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A knock-in BSEP^{E297G} mouse presents with cholestasis and is a novel translational model of PFIC2

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Introduction

Progressive familial intrahepatic cholestasis type 2 (PFIC2) is a rare pediatric liver disease caused by genetic variants in the bile salt export pump (BSEP, *ABCB11*)[1]. The E297G variant is one of the most prevalent drivers of PFIC2 (when inherited biallelically), leading to impaired membrane trafficking[2], bile acid efflux and consequential hepatic accumulation and toxicity. Here, we present a novel PFIC2 mouse model harboring the BSEP^{E297G} variant. Characterization of this mouse demonstrates its recapitulation of key translational aspects of the human disease. Furthermore, 4-PBA, which has shown therapeutic efficacy in the clinic[4], demonstrates anti-cholestatic efficacy in our model, highlighting the utility of this model as a tool to evaluate novel therapeutics for the treatment of PFIC2.

Materials and Methods

Mouse model generation: CRISPR/Cas9-mediated gene editing was used to generate a constitutive knock-in of the E297G variant in exon 9 of the *Abcb11* locus (NCBI transcript NM_021022.3). The Cas9 protein, gRNA, and oligonucleotide were injected into C57BL/6NTaczygotes. F0 founders were screened for on-target edits by PCR and BsmI digest. Selected F0 male founder was used for IVF to generate F1 animals that were screened for presence of on-target edits as well as predicted off-target edits.

Study cohorts: *Data on female mice is presented as they demonstrated a more robust phenotype than male mice.* Phenotyping animals were fasted 4 hrs prior to bi-weekly tail sampling, and fasted overnight prior to termination. The pharmacology study dosed female HOM mice with 4-PBA 1000 mg/kg BID for 7 days, and fasted overnight prior to takedown.

Serum chemistry, biliary chemistry, and bile acids: ALT activity levels were measured using the Alanine Aminotransferase Activity Assay Kit (Sigma, Catalog No. MAK052). ALP activity levels were measured using the Alkaline Phosphatase Assay Kit (Sigma, Catalog No. MAK447). Total Bile Acids Assay Kit (Diazyme, Catalog No. DZ042A) was used to measure serum and biliary bile acids according to manufacturer's instructions. Liver bile acids and were quantitated with the AbsoluteIDQ® Bile Acids kit (Biocrates, Innsbruck, Austria). Biliary phospholipids were measured using the Phospholipid Assay Kit (Sigma, Catalog No. MAK122) according to manufacturer's instructions and biliary cholesterol levels were measured using the FUJIFILM Medical Systems USA Cholesterol E kit (Fisher, Catalog No. NC9138103).

RNA-seq: RNA-Seq libraries were prepared by Novogene Co., Ltd and sequenced on an Illumina HiSeq1000 system. Differential expression analysis was performed using the DESeq2 R package of Bioconductor. The resulting *P* values were adjusted using the Benjamini-Hochberg procedure to control for the false discovery rate.

Western blots +/- PNGase: Total protein extracts were prepared from flash frozen liver tissue by homogenization in RIPA buffer supplemented with a combined protease and phosphatase inhibitor cocktail. Proteins were reduced and denatured in Laemmli sample buffer containing fresh DTT, resolved on 4%–12% Bis-Tris gels, transferred to nitrocellulose membranes, immunodetected with antibodies, and imaged using a ChemiDoc system (BioRad).

