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Cyp2b-knockdown Mice Poorly Metabolize Corn Oil and are Age-Dependent Obese

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Abstract

We previously made a RNAi-based Cyp2b-knockdown (Cyp2b-KD) mouse to determine the in vivo role of the Cyp2b subfamily in xenobiotic detoxification. Further studies reported here indicate a role for Cyp2b in unsaturated fatty acid (UFA) metabolism and in turn obesity. Mice were treated i.p. with 100 μl corn oil as a carrier or the potent Cyp2b-inducer TCPOBOP (TC) dissolved in corn oil. Surprisingly, female Cyp2b-KD mice but not male mice showed increased liver lipid accumulation. Male Cyp2b-KD mice had higher serum triacylglycerols, cholesterol, VLDL, LDL, and HDL than wildtype (WT) mice; females had higher cholesterol, LDL, and HDL. Thus, Cyp2b-KD mice are unable to clear a high bolus dose of corn oil, potentially because the Cyp2b-KD mice were unable to metabolize the UFA in the corn oil. Therefore, WT and Cyp2b-KD mice were housed for 35 weeks and necropsies performed to test whether Cyp2b-KD mice develop age onset obesity. Cyp2b-KD mice exhibited a significant increase in body weight caused by an increase in white adipose tissue deposition relative to WT mice. Serum cholesterol, triacylglycerol, LDL, and VLDL were significantly greater in 35-week old Cyp2b-KD males compared to WT males; only serum triacylglycerol and LDL were higher in females. In conclusion, changes in Cyp2b expression led to perturbation in lipid metabolism and depuration in Cyp2b-KD mice. This suggests that Cyp2b is more than a detoxification enzyme, but also involved in the metabolism of UFA, as Cyp2b-KD mice have increased body weight, fat deposition and serum lipids.

Introduction

Cyp2b members participate in the metabolism of endogenous and exogenous compounds. Xenobiotic chemicals metabolized by Cyp2b isoforms include parathion, efavirenz, chlorpyrifos, phenobarbital, nonylphenol, some PCBs, and DDT (Foxenberg et al., 2007; Hodgson and Rose, 2007; Lee et al., 1998). Evidence suggests that several endogenous chemicals are also metabolized by CYP2B members including steroid hormones, prostaglandins, and fatty acids (Du et al., 2005; Finn et al., 2009; Waxman, 1988). For example, Cyp2b's are potent arachidonic acid epoxygenases (El-Sherbeni et al., 2013;

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Keeney et al., 1998a). *Cyp2b19* in mouse and CYP2B12 in rat are primarily found in keratinocytes and important in 14,15-epoxyeicosatrienoic acid formation, which is a key factor in epithelial cornification (Du et al., 2005; Keeney et al., 1998b).

Cyp2b's are widely expressed. They are expressed in the kidney (Jarukamjorn et al., 2001), small intestine (Zhang et al., 2003), brain (Hersman and Bumpus, 2014; Johri et al., 2007), lungs (Hersman and Bumpus, 2014), heart (Isensee et al., 2007), testes, skeletal muscle (Finger et al., 2011), skin (Du et al., 2005; Keeney et al., 1998b), adipose (Yoshinari et al., 2004), and prostate (Kumagai et al., 2007). Five Cyp2b isoforms have been identified in mice (*Cyp2b9, 2b10, 2b13, 2b19, and 2b23*) compared to only one in humans (CYP2B6) (Kumar et al., 2017). However, only three Cyp2b isoforms are primarily expressed in the liver with *Cyp2b9*, *Cyp2b10*, and to a lesser extent *Cyp2b13* being the major hepatic Cyp2b isoforms (Mota et al., 2010; Peng et al., 2012). *Cyp2b19* is primarily expressed in skin (Du et al., 2005; Keeney et al., 1998b) and until recently, no study had established that *Cyp2b23* is expressed and this recent study indicates very low expression of *Cyp2b23* in the liver and only in mice younger than 21-days old (Peng et al., 2012).

