

# Identification of Taurine-Responsive Genes in Murine Liver Using the *Cdo1*-Null Mouse Model

Martha H. Stipanuk, Halina Jurkowska, Julie Niewiadomski, Kevin M. Mazor, Heather B. Roman, and Lawrence L. Hirschberger

**Abstract** The cysteine dioxygenase (*Cdo1*)-null mouse is unable to synthesize hypotaurine and taurine by the cysteine/cysteine sulfinate pathway and has very low taurine levels in all tissues. The lack of taurine is associated with a lack of taurine conjugation of bile acids, a dramatic increase in the total and unconjugated hepatic bile acid pools, and an increase in betaine and other molecules that serve as organic osmolytes. We used the *Cdo1*-mouse model to determine the effects of taurine deficiency on expression of proteins involved in sulfur amino acid and bile acid metabolism. We identified cysteine sulfinic acid decarboxylase (*Csad*), betaine:homocysteine methyltransferase (*Bhmt*), cholesterol 7 $\alpha$ -hydroxylase (*Cyp7a1*), and cytochrome P450 3A11 (*Cyp3a11*) as genes whose hepatic expression is strongly regulated in response to taurine depletion in the *Cdo1*-null mouse. Dietary taurine supplementation of *Cdo1*-null mice restored hepatic levels of these four proteins and their respective mRNAs to wild-type levels, whereas dietary taurine supplementation had no effect on abundance of these proteins or mRNAs in wild-type mice.

**Keywords** Betaine:homocysteine methyltransferase (BHMT) • Cysteine dioxygenase (CDO) • Cysteine sulfinic acid decarboxylase (CSAD) • Cytochrome P450, family 3, subfamily A, member 11 (CYP3A11) • Cholesterol 7 $\alpha$ -hydroxylase (cytochrome P450, family 7, subfamily A, member 1) (CYP7A1)

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## Abbreviations

ABCB11	ATP-binding cassette, subfamily B, member 11
BHMT	Betaine:homocysteine methyltransferase
CDO	Cysteine dioxygenase
CSAD	Cysteine sulfinic acid decarboxylase
CYP27A1	Sterol 27-hydroxylase (cytochrome P450, family 27, subfamily A, member 1)
CYP3A11	Cytochrome P450, family 3, subfamily A, member 11
CYP7A1	Cholesterol 7 $\alpha$ -hydroxylase (cytochrome P450, family 7, subfamily A, member 1)
FXR	Farnesoid X receptor
LRH1	Liver receptor homolog 1
OST $\alpha$ -OST $\beta$	Organic solute and steroid transporter
SHP	Small heterodimer partner (also known as NR0B2)
SLC6A6	Sodium- and chloride-dependent taurine transporter (also known as TAUT)

## 1 Introduction

Taurine, or 2-aminoethanesulfonic acid, is an abundant organic compound in mammalian cells, being found in the range of 10–20  $\mu$ mol per gram in murine tissues (Roman et al. 2013; Wójcik et al. 2010). Taurine has diverse biological roles and is involved in the processes of bile acid conjugation, cell volume regulation, modification of mitochondrial tRNAs, nervous and visual system development, antioxidation, and immune defense (Huxtable 1992; Ripps and Shen 2012). In general, taurine has protective effects on cells that are manifest under various types of injury as a result of its homeostatic effects.

Cysteine dioxygenase (CDO), which is encoded by the *Cdo1* gene, catalyzes the addition of oxygen to cysteine to form cysteine sulfinate. Cysteine sulfinate undergoes further metabolism, either by transamination to yield pyruvate and sulfite or by decarboxylation to hypotaurine and its further oxidation to taurine. The CDO-dependent pathway of taurine biosynthesis is the major route for taurine synthesis in mammals, and hence the *Cdo1* knockout mouse has a severely limited capacity to synthesize taurine and very low tissue taurine levels if fed a taurine-free diet.

A taurine-responsive decrease in cysteine sulfinic acid decarboxylase (CSAD) protein abundance has been reported previously for the *Cdo1* knockout mouse (Roman et al. 2013; Ueki et al. 2011), and we recently reported that BHMT mRNA and protein levels were downregulated in liver of the *Cdo1* knockout mouse (Jurkowska et al. 2016). Because of the dramatic effects of taurine of the hepatic bile acid pool and because of recent reports that CSAD and CYP7A1 (cholesterol

7 $\alpha$ -hydroxylase) may be regulated in a common fashion by bile acids (Kerr et al. 2013), we decided to use the *Cdo1*-null mouse model to identify other hepatic proteins that are expressed in a taurine-responsive manner.

## 2 Methods

### 2.1 *Animals, Diets, and Liver Collection*

*Cdo1*<sup>-/-</sup> and *Cdo1*<sup>+/+</sup> mice for this study were generated by crossing C57BL/6 *Cdo1*<sup>+/-</sup> male and female mice as described previously (Ueki et al. 2011; Roman et al. 2013). Animal studies were conducted with the approval of the Cornell University Institutional Animal Care and Use Committee (#2009-0138). Mice were housed at 23 °C and 45–50% humidity with light from 6:00 to 20:00 daily. Pups, as well as their dams, had free access to the standard semipurified taurine-free diet available in their cages from birth to postnatal day 21. Pups were weaned at day 21 and assigned to either a basal (taurine-free) diet based on the AIN93G semi-purified diet for growing mice or to the same diet supplemented with 5 g taurine/kg. Six mice were assigned to each sex/genotype/diet group (48 mice total).

After weaning, mice assigned to the same diet were housed together with two to four mice per cage. All experimental mice were fed the basal diet from weaning through postnatal day 37. From postnatal day 38 until postnatal day 62–68 when liver was collected, mice were fed the assigned treatment diet, which was either the same taurine-free diet or that diet supplemented with taurine. To obtain liver, mice were euthanized between 10:00 and 14:00 h with an overdose of isoflurane. Liver was removed, immediately frozen in liquid nitrogen, and stored at –80 °C until samples were analyzed.

### 2.2 *Determination of Hepatic Protein and Taurine Levels and Western Blotting to Measure the Relative Abundance of Proteins*

Frozen liver samples were homogenized in four volumes of lysis buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA and 0.5% Nonidet P-40] containing 1 $\times$  Complete Protease Inhibitor Cocktail (Roche) and 1 $\times$  PhosSTOP phosphatase inhibitor (Roche). The supernatant fraction was obtained by centrifuging the homogenates at 18,000  $\times$  g for 20 min at 4 °C and used for determination of total soluble protein, taurine levels, and the relative abundances of selected mRNAs and proteins.

Total cellular protein in hepatocyte lysates and soluble protein in the supernatant fractions from liver homogenates were determined using the BCA Protein Assay Kit (Thermo Scientific/Pierce) using bovine serum albumin (BSA) as the standard.

For measurement of taurine and hypotaurine, the cell lysate or the liver supernatant fraction was mixed with one volume of 5% (wt/vol) sulfosalicylic acid, and the mixture was centrifuged at  $15,000 \times g$  for 15 min at 4 °C to obtain the acid supernatant. Taurine and hypotaurine were measured by HPLC as described previously (Ueki et al. 2011). Samples were derivatized with *o*-phthaldialdehyde (OPA) and separated on a C18 column by gradient elution using 0.05 M potassium phosphate buffer (pH 7.0) with 3.5% (vol/vol) tetrahydrofuran mobile phase without or with 40% (vol/vol) acetonitrile. Detection of OPA-derivatized compounds was performed using excitation and emission peaks at 360 and 455 nm, respectively.