BSEP^{E297G} PFIC2 mouse model generation

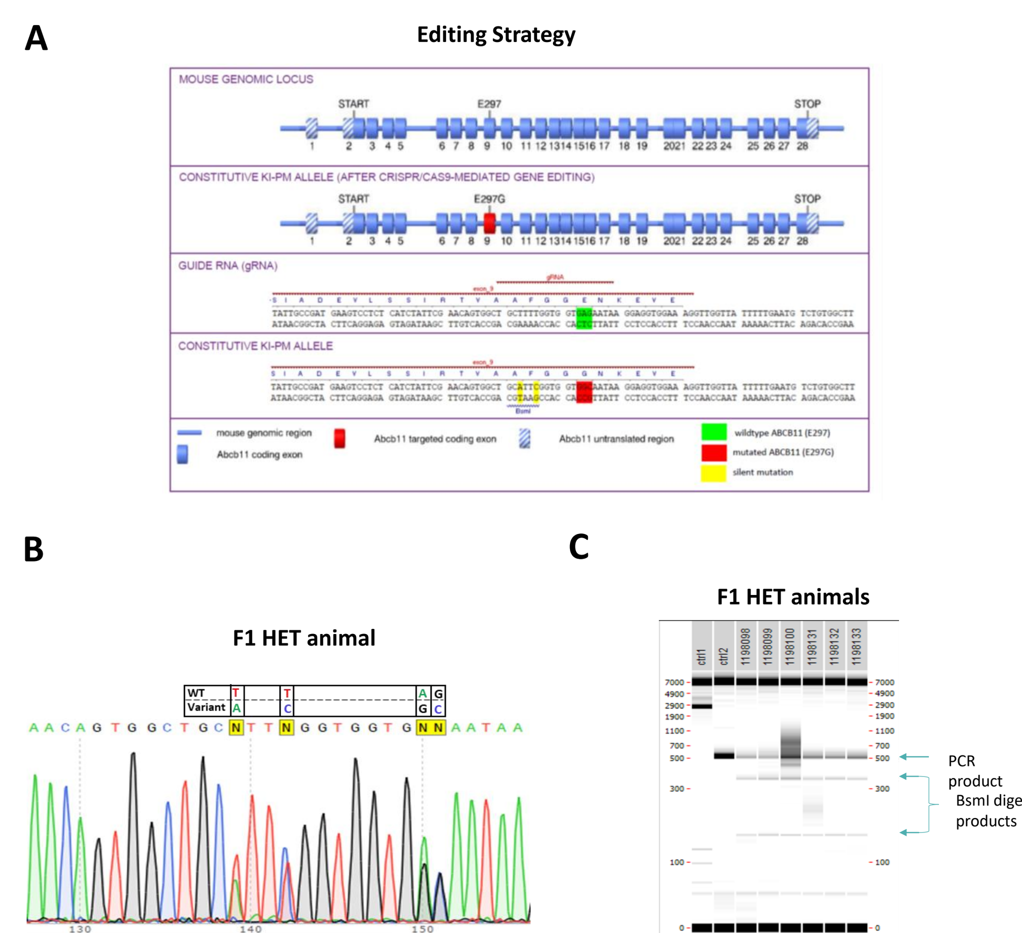
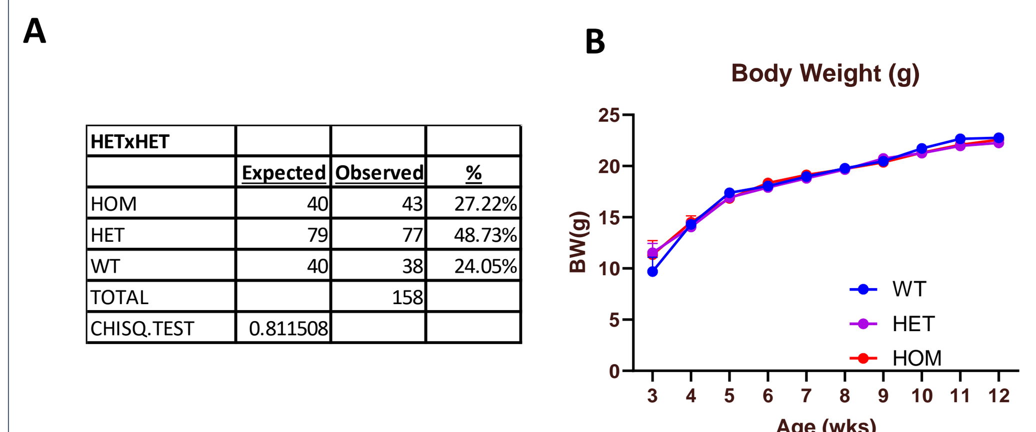


Figure 1. Introduction of E297G variant into mouse *Bsep*. (A) Editing strategy for the introduction of E297G variant and silent BsmI site into exon 9 of mouse *Bsep*. (B) Sequencing trace demonstrating the edit of WT 297 codon to variant codon and introduction of BsmI restriction site. (C) PCR and restriction analysis of F1 animals indicating presence of BsmI silent mutation by presence of both the 350 bp and 196 bp bands.

