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A mouse genetic model for familial cholestasis caused by ATP8B1 mutations reveals perturbed bile salt homeostasis but no impairment in bile secretion

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Mutations in ATP8B1, a broadly expressed P-type ATPase, result, through unknown mechanisms, in disorders of bile secretion. These disorders vary in severity from mild and episodic to progressive with liver failure. We generated Atp8b1$^{G308V/G308V}$ mutant mice, which carry a mutation orthologous to that present in homozygous form in patients from the Amish index kindred for severe *ATP8B1* disease. In contrast to human patients, Atp8b1$^{G308V/G308V}$ mice had unimpaired bile secretion and no liver damage, but showed mild abnormalities including depressed weight at weaning and elevated serum bile salt levels. We challenged the hepatobiliary metabolism of Atp8b1$^{G308V/G308V}$ mice by administering exogenous bile salts. Upon bile salt feeding, Atp8b1$^{G308V/G308V}$ mice, but not wild-types, demonstrated serum bile salt accumulation, hepatic injury and expansion of the systemic bile salt pool. Unexpectedly, this failure of bile salt homeostasis occurred in the absence of any defect in hepatic bile secretion. Upon infusion of a hydrophobic bile salt, wild-type mice developed cholestasis while Atp8b1$^{G308V/G308V}$ mice maintained high biliary output and more extensively rehydroxylated the infused bile salt. Increased bile salt hydroxylation, which reduces bile salt toxicity, may explain the milder phenotype in Atp8b1$^{G308V/G308V}$ mice compared with humans with the equivalent mutation. These results demonstrate the key role of Atp8b1 in bile salt homeostasis and highlight the importance of bile salt hydroxylation in the prevention of cholestasis. The mouse phenotype reveals that loss of Atp8b1 disrupts bile salt homeostasis without impairment of canalicular bile secretion; in humans this process is likely to be obscured by early onset of severe liver disease.

INTRODUCTION

Bile secretion is required for intestinal absorption of lipids and fat-soluble vitamins and for eliminating toxins, metabolic byproducts and cholesterol. The principal components of bile are bile salts, phospholipids and cholesterol (1). Bile salts are synthesized in hepatocytes, secreted at the canalicular (apical) membrane by the bile salt export pump (BSEP, *ABCB11*), and pass with bile through the bile canaliculi into the bile ducts, gallbladder and small intestine (2,3). Over 95% of bile salts are resorbed in the ileum, then return to the liver and are taken up into hepatocytes. The remaining 5% of bile salts is excreted in feces (4–7).

The mammalian bile salt pool consists of bile salts of varying degrees of hydrophobicity and consequently hepatotoxicity.

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Bile salt pool composition differs among species; mice have a much less hydrophobic bile salt pool than humans (8).

Cholestasis, the impairment of bile production or flow, leads to liver damage and fat and vitamin malabsorption with consequent diarrhea and growth retardation (1). In cholestasis, bile salts accumulate in liver and serum, and serum activity levels of hepatic enzymes rise, indicating liver damage.

The discovery of genetic defects responsible for inherited cholestasis syndromes has informed understanding of the etiology of cholestasis and the molecular physiology of bile salt metabolism. These syndromes range in severity from an episodic, non-progressive condition (benign recurrent intrahepatic cholestasis, BRIC) (9) to non-remitting progressive familial intrahepatic cholestasis (PFIC) which leads to liver failure (10,11). Linkage disequilibrium mapping using patients from Dutch and Amish populations isolates localized a gene for BRIC and PFIC to chromosome 18q21–q22 (12,13), then revealed that these diseases could be caused by allelic mutations in a single gene, *ATP8B1* (ATPase, Class I, type 8B, member 1; formerly *FIC1*) (14). The severity of the observed mutations' effects on protein function probably contributes to the phenotypic differences between these syndromes; however, the episodic nature of BRIC implicates unknown environmental variables in the expression of these mutations.

Defects in *BSEP* also cause PFIC (15). The forms of PFIC associated with *ATP8B1* and with *BSEP* differ in that patients with *ATP8B1* defects have milder liver damage, but suffer from extrahepatic complications, such as secretory diarrhea and pancreatitis, that are not ameliorated by liver transplantation (16–19). Consistent with these clinical observations, *ATP8B1* is broadly expressed in epithelial tissues, with particularly high expression levels in the small intestine and pancreas (14). The mouse ortholog, Atp8b1, has been localized to apical membranes of hepatocytes, bile duct and intestinal epithelial cells (cholangiocytes and ileocytes), and pancreatic acinar cells (20,21) (L. Klomp, personal communication). In contrast, BSEP is present primarily in the hepatocyte canalicular membrane in humans and rodents (2,3).

As cholestasis is the primary manifestation of BRIC and PFIC, it was surprising that ATP8B1 is not homologous to known bile salt transporters. It belongs to P-type ATPase subfamily IV (14). Some members of this subfamily flip aminophospholipids from the outer to the inner leaflet of the plasma membrane to maintain its asymmetric lipid distribution (22). ATP8B1 also may possess such activity (21). Members of subfamily IV are implicated in protein trafficking (23,24) and apoptosis (22). ATP8B1 is the first type IV P-type ATPase implicated genetically or functionally in human disease (25).

We generated mice homozygous for a mutation in *Atp8b1*, the mouse ortholog of *ATP8B1*, reproducing the ATP8B1 G308V point mutation seen in the Amish PFIC kindred (14). The Atp8b1$^{G308V/G308V}$ mice suffer from deranged bile salt homeostasis leading to an expanded bile salt pool and hepatic injury, despite the absence of any defect in canalicular bile secretion. We hypothesize that the expansion of the bile salt pool is caused by increased bile salt resorption.

RESULTS

Generation of Atp8b1$^{G308V/G308V}$ mutant mice

We isolated the mouse ortholog of *ATP8B1* and found that *Atp8b1* exhibits 86% DNA identity and 98% amino acid similarity to *ATP8B1*. We designed a targeting vector, *Atp8b1G923Tneo*, to introduce into *Atp8b1* the *G923T* mutation (resulting in a G308V substitution in a glycine conserved between human and mouse) found in many Amish PFIC patients (14). Gene targeting in mouse ES cells achieved germ-line transmission of the *Atp8b1G923Tneo* allele (Fig. 1A and B). Deletion of the *neo* selection cassette was confirmed. Heterozygous mice were intercrossed to produce Atp8b1$^{G308V/G308V}$ homozygous mutants (Fig. 1C), which were born at the expected frequency, indicating *Atp8b1* was not required for embryonic development. Atp8b1$^{G308V/G308V}$ mice were fertile; their lifespan did not differ from that of strain-matched wild-types.

The gene targeting approach was not expected to affect *Atp8b1* transcription, and indeed normal levels of *Atp8b1* mRNA were seen in liver and ileum of Atp8b1$^{G308V/G308V}$ mice (RT–PCR, data not shown). Western blotting revealed that *Atp8b1* mutant mice had no detectable Atp8b1 in the liver and only traces in the ileum (Fig. 1D). The G308V mutation thus resulted in loss of Atp8b1 in mice, reproducing the defect seen in fibroblasts from a PFIC patient with the orthologous mutation (unpublished data).

Atp8b1$^{G308V/G308V}$ mice have delayed growth and hepatic abnormalities

Loss of Atp8b1 impaired pup growth in the nursing period, as Atp8b1$^{G308V/G308V}$ pups gained weight more slowly than their wild-type and heterozygous littermates (Table 1). The weight difference resolved with age, and adult Atp8b1$^{G308V/G308V}$ mice appeared normal. Unlike human patients with the corresponding mutation (10,11,14), Atp8b1$^{G308V/G308V}$ mice did not suffer from jaundice or diarrhea and had normal serum bilirubin levels and normal liver enzyme activities, except for mildly elevated serum AST (aspartate aminotransferase) activity (Table 1). Their slightly enlarged livers suggested mild hepatic stress, but light microscopy revealed no histological abnormalities (data not shown). Atp8b1$^{G308V/G308V}$ mice showed elevated serum bile salt levels (Table 1), confirming Atp8b1 involvement in bile salt homeostasis.

