GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Hepatic *Tmem30a* Deficiency Causes Intrahepatic Cholestasis by Impairing Expression and Localization of Bile Salt Transporters

Leiming Liu,^{*,†} Lingling Zhang,[‡] Lin Zhang,^{§¶} Fan Yang,^{†||} Xudong Zhu,[‡] Zhongjie Lu,^{**,*} Yeming Yang,^{§¶} Haiqi Lu,^{*} Lifeng Feng,^{*} Zhuo Wang,^{*} Hui Chen,^{**,*} Sheng Yan,^{**,*} Lin Wang,^{††} Zhenyu Ju,^{†‡} Hongchuan Jin,^{*} and Xianjun Zhu^{§¶}

From the Laboratory of Cancer Biology,^{*} Key Laboratory of Biotherapy in Zhejiang Province, Sir Runrun Shaw Hospital, and the Key Laboratory of Combined Multi-Organ Transplantation,^{**} Ministry of Public Health, Division of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China; the Key Laboratory of Regenerative Medicine of Ministry of Education,[†] Institute of Aging and Regenerative Medicine, Jinan University, Guangzhou, China; the Institute of Aging Research,[‡] Leibniz Link Partner Group on Stem Cell Aging, School of Medicine, Hangzhou Normal University, Hangzhou, China; the Sichuan Provincial Key Laboratory for Human Disease Gene Study and School of Medicine,[§] Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, Chengdu, China; the Key Laboratory for NeuroInformation of Ministry of Education and Medicine Information Center,[¶] School of Medicine, University of Electronic Science and Technology of China, Chengdu, China; the Leibniz Institute for Age Research - Fritz Lipmann Institute,^{||} Friedrich-Schiller University of Jena, Jena, Germany; and the Department of Hepato-Biliary Surgery,^{††} Xijing Hospital, The Fourth Military Medical University, Xi'an, China

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Address correspondence to
Hongchuan Jin, M.D., Ph.D.,
Laboratory of Cancer Biology,
Sir Runrun Shaw Hospital,
#401, 3 E Qingchun Rd, Hang-
zhou, Zhejiang 310016, China;
or Xianjun Zhu, Ph.D., Key
Laboratory for Human Disease
Gene Study, Sichuan Academy
of Medical Sciences and
Sichuan Provincial People's
Hospital, 32# W Sec 2, 1st Ring
Rd, Chengdu, Sichuan 610072,
China. E-mail: jinhc@zju.edu.cn
or xjzhu@uestc.edu.cn.

Mutations in *ATP8B1* or *ATP11C* (members of P4-type ATPases) cause progressive familial intrahepatic cholestasis type 1 in human or intrahepatic cholestasis in mice. Transmembrane protein 30A (TMEM30A), a β -subunit, is essential for the function of *ATP8B1* and *ATP11C*. However, its role in the etiology of cholestasis remains poorly understood. To investigate the function of TMEM30A in bile salt (BS) homeostasis, we developed *Tmem30a* liver-specific knockout (LKO) mice. *Tmem30a* LKO mice experienced hyperbilirubinemia, hypercholanemia, inflammatory infiltration, ductular proliferation, and liver fibrosis. The expression and membrane localization of *ATP8B1* and *ATP11C* were significantly reduced in *Tmem30a* LKO mice, which correlated with the impaired expression and localization of BS transporters, such as OATP1A4, OATP1B2, NTCP, BSEP, and MRP2. The proteasome inhibitor bortezomib partially restored total protein levels of BS transporters but not the localization of BS transporters in the membrane. Furthermore, the expression of nuclear receptors, including FXR α , RXR α , HNF4 α , LRH-1, and SHP, was also down-regulated. A cholic acid-supplemented diet exacerbated the liver damage in *Tmem30a* LKO mice. TMEM30A deficiency led to intrahepatic cholestasis in mice by impairing the expression and localization of BS transporters and the expression of related nuclear receptors. Therefore, TMEM30A may be a novel genetic determinant of intrahepatic cholestasis. (*Am J Pathol* 2017, 187: 2775–2787; <https://doi.org/10.1016/j.ajpath.2017.08.011>)

Bile acids are synthesized in the liver and secreted into the gut, and they have multiple physiological functions, including bile flow, absorption of lipophilic nutrients, clearing toxic molecules, and antimicrobial and metabolic effects.¹ Disturbances in bile acid homeostasis contribute to cholestasis, gallstone disease, and malabsorption. Cholestasis causes liver damage due to the excessive accumulation of bile salts (BSs) and other toxins in the liver. Hereditary mutations in hepatobiliary transporter genes result in defects in the expression and function of the

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L.L. and Ling.Z. contributed equally to this work.

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hepatobiliary transport systems, which are implicated in the pathogenesis of cholestasis. Progressive familial intrahepatic cholestasis (PFIC) types 1 and 2 are caused by different mutations in *ATP8B1* and *ABCB11* (which encodes the BS export pump, BSEP), respectively.^{2,3} The third type of PFIC (PFIC3) is caused by a genetic defect in *ABCB4* (which encodes multidrug resistance 3).⁴ Patients with PFIC often have symptoms in the neonatal period or the first year of life, and their condition usually leads to death from liver failure at ages ranging from infancy to adolescence.⁵ In addition, mutations in *MRP2* (multidrug resistance-associated protein 2) are associated with Dubin-Johnson syndrome, which results in jaundice and intrahepatic cholestasis of pregnancy.^{6,7} Recently, mutations in *TJP2* (tight junction protein 2) and *NIRH4* (which encodes the farnesoid X receptor, FXR) were also reported to cause progressive familial intrahepatic cholestasis in humans.^{8,9}