Homozygous BSEP^{E297G} PFIC2 mice are viable without growth abnormalities



HETxHET			
	Expected	Observed	%
HOM	40	43	27.22%
HET	79	77	48.73%
WT	40	38	24.05%
TOTAL		158	
CHISQ.TEST	0.811508		

Figure 2. Introduction of BSEP^{E297G} variant into the mouse gene does not impact viability or growth. (A) Tracking the variant allele indicates it follows mendelian inheritance, and no post-natal mortality was observed. (B) Tracking weight gain of female mice indicates the model does not have a growth defect as seen in some patients.

Introduction of E297G variant disrupts BSEP protein maturation and localization

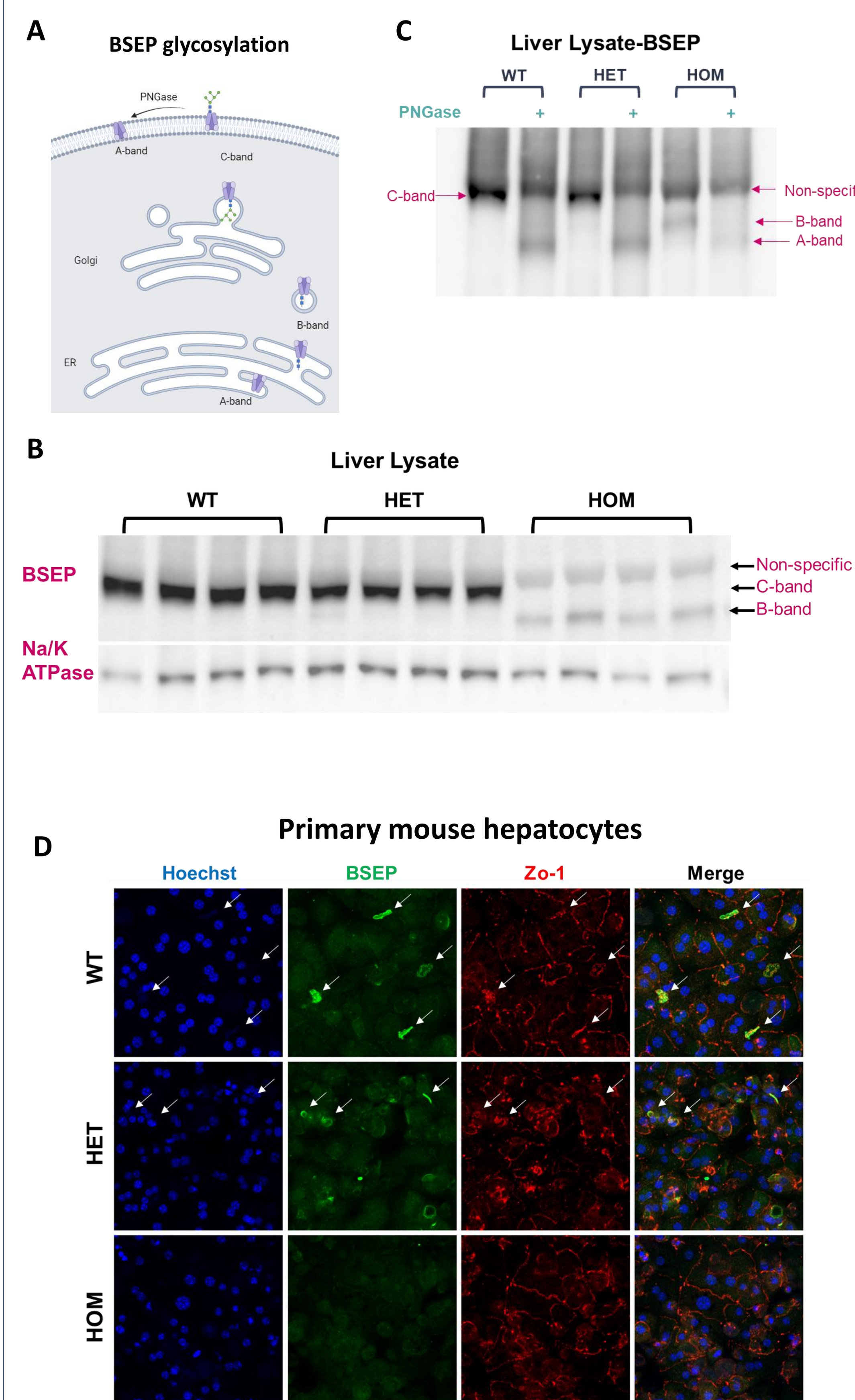


Figure 3. BSEP^{E297G} variant impacts protein maturation and localization. (A) Glycosylation is required for BSEP to be properly trafficked to the membrane. These modifications are added in a stepwise process to generate B-band (immature, partially glycosylated), C-Band (fully glycosylated mature protein) from the unmodified form A-Band. Experimentally the addition of PNGase removes all glycosylation modifications converting B-band and C-band protein into A-band. (B) western blot of liver tissue lysates from 12 week old WT, HET, and HOM BSEP^{E297G} mice demonstrating the appearance of the immature B band in HOM animals. (C) Application of PNGase converts mature c-band in WT and HET animals to unmodified A-band, while it converts B-band in HOMs to A-band demonstrating a defect in glycosylation of BSEP^{E297G}. (D) IF localization of BSEP and ZO-1 in primary mouse hepatocytes isolated from PFIC2 mice demonstrating BSEP^{E297G} does not traffic to canaliculi.