The hepatic CYP also show sexual dimorphism as female mice express more *Cyp2b9* and *Cyp2b13* than males (Hernandez et al., 2009b; Wiwi et al., 2004). The sexually dimorphic expression of *Cyp2b* members is probably regulated by several transcription factors including hepatocyte nuclear factor 4α (HNF-4α) (Wiwi et al., 2004), Forkhead box A2 (Foxa2) (Hashita et al., 2008), and the constitutive androstane receptor (CAR) (Hernandez et al., 2009b; Mota et al., 2010). Of special interest are CAR (NR1I3) and Foxa2. CAR is a xenobiotic sensor that regulates the induction of *Cyp2b10*, and several other detoxification genes after exposure to a variety of chemicals, including phenobarbital or 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TC) (Hernandez et al., 2009a; Honkakoski et al., 1998; Wei et al., 2000). Foxa2, which regulates *Cyp2b9* expression, has been implicated in age-onset obesity and sporadic cases of early onset Type II diabetes (Bochkis et al., 2013). Foxa2 is activated by fasting, fatty acids, and bile acids and inhibited by insulin (Bochkis et al., 2008; Wolfrum et al., 2004). Fatty liver and diabetes activate both CAR and Foxa2 and increase drug metabolism due in part to increased Cyp2b9 and Cyp2b10 (Dong et al., 2009a; Wolfrum et al., 2004). Interestingly recent data showed that *Cyp2b9* was the most highly induced gene in liver of diet-induced obese mice (Leung et al., 2016).

CAR much like Foxa2 is also a metabolic sensor. For example, TC-activation of CAR ameliorated diabetic activity and fatty liver disease in ob/ob mice, and double mutants (ob/ob mice that are also CAR-null) did not respond to TC-mediated amelioration of diabetes and fatty liver, indicating a role for CAR in inducing β-oxidation (Dong et al., 2009b). This provides a link between drug metabolism and energy metabolism. Additionally, the hepatic P450 oxidoreductase-null mouse (hepatic reductase null, POR-null, or HRN), which lacks hepatic CYP activity, shows profound changes in lipid homeostasis and liver size (Finn et al., 2009). This is associated with significantly elevated *Cyp2b10*, and linked to hepatic triacylglycerol accumulation and increased hepatic polyunsaturated fatty acids (PUFA) in the HRN mice, indicating a crucial role for CYP in unsaturated fatty acid metabolism. Furthermore, double HRN/CAR-null mice did not demonstrate *Cyp2b10* induction, and linoleic acid, an unsaturated fatty acid, activated CAR in transactivation

assays. The authors suggest that increases in *Cyp2b10* could be an adaptive response against unsaturated fatty acid toxicity and indicated that their study is the first evidence that P450s, and particularly *Cyp2b10*, play a major role in controlling unsaturated fatty acid homoeostasis via CAR (Finn et al., 2009).

We previously constructed a Cyp2b-knockdown (Cyp2b-KD) mouse model using RNAi technology under the control of a lentiviral promoter, and demonstrated reduced expression of all three major hepatic Cyp2b isoforms (Damiri et al., 2012). The Cyp2b-KD mice developed normally and were fertile; however, we observed enlarged livers and a putative increase in abdominal fat deposition. Furthermore, our initial studies reported here indicate that the Cyp2b-KD mice do not clear corn oil from the liver as well as wildtype (WT) mice. Therefore, we examined serum parameters associated with perturbed lipid homeostasis compared to WT mice, and followed up by investigating whether Cyp2b-KD mice show increased lipid stores as they age. The endogenous role of the Cyp2b subfamily is not known (Wang and Tompkins, 2008; Yamada et al., 2006), and this study suggests a role for Cyp2b's in fatty acid metabolism, and therefore they may act as anti-obesity CYP.