For measurement of relative protein abundance, aliquots of liver supernatant equivalent to 30 µg of total protein were separated by SDS-PAGE (12%, w/v, polyacrylamide). Protein bands were transferred onto a 0.45-µm Immobilon-FL PVDF membrane (Millipore Corp.). Immunoblotting was performed by first exposing membranes to blocking buffer for near infrared fluorescent westerns (LI-COR Biosciences) and then blotting for immunoreactive proteins. The primary antibodies included anti-CSAD at a 1:8000 dilution (gift from Dr. Marcel Tappaz, INSERM, France), anti-CYP7A1 at a 1:500 dilution (GeneTex), anti-CYP3A11/4 at a 1:1000 dilution (Cell Signaling), anti-CYP27A1 at a 1:1000 dilution (Thermo Fisher Scientific), anti-OSTβ at a 1: 400 dilution (Bioss), anti- BHMT at a 1:1000 dilution (Thermo Fisher Scientific), anti-β-tubulin at a 1:500 dilution (Santa Cruz Biotechnology), and anti-β-actin at a 1:1000 dilution (Proteintech Group). Immunoreactive bands were detected and quantified using an infrared fluorescent dye-labeled secondary antibody (IRDye, LI-COR Biosciences) and the Odyssey direct infrared imaging system and software (LI-COR Biosciences). Protein abundances were divided by β-tubulin or β-actin abundance to normalize the values, which were then expressed as fold the value for wild-type mice of the same sex fed the basal taurine-free diet.

### 2.3 PCR Measurement of mRNA Relative Abundance

The relative abundance of mRNA was measured by PCR. The RNeasy mini kit (Qiagen) was used to isolate RNA isolated from the liver samples, and complementary DNA was reverse transcribed using Applied Biosystems High Capacity cDNA kit (Applied Biosystems). Quantification of mRNA relative abundance was done using Power Sybr Green (Applied Biosystems) and a Roche 480 Lightcycler (Roche Diagnostics). The forward and reverse primer sequences were: ABCB11 forward 5'-ACTGAACTTGAAAGGGGTGT-3' reverse 5'-TCACTCAACAACCCTACAGATG-3'; BHMT forward 5'-CGGCTTCAGAAAAACATGG-3' reverse 5'-TCTGCCAGATTCCTTTCTGG-3'; CYP3A11 forward 5'-GAAGCATTGAGGAGGATCACA-3' reverse 5'-GGTCCATCCCTGCTGTTT-3'; CYP7A1 forward 5'-CACCATTCTGCAACCTTCT-3' reverse 5'-TTGGCCAGCACTCTGTAATG-3'; CYP27A1, forward 5'-GTGGACAACCTCCTTTGGGAC-3' reverse 5'-CCCTCCTGTCTCATCACTTGC-3'; CSAD forward 5'-CCAGTGCCTCTGAGAAGGTC-3'

reverse 5'-TGACACTGTAGTGAATCACAGTCC-3'; OST $\beta$  forward 5'-TGACAAGCATGTTCCCTCCTG-3' reverse 5'-TGGAGTCATCAAGATGCAGGT-3'; and SLC6A6 forward 5'-CTGCCTGGATTTGGAAGG-3' reverse 5'-GCCACTGAAGACAGGTGAGG-3'.

Values for ABCB11, BHMT, CYP7A1, CYP27A1, CYP3A11, CSAD, OST $\beta$ , and SLC6A6 mRNAs were normalized to values for  $\beta$ -actin mRNA, and the normalized values were used to calculate the fold differences relative to the average value for wild-type mice of the same sex fed the basal diet.

## 2.4 LC/MS Analysis of Liver Metabolites

Samples were prepared for LC/MS analysis by homogenizing a weight aliquot of frozen liver in ice-cold 80% methanol/water (200  $\mu$ L per 5 mg liver) and then diluting the homogenate with an additional volume of ice-cold 80% methanol/water (200  $\mu$ L per 5 mg liver). The homogenate was then vortexed, allowed to sit on ice for 10 min, and centrifuged (20,000  $\times$  g at 4  $^{\circ}$ C for 10 min) to obtain supernatant. A 200  $\mu$ L aliquot of the supernatant was transferred to a microcentrifuge tube, dried in a SpeedVac (Thermo Scientific), and stored at  $-80^{\circ}$  C until the LC-MS analysis was done.

For LC-MS, dried supernatant samples were reconstituted with 30  $\mu$ L water, diluted with an additional 30  $\mu$ L acetonitrile/methanol (1:1, v/v), and centrifuged (20,000  $\times$  g at 4  $^{\circ}$ C for 3 min) to obtain the final supernatant used for LC/MS.

A 4  $\mu$ L-aliquot of the final supernatant was injected into the LC-MS system, which was an Ultimate 3000 UHPLC (Dionex) coupled to a Q Exactive-Mass spectrometer (QE-MS, Thermo Scientific). LC was done at room temperature. For analysis of bile acids and related compounds, reversed phase LC was performed using a Luna C18 column (100  $\times$  2.0 mm i.d., 3  $\mu$ m; Phenomenex) with a gradient mobile phase system (A: 5 mM ammonium acetate in water; B: methanol). The percentage of mobile phase B was 2% B between 0 and 1.5 min, was linearly increased to 15% B between 1.5 and 3 min and to 95% between 5.5 and 14.5 min, was held at 95% between 14.5 and 15 min before being returned to 2% B between 15 and 20 min. For analysis of other polar metabolites, hydrophilic interaction LC (HILIC) was run with an Xbridge amide column (100  $\times$  2.1 mm i.d., 3.5  $\mu$ m; Waters). Details of the LC/MS analysis were published previously by Liu et al. (2014).

## 2.5 Statistical Analysis

Results of measurements are expressed as means  $\pm$  SEM for six mice. Statistical analysis was run as a full factorial least squares model using JMP version 10 (SAS, Cary, NC). Results for male and female mice were analyzed separately. Differences were accepted as significant at  $p \leq 0.05$  for main effects (genotype, treatment) and at  $p \leq 0.1$  for interactions. Post-hoc individual pairwise comparisons of least squares

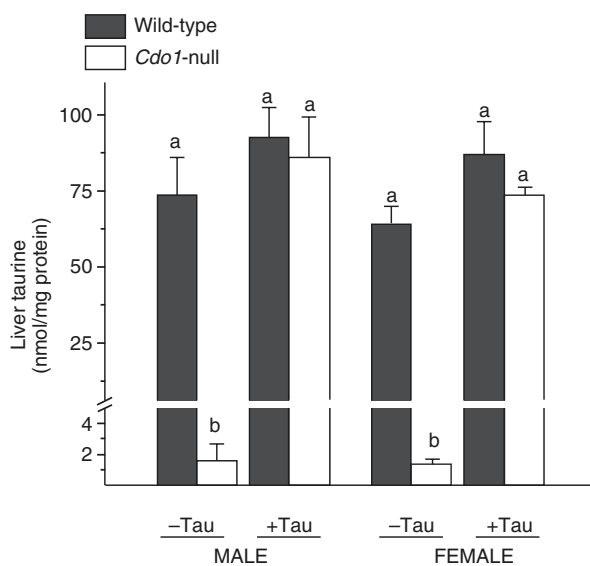
means by Tukey's procedure were considered significant at  $p < 0.05$ . Data for hepatic mRNA and protein abundances of CSAD, CYP3A11, and OST $\beta$  were square root transformed prior to statistical analysis. Data for taurine and hypotaurine levels, the hepatic abundances of BHMT mRNA and protein, and metabolite differences were log-transformed prior to statistical analyses.