Endogenous biliary bile salt secretion of Atp8b1$^{G308V/G308V}$ mice is unimpaired

To investigate bile secretion in Atp8b1$^{G308V/G308V}$ mice, we collected bile by gallbladder cannulation after distal ligation of the common bile duct. Contrary to expectation, biliary bile salt concentration was not reduced, and bile flow as well as output of principal bile components was mildly enhanced (Table 1). *Atp8b1* mutants had a slightly more hydrophilic biliary bile salt composition than wild-types, with a higher proportion of muricholate and lower proportion of cholate (Table 1).

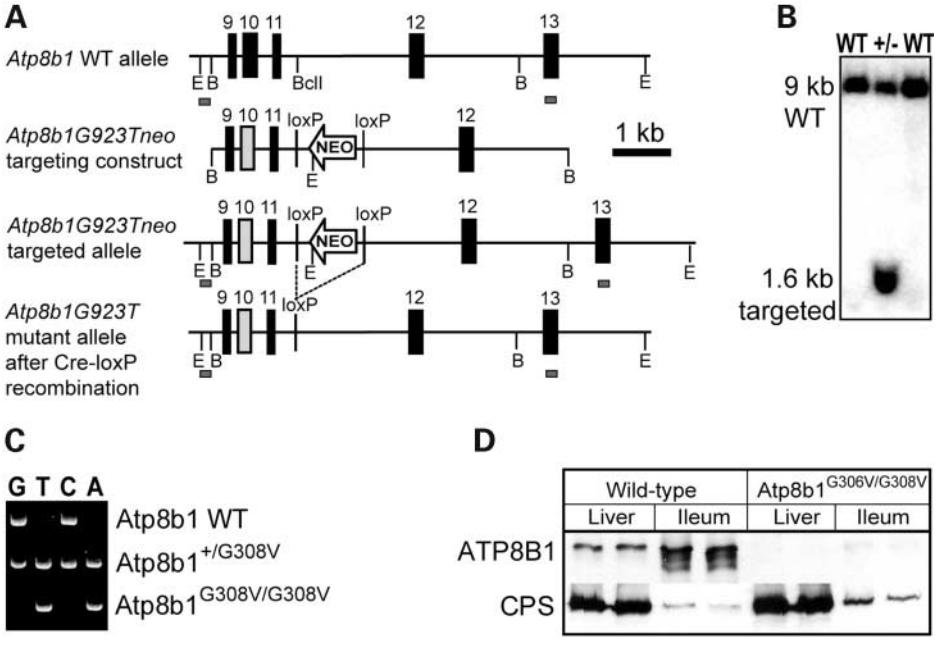

Figure 1. Generation of Atp8b1$^{G308V/G308V}$ mice. (**A**) Targeted mutagenesis of the *Atp8b1* genomic locus [wild-type (WT) allele] with the *Atp8b1G923Tneo* targeting construct, followed by Cre-loxP recombination to produce the *Atp8b1G923T* mutant allele. Black boxes represent exons; exon 10 after insertion of the *G923T* mutation is shown as a gray box. NEO, neomycin selection cassette. *Bam*HI (B), *Eco*RI (E), and *Bcl*I restriction sites and 5′- and 3′-genomic probes (small horizontal boxes) used for screening targeted embryonic stem (ES) cell clones are shown. (**B**) Southern blot analysis of wild-type and targeted heterozygous (+/−, *Atp8b1*$^{+/G923T}$) ES cell clones with the 5′-genomic probe shown in A. (**C**) PCR genotyping of *Atp8b1* mutant mice. DNA from wild-type, heterozygous (Atp8b1$^{+/G308V}$), and homozygous mutant (Atp8b1$^{G308V/G308V}$) mice was amplified using primers specific for the *G923T* mutation: G, forward wild-type; T, forward mutant; C, reverse wild-type; A, reverse mutant. (**D**) Western blot of total protein extracts (15 µg protein/lane) from liver and ileum of wild-type and Atp8b1$^{G308V/G308V}$mice. Blot probed with antibodies against ATP8B1 and carbamoylphosphate synthetase (CPS, reference).

Atp8b1$^{G308V/G308V}$ mice display unimpaired transhepatic bile salt transport and are resistant to bile salt-induced cholestasis

To characterize hepatic bile salt transport in Atp8b1$^{G308V/G308V}$ mice, we intravenously infused hydrophilic or hydrophobic bile salts into mice whose endogenous bile salt pool had been depleted by bile drainage. During bile salt pool depletion, biliary bile salt secretion was significantly higher in mutants than in wild-types. Intravenous infusion of tauroursodeoxycholate (TUDC), a hydrophilic, choleretic bile salt, confirmed that transhepatic bile salt transport and the biliary bile salt secretion capacity of *Atp8b1* mutant mice were unimpaired; biliary bile salt output was no lower in Atp8b1$^{G308V/G308V}$ mice than in wild-types even at the maximal TUDC infusion rate (Fig. 2A).

Hydrophobic bile salts are significant components of the human bile salt pool; accumulation of such hepatotoxic bile salts in cholestasis causes liver damage. Intravenous infusion of hydrophobic bile salts induces cholestasis in rodent models (26). To investigate whether loss of Atp8b1 specifically impaired transhepatic transport of hydrophobic bile salts, or increased the sensitivity of mutant mice to their cholestatic effects, we intravenously infused taurodeoxycholate (TDC). The biliary bile salt output of wild-type mice decreased upon TDC infusion. In contrast, *Atp8b1* mutants increased bile salt output with increasing TDC infusion rates (Fig. 2B), thus demonstrating a striking resistance to TDC-induced cholestasis. Analysis of bile secreted during the infusion revealed that biliary output of taurocholate increased in parallel with TDC infusion rate in *Atp8b1* mutants, but not in wild-types (Fig. 2C). Increased hepatic hydroxylation of infused TDC to taurocholate may thus protect the mutant mice from TDC-induced cholestasis.

Atp8b1$^{G308V/G308V}$ mutant mice accumulate serum bile salt upon cholate feeding

Since Atp8b1$^{G308V/G308V}$ mutant mice did not manifest defective transhepatic bile salt transport, we next investigated intestinal bile salt handling, by feeding the mice a bile salt-supplemented (0.5% cholate) diet. This experiment also allowed examination of the role of *Atp8b1* in bile salt homeostasis under conditions of chronic bile salt overload and within an uninterrupted enterohepatic circulation.

In wild-type mice, cholate feeding had no deleterious effects, but strongly up-regulated Atp8b1 expression in ileum and mildly up-regulated Atp8b1 expression in liver (Fig. 3A). Cholate-fed Atp8b1$^{G308V/G308V}$ mice lost weight (Fig. 3B). Their serum bilirubin concentration and liver enzyme activity levels rose, compared to wild-types (Fig. 3C and D), and their livers became enlarged (7.5 ± 1.1 versus 3.6 ± 0.3% body weight, $P < 0.001$), indicating liver damage. Light microscopy revealed increased hepatocyte proliferation, but no cholestasis (data not shown). Serum bile salt levels rose dramatically in *Atp8b1* mutant mice on cholate diet (Fig. 3E). Cholate conjugation by the liver was unimpaired in Atp8b1$^{G308V/G308V}$

