X-linked cholestasis in mouse due to mutations of the P4-ATPase ATP11C

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Transporters at the hepatic canalicular membrane are essential for the formation of bile and the prevention of cholestatic liver disease. One such example is ATP8B1, a P4-type ATPase disrupted in three inherited forms of intrahepatic cholestasis. Mutation of the X-linked mouse gene *Atp11c*, which encodes a paralogous P4-type ATPase, precludes B-cell development in the adult bone marrow, but also causes hyperbilirubinemia. Here we explore this hyperbilirubinemia in two independent *Atp11c* mutant mouse lines, and find that it originates from an effect on nonhematopoietic cells. Liver function tests and histology revealed only minor pathology, although cholic acid was elevated in the serum of mutant mice, and became toxic to mutant mice when given as a dietary supplement. The majority of homozygous mutant females also died of dystocia in a maternal genotype-specific manner. ATP11C therefore represents a multifunctional transporter, essential for adult B-cell development, the prevention of intrahepatic cholestasis, and parturition, and is a new candidate for genetically undiagnosed cases of cholestasis and dystocia in humans.

gallbladder | uterus | enterohepatic circulation | jaundice | bile acid

Bile is essential for the emulsification of dietary lipids and fat-soluble vitamins, for the catabolism of cholesterol, and for the expulsion of toxic metabolites such as bilirubin. The detergent properties of bile are conferred by bile acids, which are synthesized in the liver from cholesterol (1). Following their synthesis in liver, bile acids (along with other constituents of bile) are transported across the canalicular membrane of hepatocytes, drain into the gallbladder via bile ducts, and are expelled into the duodenum in response to cholecystokinin, secreted by epithelial cells of the duodenum.

Cholestasis is an acquired or inherited syndrome associated with the retention of bile constituents in the circulation. Among the inherited forms, progressive familial intrahepatic cholestasis (PFIC) is a degenerative liver disease caused by mutations affecting canalicular hepatocyte membrane transporters (2). PFIC1 is caused by mutation of *ATP8B1* (3), encoding a P4-type ATPase involved in the translocation of phosphatidylserine (PS) from the exoplasmic to cytoplasmic membrane leaflets (4). PFIC2 is caused by mutations of *ABCB11*, encoding the bile salt export pump (BSEP) (5). BSEP is the primary transporter of hydrophobic bile acids, and as a consequence, patients with PFIC2 have extremely low concentrations of bile salts in bile (6).

Finally, mutation of *ABCB4* leads to PFIC3 (7), which can be clinically distinguished from PFIC1 and PFIC2 by elevated quantities of serum γ-glutamyl transferase (GGT). *ABCB4* encodes the MDR3 protein (MDR2 in mouse), which is essential for the translocation of phosphatidylcholine from the inner to outer leaflet of the canalicular membrane (8), as highlighted by the near absence of phosphatidylcholine from the bile of PFIC3 patients and *Abcb4* mutant mice (9).

Dubin–Johnson syndrome, caused by mutations of *ABCC2*, is also caused by inactivation of a canalicular transporter (MRP2) (2). Hyperbilirubinemia is common to patients with PFIC and Dubin–Johnson syndrome, but for different reasons. In the case of PFIC, a failure of transport leads to liver damage, which in turn prevents the expulsion of bile acids and bilirubin, leading to hyperbilirubinemia. MRP2, on the contrary, is a key transporter of conjugated bilirubin, and patients with Dubin–Johnson syndrome therefore develop hyperbilirubinemia without excessive retention of bile acids.

The similar clinical outcomes of *ATP8B1* and *ABCB4* mutations, and *ABCB11* and *ABCC2* mutations, all highlight the importance of phospholipid asymmetry and its role in canalicular transport. Phospholipid asymmetry is known to be influenced by the P4-type ATPases, a group of hypothetical aminophospholipid flippases (to which ATP8B1 belongs), which are thought to enrich phosphatidylserine and/or phosphatidylethanolamine at the cytoplasmic membrane leaflet (10). However, beyond ATP8B1, little is known of the functions of the 13 other mammalian P4-type ATPases (11).

We and others have recently discovered that mutation of the P4-type ATPase ATP11C in mouse leads to a syndrome of B-cell lymphopenia associated with hyperbilirubinemia (12, 13), although the origins of hyperbilirubinemia were uncertain. Here we detail a key nonhematopoietic role for ATP11C, specifically in the prevention of intrahepatic cholestasis.