ATP8B1 is a widely expressed P4-type ATPase that functions as a phospholipid flippase to maintain phospholipid asymmetry in the membrane. Compared with patients with PFIC1, *Atp8b1*^{G308V/G308V} mice showed mild abnormalities, including decreased weight at weaning and elevated serum bile acid levels.¹⁰ In addition, mice deficient in ATP11C (which encodes a paralogous P4-type ATPase) exhibited impaired localization of BS transporters and plasma conjugated hyperbilirubinemia and unconjugated hypercholanemia.^{11–15} From these studies, the P4-ATPases have important roles in preventing intrahepatic cholestasis. Transmembrane protein 30 (TMEM30) or cell division cycle protein 50 (CDC50) binds to the P4-ATPases to form a heterodimer, and mammals express three members of the TMEM30 family: TMEM30A (CDC50A), TMEM30B (CDC50B), and TMEM30C (CDC50C). TMEM30A is ubiquitously expressed and binds 11 of 14 P4-ATPases.^{16–20} TMEM30A is required for the flippase activity and localization of these P4-ATPases. Depletion of TMEM30A in cell lines impaired the endoplasmic reticulum (ER) exit of these P4-ATPases and caused a severe defect in the formation of membrane ruffles because of defects in the inward translocation of phosphatidylethanolamine and phosphatidylserine that subsequently inhibited cell migration.^{16–18,21} TMEM30A-deficient cells are engulfed by macrophages because of the exposure of phosphatidylserine in the outer leaflet in the plasma membrane, and these cells lost their ability to induce tumor formation in nude mice.²² TMEM30A also plays a key role in the uptake of the anticancer drugs and choline phospholipids in mammalian cells.^{23,24} Knockdown of *Tmem30a* in wild-type FVB mice resulted in elevated plasma levels of unconjugated BSs but no liver damage.¹³ However, it is still unclear whether TMEM30A plays a role in cholestasis.

In this study, we investigated the *in vivo* roles of *Tmem30a* in the liver by generating *Tmem30a* liver-specific knockout (LKO) mice. Heterozygous *Tmem30a* KO first mice were crossed with *Flp* mice to generate *Tmem30a*^{fllox/+} mice, which were then crossed with Alb-Cre

mice to generate *Tmem30a*^{fllox/fllox}-Alb-Cre mice. These *Tmem30a* LKO mice were used to investigate the function of TMEM30A in cholestasis. *Tmem30a* LKO mice exhibited severe hyperbilirubinemia and hypercholanemia, inflammatory infiltration, ductular proliferation, and liver fibrosis. These pathologic phenotypes in *Tmem30a* LKO mice were exacerbated on 0.5% cholic acid (CA)-supplemented diet. Mechanistically, the expression and localization of ATP8B1, ATP11C, and several BS transporters, including basolateral uptake transporters [organic anion-transporting polypeptide 1A4 (OATP1A4), OATP1B2, and Na⁺-taurocholate cotransporting polypeptide (NTCP)] and canalicular efflux transporters (BSEP and MRP2) in hepatocytes, were severely impaired. In addition, the expression of regulatory nuclear receptors related to bile acid synthesis and transport, including FXR α , retinoid X receptor α (RXR α), hepatocyte nuclear factor 4 α (HNF4 α), liver receptor homolog-1 (LRH-1), and short heterodimer partner (SHP), was also significantly decreased. These data demonstrated that TMEM30A is essential for the expression and proper localization of BS transporters and thereby is critical for the maintenance of BS homeostasis.

Materials and Methods

Generation of *Tmem30a* LKO Mice

The original *Tmem30a* allele is of type KO first by using the FRT-Flp strategy and also is conditional potential by using the Flox-cre system. Mice carrying the two floxed alleles on the third exon of the *Tmem30a* gene were generated by conventional homologous recombination in embryonic stem cells and blastocyst injection of correctly targeted embryonic stem cell clones. *Tmem30a* KO first mice were first interbred with Flp mice²⁵ (The Jackson Laboratory, Bar Harbor, ME; strain name: B6.129S4-Gt(ROSA)26Sortm1(FLP1)Dym/RainJ; stock number 009086; alias ROSA26::FLPe knock in) to generate *Tmem30a*^{fllox/+} mice. *Tmem30a*^{fllox/+} mice were then crossed with Albumin-Cre²⁶ (alias Alb-Cre; The Jackson Laboratory) mice to generate LKO mice with the genotype *Tmem30a*^{fllox/fllox}-Alb-Cre mice (Supplemental Figure S1A). *Tmem30a*^{fllox/fllox} or Alb-Cre mice were used as littermate WT controls (genetic background: C57BL/6J). To monitor the efficiency of Cre-mediated deletion of the LoxP-flanked exon 3 in the liver, a TdTomato reporter²⁷ was used [strain name: B6.Cg-Gt(ROSA)26Sortm14(CAG-TdTomato)Hze/J; alias Ai14D]. This reporter line contains a loxP-flanked STOP cassette that prevents transcription of the downstream CAG promoter-driven red fluorescent protein variant TdTomato. In the presence of Alb-Cre recombinase, the STOP cassette in the reporter mice will be removed in the Cre-expressing hepatocyte and TdTomato will be expressed. Because this CAG promoter-driven reporter construct was inserted into the Gt(ROSA)26Sor locus, TdTomato expression pattern was determined by the tissue(s) that expressed Cre recombinase.

All animals received humane care according to the criteria outlined in the NIH's *Guide for the Care and Use of Laboratory Animals*,²⁸ and all animal experiments were approved by the Animal Care and Ethics Committee at Hangzhou Normal University and Sichuan Provincial People's Hospital.

Plasma Biochemistry, BS Species Identification and Quantification, and Bile Formation Assays

Reagents for serum biochemical analysis were purchased from DiaSys Diagnostic Systems GmbH (Holzheim, Germany). All samples were tested on a Hitachi (Tokyo, Japan) Clinical Analyzer 7180, according to the manufacturer's protocols. BS species were identified and quantified as previously described in detail.²⁹ The bile formation assays were performed as previously described in detail¹³; the concentration of phospholipid was tested according to the instruction (catalog number MAK122; Sigma-Aldrich, St. Louis, MO).

Real-Time Quantitative PCR

Total RNA was isolated from liver samples using Trizol (Invitrogen, Carlsbad, CA) and then reverse-transcribed and analyzed by real-time quantitative PCR with SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) and specific primers (Table 1) on a Bio-Rad CFX96 Touch.