Materials and Methods

Mice treatment

All studies were carried out according to NIH guidelines for the humane use of research animals and pre-approved by Clemson University's IACUC. Mice were provided water, and fed *ad libitum* with a typical chow diet (Envigo Harlan Tekland 2918) containing 3.1 kcal/g with 18% of its kcal from fat (61% PUFA/ 23% MUFA / 16% saturated fats), 58% carbohydrate, 24% protein, prior to and during treatments. Construction of Cyp2b-KD mice was described previously (Damiri et al., 2012), and the Cyp2b-KD mice were maintained on a FVB/NJ background, and bred with FVB/NJ (wild-type; WT) mice to maintain the genetic background. The shRNA construct, named Cyp2b-KD2 (GGATCCCAAGAACACTGAGGTGTACCCCTTGATATCCGGGGGTACACCTCAGTGTTCTTTTTTTTTCCAACTCGAG; underlined regions recognize the Cyp2b genes), was designed to recognize all five murine Cyp2b subfamily members. The shRNA construct was cloned into the pRNAT-U6.2/Lenti plasmid, virus produced, and injected into fertilized single cell FVB embryos as described previously (Damiri et al., 2012). The zygotes were cultured overnight and transplanted into pseudopregnant CD-1 mice the next day. shRNA integration was detected by PCR genotyping (Cyp2b-KD2; Forward-5'-GAGGGCCTATTTCCCATGAT – 3'; Reverse-5' – AGGCACAGTCGAGGCTGAT – 3') for recognition of the Cyp2b-KD construct and/or the presence of the U.6/Lentivirus (U.6/Lenti; Forward-5'- TTATCGTTTCAGACCCACCTCCCAA – 3'; Reverse-5' – TCCCATAAGGTCATGTACTGGGCA – 3') (Damiri et al., 2012).

Untreated WT and Cyp2b-KD mice at 9-weeks or 35-weeks of age were weighed, blood was collected by heart puncture, and then mice were euthanized in separate experiments. In addition, WT and Cyp2b-KD mice (9-weeks old) were treated with 3 mg/kg TCPOBOP (TC) or received 100 μl corn oil i.p. as a vehicle control. TC-treated and corn oil control mice were weighed, blood collected, and then euthanized 24 hours after treatment. Animals were weighed prior to the necropsies. During the necropsies, livers were excised and

weighed. A portion of the liver was placed in formalin for histology investigation via hemotoxylin and eosin (H&E) staining and Oil Red O evaluation of liver triacylglycerols. Another portion was snap for microsome or RNA isolation for Western blots or qPCR. Abdominal/renal/inguinal fat was also collected, pooled, and weighed.

Quantitative Real-time Polymerase Chain Reaction (qPCR):

Quantitative real-time PCR (qPCR) was performed as described previously by us and other groups (Damiri et al., 2012; Muller et al., 2002). First, Total RNA was extracted from liver tissue with Tri-Reagent according to the manufacturer's instructions (Molecular Research Center (Cincinnati, OH) followed by DNase digestion to remove residual genomic DNA (Promega Corporation, Madison, WI). RNA concentrations were determined spectrophotometrically at 260/280 nm (Molecular Devices, Ramsey, MN) prior to cDNA preparation via reverse transcription with 200 units of Moloney murine leukemia virus, 10mM of a dNTP mixture, and 0.05 mg of random hexamers (Promega). RNA was stored at −80°C and cDNA was stored at −20°C. cDNA from liver samples was diluted 1:10 prior to qPCR. To generate a standard curve and determine the PCR efficiency of each reaction, a composite sample of cDNA from FVB (WT) and Cyp2b-KD mice, both treated and untreated mice, was made and dilutions from 1:1 to $1:10^{-6}$ were prepared. During qPCR, samples were denatured at 95°C for 10 s, lowered to the appropriate annealing temperature for 30 s, and extended at 72°C for 20 s. Amplifications of the samples and the standard curve were performed in triplicate using a 96-well iQ5 Real-Time PCR Detection System (Bio-Rad) with 0.25X SybrGreen (SA Biosciences, Frederick, MD) as the fluorescent double strand-intercalating agent to quantify changes in relative gene expression as described previously using our inverted Muller's equation to determine relative quantities of each CYP (Muller et al., 2002; Roling et al., 2004). A minimum of forty cycles was run on all real-time samples to ensure a log based growth curve. 18S was used as the housekeeping gene as confirmed by taking the ratio of the quantification of the housekeeper compared to total RNA content (Bustin, 2002). Melting curve analysis was performed during qPCR runs on all of the primer sets. The primers and their annealing temperatures are presented in Table 1.