Cholestatic and hepatotoxic biomarkers are increased in PFIC2 mouse model

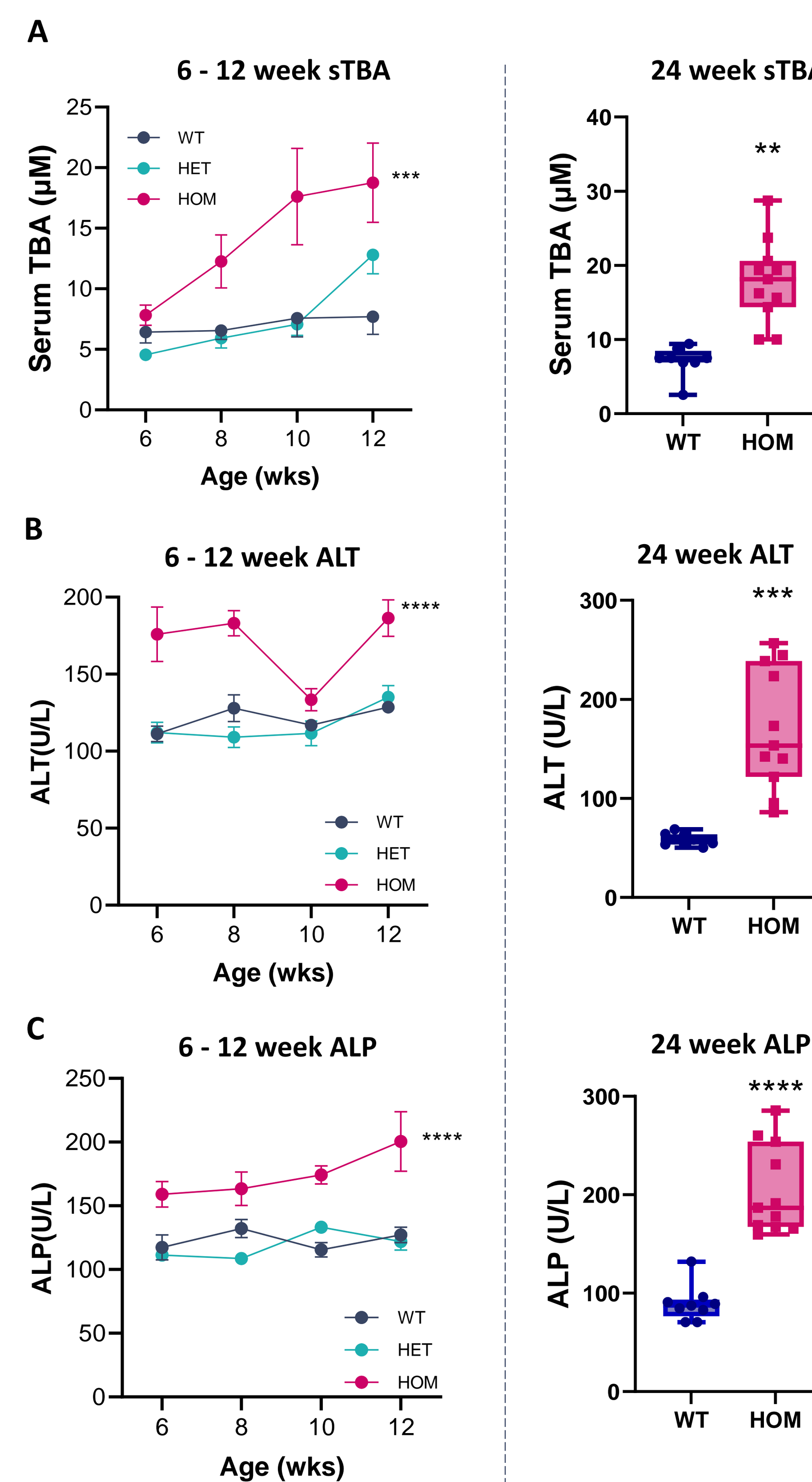


Figure 4. Blood chemistry analysis indicates that BSEP^{E297G} PFIC2 model presents with cholestasis and hepatotoxicity. (A) Compared to WT and HET animals, HOMs demonstrate a progressive increase in total serum bile acids from 6 weeks of age to 12 weeks of age (left) and remain elevated out to 6 months (right). Similarly, both ALT (B) and ALP (C) are increased at all ages tested.