Liver Histologic, Immunohistochemistry, Immunofluorescence, and Western Blot Analysis

Liver tissues were fixed in 0.01 mol/L phosphate-buffered saline (PBS; pH 7.4) containing 10% formalin and then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) and Masson blue for histologic examinations. Immunohistochemistry was performed with rabbit anti-F4/80 and cytokeratin-19 antibodies. H&E and immunohistochemistry slices were scanned and captured with a Panoramic MIDI (3DHISTECH Ltd., Budapest, Hungary). For immunofluorescence, frozen tissue sections were fixed in 4% formaldehyde. After washing the samples twice with PBS, they were incubated in PBS/fetal bovine serum (pH 7.4, containing 10% fetal bovine serum) to block nonspecific sites of antibody absorption. Then, these slices were incubated with appropriate primary and secondary antibodies in 0.1% saponin. The images were captured on a laser scanning confocal microscope (Carl Zeiss, Jena, Germany). The plasma membrane proteins were extracted according to the manufacturer's protocol (catalog number 71772; NovaGen, Los Angeles, CA). Liver samples (50 mg) were homogenized in 1 mL of radioimmunoprecipitation lysis buffer (Beyotime, Shanghai, China) containing a protease inhibitor cocktail (Roche, Mannheim, Germany). The VersaDoc Imaging System (Bio-Rad) was used to visualize the proteins and to

Table 1 Sequences of Oligonucleotides Used in This Study

Gene	Forward primer	Reverse primer
<i>Asbt</i>	5'-GGAAGTGGCTCCAATATCCTG-3'	5'-TCCAATCACAGCTATGAGCAC-3'
<i>Bsep</i>	5'-ACTAATGTTGGGATCCAGGGC-3'	5'-GGCTTTGTCCAGAGCAAGCTG-3'
<i>Cyp8b1</i>	5'-TAGCCCTGTTAGAGTGTGTGTGAC-3'	5'-AGTCAGGATCTCTCCCTGAACCTG-3'
<i>Cyp7a1</i>	5'-CAATGAAAGCAGCCTCTGAAG-3'	5'-AGCCTCCTTGATGATGCTATC-3'
<i>Cyp27a1</i>	5'-CCACAAGGGCCTCACCTATG-3'	5'-GCACCTGGTCCAGCCGGGTG-3'
<i>Fgf15</i>	5'-CCAAGTGTCTCCGGAATCC-3'	5'-TACAGTCTTCTCCGAGTAGC-3'
<i>Fgf4</i>	5'-CTCGGAAAGCCCTGGGTGA-3'	5'-AGCTTCATCACCTCCATCTCG-3'
<i>Fxrα</i>	5'-GAAAGAGTGGTATCTCTGATGAG-3'	5'-ACCGCCTCTCTGTCCCTTGATG-3'
<i>Lrh1</i>	5'-AACCTCTGAGTCTCGCACAG-3'	5'-ACCTGCTCTTGGACACCTTCC-3'
<i>Lxrα</i>	5'-AATGCCAGGAGTGTGCACTT-3'	5'-CTTGCCGCTTCAGTTTCTTC-3'
<i>Mrp2</i>	5'-AGCAGGTGTTTCGTTGTGTGT-3'	5'-AGCCAAGTGCATAGGTAGAGAAT-3'
<i>Mrp3</i>	5'-CCCTGCGTATGAACCTAGATC-3'	5'-CTGCCTCTGGCCAACTG-3'
<i>Mrp4</i>	5'-GCCACATCCTCATACTCAA-3'	5'-CTCTGCTTCCCTCGTTTCT-3'
<i>Shp</i>	5'-TCTGGAGCCTTGAGCTGGGT-3'	5'-GCCTTGGCTGGCTGGGTAC-3'
<i>Ntcp</i>	5'-ATCTGACCAGCATTTGAGGCTCT-3'	5'-CCGTGCTAGATTCTCTTGCTGT-3'
<i>Atp11c</i>	5'-CGAAGAAGAAGTGCCAGGAATCCGA-3'	5'-GCTGCAACCACGGTCAATATGCT-3'
<i>Atp8b1</i>	5'-GTCTGGGACAGAGTCATTTTC-3'	5'-CTTATCAGAGAAGATGTAATG-3'
<i>Oatp1a1</i>	5'-CAGATAAATGGATTGCGAC-3'	5'-GTCAACAAATAGTTACAGAG-3'
<i>Oatp1a4</i>	5'-ATAGCTTCAGGCGCATTTAC-3'	5'-ATAGCTTCAGGCGCATTTAC-3'
<i>Oatp1b2</i>	5'-TTCACCACAACAATGGCCTA-3'	5'-TTTCCCCACAGACAGGTTTC-3'
<i>Hnf1α</i>	5'-CTGATTGAAGAGCCCA-3'	5'-CACTCCGCCCTATTACACT-3'
<i>Hnf4α</i>	5'-AGACAAAGATAAGAGGAACCAG-3'	5'-CAGAGATGGGAGAGGTGA-3'
<i>Mdr2</i>	5'-CCCCTGTATTGATGCTTTC-3'	5'-CCTTTGATGTTGTCTGGTTT-3'
<i>Rar</i>	5'-TGAGCAAGACACAATGACC-3'	5'-CGAAGGCAAAGACCAAGT-3'
<i>Rxr</i>	5'-TACGCAAAGACCTGACCTAC-3'	5'-CTCCACCTCGTTCTCATTC-3'
<i>Gr</i>	5'-ACAGACAAGCAAGTGGAAAC-3'	5'-TGAGGAGAGAAGCAGTAAGG-3'

quantify the band densities. All primary and secondary antibodies are listed in [Table 2](#).

Proteasome Inhibition and CA-Supplemented Diet

For proteasome inhibition, mice were intraperitoneally injected with 0.5 mg/kg bortezomib (Selleck, Shanghai, China) or dimethyl sulfoxide and then sacrificed after 6 or 12 hours. The mice were fed a basal 14% protein diet with or without a 0.5% sodium cholate supplement ([Supplemental Table S1](#)), and the animals were weighed at 3-day intervals and monitored daily for survival. The mice were euthanized when they were experiencing severe distress, such as dramatic weight loss, back deformations, and slow movement.

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance test, followed by *t*-test. A *P* value ≤0.05 was considered statistically significant.