### 3 Results

#### 3.1 Taurine and Metabolite Concentration Differences

*Cdo1*-null mice fed a standard semi-purified rodent diet, which contained no taurine, had very low taurine levels as reported previously (Roman et al. 2013; Ueki et al. 2011). Hepatic taurine concentrations in the *Cdo1*-null mice fed a taurine-free diet were less than 3% of wild-type levels (Fig. 1). Supplementation of the diet with taurine restored the hepatic taurine level. Hepatic taurine levels in *Cdo1*-null and wild-type mice fed the taurine supplemented diet were similar to each other as well as similar to the level in wild-type mice fed the basal taurine-free diet.

To further explore the changes in liver metabolites in the taurine-deficient *Cdo1*-null mice and to assess the extent to which any alterations were due to taurine deficiency, metabolomic profiles were run on liver samples from male rats in each genotype-diet group. Taurine and bile acid levels exhibited the largest fold differences between the two genotypes when mice fed taurine-free diets were compared. *Cdo1*-null mice fed the standard taurine-free semipurified diet had very low hepatic taurine levels (Table 1), which was consistent with the dramatic reductions based on



**Fig. 1** Taurine concentration in the liver of male and female *Cdo1*-null and wild-type mice fed either a basal (-Tau) or taurine-supplemented (+Tau) diet. Values shown in bar graphs are means  $\pm$  SEM for six mice. Bars not denoted by the same letter are significantly different from other values for mice of the same sex ( $P < 0.05$ )

**Table 1** Fold differences in taurine, bile acids and organic osmolytes in liver of male wild-type and *Cdo1*-null mice fed a basal taurine-free or a taurine-supplemented diet

Metabolites	Basal taurine-free diet		Taurine-supplemented diet	
	Wild-type	<i>Cdo1</i> -null	Wild-type	<i>Cdo1</i> -null
Taurine	1 ± 0.10 <sup>a,†</sup>	0.048 ± 0.01 <sup>b</sup>	0.97 ± 0.18 <sup>a</sup>	1.16 ± 0.06 <sup>a</sup>
Betaine	1 ± 0.09 <sup>b</sup>	2.29 ± 0.34 <sup>a</sup>	0.91 ± 0.09 <sup>b</sup>	1.19 ± 0.22 <sup>b</sup>
Choline	1 ± 0.05 <sup>b</sup>	1.57 ± 0.04 <sup>a</sup>	0.96 ± 0.11 <sup>b</sup>	0.80 ± 0.32 <sup>b</sup>
<i>sn</i> -Glycerol-3-phosphocholine	1 ± 0.23 <sup>b</sup>	1.82 ± 0.27 <sup>a</sup>	0.92 ± 0.10 <sup>b</sup>	0.81 ± 0.12 <sup>b</sup>
Carnosine	1 ± 0.11 <sup>b</sup>	2.12 ± 0.14 <sup>a</sup>	0.87 ± 0.11 <sup>b</sup>	0.76 ± 0.06 <sup>b</sup>
<i>myo</i> -Inositol	1 ± 0.04	1.42 ± 0.16	1.12 ± 0.06	1.20 ± 0.09
Taurocholate/taumuricholate	1 ± 0.26 <sup>a</sup>	0.23 ± 0.04 <sup>b</sup>	0.96 ± 0.11 <sup>a</sup>	1.11 ± 0.32 <sup>a</sup>
Taurodeoxycholate/ taurochenodeoxycholate/ tauroursodeoxycholate	1 ± 0.23 <sup>a</sup>	0.18 ± 0.03 <sup>b</sup>	2.40 ± 0.10 <sup>a</sup>	1.02 ± 0.21 <sup>a</sup>
Taurolithocholate	1 ± 0.18 <sup>a</sup>	0.15 ± 0.04 <sup>b</sup>	0.92 ± 0.11 <sup>a</sup>	0.84 ± 0.11 <sup>a</sup>
Glycocholate/glycomuricholate	1 ± 0.64 <sup>b</sup>	110 ± 38 <sup>a</sup>	0.88 ± 0.27 <sup>b</sup>	1.45 ± 0.70 <sup>b</sup>
Glycodeoxycholate/ glycochenodeoxycholate/ glycoursodeoxycholate	1 ± 0.44 <sup>b</sup>	49.6 ± 7.6 <sup>a</sup>	0.89 ± 0.36 <sup>b</sup>	0.67 ± 0.28 <sup>b</sup>
Cholate/muricholate	1 ± 0.27 <sup>b</sup>	86 ± 12 <sup>a</sup>	3.20 ± 2.29 <sup>b</sup>	0.36 ± 1.51 <sup>b</sup>
Deoxycholate/chenodeoxycholate/ ursodeoxycholate	1 ± 0.13 <sup>b</sup>	39.7 ± 4.7 <sup>a</sup>	2.26 ± 1.24 <sup>b</sup>	1.13 ± 0.21 <sup>b</sup>
Lithocholate	1 ± 0.15 <sup>b</sup>	14.0 ± 1.3 <sup>a</sup>	1.22 ± 0.09 <sup>b</sup>	1.14 ± 0.27 <sup>b</sup>
3 $\alpha$ -7 $\alpha$ -12 $\alpha$ -Trihydroxy-5 $\beta$ - cholestanoate	1 ± 0.14 <sup>b</sup>	3.40 ± 0.63 <sup>a</sup>	1.03 ± 0.10 <sup>b</sup>	0.72 ± 0.12 <sup>b</sup>
3 $\alpha$ -7 $\alpha$ -Dihydroxy-5 $\beta$ -cholestanoate	1 ± 0.23 <sup>b</sup>	2.27 ± 0.43 <sup>a</sup>	0.90 ± 0.13 <sup>bc</sup>	0.65 ± 0.17 <sup>c</sup>
7 $\alpha$ -Hydroxy-3-oxo-4-cholestenoate	1 ± 0.20 <sup>b</sup>	2.70 ± 0.68 <sup>a</sup>	0.71 ± 0.06 <sup>b</sup>	1.04 ± 0.22 <sup>b</sup>

<sup>a</sup>All values for metabolite concentrations are expressed relative to the average value for the wild-type/basal diet group, which was set as 1.0. Data are expressed as means ± SEM

<sup>†</sup>All values were transformed to log<sub>10</sub> values prior to statistical analysis by a general linear model for the two categorical variables and their interaction using JMP, version 11 (SAS, Cary, NC). Values not followed by the same superscript letter are significantly different at  $p < 0.05$  as determined by Tukey's post-hoc comparison test