Table 1. Phenotypic characteristics of Atp8b1$^{G308V/G308V}$ mice

Parameter	Unit	Wild-type (*n*)	Atp8b1$^{G308V/G308V}$ (*n*)	*P*[a]
Weight at weaning[b]	g/g mean pup weight	1.01 ± 0.079 (73)	0.97 ± 0.098 (34)	0.0001
Hepatic function parameters				
Liver weight	g/100 g body weight	3.8 ± 0.4 (14)	4.5 ± 0.5 (17)	0.008
Serum total bilirubin	μM	0.6 ± 0.8 (12)	0.7 ± 1.1 (15)	0.8
Serum AST	U/l	92 ± 8 (12)	225 ± 179 (15)	0.006
Serum ALP	U/l	52 ± 16 (12)	69 ± 36 (15)	0.3
Total serum bile salt	μM	2.0 ± 0.92 (10)	18.5 ± 29.8 (11)	0.0006
Endogenous bile secretion parameters[c]				
Biliary bile salt concentration	mM	60.4 ± 15.3 (11)	74.7 ± 25.7 (11)	0.08
Bile flow	μl/min/100 g	8.0 ± 1.6 (11)	12.4 ± 3.6 (11)	0.001
Bile salt output	nmol/min/100 g	498 ± 278 (11)	868 ± 320 (11)	0.0003
Phospholipid output	nmol/min/100 g	58.5 ± 17.4 (8)	66.4 ± 27 (8)	0.03
Cholesterol output	nmol/min/100 g	4.2 ± 1.0 (8)	5.9 ± 3.7 (8)	0.002
Biliary bile salt pool composition				
Percentage tauromuricholate	Percentage total bile salt	39 ± 0.6% (4)	50 ± 4.2% (4)	0.03
Percentage taurocholate	Percentage total bile salt	59 ± 0.7% (4)	49 ± 4.9% (4)	0.03
Percentage other bile salt species	Percentage total bile salt	2.6 ± 0.5% (4)	0.9 ± 0.7% (4)	0.03

All data except for weights are from male mice aged 3–7 months.
[a]Significance (*P*) was evaluated with the Mann–Whitney non-parametric test, except for bile secretion parameters, which were evaluated with two-way repeated measures ANOVA with Tukey's correction for multiple testing.
[b]Weight at weaning (3 weeks) was determined in pups of both genders from mixed litters (heterozygote intercrosses) and normalized to mean pup weight in litter to account for litter size variation. As weight did not differ between WT and heterozygotes ($P = 0.3$), these genotypes were combined for the comparison.
[c]Bile secretion parameters at the initial (10 min) collection time point are quoted.

mice, as taurocholate predominated among accumulated bile salts (Fig. 3F).

Chronic bile salt overload does not impair hepatic bile salt secretion in Atp8b1$^{G308V/G308V}$ mice

We compared biliary bile salt output between wild-type and Atp8b1$^{G308V/G308V}$ mice on control (Fig. 2A) and cholate-supplemented (Fig. 4) diets. In wild-type mice, cholate feeding did not significantly affect biliary bile salt output. In contrast, cholate-fed mutants increased their bile salt output, compared with mutants fed a control diet, as well as with wild-types on a cholate-supplemented diet. Subsequent intravenous infusion of TUDC induced a parallel rise in bile salt output in cholate-fed mice of both genotypes (Fig. 4). These data demonstrate that serum bile salt accumulation in cholate-fed Atp8b1$^{G308V/G308V}$ mice does not result from a defect in either hepatic uptake or canalicular secretion.

Cholate-fed Atp8b1$^{G308V/G308V}$ mice retain high levels of orally administered ^{3}H-taurocholate

To investigate why Atp8b1$^{G308V/G308V}$ mice accumulate bile salts upon cholate feeding, we administered an oral dose of ^{3}H-labeled taurocholate to mice equilibrated to control or cholate-supplemented diets, and determined total residual ^{3}H levels after 4 days. ^{3}H levels were similar in wild-types and mutants on control diet. However, on cholate-supplemented diet, total residual ^{3}H was 16-fold higher in *Atp8b1* mutants than in wild-types, confirming excessive bile salt retention (Fig. 5A). Cholate feeding significantly decreased ^{3}H retention in wild-types, demonstrating increased turnover of the bile salt pool upon dietary bile salt overload. In contrast, this increased turnover was absent in Atp8b1$^{G308V/G308V}$ mice, as cholate-fed mutants retained more ^{3}H than control-fed mutants.

We also compared proportional distributions of ^{3}H among the organs of the enterohepatic circulation between wild-type and mutant mice on cholate diet (Fig. 5B). If canalicular bile salt secretion was defective, accumulation of label in the liver of mutant mice would be expected. However, a much larger proportion of the label was found in the gallbladder bile of Atp8b1$^{G308V/G308V}$ mice than in the gallbladder bile of wild-type mice, and a relatively smaller proportion in mutant mouse liver. These experiments confirmed, in the physiologically relevant context of an intact enterohepatic circulation, that hepatic secretion is not impaired in *Atp8b1* mutant mice.

Atp8b1$^{G308V/G308V}$ mice have an enlarged bile salt pool

To characterize further the deranged bile salt homeostasis in Atp8b1$^{G308V/G308V}$ mice, we measured the size of the bile salt pool. Under control conditions, the bile salt pool of mutant mice was enlarged more than 2-fold compared with wild-types. Bile salt feeding increased pool size in all mice, but the increase was more dramatic in Atp8b1$^{G308V/G308V}$ mice, resulting in a bile salt pool more than 4-fold larger than in cholate-fed wild-types (Fig. 6A). *Atp8b1* mutant mice were thus unable to maintain an appropriate bile salt pool size upon dietary bile salt challenge.

We analyzed the fecal bile salt composition of control and cholate-fed mice, which provides an indirect measure of bile salt synthesis. Total fecal bile salt output was similar in mutant and wild-type mice under both conditions; cholate feeding increased the fecal bile salt output 10-fold in both genotypes (Fig. 6B). Under control conditions, the fecal bile salt composition of Atp8b1$^{G308V/G308V}$ mice was more