Results

Hyperbilirubinemia and Hypercholanemia in *Tmem30a* LKO Mice

Tmem30a LKO mice were viable and fertile and had normal life spans. The specific depletion of TMEM30A in hepatocytes was confirmed by immunostaining and Western blot

analysis with the use of specific TMEM30A antibody³⁰ ([Supplemental Figure S1](#), B and C), and the expression of TMEM30A in kidney and intestine was similar between littermate controls and *Tmem30a* LKO mice ([Supplemental Figure S1D](#)). Strong yellow pigmentation was observed in the sera from *Tmem30a* LKO mice compared with littermate controls at 2 months of age ([Figure 1A](#)). Serum total bile acid (TBA) levels ($168.8 \pm 66.6 \mu\text{mol/L}$; *n* = 10) were approximately 20-fold higher in *Tmem30a* LKO mice than levels in the littermate controls ($7.4 \pm 1.6 \mu\text{mol/L}$; *n* = 11) ([Figure 1B](#)). Serum total bilirubin (TBIL) levels ($20.1 \pm 6.7 \mu\text{mol/L}$; *n* = 12) were approximately 15-fold higher in *Tmem30a* LKO mice than levels in littermate controls ($1.4 \pm 0.2 \mu\text{mol/L}$; *n* = 12) ([Figure 1C](#)). TMEM30A deficiency primarily resulted in a dramatic increase in direct bilirubin (DBIL) levels, whereas indirect bilirubin (IBIL) levels were slightly increased ([Figure 1C](#)). Serum γ -glutamyl transferase levels were comparable between the two groups ([Figure 1D](#)). Next, TBA, TBIL, and DBIL levels were examined at various ages, and it was found that TBA ($217.2 \pm 53.1 \mu\text{mol/L}$; *n* = 14) and TBIL ($23.7 \pm 6.3 \mu\text{mol/L}$; *n* = 12) levels peaked at 4 months of age ([Figure 1](#), E–G). According to the assay used to identify and quantify BS species, serum unconjugated and conjugated bile acid levels were dramatically increased ([Figure 2A](#)). Only small changes were detected in the hepatic and biliary BS species ([Figure 2](#), B and C). Thus, hepatic TMEM30A deficiency induced intrahepatic cholestasis in mice.

Table 2 The Primary and Secondary Antibodies Used in This Study

Name	Catalog	Manufacturer
SHP	sc-30169	Santa Cruz Biotechnology, Santa Cruz, CA
ATP8B1	sc-134967	Santa Cruz Biotechnology
OATP1A4	sc-376424	Santa Cruz Biotechnology
BSEP	ab112494	Abcam, Cambridge, MA
NTCP	ab131084	Abcam
LRH-1	ab125034	Abcam
HNF4 α	ab199431	Abcam
FXR α	ab28480	Abcam
RXR α	ab125001	Abcam
F4/80	ab100790	Abcam
PKC ζ	9368p	Cell Signaling Technology, Danvers, MA
Phospho-PKC ζ /λ (Thr410/403)	9378p	Cell Signaling Technology
CK-19	12434s	Cell Signaling Technology
β-Actin	A5316	Sigma-Aldrich, St Louis, MO
Anti-rabbit IgG HRP-linked antibody	7074s	Cell Signaling Technology
Anti-mouse IgG HRP-linked antibody	7076s	Cell Signaling Technology
Donkey anti-goat IgG Fab secondary antibody, HRP	PA1-29617	Thermo Fisher Scientific, Rockford, Illinois
Donkey anti-goat IgG (H+L) secondary antibody, Alexa Fluor 546	A11056	Thermo Fisher Scientific
Donkey anti-goat IgG (H+L) secondary antibody, Alexa Fluor 546	A11010	Thermo Fisher Scientific
Goat anti-mouse IgG (H+L) secondary antibody, Alexa Fluor 488	A11001	Thermo Fisher Scientific
Rabbit anti-mouse IgG (H+L) secondary antibody, Alexa Fluor 594	A21205	Thermo Fisher Scientific
Donkey anti-rabbit IgG (H+L) secondary antibody, Alexa Fluor 488	A21206	Thermo Fisher Scientific

BSEP, bile salt export pump; CK, cytokeratin; FXR, farnesoid X receptor; HNF, hepatocyte nuclear factor; HRP, horseradish peroxidase; LRH, liver receptor homolog; NTCP, Na⁺-taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide; PKC, protein kinase C; RXR, retinoid X receptor; SHR, short heterodimer partner.

Figure 1 Hyperbilirubinemia and hypercholanemia in *Tmem30a* liver-specific knockout (LKO) mice. **A:** *Tmem30a* LKO mice showed strong yellow pigmentation in their sera. **B:** Serum total bile acid (TBA) levels were dramatically elevated in *Tmem30a* LKO mice compared with the littermate controls. **C:** Serum total bilirubin (TBIL), direct bilirubin (DBIL), and indirect bilirubin (IBIL) levels were dramatically increased in *Tmem30a* LKO mice compared with their littermate controls. **D:** Serum γ -glutamyl transferase (GGT) levels were comparable between the two groups. **E–G:** Serum TBA ($217.2 \pm 53.1 \mu\text{mol/L}$) (**E**), DBIL ($18.7 \pm 5.5 \mu\text{mol/L}$) (**F**), and TBIL ($23.7 \pm 6.3 \mu\text{mol/L}$) (**G**) levels peaked at 4 months of age, and their increased levels decreased progressively with age in *Tmem30a* LKO mice. All mice were at 2 months of age except for special indication. Data are expressed as means $\pm$ SEM. $n = 10$ *Tmem30a* LKO mice (**B**); $n = 11$ littermate control mice (**B** and **C**); $n = 12$ *Tmem30a* LKO mice (**C**); $n = 6$ to 8 mice in both groups (**D**); $n = 14$ TBA samples (**E**); $n = 13$ samples (**F**), $n = 12$ samples (**G**). * $P < 0.05$, ** $P < 0.01$ versus WT (*t*-test). WT, wild-type.

TMEM30A Deficiency Induces Liver Damage and Impairs Liver Function

Next, the liver histologic findings and serum biochemistry variables were examined because intrahepatic cholestasis often induces liver damage and impairs liver function. Liver-to-body weight ratios of *Tmem30a* LKO mice were comparable with the littermate controls (Supplemental

Figure S2A). H&E staining and the F4/80 immunohistochemistry analysis revealed significant lymphocyte and macrophage infiltration in *Tmem30a*-deficient livers (Supplemental Figure S2, B and C). Serum levels of inflammatory cytokines, such as IL-6, IL-18, IL-10, interferon- γ , and tumor necrosis factor- α were consistently increased in *Tmem30a* LKO mice (Supplemental Figure S2D). Masson blue staining showed that *Tmem30a*

Figure 2 Identification and quantification of bile salt (BS) species in wild-type (WT) and *Tmem30a* liver-specific knockout (LKO) mice. **A:** The plasma levels of unconjugated (MCA, CA, DCA, UDCA, CDCA) and conjugated (TMCA, TCA, TCDCA, TDCA, TLCA, GCA) BS species were significantly increased in *Tmem30a* LKO mice. **B:** The levels of MCA, cholic acid (CA), and TCA in *Tmem30a*-deficient livers were slightly increased. **C:** The levels of muricholate (MCA) and TUDCA in bile were slightly changed in *Tmem30a* LKO mice. All samples were from mice aged 2 to 3 months. Plasma levels are expressed in $\mu\text{mol/L}$, biliary levels in nmol/mL, hepatic levels in nmol/g liver, respectively. Error bars represent SEM. $n \geq 6$ mice per group (**A–C**). * $P < 0.05$, ** $P < 0.01$ (*t*-test). CDCA, chenodeoxycholate; DCA, deoxycholate; G, glycol; LCA, lithocholic acid; T, tauro; UDCA, ursodeoxycholate.