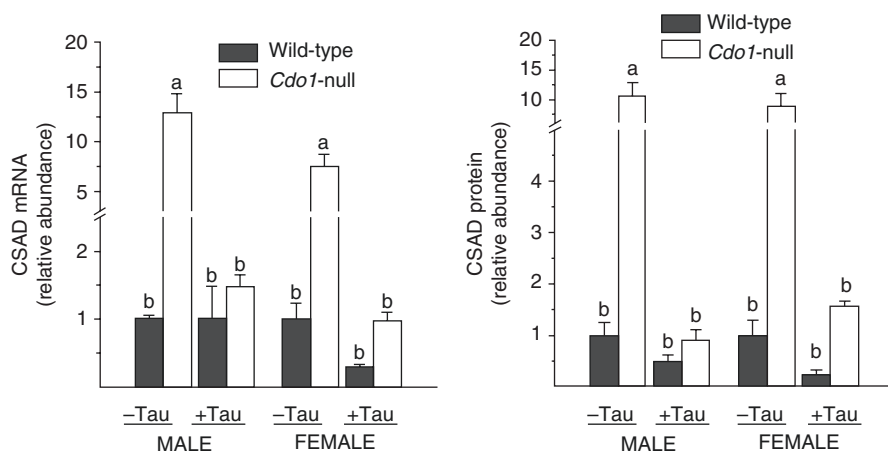
HPLC analysis reported in Fig. 1. The hepatic levels of taurine-conjugated bile acids in taurine-deficient *Cdo1*-null mice were low (15–23% of wild-type for mice fed the basal diet). Levels of unconjugated bile acids and glycine-conjugated bile acids were elevated in *Cdo1*-null mice fed the basal diet, and the abundances of several other molecules that can function as organic osmolytes (betaine, choline, glycerophosphocholine, and carnosine) were significantly higher in the *Cdo1*-null mice fed the basal diet (Table 1). In addition, the concentration of 7 $\alpha$ -hydroxycholest-4-en-3-one, an early metabolite of cholesterol in the CYP7A1-initiated neutral pathway of bile acid synthesis; 3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -trihydroxy-5 $\beta$ -cholestanoate, a subsequent intermediate in the pathway for cholate synthesis; and 3 $\alpha$ ,7 $\alpha$ ,dihydroxy-5 $\beta$ -cholestanoate, a subsequent intermediate in the pathway for chenodeoxycholate/muricholate synthesis were all significantly elevated (to ~2.3 to 3.4-fold wild-type levels) in liver of *Cdo1*-null mice fed the taurine-free basal diet. Notably, all of these

differences disappeared when taurine was added to the diet, indicating that the lack of taurine biosynthesis in the *Cdo1*-null mouse was responsible for the changes in levels of both bile acid metabolites and organic osmolytes.

### 3.2 Effect of Taurine Status on Gene Expression

Hepatic levels of CSAD mRNA and protein have been reported by several investigators to be strongly influenced by taurine status and were examined to verify the usefulness of the *Cdo1*-null mouse model for detecting taurine-responsive genes. CSAD mRNA abundance was increased to 12.8-fold control in male and 7.5-fold control in female *Cdo1*-null mice fed the taurine-free basal diet, and this was reversed when the *Cdo1*-null mice were given the taurine-supplemented diet (Fig. 2). Similarly, CSAD protein abundance was increased to 10.5-times control levels in male and to 8.8-times control levels in female *Cdo1*-null mice fed the taurine-free diet.

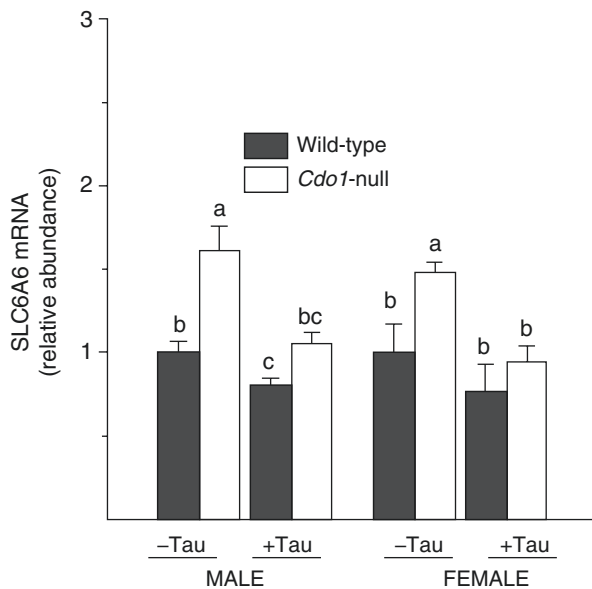
The mRNA abundance for the sodium- and chloride-dependent taurine transporter SLC6A6 was also significantly elevated by 50–60% in liver of taurine-deficient *Cdo1*-null mice, but not in the liver of taurine-supplemented *Cdo1*-null mice (Fig. 3). We were unsuccessful in detecting SLC6A6 protein in liver due to nonspecificity of the antibody we used. Expression of *Slc6a6* (*Taut*) has been shown to be sensitive to hypertonic stress and to be transcriptionally regulated by the tonicity-responsive element (TonE) and the TonE-binding protein (TonEBP) in



**Fig. 2** CSAD mRNA and protein abundance in liver of male and female *Cdo1*-null and wild-type mice fed either a basal (–Tau) or taurine-supplemented (+Tau) diet. Values shown in bar graphs are means  $\pm$  SEM for six mice and are expressed as fold the value for wild-type mice of the same sex fed the basal diet; bars not denoted by the same letter are significantly different from other values for mice of the same sex ( $P < 0.05$ )



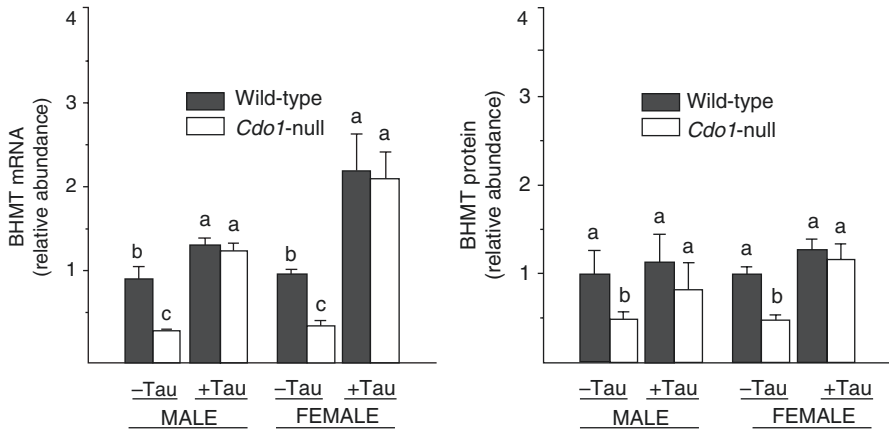
**Fig. 3** SLC6A6 mRNA abundance in liver of male and female *Cdo1*-null and wild-type mice fed either a basal (-Tau) or taurine-supplemented (+Tau) diet. Values shown in bar graphs are means  $\pm$  SEM for six mice and are expressed as fold the value for wild-type mice of the same sex fed the basal diet; bars not denoted by the same letter are significantly different from other values for mice of the same sex ( $P < 0.05$ )



HepG2 cells (Ito et al. 2004). Both the mRNA and protein abundance for SLC6A6 increased in HepG2 cells cultured in hypertonic medium (Ito et al. 2004; Satsu et al. 2003). In addition, culturing HepG2 cells in taurine-rich medium resulted in down-regulation of both SLC6A6 mRNA and protein abundance (Satsu et al. 2003). The metabolomics results shown in Table 1 show increases in other organic osmolytes, especially betaine, in the *Cdo1*-null mouse liver, which supports the likelihood of osmotic stress (cell shrinkage) in the taurine-deficient *Cdo1*-null mice. Whether the upregulation of SLC6A6 mRNA abundance was due to hypertonicity or low taurine levels, or both, cannot be distinguished, but taurine supplementation of the diet returned the hepatic SLC6A6 mRNA level to that of wild-type taurine-sufficient mice.