Results

Intrahepatic Hyperbilirubinemia in *Atp11c* Mutant Mice. While investigating B-cell lymphopenia in *Atp11c* mutant mice (12), we observed strong yellow pigmentation in the plasma of mutant mice (Fig. 1*A*). Yellow plasma pigmentation segregated exclusively with B-cell lymphopenia in the *Atp11c^emptyhive* pedigree (Fig. 1*B*), and was also apparent in mice with an independent chemically induced allele, *Atp11c^spelling* (Fig. 1*B*). Compound heterozygosity for both alleles also led to plasma pigmentation (Fig. 1*C*), indicating that it was not caused by a genetically linked incidental mutation, but by mutation of the *Atp11c* locus itself. Measurement of plasma bilirubin revealed a 10-fold increase in both total and direct bilirubin (Fig. 1*D*). Hyperbilirubinemia was most dramatic in young mice, and declined progressively with age (Fig. 1*E*).

Hyperbilirubinemia may be a consequence of several physiological defects, including increased hemolysis (prehepatic), autoimmune hepatitis or hepatocyte death (intrahepatic), or biliary obstruction (posthepatic). Combining the *Atp11c* mutation with a null allele of *Rag1* allowed us to exclude autoreactive lymphocytes as a cause (Fig. 2*A*), as did bone marrow transplantation experiments, which further established that plasma pigmentation was caused by radioresistant cell lineages (Fig. 2*B*), rather than increased hemolysis. These findings were consistent with the increase of direct (conjugated) bilirubin (Fig. 1*D*), which is not expected to occur in prehepatic hyperbilirubinemia.
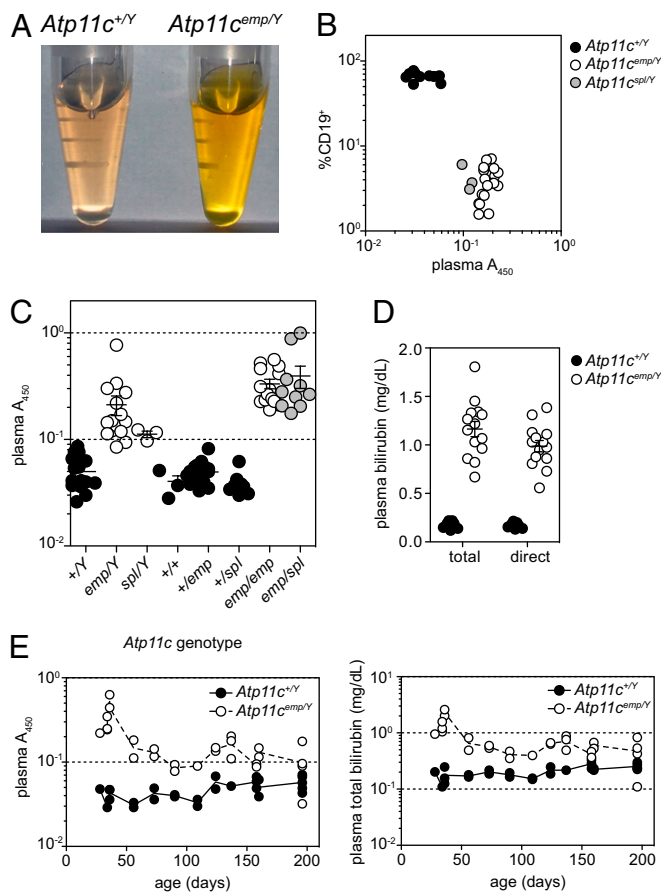
Fig. 1. Hyperbilirubinemia caused by mutations of *Atp11c*. Plasma pigmentation (*A*) and its relationship with B-cell lymphopenia (*B*) in *Atp11c* mutant mice. (*C*) Allelism testing of *emptyhive* and *spelling* alleles of *Atp11c*. (*D*) Quantitation of total and direct bilirubin in plasma, and the severity of plasma hyperbilirubinemia and pigmentation across various ages (*E*). Error bars in *C* and *D* represent SEM. Data are representative of at least three independent experiments.