Western Blots:

Microsomes were prepared from diced livers by dounce homogenization followed by differential centrifugation including high speed microsome separation from cytosol at 100,000XG for 45 minutes as described previously (Van der Hoeven and Coon, 1974). The Bio-Rad (Hercules, CA) protein assay based on the Bradford method was used to determine protein concentrations. Microsomes were stored at −80°C. Western blots were performed with 30 μg of hepatic microsomal protein as described previously using our rabbit-anti-mouse CYP2B antibody and a goat anti-rabbit IgG (Bio-Rad) alkaline-phosphatase coupled secondary antibody (Mota et al., 2010). β-actin (Sigma Aldrich, St. Louis, MO) was used as a housekeeper to ensure equal loading of samples. Bands were visualized via chemiluminescent detection (Bio-Rad) and quantified with the Chemi-Doc XRS HQ using Quantity One 4.6.5 software (Bio-Rad). Density was determined relative to β-actin (Kumar et al., 2017).

Histopathology and Oil Red O staining

Samples fixed in 10% formalin were processed and stained with hemotoxylin and eosin or Oil Red O at Colorado Histo-Prep for blind histopathological evaluation (Fort Collins, CO). Standard toxicology pathology criteria and nomenclature for mice was used to categorize microscopic tissue changes of H&E and Oil Red O stained samples (Banks, 1993; Percy and Barthold, 2001). Individual parameters (i.e. hepatocellular swelling, necrosis, hypertrophy, hyperplasia, inflammation, bile duct hyperplasia, mineralization, hepatocellular involvement) were scored 0–4 (0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe) and then summed. Total scores for the hepatic histopathology lesions or Oil Red O staining in each mouse were ranked and significance determined by Kruskal–Wallis followed by Dunn's post hoc test.

Serum Chemistry

Serum was prepared by centrifugation as described previously (Tuck et al., 2009) and analyzed at the Comparative Pathology Laboratory at Baylor College of Medicine (Houston, TX) for levels of non-fasting cholesterol, triacylglycerols, high density lipoporotein (HDL), low density lipoporotein (LDL), very low density lipoprotein (VLDL), phosphorus, albumin, and glucose using a Beckman-Coulter AU480 analyzer and the appropriate Beckman-Coulter biochemical kits according to the manufacturer's instructions (n = 4–6).

Statistical Significance:

Data are presented as mean ± SEM. Statistical analysis was performed by ANOVA followed by Tukey's multiple comparison test as the post-hoc test when comparing more than two groups with the exception of the histopathology data that was examined by Kruskal-Wallace followed by Dunn's post-hoc test. Student's t-tests were used when comparing two groups. Statistical analysis was performed using Graphpad Prism version 4.0 (La Jolla, CA USA). A p-value ≤ 0.05 was considered statistically significant.

Results

Corn oil treated Cyp2b-KD mice have higher hepatic lipid levels:

Corn oil is a commonly used carrier for chemical treatments as it dissolves lipid soluble chemicals in a non-toxic digestible form. We injected WT and Cyp2b-KD mice with corn oil or TC dissolved in corn oil i.p., and then euthanized the mice 24 hours later. Surprisingly, we observed a significant increase in hepatic lipids within the hepatocytes (steatosis) of female Cyp-KD mice (but not male Cyp2b-KD mice) as indicated by Oil Red O staining (Fig. 1). TC provided protection from steatosis in previous studies (Dong et al., 2009b), however, TC-treatment did not provide significant protection from corn oil treatment in Cyp2b-KD female mice (Fig. 1); but the number of mice with severe effects dropped.