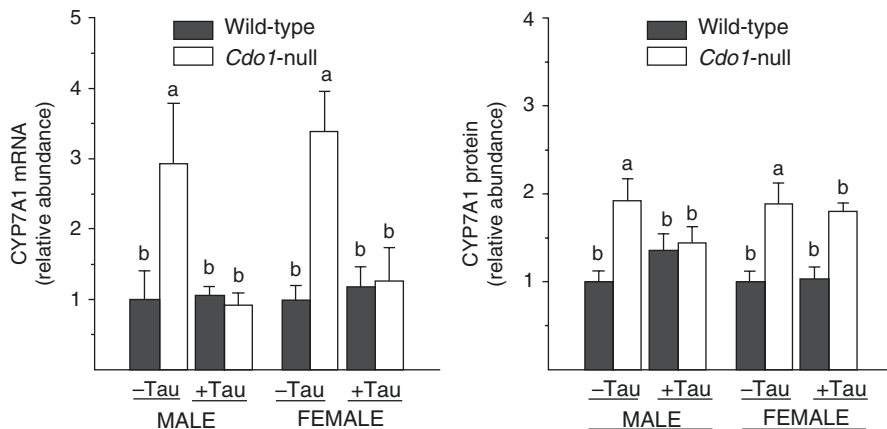
A third taurine-responsive gene, *Bhmt*, was recently identified by our laboratory (Jurkowska et al. 2016). BHMT encodes the betaine: homocysteine methyltransferase, which consumes betaine as a source of methyl groups for remethylation of homocysteine in the liver. As shown in Fig. 4, BHMT mRNA and protein abundances were suppressed in the *Cdo1*-null mice fed the taurine-free basal diet, with mRNA levels being reduced to 37% of control in males and 42% of control in females and protein levels being reduced to 48% of control in both male and female mice. Taurine supplementation reversed the low BHMT mRNA and protein levels in *Cdo1*-null mice. It is notable that the decrease in BHMT mRNA and protein levels in *Cdo1*-null mice fed the basal taurine-free diet was associated with elevated hepatic betaine levels that were 2.3-times those of wild-type mice fed the same diet (Table 1).

Because of the dramatic changes in the hepatic bile acid metabolite profile in liver of *Cdo1*-null mice fed the taurine-free diet (Table 1) and because Kerr et al. (2013)

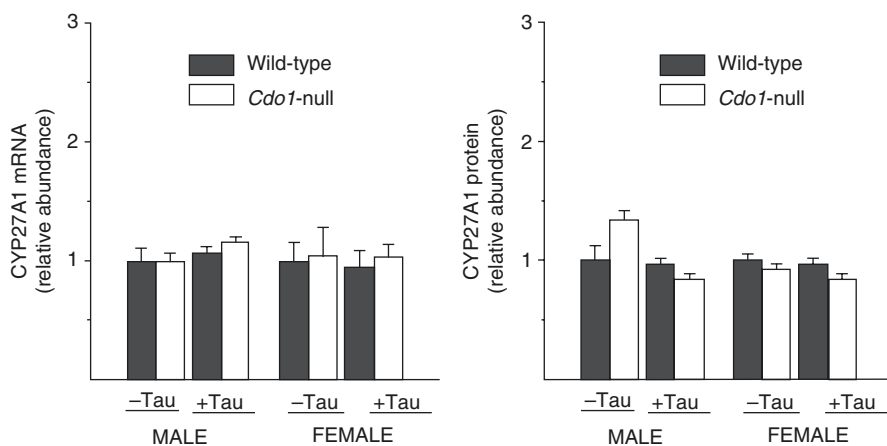


**Fig. 4** BHMT mRNA and protein abundance in liver of male and female *Cdo1*-null and wild-type mice fed either a basal (-Tau) or taurine-supplemented (+Tau) diet. Values shown in bar graphs are means  $\pm$  SEM for six mice and are expressed as fold the value for wild-type mice of the same sex fed the basal diet; bars not denoted by the same letter are significantly different from other values for mice of the same sex ( $P < 0.05$ )

reported similarities between the mechanisms of *Csad* and *Cyp7a1* induction via farnesoid X receptor (FXR) and small heterodimer partner (SHP)-dependent signaling, we decided to examine the expression of several proteins involved in bile acid synthesis and transport. [SHP is the same as nuclear receptor subfamily 0, group B, member 2 (NR0B2).] First, we looked at the expression of several cytochrome P450 (CYP) enzymes involved in bile acid synthesis, CYP7A1, CYP27A1, and CYP3A11. CYP7A1 (cholesterol 7 $\alpha$ -hydroxylase) catalyzes the initial step in the major pathway for bile acid synthesis from cholesterol, whereas CYP27A1 (sterol 27-hydroxylase) catalyzes the initial step in an alternate pathway for bile acid biosynthesis. The major bile acids synthesized by human liver are cholic acid and chenodeoxycholic acid, but in mice chenodeoxycholic acid is readily further converted to muricholic acid by CYP3A11. In rodent liver, CYP3A11 hydroxylates chenodeoxycholate at the 6 $\beta$ -position to form the more hydrophilic  $\alpha$ -muricholate, which can be further converted to  $\beta$ -muricholate by epimerization of its 7 $\alpha$ -OH to 7 $\beta$ -OH (Martignoni et al. 2006; Gardès et al. 2013). In *Cdo1*-null mice, CYP7A1 mRNA was 3.0–3.4-times that in liver of wild-type mice and CYP7A1 protein abundance was 2-times that in liver of wild-type mice (Fig. 5). Supplementation of *Cdo1*-null mice with taurine returned CYP7A1 to wild-type levels. Results were identical for male and female mice. CYP27A1 mRNA and protein abundances were not affected by the *Cdo1* genotype or by taurine supplementation, indicating the absence of regulation of the alternative pathway of bile acid synthesis by taurine (Fig. 6). CYP3A11 mRNA and protein abundances were markedly increased in male *Cdo1*-null mice, with CYP3A11 mRNA and protein being 6-times and 15-times wild-type levels, respectively (Fig. 7). In female mice,

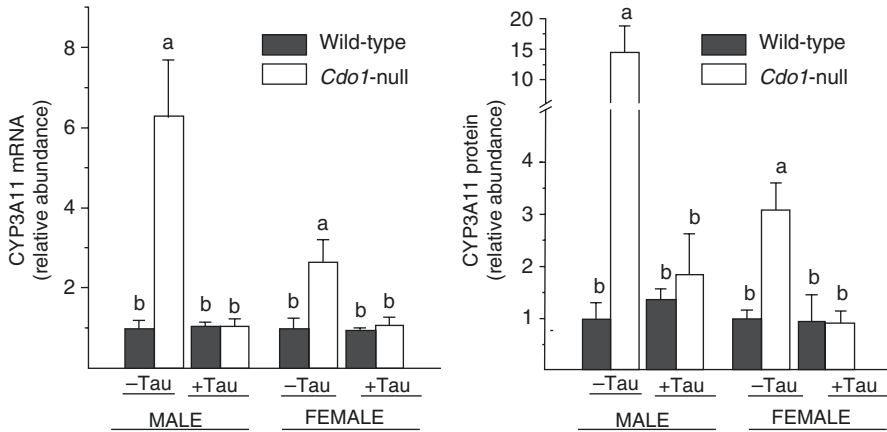


**Fig. 5** CYP7A1 mRNA and protein abundance in liver of male and female *Cdo1*-null and wild-type mice fed either a basal (-Tau) or taurine-supplemented (+Tau) diet. Values shown in bar graphs are means  $\pm$  SEM for six mice and are expressed as fold the value for wild-type mice of the same sex fed the basal diet; bars not denoted by the same letter are significantly different from other values for mice of the same sex ( $P < 0.05$ )