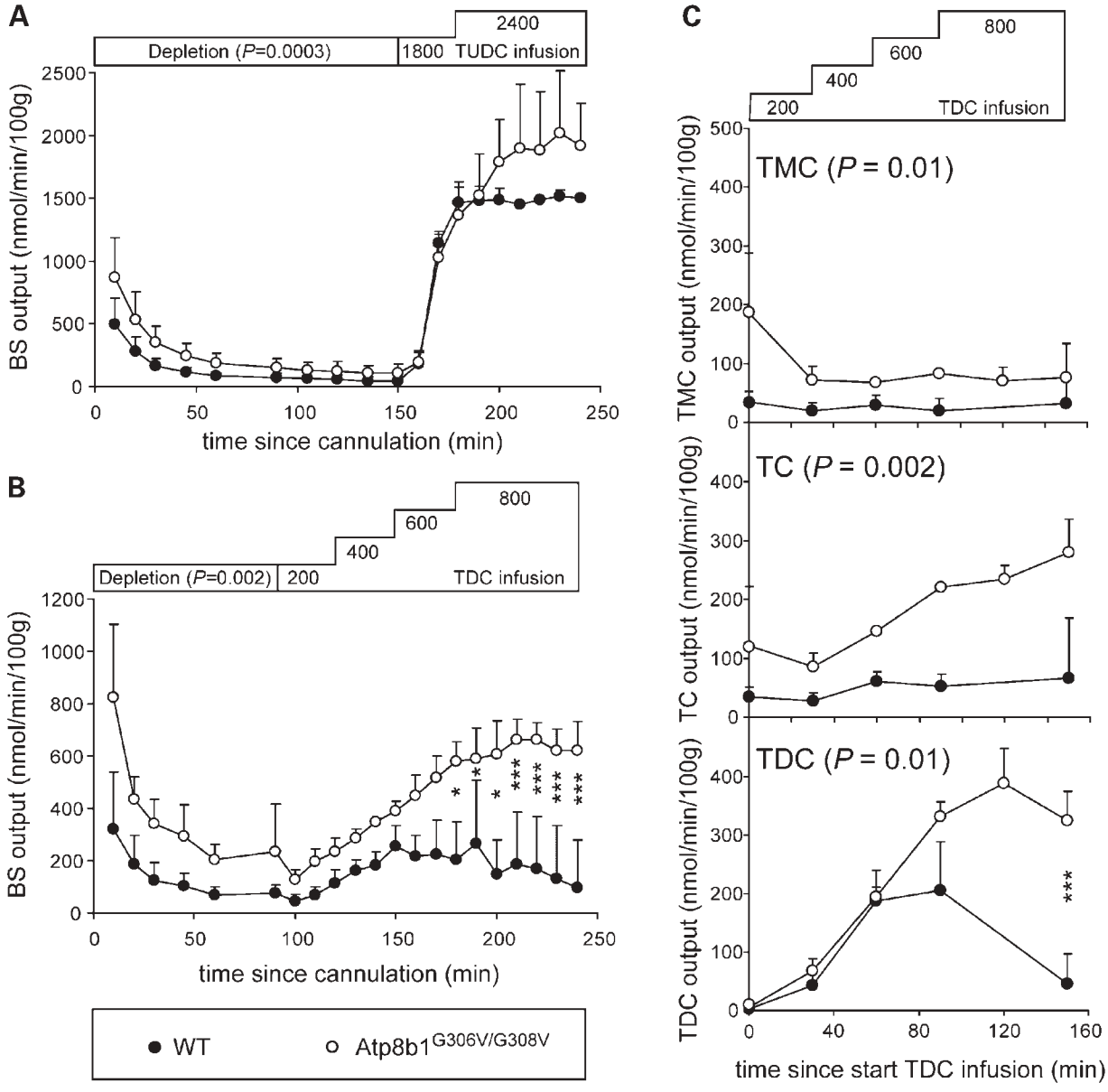

Figure 2. Intravenous bile salt infusion reveals no impairment of bile salt secretion capacity and resistance to TDC-induced cholestasis in Atp8b1$^{G308V/G308V}$ mutant mice. Gallbladders were cannulated and bile collected to deplete the endogenous bile salt pool. Subsequently, bile salts were infused intravenously at a stepwise increasing rate terminating with 60 min at the maximal rate of 2400 nmol/min/100 g (TUDC), or 800 nmol/min/100 g (TDC). Significance (P) was evaluated with two-way repeated measures ANOVA with Tukey's correction for multiple testing. Wild-type, filled symbols; Atp8b1$^{G308V/G308V}$, open symbols. (**A**) Total biliary bile salt (BS) output during 150 min of bile salt pool depletion and under TUDC infusion ($n = 3$–8). Depletion period: overall difference between wild-type and Atp8b1$^{G308V/G308V}$ mice was significant ($P = 0.0003$). Infusion period: no significant difference. (**B**) Total biliary bile salt output during 90 min of bile salt pool depletion and under TDC infusion ($n = 4$–6). Depletion period: overall difference between wild-type and Atp8b1$^{G308V/G308V}$ mice was significant ($P = 0.002$). TDC infusion period: no significant difference between wild-types and mutants for times 100–170 min. From 180–240 min significance levels were: $t = 180$–210 min, $^{*}P < 0.05$; $t = 220$–240, $^{***}P < 0.0001$. (**C**) Biliary output of tauromuricholate (TMC), taurocholate (TC), and TDC under TDC infusion (n = 2–4). Overall difference between wild-type and Atp8b1$^{G308V/G308V}$ mice was significant: TMC, $P = 0.01$; TC, $P = 0.002$; TDC, $P = 0.01$. TDC output: $t = 150$ min, $^{***}P < 0.0001$.

hydrophilic than in wild-types, consistent with the mild changes seen in biliary and serum bile salt pool composition (Fig. 6C). Cholate-fed wild-type and mutant mice excreted similar fecal levels of cholate and deoxycholate; dietary cholate intake and intestinal dehydroxylation of cholate to deoxycholate were thus equivalent in *Atp8b1* mutants and wild-types (Fig. 6D). However, cholate-fed *Atp8b1* mutants had 9-fold higher fecal levels of taurocholate than wild-types, as well as higher levels of other tauro-conjugated bile salts. This indicated that enhanced resorption and hepatic conjugation of the fed cholate occurred in the *Atp8b1* mutants. All mice had only trace amounts of lithocholate in their feces. However, cholate-fed Atp8b1$^{G308V/G308V}$ mice excreted less lithocholate and taurolithocholate than wild-types.