Histological and Biochemical Anomalies. Histological analysis of H&E-stained liver sections from 3-mo-old mice indicated occasional portal triad-associated inflammation in mutant livers (Fig. S1*A*). Both Sirius red and trichrome blue staining were slightly more pronounced in mutant portal regions compared with lit-
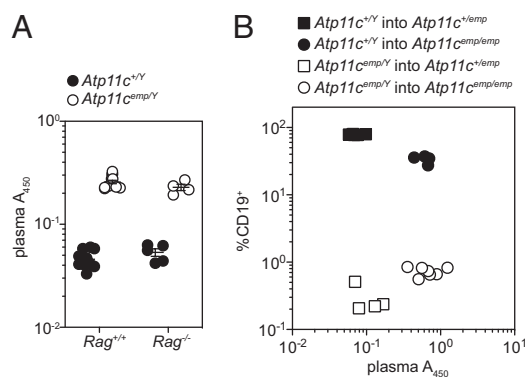

Fig. 2. Plasma hyperpigmentation is independent of B and T lymphocytes. Plasma pigmentation of *Rag1/Atp11c* double mutant mice (*A*) and reciprocal bone marrow chimeric mice (*B*). Error bars in *A* represent SEM. Data are representative of three (*A*) or two (*B*) independent experiments.

termate controls, which indicated an increased deposition of extracellular matrix (ECM), and suggested chronic, albeit mild, damage (Fig. S1*B*). Oil red O staining did not reveal an increase in lipid deposition, although apoptotic cells, as revealed by TUNEL staining, were more prevalent in 6-mo-old mutant livers (but not in 3-mo-old mutants; Fig. S1*B*). Variation in nuclear volume suggested that TUNEL-positive cells were of both pa-renchymal and nonparenchymal origin. Plasma pigmentation was nonetheless independent of the death domain-containing re-ceptor Fas and the proapoptotic Bcl2 family member Bim (14, 15), as combined mutation of *Fas* or *Bcl2l11* and *Atp11c* did not affect it (Fig. S2).

Quantities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), GGT, and alkaline phosphatase in plasma were also equivalent between 3-mo-old *Atp11c* mutants and controls, although total cholesterol was approximately 30% higher in mutant plasma, predominantly because of an increase in the LDL form (Table S1).

The collective implications of these data were that hyper-bilirubinemia in *Atp11c* mutant mice appeared to be of hepato-cellular origin. This appears consistent with the predominant expression of ATP11C-encoding mRNA in the liver (Fig. S3*A*), primarily in hepatocytes and liver endothelial cells (Fig. S3*B*).

High Incidence of Dystocia in Homozygous *Atp11c* Mutant Females. An additional pathological effect of the *Atp11c* mutation was a high frequency of dystocia in homozygous mutant females (Fig. 3*A*). More than 50% of *Atp11c^{emp/emp}* females suffered dys-tocia in their first pregnancy, compared with fewer than 5% in *Atp11c^{+/emp}* females. This phenomenon depended upon the geno-type of the mother, rather than that of the pups, as *Atp11c^{+/Y}* × *Atp11c^{emp/emp}* matings had as many (if not more) cases of dystocia than *Atp11c^{emp/Y}* × *Atp11c^{emp/emp}*. Surviving *Atp11c^{emp/emp}* females almost always gave additional litters without dystocia, with an av-erage litter size of 4.05 (n = 19, SD = 4.55 from 13 *Atp11c^{emp/emp}* females) compared with 4.79 for *Atp11c^{+/emp}* females (n = 78, SD = 4.79 from 35 females). Neither plasma pigmentation nor hyper-bilirubinemia was more severe in *Atp11c^{emp/emp}* females at 18.5 d post coitum, compared with their virgin counterparts (Fig. 3*B*).