**Fig. 6** CYP27A1 mRNA and protein abundance in liver of male and female *Cdo1*-null and wild-type mice fed either a basal (-Tau) or taurine-supplemented (+Tau) diet. Values shown in bar graphs are means  $\pm$  SEM for six mice and are expressed as fold the value for wild-type mice of the same sex fed the basal diet; bars not denoted by the same letter are significantly different from other values for mice of the same sex ( $P < 0.05$ )

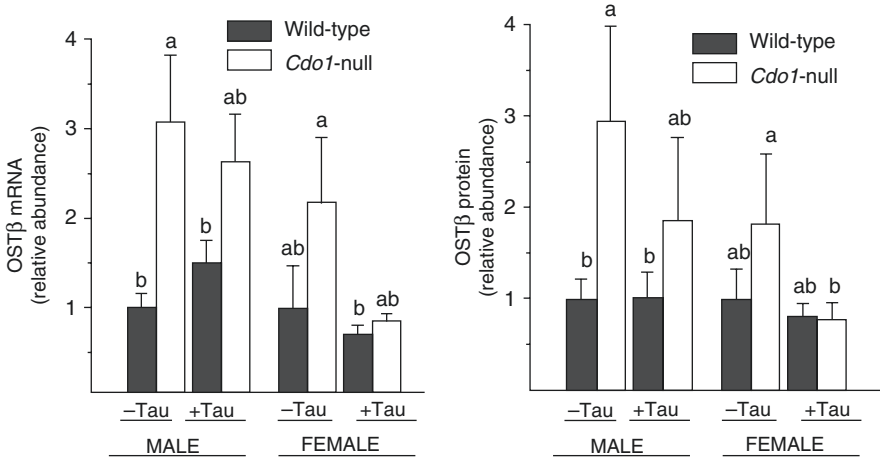
CYP3A11 mRNA and protein expression were both 3-times those in wild-type mice. Supplementation of *Cdo1*-null mice with taurine returned CYP3A11 mRNA and protein abundances to the lower wild-type levels. Thus, both CYP7A1 and CYP3A11 are sensitive to taurine status, being elevated in the taurine-deficient



**Fig. 7** CYP3A11 mRNA and protein abundance in liver of male and female *Cdo1*-null and wild-type mice fed either a basal (–Tau) or taurine-supplemented (+Tau) diet. Values shown in bar graphs are means  $\pm$  SEM for six mice and are expressed as fold the value for wild-type mice of the same sex fed the basal diet; bars not denoted by the same letter are significantly different from other values for mice of the same sex ( $P < 0.05$ )

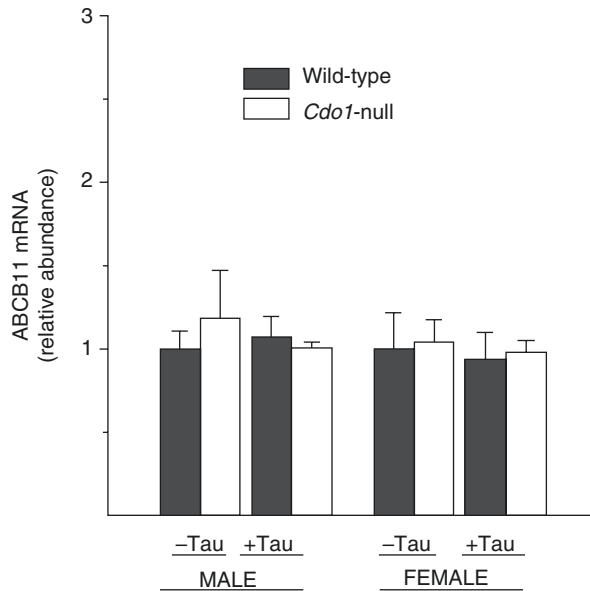
*Cdo1*-null mice but reduced to control levels by taurine supplementation. The greater expression of these two enzymes involved in bile acid synthesis is consistent with the fold increases in abundance of cholestanolate and cholestenoate intermediates in bile acid synthesis (Table 1). These responses may be seen as an attempt of the liver to increase bile acid synthesis when taurine conjugation of bile acids is deficient.

Finally, we looked at the expression of two bile acid transporters, OST $\beta$  and ABCB11. OST $\beta$  is a subunit of the heteromeric organic solute and steroid transporter (OST $\alpha$ -OST $\beta$ ), which transports bile acids, conjugated steroids and structurally-related molecules according to their electrochemical gradients. Hepatic expression of OST $\beta$  subunit mRNA and protein in *Cdo1*-null male mice was 3-times that of wild-type male mice, but taurine-supplementation did not reverse this effect, suggesting it might be due to some aspect of the *Cdo1*-null genotype other than taurine depletion (Fig. 8). In female *Cdo1*-null mice, OST $\beta$  mRNA and protein abundances were not significantly greater than those of wild-type mice, regardless of diet. Overall these results suggest a more complicated regulation of OST $\beta$  expression that is not solely responsive to taurine, although taurine appears to have some affect. For *Abcb11* expression, we only looked at mRNA levels, and these were not affected by *Cdo1* genotype or taurine supplementation (Fig. 9). The *Abcb11* gene encodes the ATP-binding cassette, subfamily B, member 11, which is also known as the bile salt export pump (BSEP) because of its role in the transport of taurocholate and other bile acids from hepatocytes into the bile.



**Fig. 8** OSTβ mRNA and protein abundance in liver of male and female *Cdo1*-null and wild-type mice fed either a basal (-Tau) or taurine-supplemented (+Tau) diet. Values shown in bar graphs are means ± SEM for six mice and are expressed as fold the value for wild-type mice of the same sex fed the basal diet; bars not denoted by the same letter are significantly different from other values for mice of the same sex ( $P < 0.05$ )

**Fig. 9** ABCB11 mRNA in liver of male and female *Cdo1*-null and wild-type mice fed either a basal (-Tau) or taurine-supplemented (+Tau) diet. Values shown in bar graphs are means ± SEM for six mice and are expressed as fold the value for wild-type mice of the same sex fed the basal diet; bars not denoted by the same letter are significantly different from other values for mice of the same sex ( $P < 0.05$ )



## 4 Discussion

*Unconjugated bile acids accumulate in liver of Cdo1-null mice as a result of a lack of taurine for bile acid conjugation.* Murine bile acids undergo conjugation with taurine in the liver prior to their secretion in the bile. Mice differ from humans and rats in that they conjugate bile acids almost solely with taurine instead of with either glycine or taurine. This is due to the specificity of bile acid CoA:amino acid *N*-acyltransferase in different species of mammals, with murine bile acid CoA:amino acid *N*-acyltransferase almost exclusively conjugating bile acids with taurine (Falany et al. 1997). Both newly synthesized bile acids and deconjugated bile acids returning to the liver via the enterohepatic circulation are normally conjugated with taurine prior to secretion into the bile.