Corn Oil Increases Serum Lipids in Cyp2b-KD Mice:

Cyp2b-KD mice also displayed a significant increase in several serum lipid parameters, including total serum cholesterol, triacylglycerol, HDL, LDL, and VLDL compared to the corresponding WT controls (Fig. 2). Other than phosphorous, which increased 44% in corn

oil treated Cyp2b-KD males and 42% in TC-treated Cyp2b-KD males compared to WT males, no other changes (glucose, albumin) were observed between WT and Cyp2b-KD mice following exposure to corn oil or TC (data not shown).

Serum triacylglycerol and VLDL concentrations were significantly increased in male (53%), but not female Cyp2b-KD mice (21%)(Fig. 2), potentially because the lipids were stored in the livers of the females (Fig. 1). Females and males exhibited a significant increase in cholesterol (16.5%; 11.1%), HDL (42.6%; 17.3%), and LDL (48.7%; 42.1%)(Fig. 2).

The CAR activator and Cyp2b inducer, TC, reversed the effects of corn oil on cholesterol and HDL levels in male Cyp2b-KD mice. The effects of TC in female Cyp2b-KD mice were not as prominent as in male mice (Fig. 2). In addition, male TC-treated Cyp2b-KD mice did not show significant increases in triacylglycerols, LDL, and VLDL unlike the male corn oil treated Cyp2b-KD mice (Fig. 2). TC-treatment led to an increase in serum triacylglycerols (60%) and VLDL levels (67%) in TC-treated Cyp2b-KD female mice compared to TC-treated WT mice.

Changes in Cyp and metabolic transcription factor expression in Corn Oil and TC-treated WT and Cyp2b-KD Mice:

Previously we demonstrated that Cyp2b-KD mice showed repression of Cyp2b expression (Damiri et al., 2012); however, we did not investigate whether there was compensatory changes in other CYP regulated by CAR or if TC-treatment could overcome RNAi-mediated repression of Cyp2b expression. Surprisingly, corn oil-treated mice showed no significant changes in Cyp2b mRNA expression (Table 2–3), indicating that both corn oil and TC could overcome RNAi-mediated repression of Cyp2b RNA expression. Therefore, we examined Cyp2b protein expression by Western blotting, and found significant repression of Cyp2b protein levels (Fig. 3). The lack of RNA repression in Cyp2b-KD mice was not observed previously in untreated Cyp2b-KD mice where repression varied from 0 – 80% depending on the Cyp2b isoform and gender (Damiri et al., 2012). However, TC-treated mice previously showed no repression or slight induction of Cyp2b RNA expression along with repressed protein levels, indicating that the lack of Cyp protein was causing a compensatory transcriptional response and increased *Cyp2b* mRNA (Damiri et al., 2012). A similar response from corn oil suggests its components such as linoleic acid also activate CAR, which was shown previously (Finn et al., 2009). Studies have also shown that the monounsaturated fatty acid, olive oil can induce *Cyp2b9* and *Cyp2b13* (Guillen et al., 2009). Taken together, TC and unsaturated fatty acid components of corn oil are probably inducing *Cyp2b* expression; however, the shRNA is still able to cause enough destruction of the mRNA to reduce protein. In addition, there are no significant differences between WT and Cyp2b-KD female and male mice after corn oil or TC-treatment as to the expression of *Car*, *Foxa2*, *Cpt1a*, and a number of *Cyp* (Table 2–3).