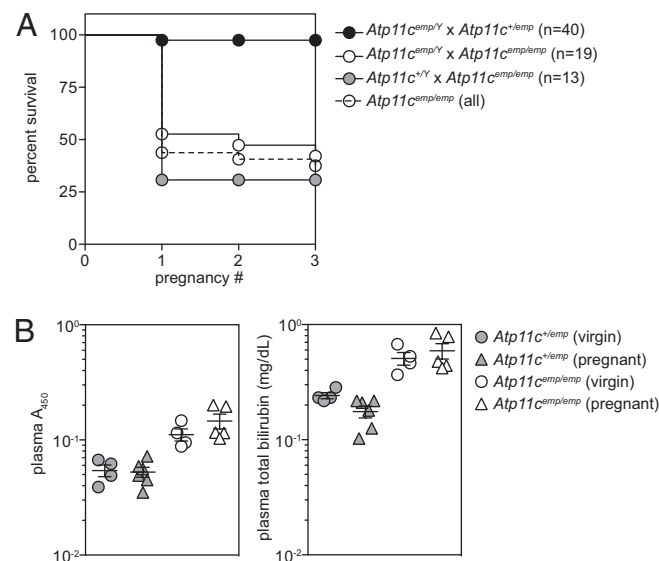**Fig. 3.** High frequency of dystocia in *Atp11c^{emp/emp}* females. (*A*) Survival curve of homozygous and heterozygous *Atp11c* mutant female littermates follow-ing pregnancies to hemizygous mutant or WT males. (*B*) Plasma absorbance (A_{450}) and total bilirubin measurements from the plasma of age-matched vir-gin or 18.5 d post coitum pregnant females. Error bars in *B* represent SEM.

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Table 1. Ions on LC/MS with the greatest difference between WT and *Atp11c* mutant serum

No.	m/z	Retention time	Group	Atp11c+/emp ($n = 4$)		Atp11cemp/emp ($n = 3$)		Mean difference	Fold change	P value
---	---	---	---	Mean	SD	Mean	SD			
1	373.289	1,039.625	152	2.30×10^6	3.70×10^6	2.36×10^7	7.29×10^6	2.13×10^7	10	3.6×10^{-3}
2	817.603	1,004.24	290	2.73×10^5	4.20×10^5	9.69×10^6	5.67×10^6	9.42×10^6	36	1.9×10^{-2}
3	546.309	1,136.01	20	3.81×10^5	1.10×10^5	8.59×10^6	1.07×10^6	8.21×10^6	23	1.9×10^{-5}
4	761.323	1,098.94	203	3.25×10^4	1.71×10^4	7.71×10^6	1.82×10^6	7.67×10^6	237	3.3×10^{-4}
5	1031.612	882.73	192	4.32×10^5	6.62×10^5	6.68×10^6	4.14×10^6	6.25×10^6	15	2.8×10^{-2}
6	584.269	1,680.245	331	4.66×10^5	6.49×10^5	4.68×10^6	1.11×10^6	4.22×10^6	10	1.4×10^{-3}
7	257.156	56.77	18	4.19×10^5	2.38×10^5	4.24×10^6	1.23×10^6	3.82×10^6	10	1.5×10^{-3}
8	761.326	1,077.81	343	1.13×10^4	7.38×10^3	2.55×10^6	9.61×10^5	2.54×10^6	225	2.8×10^{-3}
9	373.285	927.82	100	1.20×10^4	1.32×10^4	2.05×10^6	6.72×10^5	2.04×10^6	171	1.5×10^{-3}
10	599.307	1,313.93	229	1.76×10^3	3.08×10^3	2.03×10^6	8.50×10^5	2.03×10^6	1,155	4.3×10^{-3}

Ions with a fold change greater than or equal to 10 and a mean peak intensity difference greater than 1 million were ranked by mean peak intensity difference. Multiple ions from a common retention group (and therefore likely to originate from the same molecule) are represented only once.

***Atp11c* Mutant Mice Are Cholestatic.** As a result of the absence of severe liver pathology, we screened for further metabolic abnormalities (adapted from ref. 16), subjecting mutant and WT serum to liquid chromatography/mass spectrometry (LC/MS). Ion peaks were sorted into groups based on ion retention time, and groups containing ions with the greatest difference in mean peak intensity were scrutinized further (Table 1). Ion molecular weights were cross-referenced with METLIN database and Human Metabolome Database, and one candidate molecule (cholic acid) was selected for validation by tandem MS (MS/MS; Fig. S4). LC/MS confirmed that cholic acid was present in higher quantities in *Atp11c* mutant serum (Fig. 4 *A* and *B*), with an independent assay confirming that total bile acid concentrations were also increased (Fig. 4*C*).