Hepatobiliary dysfunction is evident in PFIC2 mice

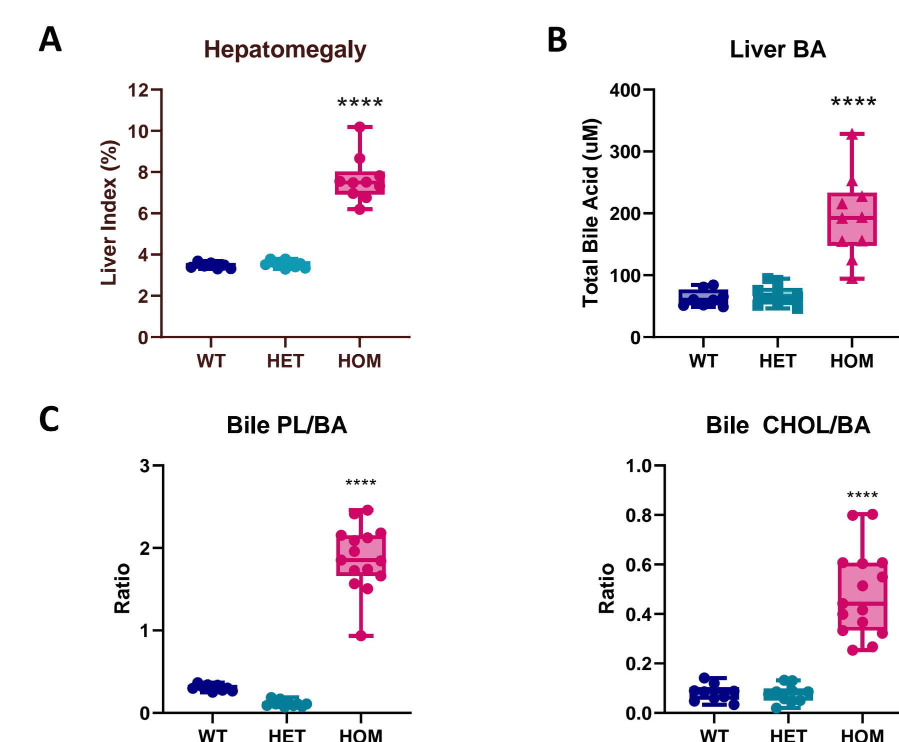


Figure 5. Hepatobiliary bile homeostasis is disrupted in BSEP^{E297G} mice (A) Liver Index is increased in PFIC2 HOM mice compared to WT and HET. (B) Consistent with increased serum bile acids, the livers of HOM animals contain more bile acids compared to littermate controls. (C) Biliary ratios of phospholipid (PL) and cholesterol (CHOL) to biliary bile acids are increased indicating disruption of hepatobiliary system to generate normal bile.