LKO mice exhibited liver fibrosis, which was further confirmed by the increased levels of collagen type 1 α 1 chain (Col1a1), Col3a1, and transforming growth factor- β mRNAs (Supplemental Figure S2E). Immunohistochemistry and real-time PCR of cytokeratin-19 indicated significantly ductular proliferation in *Tmem30a* LKO mice (Supplemental Figure S2F). Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) levels were elevated in *Tmem30a* LKO mice at various ages (Supplemental Figure S3, A–C). However, serum total protein and albumin were slightly reduced (Supplemental Figure S3, D and E). Thus, hepatic deletion of TMEM30A resulted in inflammatory infiltration, ductular proliferation, and liver fibrosis.

Decreased Expression of ATP11C, ATP8B1, BS Transporters, and Nuclear Receptors in *Tmem30a* LKO Mice

Because TMEM30A is required for the localization and functions of P4-ATPases, the expression and localization of ATP11C and ATP8B1 were examined in *Tmem30a* deficient livers. Although the mRNA levels of ATP8B1 and ATP11C were not changed (Figure 3A), their protein levels were reduced in *Tmem30a*-deficient livers (Figure 3B). Considering that the protein levels of BS uptake transporters OATPs and NTCP are decreased in *Atp11c*-deficient mice,^{12,13} the expression of BS transporters was analyzed in *Tmem30a*-deficient livers. The mRNA levels of OATP1B2, NTCP, and BSEP were significantly reduced in *Tmem30a*-deficient livers (Figure 3A). The protein levels of

BS uptake transporters (OATP1A4, OATP1B2, and NTCP) and canalicular efflux proteins (BSEP and MRP2) were significantly reduced in *Tmem30a*-deficient livers (Figure 3B). These results suggested that the reduced expression of BS transporters was partially mediated by a defect in transcriptional regulation.

The expression of BS transporters and synthetic enzymes is transcriptionally regulated by hepatic nuclear receptors. Real-time PCR analysis demonstrated that the mRNA levels of nuclear receptors, including *Fxr*, *Shp*, *Hnf4 α* , *Lrh-1*, *Rxra*, liver X receptor α , constitutive androstane receptor, pregnane X receptor, and the enzymes cytochrome P450 family 8 subfamily B member 1 and cytochrome P450 family 27 subfamily A member 1 were decreased (Figure 3A). Moreover, the protein levels of FXR α , RXR α , LRH-1, HNF4 α , and SHP were also significantly reduced in *Tmem30a*-deficient livers (Figure 3C). ATP8B1 has been shown to regulate the FXR α pathway by down-regulating the phosphorylation of protein kinase C (PKC) ζ .^{31–35} Consistent with this result, it was observed that a significant reduction occurred in the levels of phosphorylated PKC ζ in *Tmem30a*-deficient livers (Figure 3D). From these findings, the decreased expression of ATP11C, ATP8B1, BS transporters, and hepatic nuclear receptors may be responsible for hyperbilirubinemia and hypercholanemia in *Tmem30a* LKO mice.

Mislocalization of ATP11C, ATP8B1, and BS Transporters in *Tmem30a* LKO Mice

The proper membrane localization of ATP8B1, ATP11C, and BS transporters is important for their functions. The

Figure 3 Analysis of mRNA and protein levels of ATP8B1, ATP11C, bile salt (BS) transporters and related nuclear receptors. **A:** Mean relative mRNA levels of BS transporters (*Oatp1b2*, *Ntcp*, *Bsep*), *Atp8b1*, *Atp11c*, related nuclear receptors (*Fxr*, *Shp*, *Hnf4 α* , *Lrh-1*, *Rxra*, *Lxra*, *Car*, *Pxr*), and enzymes (*Cyp8b1*, *Cyp27a1*) in livers were reduced in *Tmem30a* LKO mice compared to the littermate controls (relative mRNA levels were normalized to β -actin). **B:** The protein levels of ATP8B1, ATP11C, and BS transporters in *Tmem30a*-deficient livers were significantly reduced compared with the littermate controls. **C:** The protein levels of related nuclear receptors (FXR α , RXR α , HNF4 α , LRH-1, SHP) were also significantly reduced in *Tmem30a*-deficient livers. **D:** Phosphorylation of protein kinase C (PKC) ζ was also dramatically reduced in *Tmem30a* liver-specific knockout (LKO) mice. All samples were from mice aged 2 to 3 months. Protein levels were quantified by densitometry and normalized to β -actin. Error bars represent SEM. $n \geq 4$ samples (**A**); $n = 5$ WT and LKO (**B** and **C**); $n \geq 4$ samples per group (**D**). * $P < 0.05$, ** $P < 0.01$ (t -test). BSEP, bile salt export pump; FXR, farnesoid X receptor; HNF, hepatocyte nuclear factor; LRH, liver receptor homolog; MRP, multidrug resistance-associated protein; NTCP, Na⁺-taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide; RXR, retinoid X receptor; SHP, small heterodimer partner; WT, wild-type.