Exogenous Bile Acid Toxicity in *Atp11c* Mutant Mice. These findings, along with earlier data, were indicative of intrahepatic cholestasis, and led us to suspect that feeding mutant mice with a cholic acid-supplemented diet might exacerbate the phenotype, as is observed in mice with mutation of *Atp8b1* (17) or *Abcb11* (18). Indeed, *Atp11c* mutants lost weight more rapidly than their WT littermates (Fig. 5*A*), culminating in uniform lethality in mutant males by day 15 (Fig. 5*B*). Weight loss was also associated with dramatic increases in plasma bilirubin (Fig. 5*C*).

Two striking observations made in cholic acid-fed mutant mice at necropsy were a pronounced enlargement of the gallbladder (Fig. 5*D*) and jaundiced skin (Fig. 5*E*), neither of which was observed in *Atp11c* mutants on a normal diet. Sirius red staining was also more apparent in mutant livers (Fig. 5*G*). Gallbladder enlargement did not appear to be caused by biliary obstruction,

as no precipitates were apparent within the gallbladder, nor was there any histological evidence of ductular proliferation.

This potential disruption of enterohepatic bile circulation seen in *Atp11c* mutant mice could be explained by ATP11C acting in bile acid transport, either as a pump, channel, or sensor. If this were the case, one would expect that dietary supplementation of cholic acid would fail to suppress expression of cholesterol 7α-hydroxylase (encoded by *Cyp7a1*), a rate-limiting enzyme in bile acid synthesis from cholesterol, which occurs in a farnesoid X receptor-dependent manner (19). This was not the case, however, as *Cyp7a1* expression was equally low in mutants as in WT littermates after cholic acid feeding (Fig. 5*F*). *Atp11c* mRNA was nonetheless increased in *Atp11c*$^{emp/Y}$ mutant mice and in mice fed a cholic acid-supplemented diet (Fig. 5*F*), suggesting that *Atp11c* is transcriptionally induced in response to cholestasis.

Discussion

The present findings reveal an additional, extrahematopoietic function for the P4-type ATPase ATP11C, namely in the prevention of cholestasis. This mild cholestatic phenotype of *Atp11c* mutant mice resembles other mouse models of PFIC, but with notable distinctions. Chief among them is hyperbilirubinemia, which was greatest in younger mutant mice, and declined progressively with age. The reason for this is yet to be determined, but may reflect an increased dependence on ATP11C-facilitated bilirubin excretion in younger mice, perhaps because of behavioral differences (20) or competition between substrates of ATP11C. Hyperbilirubinemia is observed in *Atp11c* and *Abcb4* mutants (9), but not in *Abcb11* or *Atp8b1* mutants (17, 21), although, unlike in *Abcb4* mutants, plasma ALT and AST were not
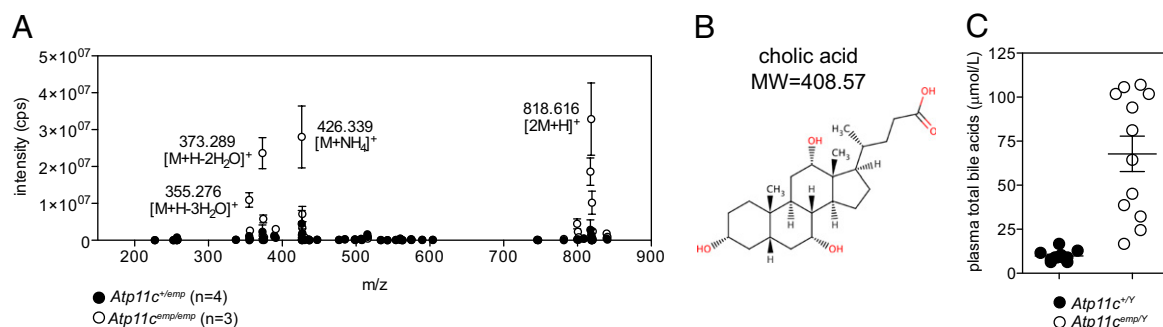
Fig. 4. *Atp11c* mutant mice are cholestatic. (*A*) LC/MS ion intensity profile for the 1039.625s retention group. The ion at 373.289 (*m/z*) showed the greatest mean difference between *Atp11c* mutant (*n* = 3) and littermate control (*n* = 4) serum. Hypothetical ion identities are shown in parentheses below each *m/z* value, which are all consistent with the chemical structure and molecular weight of cholic acid (*B*). (*C*) Total bile acids as measured from the plasma of *Atp11c* mutant and littermate control mice. Error bars in *A* and *C* represent SEM.