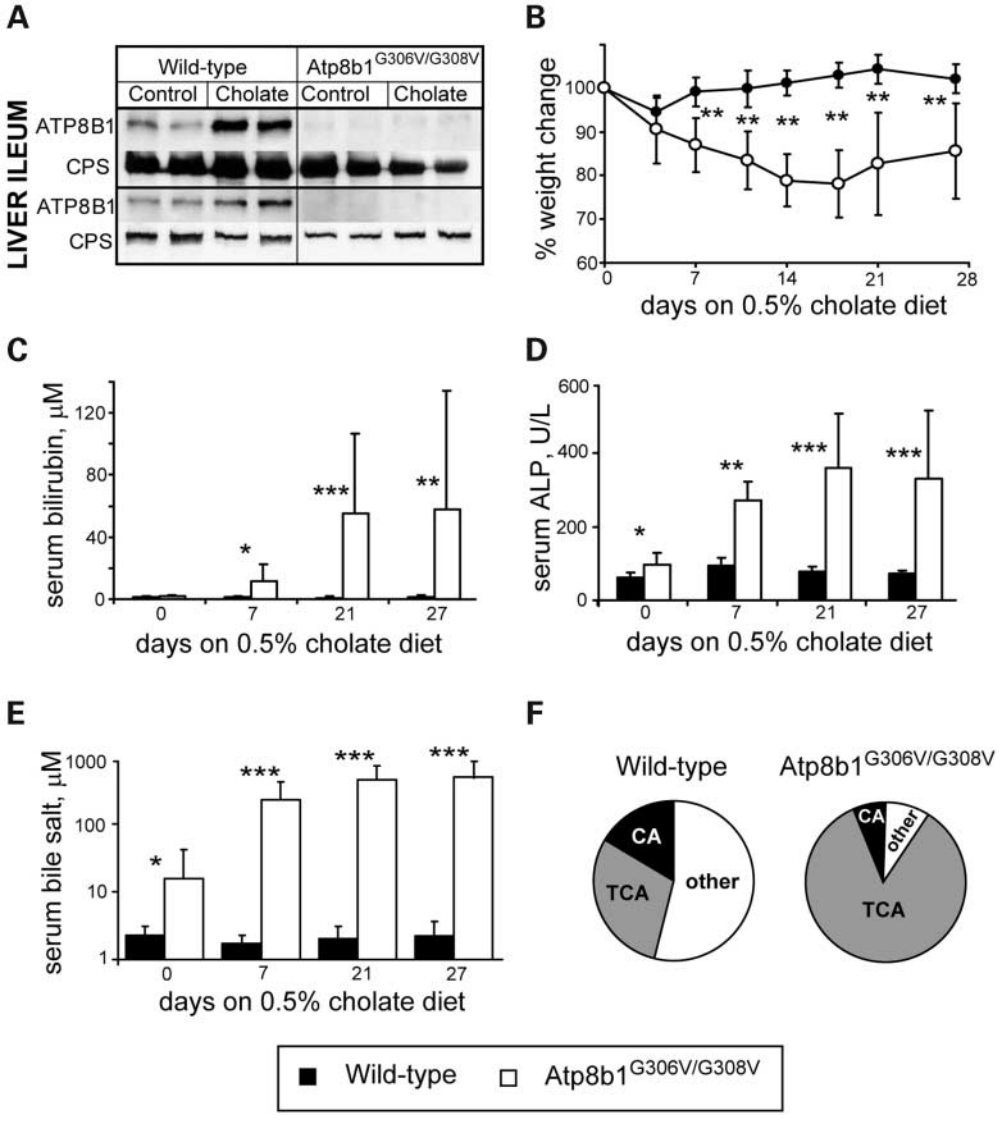

Figure 3. Exacerbation of Atp8b1$^{G308V/G308V}$ mutant phenotype upon cholate feeding. Mice were fed control or 0.5% cholate supplemented diets for 4 weeks. (**A**) Western blot analysis of protein expression levels of Atp8b1 and carbamoylphosphate synthetase (CPS, reference) in total protein extracts (15 μg protein/lane) from the ileum (upper panels) and liver (lower panels) of wild-type and Atp8b1$^{G308V/G308V}$ mice on control and 0.5% cholate diet ($n = 2$). (**B**) Variation in body weight in wild-type and Atp8b1$^{G308V/G308V}$ mice on 0.5% cholate diet ($n = 8$), normalized to weight on day 0. $^{*}P < 0.0005$, $^{***}P < 0.0001$ (two-way repeated measures ANOVA). (**C**) Serum total bilirubin levels (μM) in wild-type and Atp8b1$^{G308V/G308V}$ mice on 0.5% cholate diet ($n = 3$–8). $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0001$ (two-way repeated measures ANOVA). (**D**) Serum alkaline phosphatase activity (ALP, U/l) in wild-type and Atp8b1$^{G308V/G308V}$ mice on 0.5% cholate diet ($n = 3$–8). $^{*}P < 0.01$, $^{**}P < 0.0005$, $^{***}P < 0.0001$ (two-way repeated measures ANOVA). (**E**) Total serum bile salt (BS) levels (μM) in wild-type and Atp8b1$^{G308V/G308V}$ mice on 0.5% cholate diet ($n = 6$–8). $^{*}P < 0.05$, $^{***}P < 0.001$ (two-way repeated measures ANOVA). (**F**) Serum bile salt composition of wild-type and Atp8b1$^{G308V/G308V}$ mice on 0.5% cholate diet ($n = 8$). Percentage taurocholate: wild-type 28% ± 14%; Atp8b1$^{G308V/G308V}$ 86 ± 11%; $P < 0.0003$ (Mann–Whitney).

Effects of Atp8b1 mutation on expression levels of genes involved in bile salt homeostasis

To investigate the molecular mechanisms underlying the perturbed bile salt homeostasis in Atp8b1$^{G308V/G308V}$ mice, we employed real-time quantitative PCR to evaluate hepatic and ileal mRNA expression of genes involved in the regulation of bile salt synthesis and transport.

Examination of hepatic mRNA expression levels of nuclear receptors implicated in bile salt sensing and regulation of bile salt homeostasis [*Fxr* (farnesoid X receptor), *Lxr*-α (liver X receptor-α), *Pxr* (pregnane X receptor) and *Car* (constitutive androstane receptor)] (27–30) revealed little difference between mutant and wild-type mice under control conditions (Fig. 7A). However, in Atp8b1$^{G308V/G308V}$ mice, but not in wild-types, cholate feeding resulted in 2-fold down-regulation of *Fxr* expression and 2-fold up-regulation of *Pxr* expression. The vitamin D receptor (*Vdr*), recently implicated in intestinal bile salt sensing (31), is absent from hepatocytes, but expressed in other hepatic cells, including cholangiocytes (32). Although hepatic *Vdr* expression was very low in all mice, cholate-fed *Atp8b1* mutants had 5-fold higher *Vdr* expression than wild-

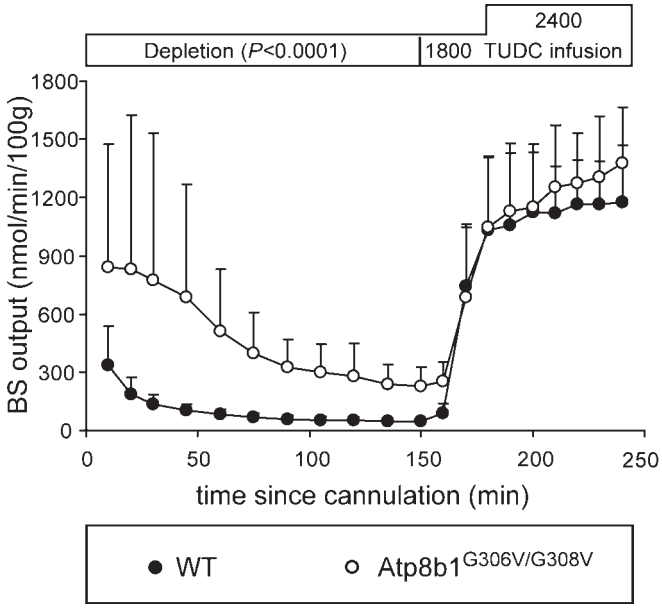

Figure 4. Cholate feeding enhances biliary bile salt output in Atp8b1$^{G308V/G308V}$ mice. Gallbladders were cannulated at $t = 0$ and bile collected during 150 min of bile salt depletion, followed by 90 min of TUDC intravenous infusion, as in Figure 2A. Biliary BS output of wild-type and Atp8b1$^{G308V/G308V}$ mice after 4 weeks on 0.5% cholate diet is shown ($n = 6$–8). Depletion phase: overall $P < 0.0001$ (two-way repeated measures ANOVA with Tukey's correction for multiple testing), TUDC infusion: no significant difference between wild-types and mutants.

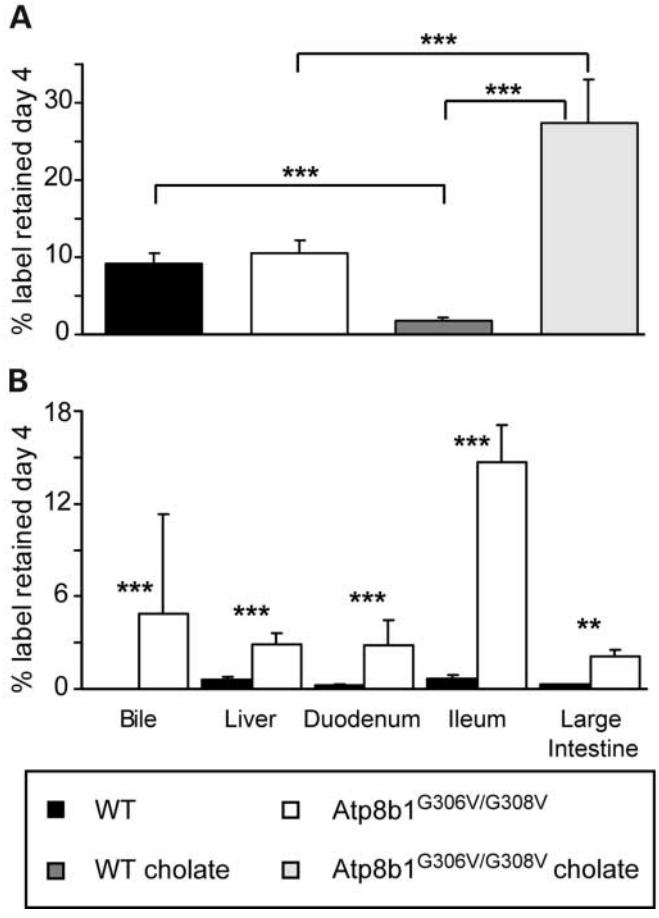

Figure 5. Excess retention of orally administered ^{3}H-taurocholate in cholate-fed Atp8b1$^{G308V/G308V}$ mice. (**A**) Total ^{3}H levels (bile, liver, intestines) in wild-type (WT) and Atp8b1$^{G308V/G308V}$ mice on control and 0.5% cholate diet, 4 days after oral administration of 1 μCi ^{3}H-taurocholate ($n = 6$). ***$P < 0.0001$ (two-way ANOVA). (**B**) Retained ^{3}H label levels in organs of the enterohepatic circulation in wild-type and Atp8b1$^{G308V/G308V}$ mice on 0.5% cholate diet (n = 6). **$P = 0.0001$, ***$P < 0.0001$ (two-way repeated measures ANOVA). Ileum = ileum with jejunum, large intestine = colon with cecum.

types, which may reflect regulation of *Vdr* expression occurring in *Atp8b1* deficient cholangiocytes under conditions of high bile salt flux.

Hepatic mRNA expression levels of three enzymes involved in bile salt biosynthesis [*Cyp7a1* (cholesterol 7-α-hydroxylase), *Cyp7b1* (oxysterol 7-α-hydroxylase) and *Cyp8b1* (sterol 12-α-hydroxylase)] were similar in Atp8b1$^{G308V/G308V}$ and wild-type mice under control conditions, indicating that the increased bile salt pool of *Atp8b1* mutants under normal conditions is not due to increased bile salt synthesis. However, compared with cholate-fed wild-types, cholate-fed mutants had profoundly decreased expression of all three enzymes, consistent with near-complete down-regulation of hepatic bile salt synthesis in response to dramatically elevated systemic bile salt levels (Fig. 7B).