Figure 4 The localization of ATP8B1, ATP11C, and bile salt (BS) transporters was altered in the *Tmem30a* liver-specific knockout (LKO) mice. **A:** Western blot analysis of membrane proteins extracted from the livers of the *Tmem30a*-deficient mice and littermate controls. **B:** Immunofluorescence images showing the reduced expression and localization of ATP8B1 and ATP11C in *Tmem30a*-deficient livers. **C–E:** Confocal images demonstrated that Na⁺-taurocholate cotransport protein (NTCP) (**C**), multidrug resistance-associated protein (MRP)2 (**D**), and BS export pump (BSEP) (**E**) in *Tmem30a* LKO mice were mainly localized intracellularly but not on the membrane compared with the littermate controls. **White arrows** indicate the membrane localization of NTCP, MRP2, and BSEP. All samples were from mice aged 2 to 3 months. Green: ATP8B1 antibody; red: primary antibodies as indicated; blue: DAPI. $n \geq 3$ samples per group (**C–E**). * $P < 0.05$, ** $P < 0.01$ (t -test). Scale bars: 100 μ m (**B–E**). OATP, organic anion-transporting polypeptide; WT, wild-type.

membrane proteins were extracted from the livers, and Western blot analysis showed a dramatic reduction in the levels of ATP8B1, ATP11C, OATP1A4, OATP1B2, NTCP, BSEP, and MRP2 in *Tmem30a*-deficient livers (Figure 4A). Immunofluorescence images confirmed that the localization of these proteins was also significantly reduced on the membrane in *Tmem30a*-deficient hepatocytes (Figure 4B and Supplemental Figure S4). Confocal images demonstrated that the localization of NTCP, BSEP, and MRP2 was mainly intracellular but not on the membrane (Figure 4, C–E). Interestingly, TMEM30A deficiency resulted in a significant reduction of NTCP levels in both portal and central hepatocyte membrane (Supplemental Figure S4), whereas *Atp11c*-deficient mice show only a reduction of NTCP levels in the central area.¹³ It is plausible that loss of TMEM30A impaired the localization of ATP8B1, ATP11C, and BS transporters in hepatocytes.

From the reduced levels of BSEP and MRP2, it was speculated that bile formation was defective in *Tmem30a* LKO mice. During BS depletion, the biliary outputs of BSs, cholesterol, and phospholipids were comparable between the two groups (Figure 5, A–C). On infusion of taurocholate, the biliary outputs of BSs, cholesterol, and phospholipids were decreased in *Tmem30a* LKO mice (Figure 5, A–C). The biliary outputs of glutathione and ALP were

reduced during bile depletion and taurocholate infusion in *Tmem30a* LKO mice (Figure 5, D and E). Thus, *Tmem30a* LKO mice exhibited defects in bile formation.

Normal Intestinal BS-Related Proteins and Other Organ Function

Enterohepatic circulation is also important in BS homeostasis. After being released in the gut, BSs are mainly reabsorbed in the terminal ileum by apical sodium-dependent BS transporters (ASBT) and other related proteins. *Tmem30a* deficiency did not affect the mRNA levels of Abst, fibroblast growth factor-15, Fxr α , and Shp in the intestine (Supplemental Figure S5A), and the protein levels of ABST, ATP8B1, and FXR α were also no different between the two groups (Supplemental Figure S5B). The plasma creatinine, blood urea nitrogen, and uric acid levels in *Tmem30a* LKO mice were comparable with the littermate controls at various ages (Supplemental Figure S5, C–E). H&E staining of heart, kidney, lung, spleen, muscle, pancreas, and intestine showed no significant differences at 12 months of age between littermate controls and *Tmem30a* LKO mice (Supplemental Figures S6–S8). Thus, the intestinal BS-related proteins and functions of other organs were not affected in *Tmem30a* LKO mice.

Figure 5 Bile formation was impaired in *Tmem30a* liver-specific knockout (LKO) mice. The endogenous bile salt (BS) pools were depleted from *Tmem30a* LKO mice and littermate controls for 90 minutes. Taurocholate (TC) was infused through the jugular vein, and the infusion rates increased in a stepwise manner every 30 minutes at 400 nmol/min per 100 g for each step (400, 800, 1200, 1600 nmol/min per 100 g). **A–C:** No difference was found in the biliary outputs of BS (**A**), cholesterol (**B**), and phospholipids (**C**) between *Tmem30a* LKO mice and littermate controls during BS depletion. However, the biliary outputs of BS, cholesterol, and phospholipids were significantly decreased in *Tmem30a* LKO mice with the increased infusion rates. **D and E:** The biliary outputs of glutathione (**D**) and alkaline phosphatase (ALP) (**E**) were significantly reduced in *Tmem30a* LKO mice during both bile depletion and TC infusion. All male mice were tested at 2 to 3 months age. Error bars represent SEM. $n \geq 3$ male mice per group (**D** and **E**). * $P < 0.05$ (one-way analysis of variance analysis). WT, wild-type.

Enhanced Proteasomal Degradation of ATP11C, ATP8B1, and BS Transporters in *Tmem30a* LKO Mice

TMEM30A-deficient cells exhibited defects in the ER exit,^{16–18} and it was speculated that this deficiency may lead to the accumulation of ATP8B1, ATP11C, and BS transporters in the ER and may induce proteasomal degradation. On proteasome inhibition with bortezomib, the protein levels of ATP8B1, ATP11C, OATP1B2, NTCP, BSEP, and MRP2 were partially restored in *Tmem30a* LKO mice (Figure 6, A and B). However, immunofluorescence staining indicated that the membrane localization of ATP8B1, ATP11C, and BS transporters was not restored. Instead, these proteins were co-localized with protein disulfide isomerase in the ER after the bortezomib treatment, suggesting that they were accumulated in the ER (Supplemental Figure S9). Interestingly, the protein levels of FXR α , RXR α , HNF4 α , LXR-1, and SHP were also increased in *Tmem30a*-deficient livers on proteasome inhibition (Figure 6, C and D). These increased nuclear receptors may partially up-regulate the mRNA levels of BS transporters compared with dimethyl sulfoxide controls in the *Tmem30a* LKO mice group (Supplemental Figure S10). Therefore, the reduction of BS transporters in *Tmem30a* LKO mice was not only attributed to the proteasomal degradation induced by the impaired trafficking of ATP8B1, ATP11C, and BS transporters but also the decreased transcriptional activation.