Many studies have demonstrated that the percentage of the hepatic or biliary bile acid pools present as taurine-conjugates reflects hepatic taurine concentrations (Stephan et al. 1981; Stipanuk et al. 1984; De la Rosa and Stipanuk 1985). Similarly, in *Cdo1*-null mice in this study, in which hepatic taurine was depleted to 3–5% of wild-type levels, there was a dramatic decrease in the hepatic concentration of taurine-conjugated bile acids and there were large fold elevations in unconjugated and glycine-conjugated bile acids. These changes were clearly related to taurine deficiency because the hepatic bile acid pools of *Cdo1*-null mice fed the taurine-supplemented diet were similar to those of wild-type mice.

Although bile acid concentrations were not directly measured in our study, we estimated the bile acid pool size in *Cdo1*-null mouse liver using the fold differences we observed along with the molar concentrations reported by Alnouti et al. (2008) and García-Cañaveras et al. (2012). Compared to their observations of ~97–98% taurine-conjugated, ~0.1% glycine-conjugated, and ~2–3% unconjugated bile acids in murine liver, we estimate that the total hepatic bile acid pool in the *Cdo1*-null mice was 30–40% higher than in wild-type mice and comprised about 3.9% taurine-conjugated, 0.7% glycine-conjugated, and 95.4% unconjugated bile acids. Clearly, the absolute concentrations of unconjugated bile acids were elevated many-fold, with unconjugated bile acids replacing taurine-conjugated bile acids as the dominant species, due to both the dramatically lower formation of taurine conjugates and the somewhat larger total bile acid pool. Although glycine-conjugated bile acids were elevated in *Cdo1*-null mice, they accounted for little of the total murine bile acid pool due to the mouse's lack of glycine conjugating activity.

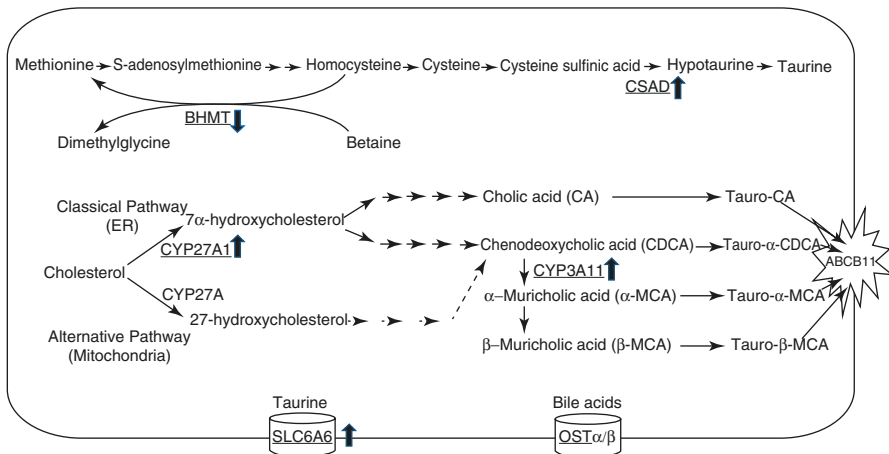
Either an increase in synthesis or a decrease in biliary secretion, or both, could account for the 30–40% increase in the hepatic bile acid pool. Because we observed approximately twofold increases in key intermediates in the pathway for bile acid synthesis from cholesterol (i.e., 7 $\alpha$ -hydroxycholest-4-en-3one; 3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -trihydroxy-5 $\beta$ -cholestanoate and 3 $\alpha$ ,7 $\alpha$ ,dihydroxy-5 $\beta$ -cholestanoate) as well as an 80% increase in CYP7A1 abundance, the rate of bile acid synthesis was almost certainly elevated in liver of *Cdo1*-null mice fed taurine-free diets. The observed upregulation of bile acid synthesis and the hepatic bile acid pool size in response to taurine depletion has not been reported previously and it is not clear whether this

response might be unique to mice. Although a diminished ability of the liver to secrete bile acids in the bile cannot be ruled out, bile acid secretion was sufficient for essentially complete fat digestion and absorption because no fat (triglyceride assay) was detectable in the colonic fecal pellets of either *Cdo1*-null or wild-type mice (data not shown). In addition, beyond the modest increase in the hepatic bile acid pool, there was no evidence for cholestasis in the *Cdo1*-null mice.

*Csad*, *Cyp7a1*, *Cyp3a11*, and *Bhmt* are genes whose expression appears to be regulated by taurine status. We have identified four genes whose hepatic expression appears to be strongly regulated by taurine status in murine liver (Fig. 10). These include *Csad*, which was highly upregulated (>6-fold) by taurine deficiency; *Cyp3A11*, which was upregulated to 2- to 6-times basal levels by taurine deficiency; *Cyp7A1*, which was upregulated to two- to threefold basal levels by taurine deficiency; and *Bhmt*, which was downregulated to less than 50% of control (~2-fold difference) by taurine deficiency. Whether *Slc6A6*, which was only mildly upregulated by about 50% by taurine deficiency, and *Ostβ*, which was variably affected by *Cdo1* genotype and taurine status in male and female mice, should be included in the list of taurine-responsive genes in mouse liver requires further study.

Although the mechanisms by which taurine deficiency regulates the expression of these genes is not known, it is clear that they are not related to tissue hypotaurine pools, as dietary taurine increases hepatic levels of taurine but not of hypotaurine (Roman et al. 2013). Mechanisms related to hepatic levels of taurine, hepatic levels of various bile acids or bile acid metabolites, or the effects of taurine depletion on cell volume are possibly involved.

Studies in several models (3T3-L1 adipocytes, MEFs, HepG2 cells, rat kidney, and rat brain) have shown that *Slc6A6* (*Taut*) expression is upregulated by hyperto-



**Fig. 10** Diagram illustrating functions of BHMT, CSDAD, CYP7A1, CYP27A, AYP3A11, SLC6A6, OSTα/β, and ABCB11. Those proteins whose expression was clearly responsive to taurine status are indicated by an arrow that also shows the direction of change in response to taurine deficiency

nicity and/or downregulated by taurine-rich conditions (Takasaki et al. 2004; Oh et al. 2006; Schaffer et al. 2000; Satsu et al. 2003; Bitoun et al. 2001; Bitoun and Tappaz 2000). In one study, SLC6A6 (TAUT) mRNA was shown to be upregulated by cholate infusion in both wild-type and *Fxr*-null mice, suggesting FXR is not required for bile acid-mediated upregulation of *Slc6a6* expression (Miyata et al. 2005). Although CSAD was not upregulated in rat kidney or rat brain in response to salt loading (Bitoun et al. 2001; Bitoun and Tappaz 2000), substantial increases in hepatic CSAD abundance in response to taurine deficiency were observed in mice and other species (Roman et al. 2013; Rentschler et al. 1986; De la Rosa and Stipanuk 1985). Recently, both CSAD mRNA and CYP7A1 mRNA levels were shown to be downregulated in mice given dietary cholate, upregulated in mice that received cholestyramine, downregulated in mice dosed with FXR agonist GW4064, and upregulated in *Shp*-null mice compared to wild-type mice (Kerr et al. 2013). These observations suggested that the FXR/SHP signaling may regulate taurine biosynthesis as well as bile acid synthesis. The possibility that *Bhmt* expression may also be regulated by FXR/SHP signaling pathways was suggested by recent reports that *Bhmt* expression was lower in mice supplemented with dietary cholate but higher in mice that received cholestyramine (Tsuchiya et al. 2015). Additionally, hepatic *Bhmt* expression in liver of *Shp*-null mice was 2.8-fold the wild-type level, and *Shp* re-expression in the *Shp*-null mice lowered the BHMT mRNA abundance back to wild-type levels (Tsuchiya et al. 2015). Overall, these studies suggest that *Csad*, *Cyp7a1*, and *Bhmt* expression could be regulated by a common mechanism involving FXR/SHP, with excess bile acid activating ligands for FXR leading to downregulation of expression of all three genes.