We also evaluated expression of hepatic bile salt transporters. Loss of Atp8b1 did not significantly affect hepatic *Bsep* expression, consistent with the unimpaired biliary bile salt output in Atp8b1$^{G308V/G308V}$ mutant mice. Mutant mice had slightly lower *Ntcp* (Na^{+}/taurocholate cotransporting peptide) expression than wild-types under both control and cholate-fed conditions, suggesting mild repression of hepatocyte bile salt uptake, consistent with response to increased serum bile salt levels. Expression of *Asbt* (apical sodium-dependent bile salt transporter; present in cholangiocytes, but not in hepatocytes) was not significantly different between *Atp8b1* mutants and wild-types under either condition (Fig. 7B). However, hepatic *Asbt* expression was three times lower in cholate-fed mutants than in control-fed wild-types, suggesting an interaction between *Atp8b1* genotype and cholate diet in cholangiocytes.

In the terminal ileum, *Atp8b1* mutation had no significant effect on mRNA expression of four receptors involved in bile salt sensing (*Fxr*, *Lxr*, *Car* and *Vdr*) under control or cholate-fed conditions (Fig. 7C). Ileal *Pxr* expression in Atp8b1$^{G308V/G308V}$ mice was mildly increased upon cholate feeding; this effect was less pronounced than in liver. There was no difference in ileal expression of *Asbt*, the primary transporter mediating ileal resorption of conjugated bile salts (4–7), between wild-type and Atp8b1$^{G308V/G308V}$ mice on either diet (Fig. 7C). This observation was confirmed by western blot (data not shown). As expected, expression of the ileal lipid binding protein *Ilbp* increased in response to cholate feeding in all mice (28,33,34), but we detected no difference between Atp8b1$^{G308V/G308V}$ mutants and wild-types (Fig. 7C).

DISCUSSION

We generated mice homozygous for the G308V point mutation in *Atp8b1*, the first targeted mouse mutants for a member of

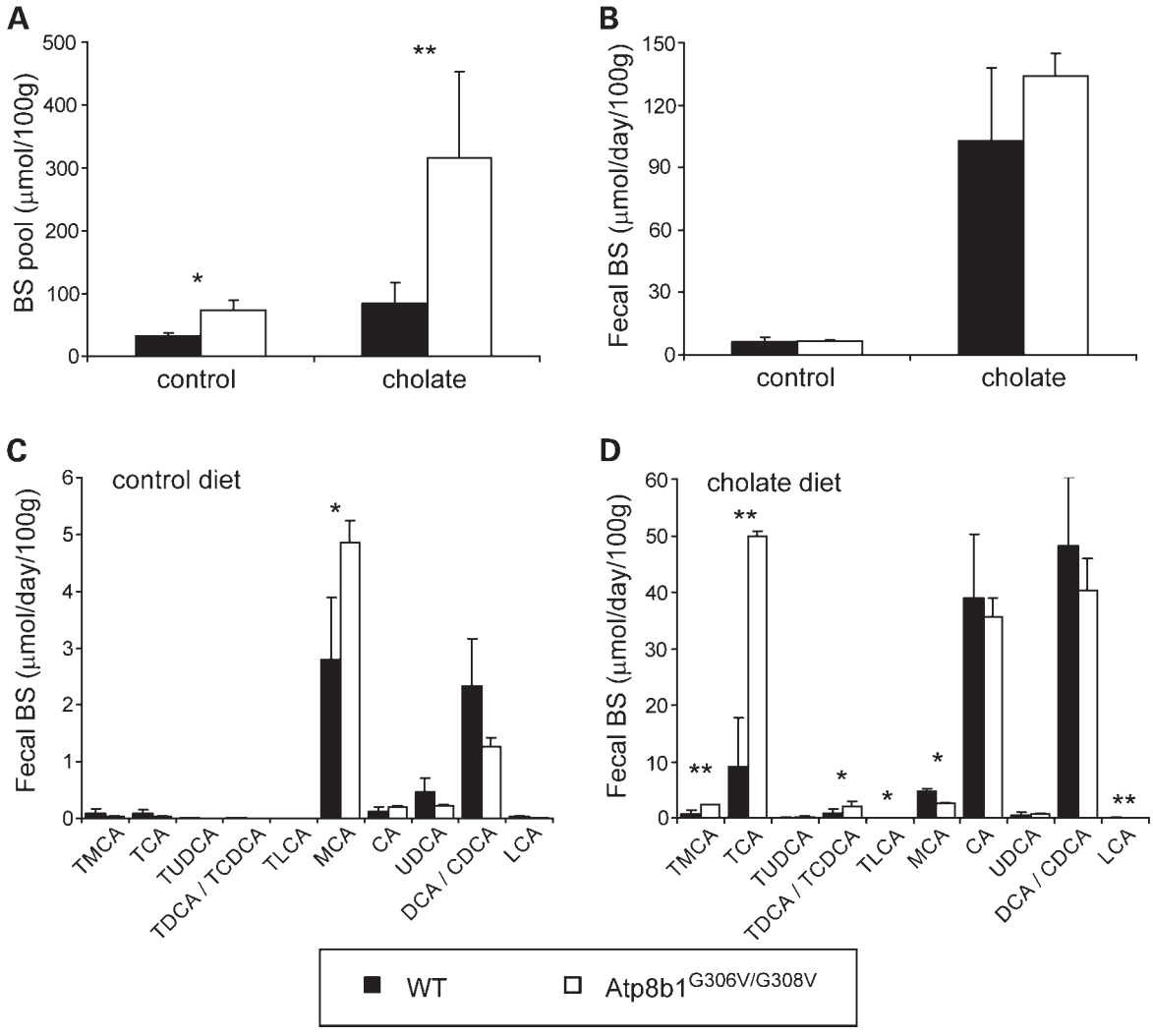

Figure 6. Total bile salt pool and fecal bile salt output of Atp8b1$^{G308V/G308V}$ mutant mice on control and 0.5% cholate diets ($n = 4$). (**A**) Total bile salt pool. (**B**) Total fecal bile salt output in 24 h. (**C**) Fecal bile salt composition after 14 days on control diet. (**D**) Fecal bile salt composition after 14 days on 0.5% cholate diet. T, tauroconjugated bile salt species; MCA, muricholate; CA, cholate; UDCA, ursodeoxycholate; DCA/CDCA, deoxycholate + chenodeoxycholate; LCA, lithocholate. *$P < 0.05$, **$P < 0.005$ (two-way ANOVA).

P-type ATPase subfamily IV. This mutation probably results in a structurally unstable and rapidly degraded protein. The orthologous mutation, present in the Amish pedigree in which PFIC was first described (10,14), results in an analogous loss of ATP8B1, indicating that G308V is a functional null mutation in both mice and humans.

The deleterious consequences of Atp8b1 loss in mice recall characteristic phenotypic features of human *ATP8B1* disease, but in milder form. Furthermore, unlike humans with the orthologous mutation, *Atp8b1* mutant mice do not suffer from progressive cholestatic liver disease. Dietary bile salt overload aggravates the *Atp8b1* mutant phenotype; the resulting serum bile salt accumulation, weight loss, jaundice and hepatomegaly parallel findings in human *ATP8B1* disease. Thus, loss of Atp8b1 disturbs bile salt homeostasis in mice as loss of ATP8B1 does in humans.

Manifestations of cholestasis, including serum bile salt accumulation, usually correlate with diminished biliary bile salt secretion in rodents and humans. Indeed, early hypotheses (10,11,14) ascribed *ATP8B1* disease to a primary defect in canalicular bile salt secretion or its regulation. However, contrary to our expectations, *Atp8b1* mutant mice demonstrated unimpaired biliary bile salt secretion, even upon bile salt challenge. Down-regulation of *Cyp7a1*, *Cyp8b1* and *Ntcp* confirmed the unimpaired regulatory response of the *Atp8b1* mutant liver to bile salt challenge; these genes are directly or indirectly regulated by Fxr, the nuclear receptor that has bile salts as ligand (33,34). In *Atp8b1* mutants, but not in wild-types, bile salt feeding also induced significant changes in hepatic expression of the bile salt sensing receptors *Fxr* and *Pxr*. Thus, although experimental evidence was consistent with the absence of any defect in the regulation of bile salt transport and synthesis in the hepatocytes of *Atp8b1* mutant mice, changes in gene expression suggest an increased flux of bile salts through the *Atp8b1* mutant liver.