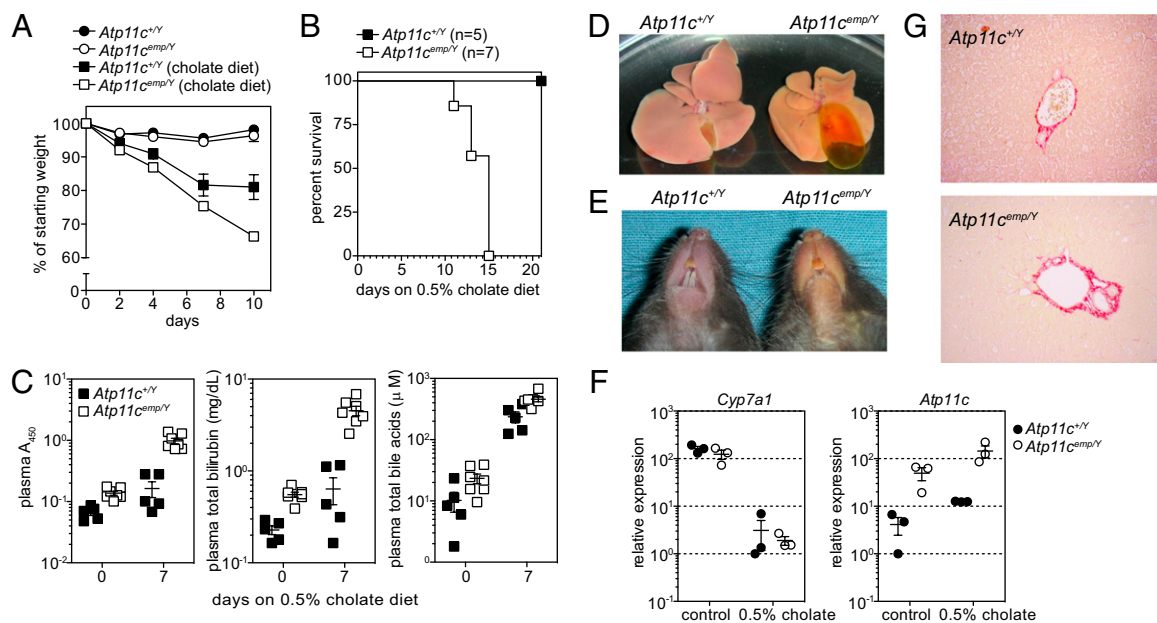**Fig. 5.** Severe pathology in Atp11c mutant mice induced by a cholic acid-supplemented diet. *Atp11c* mutant and control males were fed with a cholic acid-supplemented (*n* = 7 and *n* = 5, respectively) or control diet (*n* = 7 and *n* = 5, respectively), and monitored for weight loss (*A*), mortality (*B*), and changes in plasma constituents (*C*). Gallbladder distention (*D*) and jaundice (*E*) as observed in littermates on a cholic acid diet for 13 d. (*F*) Relative expression of *Cyp7a1* and *Atp11c* mRNA in the livers of mice after 7 d of a cholic acid-supplemented or control diet (normalized to *Actb*). (*G*) Sirius red staining of liver sections after 13 d of a cholic acid-supplemented diet. Error bars in *A*, *C*, and *F* represent SEM.

elevated in 3-mo-old *Atp11c* mutants. TUNEL staining, however, was more prevalent in 6-mo-old *Atp11c* mutants, suggesting that liver damage is a mild but progressive consequence of the mutation. Ductular proliferation was also not apparent in *Atp11c* mutant sections, indicating that hepatocyte death and biliary obstruction are more prevalent in *Abcb4* mutant mice (9).

More pathological distinctions emerged after dietary supplement of cholic acid, notably an enlargement of the gallbladder in *Atp11c*, but not *Atp8b1* or *Abcb11*, mutant strains (17, 22). This does not seem to be a result of a physical obstruction such as gallstones or acalculous cholecystitis ("sludge"), as bile duct proliferation is not observed (as it is following bile duct ligation) (23). Other explanations could include a failure of cholestokinin signaling (with failure of gallbladder contraction or relaxation of the sphincter of Oddi), or an inability to control FGF15-mediated gallbladder filling (24).