Exogenous Bile Acids Exacerbated the Phenotypes in *Tmem30a* LKO Mice

The mice were challenged with a 0.5% CA-supplemented diet to further substantiate these findings. Jaundice was only observed in *Tmem30a* LKO mice (Supplemental Figure S11A). The plasma levels of TBA, TBIL, DBIL, ALT, AST, and ALP were dramatically increased in *Tmem30a* LKO mice after 9 days on the CA-supplemented diet (Figure 7, A–C). The serum total protein and albumin levels were obviously reduced in *Tmem30a* LKO mice (Supplemental Figure S11B). No obvious difference was observed in serum creatinine, blood urea nitrogen, and uric acid levels (Supplemental Figure S11C). Male *Tmem30a* LKO mice lost weight more rapidly than the littermate controls on CA-supplemented diet for 2 weeks (Figure 7D). Moreover, more than one-half of the male *Tmem30a* LKO mice died within 2 weeks, whereas all male littermate controls survived (Figure 7E). Morbid *Tmem30a* LKO mice, which showed dramatic weight loss, back deformations, and slow movements, were sacrificed for further analysis. Liver-to-body weight ratios of the morbid mice were dramatically reduced compared with the mice with normal feeding (Supplemental Figures S2A and S11D). H&E staining showed more multinucleate hepatocytes and smaller hepatocyte sizes in morbid *Tmem30a* LKO mice (Figure 7F). The increased levels of Col1a1, Col3a1, and transforming growth factor- β mRNAs indicated severe liver fibrosis, and

Figure 6 Protein levels of bile salt (BS) transporters and related nuclear receptors were partially restored in *Tmem30a* liver-specific knockout (LKO) mice after treatment with the proteasome inhibitor. All mice were sacrificed 6 and 12 hours after treatment with the proteasome inhibitor bortezomib (0.5 mg/kg). **A** and **B**: Compared with their dimethyl sulfoxide (DMSO) control groups, wild-type (WT) mice treated with bortezomib showed no difference in the protein levels of ATP8B1, ATP11C and BS transporters (**A**), whereas *Tmem30a* LKO mice treated with bortezomib showed dramatically increased levels of these proteins (**B**). **C** and **D**: Compared with their DMSO control groups, WT mice treated with bortezomib showed no difference in the protein levels of farnesoid X receptor (FXR) α , retinoid X receptor (RXR) α , hepatocyte nuclear factor (HNF4) α , liver receptor homolog (LRH)-1, and small heterodimer partner (SHP) (**C**), whereas *Tmem30a* LKO mice treated with bortezomib showed dramatically increased levels of these nuclear receptors (**D**). All mice were tested at 2 to 3 months age. Protein levels were quantified by densitometry and normalized to β -actin. $n \geq 3$ mice per group. * $P < 0.05$, ** $P < 0.01$ (t -test). BSEP, bile salt export pump; MRP, multidrug resistance-associated protein; NTPC, Na⁺-taurocholate cotransport protein; OATP, organic anion transporting polypeptide.

Masson blue staining confirmed this result in *Tmem30a* LKO mice (Supplemental Figure S11, E and F). H&E staining showed that there were no obvious differences in other organs, such as heart, kidney, brain, intestine, and spleen (Supplemental Figures S12 and S13). Interestingly, serum TBA, TBIL, and DBIL levels and serum ALT, AST, and ALP levels were also increased in surviving *Tmem30a* LKO mice (Supplemental Figure S14, A and B) but not as dramatically as morbid *Tmem30a* LKO mice (Figure 7, A–C). In addition, H&E staining did not reveal any more difference except for unclear cell boundaries in surviving *Tmem30a* LKO mice (Supplemental Figure S14C). In summary, the CA-supplemented diet exacerbated the phenotypes and led to more severe liver damage in *Tmem30a* LKO mice. However, the molecular mechanisms underlying this phenotype are not clear, and further investigation is warranted.

Discussion

As a β -subunit, TMEM30A is essential for the function of both ATP8B1 and ATP11C.^{16,20} *Atp8b1* mutant mice

exhibited mild abnormalities, and the cholestatic phenotypes were only revealed in *Atp8b1* mutant mice challenged with a CA-supplemented diet.^{10,36} In contrast, *Atp11c*-deficient mice exhibited conjugated hyperbilirubinemia and unconjugated hypercholanemia. Similar to the *Atp11c*-deficient mice, *Tmem30a*-deficient mice showed a dramatic reduction in the levels of OATP1A4 and OATP1B2, which may be responsible for the increased levels of conjugated bilirubin and unconjugated bile acid. Consistently, these phenotypes were also observed in *Oatp1a/1b*^{-/-} mice.³⁷ Furthermore, the distribution of BS species (approximately 80% of the total plasma BS species were unconjugated) in *Tmem30a* LKO mice was also comparable with the distribution observed in *Atp11c*-deficient mice. Therefore, the two mouse models have several similar phenotypes in intrahepatic cholestasis. Nevertheless, *Tmem30a*-deficient mice exhibited more severe defects than *Atp11c*-deficient mice, as indicated by the 15-fold versus sixfold increase in plasma bilirubin levels as well as the 20-fold versus 10-fold increase in plasma total bile acid levels.

There are several explanations for the stronger phenotypes observed in *Tmem30a* LKO mice. First, in addition to

Figure 7 Exacerbation of the *Tmem30a*-deficient phenotypes on cholic acid (CA)-supplemented diet. Male *Tmem30a*-deficient mice and male littermate controls (2 to 3 months) were fed a 0.5% CA-supplemented diet. **A–C:** Serum total bile acid (TBA) levels (**A**); serum total bilirubin (TBIL), direct bilirubin (DBIL), and indirect bilirubin (IBIL) levels (**B**); and serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) levels (**C**) were dramatically increased in *Tmem30a* liver-specific knockout (LKO) mice compared with the littermate controls after 9 days on CA-supplemented diet. **D:** The body weight ratios of male *Tmem30a* LKO mice were dramatically decreased compared with male littermate controls after 3 weeks on CA-supplemented diet. The body weight ratios were normalized to the weight on day 0 (weight before receiving CA). The mice were weighed at 3-day intervals. **E:** Death of male *Tmem30a* LKO mice and male littermate controls after 3 weeks on CA-supplemented diet. Mice were monitored every day for the survival curve. **F:** Hematoxylin and eosin (H&E) staining showed the presence of more multinucleate hepatocytes and smaller hepatocyte sizes in morbid male *Tmem30a* LKO mice but not the littermate controls on CA-supplemented diet. Error bars represent SEM. $n = 15$ male *Tmem30a*-deficient mice; $n = 10$ male littermate control mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (t -test). Scale bars = 100 μm. Original magnification, $\times 200$.