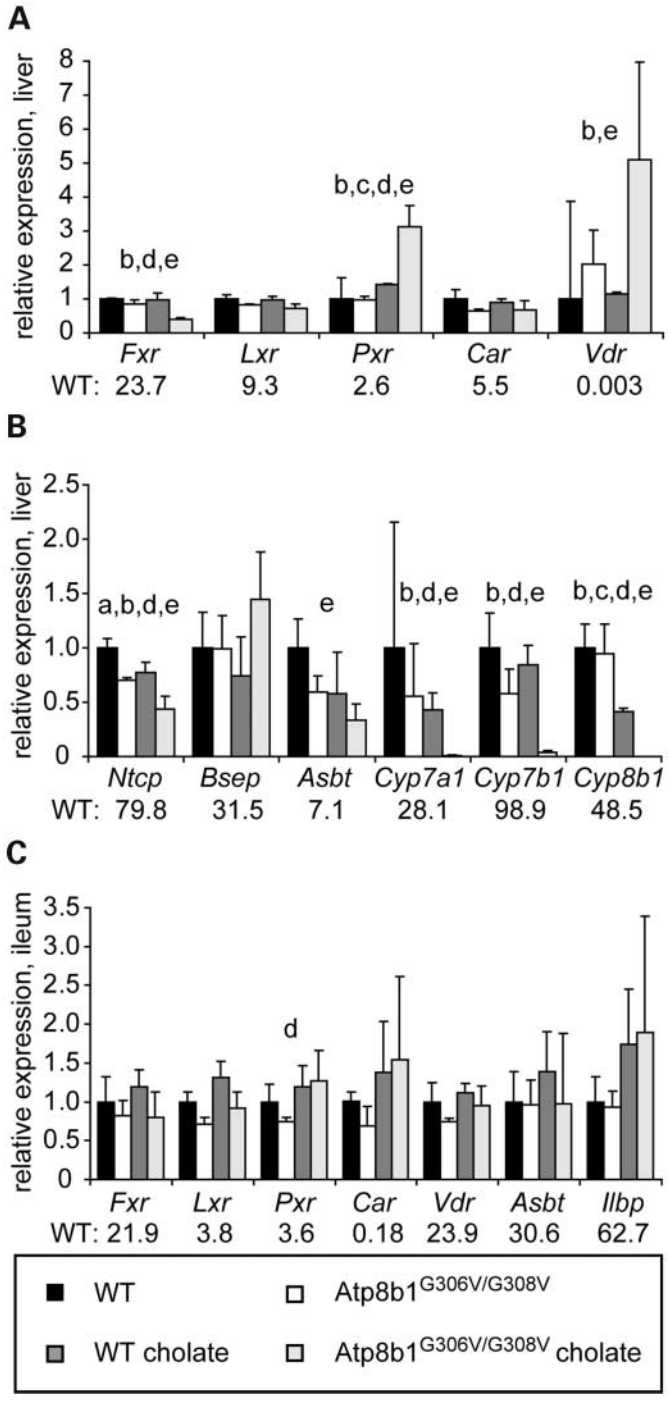

Figure 7. Effect of *Atp8b1* mutation and 0.5% cholate diet on mRNA expression of genes involved in regulation of bile salt transport and synthesis. Real-time quantitative PCR was performed on cDNA generated from mRNA extracted from tissue samples from the liver and the terminal ileum ($n = 4$). Each sample was assayed in triplicate. Relative expression levels were obtained using *Gapdh* as the endogenous control gene; values (percentage *Gapdh* expression) for wild-types on control diet are shown underneath each panel. Expression in wild-type mice on control diet was set to 1. Significance was evaluated with two-way ANOVA; statistically significant ($P < 0.05$) differences in pair-wise comparisons are denoted as follows: a, control diet—wild-types versus mutants; b, cholate diet—wild-types versus mutants; c, wild-types—control versus cholate, d, mutants—control versus cholate; e, wild-types/control versus mutants/cholate. (**A**) Hepatic expression of regulatory genes. (**B**) Hepatic expression of bile salt transporters and synthetic enzymes. (**C**) Expression in terminal ileum.

In the absence of a hepatic bile salt handling defect, we sought alternative explanations for the increased serum bile salt levels in *Atp8b1* mutant mice. The combination of an enlarged bile salt pool with normal hepatic transport and with markedly down-regulated expression of bile salt-synthetic enzymes strongly suggests increased bile salt resorption. Increased systemic retention of orally administered ^{3}H-labeled bile salt and the large proportion of taurocholate in fecal bile salts of cholate-fed *Atp8b1* mutant mice provided further evidence of such excess resorption. However, we detected no difference between wild-type and mutant mice in ileal expression levels of Asbt and Ilbp, the two proteins responsible for bile salt uptake and transport across the ileocyte. Since intestinal bile salt levels regulate Ilbp levels, these observations suggest that bile salt flux across the ileocyte is similar in wild-type and mutant mice. Therefore, the increased resorption may occur elsewhere in the enterohepatic circulation or involve a different mechanism.

In the context of increased bile salt resorption, the observed increased hydroxylation of TDC and consequent resistance to TDC-induced cholestasis in *Atp8b1* mutant mice are striking. The increased rehydroxylation is likely a response to increased levels of circulating bile salts. The inability of humans to rehydroxylate secondary bile salts may underlie the severe consequences of *ATP8B1* disease in humans. Significantly, *ATP8B1* is expressed in the colon (35), where resident bacteria produce secondary bile salts. Increased absorption of secondary bile salts in humans may thus account for the observed increased levels of hydrophobic bile salts in the bile and/or serum of patients with *ATP8B1* mutations (11,36); abnormal transhepatic flux of these toxic bile salts probably induces cholestasis and liver damage.

The possibility of increased intestinal bile salt resorption in *Atp8b1* mutant mice is consistent with observed extrahepatic abnormalities in patients with severe *ATP8B1* disease. After successful liver transplantation, many such patients suffer from persistent diarrhea, unlike *BSEP* disease patients (17–19,37). Transplanted *ATP8B1* disease patients (19), but not *BSEP* disease patients (A.S. Knisely, unpublished data), develop steatosis in allograft livers; the transplanted normal liver thus appears susceptible to damage when placed downstream of potentially deranged bile salt resorption in the ATP8B1-deficient intestine.

In addition to the intestine, increased bile salt resorption can occur in the biliary tract, including bile ducts and the gallbladder. ATP8B1 is expressed in bile duct epithelial cells (cholangiocytes) more strongly than in hepatocytes (20). Cholangiocytes lacking ATP8B1 might permit excessive shunting of hydrophobic biliary bile salts back to hepatocytes via the cholangioportal circulation (38,39); our observation of increased *Vdr* expression in the *Atp8b1* mutant liver upon cholate feeding indirectly supports this hypothesis, as Vdr is activated by hydrophobic bile salts (31). As *VDR/Vdr* expression is nearly absent in human and rodent hepatocytes, but relatively high in cholangiocytes (32), the observed increase in hepatic *Vdr* levels in cholate-fed *Atp8b1* mutant mice could be a marker for the effects of bile salt challenge on Atp8b1-deficient bile duct epithelial cells. Since *Asbt* is also present in cholangiocytes, but not in hepatocytes, the reduced hepatic *Asbt* expression observed in cholate-fed mutants compared to control-fed wild-types is also consistent with

changes in cholehepatic bile salt shunting in *Atp8b1* mutant mice.