Similar to *Abcb11* mutants, cholestatic disease is very mild in *Atp11c* mutant mice, yet mice that consume cholic acid as a dietary supplement die within a matter of days (22). BSEP is known to be a major canalicular transporter of hydrophobic bile acids, including cholic acid (21), and this sensitivity to dietary cholic acid can be attributed to the strong cholestatic effects of cholic acid, but not the primary murine bile acids (α- and β-muricholic acid). This raises the possibility that ATP11C is involved in the transport of cholestatic bile acids, perhaps in concert with BSEP, that are not normally prevalent in mice. Other transporters are likely to be affected also, such as MRP2 (encoded by *Abcc2*), which transports conjugated bilirubin (but not cholic acid) (25).

We have excluded the possibility that ATP11C acts as a non-redundant sensor or transporter of cholic acid between the portal circulation and the hepatocyte, as farnesoid X receptor-mediated repression of *Cyp7a1* in the liver remains intact after cholic acid feeding (19). We cannot, however, exclude that ATP11C acts as a transporter between the hepatocyte and the bile canaliculi, and it will be critical to determine the subcellular localization of ATP11C, and how mutation of it affects the flow of bile constituents between the hepatocyte and bile ducts.

Why is there such a high frequency of dystocia in *Atp11c* mutant females? Liver pathology did not appear to be more severe during late pregnancy, although the profile of bile acids in *Atp11c* mutant plasma might somehow be toxic to late-term embryos, or preclude their delivery. Another possibility is that smooth muscle function is compromised both in the uterus (where *Atp11c* transcript is detected; Fig. S3*A*) and in the gallbladder, during parturition and cholic acid feeding, respectively. Although dystocia has not been reported in other mouse models of PFIC, and is not characteristic of intrahepatic cholestasis of pregnancy in the clinic (26), *Atp11c* mutants may represent a unique model to understand the pathology of dystocia and other defects of parturition.

An important difference between our *Atp11c*^emptyhive^ mutant strain (12) and an independent strain [*Atp11c*^ambrosius^ (13)] is the observation of hepatocellular carcinoma (HCC) in 6 mo-old *ambrosius* mutant mice. We have not observed this in our strain, even in mice beyond 1 y of age. The inbred genetic background is consistent in each case (C57BL/6J), although modifying effects of incidental mutations induced in either stock have not been excluded. As HCC is apparent in older *Abcb4* mutant mice (27), perhaps a more likely explanation is that HCC in *ambrosius* mutant mice has an environmental cue (of microbial or dietary origin) present in one facility but not the other.

How might ATP11C function in both hepatocytes and adult B-cell progenitors? Canalicular membrane asymmetry is known to be critical for the formation of bile (9) and for hepatocytes to resist the detergent effects of bile (28), and ATP11C is presumably involved in one or both of these processes. Another conceivable possibility is that ATP11C mediates trafficking of other canalicular transporters. As much less is known of the consequences of altered membrane asymmetry in B-cell progenitors, understanding the hepatic function of ATP11C could provide important insights into lymphocyte development. Studies of canalicular membrane structure and function, by EM and functional analysis of other known canalicular transporters, will

be important in this respect. Bile acid metabolites are also known to affect B-cell function. For instance, 25-hydroxycholesterol, an oxysterol metabolite of cholesterol and precursor for bile acid synthesis, can suppress secretion of IgA (29). The observation that $Atp11c^{emp/Y}$ mutant mice also have a significant deficiency of IgA (12) might reflect a surplus of 25-hydroxycholesterol, although whether this also accounts for the development defects remains to be determined.

What about the substrate of ATP11C? These mutant strains establish a previously unappreciated biochemical link between hepatocytes and B-cell progenitors, and identification of the substrate(s) of ATP11C will be of central importance to resolving its function. Yabas et al. have shown that mutant B-cell precursors are less efficient at internalizing exogenous PS at the cell membrane (13), but whether PS is the critical substrate remains to be seen. PS asymmetry is known to important not just at the cell membrane, but also at other organellar membranes (30), any number of which could affect pathways required for B-cell development. ATP8B1 also appears to be vital for inward translocation of PS at the cell surface, although other substrates have been proposed (31). Whatever their substrate, these mutants promise to provide new insight into disorders of lymphopoiesis, parturition, and cholestatic disease.