PFIC2 mouse liver bile acid composition is altered due to changes in gene expression

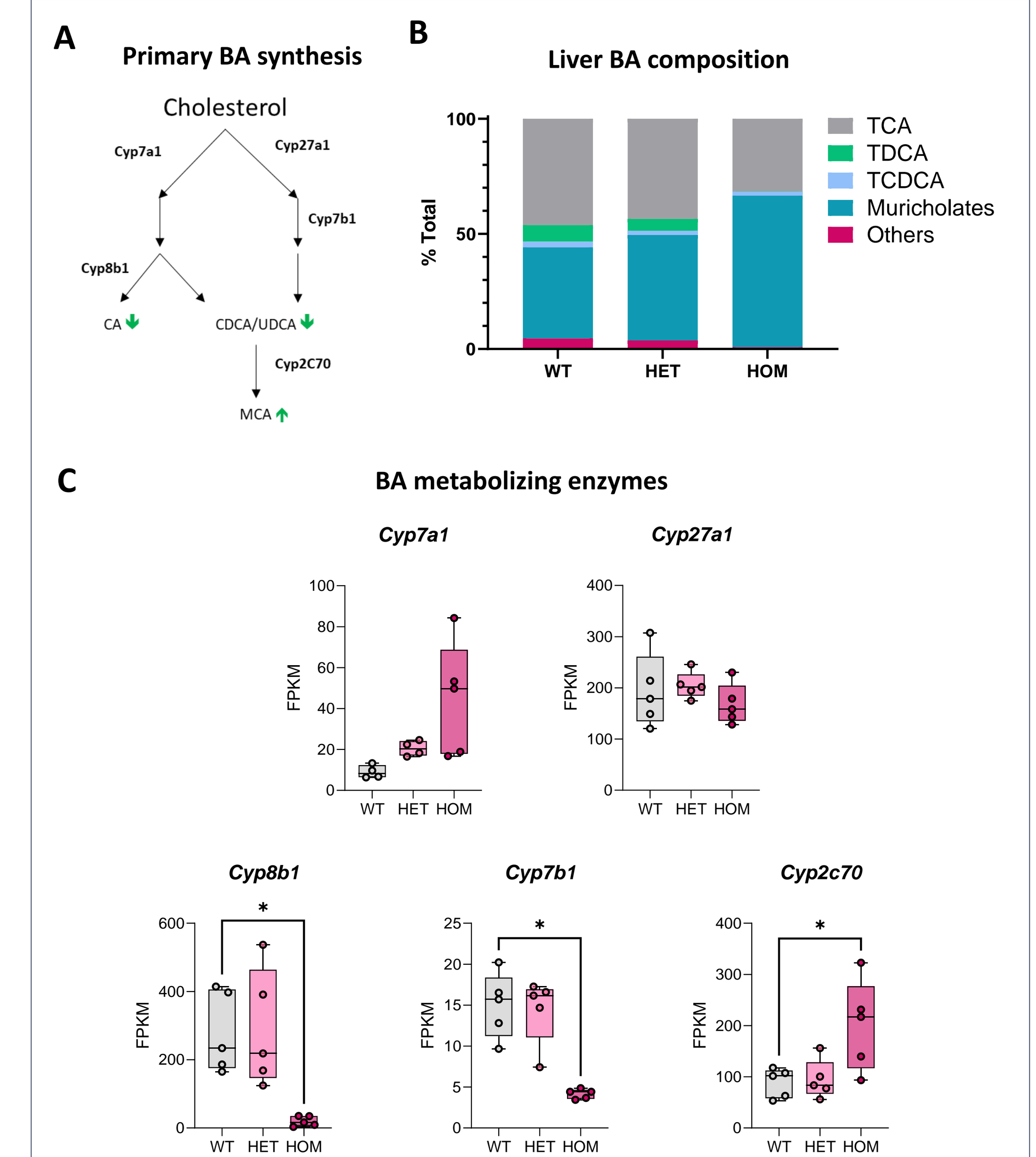


Figure 6. PFIC2 mouse liver bile acid composition shifts toward muricholate (MCA) production. (B) Metabolic pathway for conversation of cholesterol to primary bile acids in hepatocytes. (A) Bile acid composition is similar between WT and HET animals, while HOM have increased MCAs. Green arrows in A indicate changes for HOM vs WT. (C) Gene expression changes in bile acid metabolizing genes is consistent with decreased synthesis of CA, and all CDCA/UDCA is converted to MCAs.

Therapeutic treatment with 4-PBA ameliorates bile acid phenotype in PFIC2 mouse

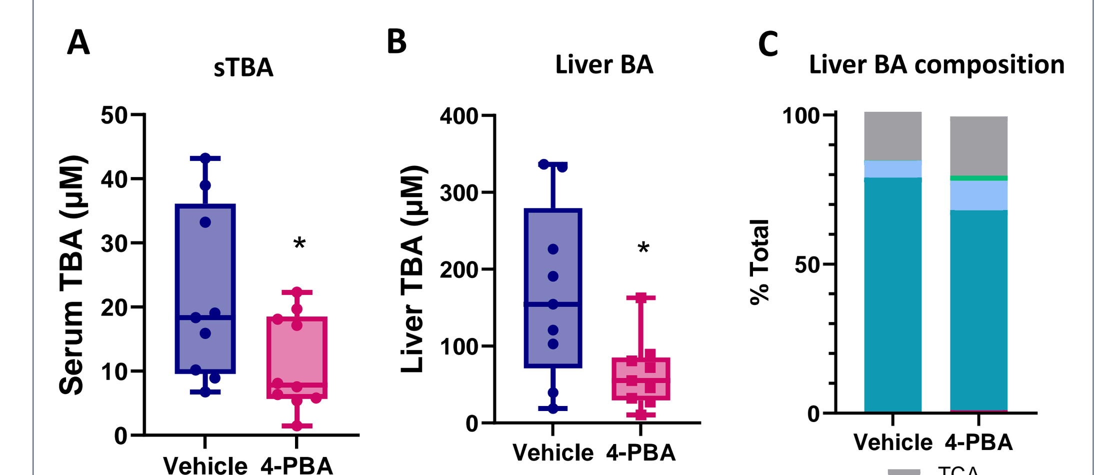


Figure 7. Treatment with 4-PBA, a clinically efficacious compound, attenuates bile acid phenotype in PFIC2 animals. Treatment with 1000 mg/kg 4-PBA for 7 days decreased serum bile acids (A) and liver bile acids (B) in the BSEP^{E297G} mouse model of PFIC2. Composition of liver bile acids shifts in HOM mice towards WT composition presented in figure 6A.

Conclusion

Introduction of the PFIC2 pathogenic BSEP variant E297G into the mouse gene results in a model recapitulates mechanistic and pathophysiological aspects of the human disease. Homozygous E297G animals have defective BSEP protein maturation and localization, and as a result demonstrate progressive increase in serum markers for cholestasis and hepatotoxicity. Moreover, liver bile acids are increased and biliary bile acids are decreased, indicating disruption of hepatobiliary homeostasis. Bile acid composition shifts towards a more hydrophilic state, similar to what is reported in the BSEP KO mouse[3]. Finally, 4-PBA has demonstrated efficacy in PFIC2 patients[4]. Our novel PFIC2 mouse responds to 4-PBA highlighting this model provides a powerful *in vivo* platform to evaluate potential disease modifying therapeutics for the treatment of PFIC2.

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4. Gonzales, E. et al. Targeted pharmacotherapy in progressive familial intrahepatic cholestasis type 2: Evidence for improvement of cholestasis with 4-phenylbutyrate. Hepatology 62, 558–566 (2015).