In *Cdo1*-null mice fed a taurine-free diet, we observed a robust increase in the abundance of both CSAD and CYP7A1, but a decrease in the abundance of BHMT. Several observations are immediately obvious compared to the suggested regulation of these genes by FXR/SHP. First, *Bhmt* expression was regulated in a different direction than *Csad* and *Cyp7a1*. Second, based on our estimation that the total bile acid pool was increased in taurine-deficient *Cdo1*-null mice, we would have expected the expression of all three genes to be downregulated, but only expression of *Bhmt* was repressed. It is not known whether the conjugation state of bile acids affects their ability to regulate expression of *Cyp7a1* or other genes, but *Cdo1*-null murine liver was dramatically depleted of taurine-conjugated bile acids even though the total bile acid concentration was not reduced. Sayin et al. (2013) recently reported that taurine conjugation is essential for the antagonistic activity of  $\alpha$ - and  $\beta$ -muricholate in FXR activation assays. Whether taurine conjugation affects the agonistic activity of any of the bile acids has not been studied in any detail. Even if the taurine-conjugation status of bile acids affects their interaction with FXR, the fact that *Bhmt* expression was regulated in the opposite direction than *Csad* and *Cyp7a1* by taurine status suggests that a mechanism other than the FXR/SHP signaling pathway may be responsible for responses to taurine deficiency.

Of the genes studied, the expression of *Csad* was the most highly regulated in response to taurine status. Unfortunately, almost nothing is known about the tran-



scriptional regulation of the *Csad* gene. *Csad* also is the only one of the four genes for which a dose-response effect related to taurine status, albeit indirectly, has been reported (Stipanuk et al. 2002). Hepatic CSAD mRNA and protein abundances responded in a dose-responsive manner to changes in dietary protein intake which were in turn associated with changes in hepatic taurine concentration.

*Cyp7a1* expression is well-known to be inhibited by bile acids (e.g., cholate, chenodeoxycholate), providing a feedback mechanism to control the rate of bile acid production (Chiang 2009; Gardès et al. 2013; Davis et al. 2002). Consistent with this, Kerr et al. (2013) suggested that bile acid binding to FXR was responsible for the repression of *Cp7a1* expression in mice given cholate or an FXR agonist, acting through FXR-induced expression of *Shp*, with SHP in turn negatively interacting with LRH-1 or other nuclear receptors that are known to regulate *Cyp7a1* expression. Clearly, this does not explain observations in the *Cdo1*-null mouse in which elevated hepatic bile acid pools were associated with upregulation, rather than downregulation, of *Cyp7a1* expression, and in which taurine-deficiency presumably was the change that promoted upregulation of bile acid synthesis, perhaps in response to a lack of taurine-conjugated bile acids.

We have no clear hypothesis for the mechanism underlying the elevation of hepatic CYP7A1 abundance in taurine-deficient *Cdo1*-null mice. In general, the regulation of bile acid synthesis is complex, being regulated by hormones, oxysterols, bile acids, drugs, and other factors including factors secreted by the intestine. The fact that muricholic acid, which comprises about half of the murine bile acid pool (Alnouti et al. 2008; García-Cañaveras et al. 2012), is an FXR-antagonistic bile acid (Hu et al. 2014) and the observation that taurine conjugation is essential for the antagonistic activity of  $\alpha$ - and  $\beta$ -muricholic acid in FXR activation assays (Sayin et al. 2013) suggest that a lack of antagonism of FXR by tauromuricholic acid is a possible explanation. Because hepatic bile acid metabolism also depends upon intestinal FXR signaling (Sayin et al. 2013; Kuribayashi et al. 2012; Miyata et al. 2013; Li et al. 2013), any effects of taurine deficiency on the secretion of bile acids in the bile, their metabolism by gut microbiota, or their reabsorption by the enterohepatic circulation could also impact bile acid-mediated regulation of gene expression.

CYP3A11, which is homologous to CYP3A4 in humans, is responsible for the conversion of chenodeoxycholic acid to muricholic acid in mice and is also responsible for metabolism of many xenobiotics. The *Cyp3a11* gene contains a pregnane X receptor (PXR), which can be activated by bile acid metabolites and a variety of other compounds. Increased abundance and activity of CYP3A11 protein and activity has been reported in mice given cholic acid (Hrycay et al. 2014), and increased expression of *Cyp3a11* was observed in response to cholic acid or ursodeoxycholic acid in mice in an FXR-independent manner (Zollner et al. 2006).

BHMT mRNA and protein levels previously have been reported to be responsive to osmotic conditions. Although not present in as high a concentration as taurine, betaine is a relatively abundant organic osmolyte in cells (Jurkowska et al. 2016; Hoffmann et al. 2013; Mong et al. 2011). In H4IIE rat hepatoma cells, *Bhmt* expres-

sion was suppressed by hyperosmotic conditions that caused cell shrinkage but enhanced by hypoosmotic conditions which also led to a decrease in intracellular betaine (Schäfer et al. 2007). This study along with our observations for liver of intact mice suggest that betaine likely functions as an organic osmolyte in cells and that its hepatic abundance can be altered by regulating the expression of BHMT.

It is interesting that bile acid status and SHP (NROB2) have been reported, independently, to have effects on both *Csad* and *Bhmt* expression. A link with bile acid metabolism is suggested by the observations that *Bhmt* expression and *Csad* expression were lower in mice supplemented with dietary cholate but higher in mice that received cholestyramine (Tsuchiya et al. 2015; Kerr et al. 2013). A link with SHP is suggested by observations of increased hepatic CSAD mRNA and hypotaurine levels (Kerr et al. 2013) and increased hepatic BHMT mRNA and protein abundances and an elevated betaine concentration (Tsuchiya et al. 2015) in liver of *Shp* (*Nr0b2*)-null mice. However, despite this suggestion that there might be a common mechanism, acting through SHP, for regulation of *Bhmt* and *Csad* expression in response to changes in bile acid metabolism, this mechanism would not account for our observations because *Csad* and *Bhmt* expression in liver of *Cdo1*-null mice changed in opposite directions in response to taurine deficiency and taurine supplementation rather than in the same direction as the SHP mechanism would predict.

## 5 Conclusion

In conclusion, we identified cysteine sulfinic acid decarboxylase (*Csad*), betaine:homocysteine methyltransferase (*Bhmt*), cholesterol 7 $\alpha$ -hydroxylase (*Cyp7a1*), and cytochrome P450 3A11 (*Cyp3a11*) as genes whose hepatic expression is strongly regulated in response to taurine depletion in the *Cdo1*-null mouse. It seems that there are a group of genes whose expression, at the level of mRNA and protein, is sensitive to taurine status. Further exploration of the ability of taurine status to regulate gene expression is needed to discern the mechanism by which taurine exerts these effects and whether it is a direct response to taurine. Exploration of the taurine sensitivity of *Csad* expression in nonhepatic tissues might be helpful, particularly because it would remove the potentially confounding effects of dramatic changes in bile acid conjugation. Given the widespread effects of taurine on diverse physiological processes, an effect of taurine on gene transcription would seem to potentially be an important avenue by which taurine exerts those effects.

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