Materials and Methods

Mice. All animal procedures were in accordance with institutional guidelines. $Atp11c^{emptyhive}$, $Atp11c^{spelling}$, and $Rag1^{maladaptive}$ (Mouse Genome Informatics database no. 3851764) alleles were generated on a pure C57BL/6J background by N-ethyl-N-nitrosourea mutagenesis as previously described (12), and are described at http://mutagenetix.scripps.edu. Fas^{lpr} mice were obtained from Jackson Laboratories, and $Bcl2l11^{tm1.1Ast}$ mice have been described previously (32). The emptyhive strain was maintained by hemizygote–to-heterozygote breeding, with male littermates used for experiments unless otherwise indicated. Mice were maintained on a PicoLab Mouse Diet 20 (LabDiet 5058), and sentinels present in the same room were routinely negative for serum antibodies against Mycoplasma pulmonis, mouse rotavirus, mouse hepatitis virus, murine norovirus, mouse parvovirus, minute virus of mice, Sendai virus, and Theiler murine encephalomyelitis virus.

Bone Marrow Chimeras. Recipient mice were γ-irradiated with a dose of 10 Gy (^{137}Cs source). The following day, mice received 5×10^6 bone marrow cells pooled from two donors via tail vein injection, and were maintained on trimethoprim/sulfamethoxazole antibiotic water until they were killed for analysis 8 wk later.

Cholate-Supplemented Diet. Mice were fed a basal 14% protein diet (TD.00357; Harlan) with or without a supplement of 0.5% sodium cholate. Mice were weighed at biweekly intervals, bled weekly for serum analysis, and monitored daily for survival.

Quantitative PCR. Total RNA was prepared from liver (RNeasy kit; Qiagen) and reverse-transcribed into cDNA (Retroscript; Ambion). Quantitative PCR reactions (SYBR Master Mix; BioPioneer) were analyzed on a 7300 Real-Time PCR System (Applied Biosystems). Relative quantities of Cyp7a1 cDNA were calculated by reference to Actb using the comparative C_T method. Isolation of cDNA from MACS-sorted liver cell fractions has been described previously (23). Primer sequences were as follows: Actb, GGCTGTATTCCCCTCCATCG (forward); Actb, CCAGTTGGTAACAATGCCATGT (reverse); Atp11c, CTCGTTTCTCTTCTC-CAGCA (forward); Atp11c, AGTGTGTTTTGTGGACGGC (reverse); Cyp7a1, GG-GATTGCTGTGGTAGTGAGC (forward); and Cyp7a1, GGTATGGAATCAACCC-GTTGTC (reverse).

Histology. Livers were fixed in 10% neutral buffered formalin and embedded in paraffin wax, and 5-μm sections were stained with H&E, Sirius red, or trichrome blue. Oil red O staining was performed on frozen sections. TUNEL staining was performed on paraffin sections according to the manufacturer's instructions (Roche).

Plasma Analysis. Blood from the retroorbital plexus of isoflurane-anesthetized mutant mice was collected in cluster tubes (Costar) containing 20 μL of 6% EDTA solution (wt/vol in water) or allowed to clot in serum separator tubes and centrifuged for 5 min at $10,000 \times g$. Mice were fasted overnight before bleeding where indicated. Serum absorbance was measured at 450 nm (subtracting absorbance at 540 nm) on a MAXline Emax Microplate Reader (Molecular Devices). Total and direct bilirubin (StanBio) and total bile acids (Diazyme) were measured according the manufacturer's instructions. All other plasma chemistry parameters, including ALT, alkaline phosphatase, and GGT were measured by standard clinical chemistry (University of California, San Diego, Murine Hematology Core).

Serum MS. Mutant and WT littermates were fasted overnight, and the following morning blood was collected from the retroorbital plexus into serum separator tubes and allowed to clot. Serum was then subjected to positive ion-mode LC/MS on an Agilent 6520 Q-TOF mass spectrometer, with the output annotated by XCMS software (33). Ion peak intensities for mutant and WT groups were ranked by mean peak difference, and those with a peak difference greater than 1 million were ranked according to fold difference. Ions with the largest peak differences were then compared with the METLIN database (http://metlin.scripps.edu/) and Human Metabolome Database (http://www.hmdb.ca/) for potential candidate metabolites. For candidate validation, standards (including cholic acid, C1129; Sigma) were run in parallel with mutant serum on an Agilent 6520 Q-TOF in tandem mode (MS/MS).

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