Physiologic differences between mice and humans could explain the paradox of an identical mutation producing contrasting phenotypes—decreased bile salt secretion and severe cholestasis in humans, but enhanced bile salt secretion and resistance to the cholestatic effects of hydrophobic bile salts in mice. As in *Atp8b1* mutant mice, in humans with *ATP8B1* mutations, enhanced bile salt absorption by bile duct, gallbladder and intestinal epithelial cells may initially increase bile salt flux through the hepatobiliary system. In humans this would occur in the context of a more hydrophobic and hepatotoxic bile salt pool, leading to accumulation of these toxic bile salts and cholestasis. In contrast, mice counteract bile salt accumulation by enhanced rehydroxylation of excess bile salts. These differences between mice and humans highlight the importance of the bile salt pool composition in the initiation and progression of disease. With mutant mice, the mildness of the baseline hepatic phenotype revealed that loss of Atp8b1 disrupts bile salt homeostasis without impairing bile salt secretion. In contrast, in humans, the severity and early onset of PFIC conflate primary and secondary manifestations of bile salt mishandling and liver damage and therefore impede elucidation of disease etiology.

Previous studies (14,21) suggest that ATP8B1 is an aminophospholipid flippase, which maintains the asymmetrical distribution of plasma membrane lipids. Such a function is consistent with our observations in *Atp8b1* mutant mice. Vesicular trafficking of membrane components is sensitive to membrane lipid distribution (40,41). Functional studies in yeast implicate type IV P-type ATPases in secretory vesicle formation and trafficking (23,24); membrane lipid imbalance caused by ATP8B1 malfunction could thus disrupt intracellular trafficking or localization of proteins important in bile salt transport. Deranged membrane lipid composition could also permit excessive non-specific entry of bile salts. A general role for ATP8B1 in apical membrane maintenance and protein trafficking is also consistent with extrahepatic manifestations of *ATP8B1* disease (17,18,37,42), particularly in epithelial secretory cells whose apical domains may be especially sensitive to perturbations in membrane lipid composition.

MATERIALS AND METHODS

Isolation of the mouse ortholog of *ATP8B1*

BLAST homology searching identified a mouse EST (GenBank AA242626) with 98% protein sequence identity to bp 3229–3694 of human *ATP8B1*. The full-length Atp8b1 cDNA sequence was assembled using primer walking and RACE (rapid amplification of cDNA ends) on mouse liver cDNA and was deposited in GenBank (accession number AY506548).

Generation of Atp8b1$^{G308V/G308V}$ mutant mice

We constructed the targeting vector from a 7.1 kb *Bam*HI genomic DNA fragment, derived from a P1 clone [mouse 129/Sv embryonic stem (ES) cell DNA library, Genome Systems, St Louis, MO, USA], encompassing exons 9–12 of *Atp8b1*. The G923T point mutation was introduced into exon 10 by site-directed mutagenesis. A loxP–*neo*-loxP resistance cassette (43) was inserted into an intronic *Bcl*I restriction site. Linearized vector was electroporated into strain129/SvJae PC-3 ES cells carrying a male-germ-line specific *Cre* transgene (44). Recombinant colonies were screened by Southern blot using 5′- and 3′-genomic probes and a *neo* probe and by PCR using primers specific for the G923T point mutation. Two independent heterozygous mutant ES cell clones injected into strain C57Bl/6 blastocysts yielded chimeric male mice, which transmitted the targeted allele when bred to C57Bl/6 females. Deletion of the loxP-flanked *neo* selection cassette from the targeted allele during germline transmission was confirmed by PCR. Chimeric founders bred to strain 129/SvImJ female mice (JAX, Bar Harbor, ME, USA) produced mutant heterozygotes, which were intercrossed to produce Atp8b1$^{G308V/G308V}$ mutants and wild-type controls.

In vivo experiments

Age-matched male mice aged 2–7 months were used in all experiments. Isoflurane anesthesia was used for blood collection by orbital puncture. Mice were fed standard rodent chow or, for diet experiments, a commercial purified diet (K4068.02, Hope Farms, Woerden, The Netherlands) with or without 0.5% w/w sodium cholate (Merck, Darmstadt, Germany). Food and water were supplied *ad libitum*.

For bile collection and organ isolation, mice were anesthetized with an intraperitoneal injection of Hypnorm/Diazepam (45). Gallbladder cannulation, bile collection for up to 240 min, and intravenous bile salt infusion were performed as described (45). Depletion of the endogenous bile salt pool by bile collection for 90 or 150 min preceded intravenous bile salt infusions. TUDC (Calbiochem, La Jolla, CA, USA) was infused for 30 min at 1800 nmol/min/100 g followed by 60 min at 2400 nmol/min/100 g. TDC (Sigma-Aldrich, St Louis, MO, USA; 15 mM in PBS pH 7.4) was infused for 150 min at a stepwise increasing rate: 30 min at 200 nmol/min/100 g, 30 min at 400 nmol/min/100 g, 30 min at 600 nmol/min/100 g, and 60 min at 800 nmol/min/100 g. Bile aliquots for analysis were collected every 10 min during the infusion.

One micro-curie of ^{3}H-taurocholate (Amersham) in 100 µL PBS was administered by gavage to mice fed control or cholate diets for 10 days and then fasted overnight. Mice were maintained on their respective diets and sacrificed 2 or 4 days after ^{3}H-taurocholate administration. Residual ^{3}H levels were determined by scintillation counting in tissue samples treated with Soluene (Packard, Meriden, CT, USA). For bile salt pool size determination by stable isotope dilution, bile salt concentration in gallbladder bile was measured enzymatically (45) as well as by liquid scintillation counting. The obtained specific radioactivity was used to calculate the bile salt pool size from the total systemic radioactivity (estimated as total radioactivity in gallbladder, liver, intestines and serum). All animal experiments were performed under approved protocols of the UCSF or AMC Committees on Animal Research.

Assays

Phospholipid, cholesterol and bile salt concentrations in bile were determined enzymatically (45). Serum bilirubin levels and liver enzyme activities were measured by routine clinical chemistry. Serum, biliary and fecal bile salt composition was determined by HPLC/MS.

Western blotting

Protein extract preparation, SDS–PAGE and western blotting were performed as described (20). L. Klomp provided anti-ATP8B1 polyclonal antibody (20). Anti-CPS (carbamoylphosphate synthetase) antibody, used as a reference, was a gift from W. Lamers.

Quantitative PCR analysis of gene expression

Tissue samples (liver and terminal ileum) were stored at −80°C in RNAlater™ (Qiagen, Hilden, Germany). Total RNA was extracted with Trizol reagent (Gibco BRL). cDNA was transcribed using random hexamers and MuLV reverse transcriptase (Gibco BRL). Real-time quantitative PCR was performed in triplicate on the ABI Prism 7900 analyzer at the UCSF Cancer Center Genome Analysis Core Facility as previously described (46,47). We purchased Assays-on-Demand probe/primer sets (Applied Biosystems, Foster City, CA, USA) to quantify expression of *Asbt* (*Slc10a2*), *Bsep* (*Abcb11*), *Car* (*Nr1i3*), *Cyp7a1*, *Cyp7b1*, *Cyp8b1*, *Fxr* (*Nr1h4*), *Ilbp* (*Fabp6*), *Lxr*-α (*Nr1h3*), *Ntcp* (*Slc10a1*), and *Vdr.* We designed the assay (spanning an exon–exon boundary) for *Pxr* (*Nr1i2*); as follows: forward primer, TGATGGACGCT CAGATGCA, reverse primer, TGGAAGCTCACAGCCACT GT, TaqMan® probe: 5′-*FAM*-CAAGGATTTCCGGCTGCCT GCA-*BHQ1*-3′. Expression levels were calculated relative to the control gene *Gapdh*; similar results were obtained with two other control genes.

Statistical analysis

Values are reported as means ± SD. Comparisons between two groups were evaluated with the Mann–Whitney non-parametric test. Data in Figures 5A, 6 and 7 were $\log10(x + 1)$-transformed and analyzed by two-way ANOVA with Tukey's correction for multiple testing. Data in Figures 2, 3B–E, 4 and 5B and Table 1 were $\log10(x + 1)$-transformed and analyzed with two-way ANOVA with repeated measures with Tukey's correction for multiple testing. We used a compound symmetry model for covariance between repeated measures on the same mouse. The repeated factor in Figure 5B was the organ assayed; elsewhere, the repeated factor was time.

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