the defect in the re-uptake of conjugated bilirubin due to reduced OATP levels,^{37,38} *Tmem30a* LKO mice but not *Atp11c*-deficient mice showed an extra defect in the expression and localization of MRP2. MRP2 primarily secretes conjugated bilirubin into the bile, and its mutations result in Dubin-Johnson syndrome, which is characterized by conjugated hyperbilirubinemia.^{38,39} Therefore, the reduced MRP2 levels could explain the higher conjugated bilirubin levels in *Tmem30a* LKO mice. Second, the reduced expression and localization of BSEP were only observed in *Tmem30a* LKO mice but not in *Atp11c*-deficient mice, possibly explaining higher total bile acid levels in *Tmem30a* LKO mice. Third, the localization of NCTP in the portal and central hepatocyte membranes was reduced in *Tmem30a* LKO mice, whereas *Atp11c*-deficient mice only lacked NCTP in the central hepatocyte membrane.¹³ This outcome may contribute to the increased tauro- and glyco-conjugated BS levels in *Tmem30a* LKO mice. Finally, biliary outputs of cholesterol, ALP, and glutathione were consistent with BSs output, which was decreased in *Tmem30a* LKO mice, and unchanged in *Atp11c*-mutant mice, but increased in *Atp8b1*-deficient mice.^{13,36} The reduction of BA synthetic enzymes CYP8B1 and CYP27A1 might be involved in the decrease in BSs output. Both BSEP and MRP2 functions are important for bile formation^{40,41}; therefore, the defects in bile formation in

Tmem30a KO mice could be due to the reduced expression of BA synthetic enzymes and transporter proteins.

BS transporters are transcriptionally regulated by a complex interacting network of nuclear receptors.^{1,42,43} In *Tmem30a* LKO mice, the mRNA and protein levels of these nuclear receptors, including FXR α , were markedly reduced. FXR α is a critical nuclear receptor for bile acids and regulates the expression of basolateral BS uptake and canalicular efflux transporters, including NTCP, OATPs, BSEP, and MRP2. In addition, FXR α also regulates the activity and/or expression of other nuclear receptors, which subsequently regulate the expression of BS transporters.^{1,42,43} On proteasome inhibition with bortezomib, the protein levels of hepatic nuclear receptors and BS transporters were partially restored in *Tmem30a* LKO mice, indicating that the reduction of these proteins was mediated by proteasomal degradation. The mRNA levels of BS transporters were also partially restored, suggesting that the reduced hepatic nuclear receptors were partly responsible for the decreased expression of BS transporters. Nevertheless, serum elevated BSs could also influence the expression of nuclear receptors, BS transporters and synthetic enzymes as a compensatory mechanism. Mutations in ATP8B1 have been suggested to suppress the expression and activity of FXR α by decreasing PKC phosphorylation.^{31–35,44} Consistent with this finding, *Tmem30a*-deficient livers exhibited a marked decrease in the

levels of phosphorylated PKC. Thus, the impaired expression and localization of ATP8B1 may be responsible for the decreased activity of the FXR α pathway in *Tmem30a*-deficient livers. Taken together, the reduced expression of BS transporters was not only attributed to the proteasomal degradation but also to the reduced expression of the nuclear receptors.

TMEM30A deficiency impaired the ER exit and catalytic phospholipid flippase activity of the P4 ATPase, which maintains an asymmetric distribution of phospholipids on the membrane to ensure membrane fluidity and constitutive signal transduction.^{16,21,45} In addition, the phospholipid flippase complex serves as a molecular scaffold to recruit signaling molecules and to provide a docking platform for proteins of the vesicle-generating machinery.^{13,21} It is plausible that *Tmem30a* deficiency blocked vesicle assembly and led to the accumulation of cargo proteins. Consequently, cargo proteins, including OATPs, NTCP, MRP2, BSEP, ATP8B1, and ATP11C, were targeted for proteasomal degradation. In line with this, proteasome inhibition by bortezomib led to a partial restoration of the levels of ATP8B1, ATP11C, and BS transporters. Confocal images demonstrated that the restored ATP8B1, ATP11C, and BS transporters were stained with protein disulfide isomerase in the ER, suggesting that the defective trafficking of vesicles containing these proteins was induced by TMEM30A deficiency. In addition, the phosphorylation of PKC ζ has been implicated in the transportation of NTCP vesicles and the canalicular localization of BSEP and MRP2^{46–48}; therefore, the insufficient phosphorylation of PKC ζ in *Tmem30a*-deficient mice might also contribute to the impaired localization of BS transporters. From these results, the impaired expression and localization of P4-ATPases induced by *Tmem30a* deficiency may affect the intracellular transport of vesicles containing BS transporters.

The present *Tmem30a* LKO mouse model displayed several interesting pathologic features that have not been reported in the previous *Atp8b1*- and *Atp11c*-deficient mice. Histologic and biochemical analyses showed dramatically increased inflammatory infiltration and liver damage, which may be attributed to more severe intrahepatic cholestasis in *Tmem30a* LKO mice. Another possibility is that the exposure of phosphatidylserine on the surface of *Tmem30a*-deficient hepatocytes elicited an eat me signal for the macrophages.²² The impaired bile formation in *Tmem30a*-deficient mice also caused the accumulation of BSs in hepatocytes, which then exacerbated the liver damage. With the CA-supplemented diet, the reduction of OATPs and NTCP could reduce the uptake of cholic acid by hepatocytes, but hydrophobic cholic acid could still enter into the hepatocytes by passive diffusion.^{49,50} In contrast to *Atp11c*-deficient mice, only 60% of *Tmem30a*-deficient mice died after consuming the CA-supplemented diet. It is speculated that these discrepancies may be due to the differences in gene deletion strategies. In contrast to the whole-body deletion of ATP11C, our mouse model was a LKO. Other

organ and tissue in *Tmem30a*-deficient mice may compensate for the CA-challenge and make a few survivals. Except for intrahepatic cholestasis, *Atp11c* mutations in mice have been reported to cause macrophage infiltration, B-cell deficiency, dystocia, hepatocellular carcinoma,^{11,14,15,22,51} which could promote the deterioration of overall viability, particularly after severe challenges.

Conclusions

This study provides a clinically relevant mouse model with phenotypes of intrahepatic cholestasis. Hepatocyte-specific *Tmem30a* KO mice present many manifestations of human intrahepatic cholestasis. TMEM30A may be a novel genetic determinant of liver disorders characterized by hyperbilirubinemia and hypercholanemia. Mutations in *ATP8B1*, *BSEP*, and *FXRA* have been shown to cause hereditary intrahepatic cholestasis in humans, and screening for *TMEM30A* mutations in patients with hereditary intrahepatic cholestasis could yield interesting findings. Taken together, this study demonstrated a critical role of TMEM30A in hepatic BS homeostasis and provided new insights into the molecular mechanisms of intrahepatic cholestasis.

Acknowledgments

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2017.08.011>.

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