Weight gain, Hepatic Histopathology, and Serum Lipids in 35-week old Cyp2b-KD mice

Because we observed what appeared to be a putative increase in adipose deposition in our initial studies (Damiri et al., 2012), and corn oil was not as quickly depurated from the liver of Cyp2b-KD mice (Fig. 1), we examined whether older Cyp2b-KD mice developed age-

onset obesity, including weight gain, adipose deposition, and serum lipids in 35-week old mice. Adipose weight from the inguinal, abdominal, and renal regions of Cyp2b-KD mice was 2.12-fold greater in males and 2.88-fold greater in females than WT controls (Fig. 4A). In addition, the Cyp2b-KD mice weighed more with females weighing 10.9% more and male weighing 22.3% more than WT counterparts (Fig. 4B), indicating a change in body composition.

To test whether Cyp2b repression caused significant perturbations in serum lipid concentrations in older mice; cholesterol, triacylglycerols, HDL, LDL, and VLDL were measured. The male 35-week old Cyp2b-KD mice exhibited a significant increase in serum cholesterol compared to WT mice (Fig. 4C). Both female and male Cyp2b-KD mice showed an increase in serum triacylglycerols (62%) compared to WT mice (Fig. 4D). Healthy HDL serum concentrations (Fig 4E) showed no change; however, serum LDL concentrations increased in males (Fig. 4F), and serum VLDL concentrations increased in both genders (Fig. 4G). Interestingly, Cyp2b-KD male mice also showed increased serum phosphorus similar to observed in corn oil treated Cyp2b-KD mice (26.6%). Overall, the age-related changes in serum lipids were more profound in Cyp2b-KD males than Cyp2b-KD females. Hypertriglyceremia is a key biomarker of metabolic disorder and the pre-diabetic condition. Therefore, we tested non-fasting blood sugar in both young and old mice. No significant changes in blood sugar or albumin were observed in the 35-week old Cyp2b-KD mice (data not shown).

There are few differences in the hepatic histopathology results between the WT and Cyp2b-KD mice. Hepatocytes from 35-week old Cyp2b-KD-female and male mice are more likely to show increased periportal hypertrophy as all of the Cyp2b-KD mice showed periportal hypertrophy and none of the WT mice showed periportal hypertrophy (Fig. 4H). However, the score was one (minimal) in each individual (Fig. 4H). In addition, Oil Red O staining results indicated no significant differences in fat accumulation in Cyp2b-KD mice compared to WT mice (data not shown).

Changes in Cyp and metabolic transcription factor expression in young and old mice:

We also examined *Cyp2b* expression for RNAi-mediated repression, and subsequent effects on other *Cyp* and metabolic transcription factors in 35-week old WT and Cyp2b-KD mice. To provide some perspective, young (9-week old) mice were also measured. As observed previously, *Cyp2b* expression is repressed in young Cyp2b-KD mice (Damiri et al., 2012) (Table 4–5); however, this repression was lost in the older mice regardless of sex (Table 4–5). Other than a similar drop in *Cyp2a4* expression in Cyp2b-KD male mice, no other significant changes in gene expression were observed for *Cyp* or metabolic transcription factors in young or old mice.

Western blots corroborate that CYP2B expression is no longer significantly repressed in the Cyp2b-KD mice as they reached 35-weeks of age (Fig. 5), indicating either the RNAi was no longer repressing expression, or more likely compensatory changes occurred such as increased lipids that led to a loss of Cyp2b suppression. While not statistically significant, the latter appears plausible based on an 8-fold increase in *Cyp2b9* and *Cyp2b13* expression in the male Cyp2b-KD mice (Table 5). This also suggests that much of the age-onset obesity

either occurred in mice at a younger age or the early effects of Cyp2b loss led to increased susceptibility to obesity as the mice aged.

DISCUSSION

Corn oil is primarily made of UFA, and contains approximately 55–57% linoleic acid (polyunsaturated), 1% linolenic acid, 30% oleic acid (monounsaturated) (85–88% total UFA), and 12–15% saturated fatty acids (US Department of Agriculture, 2007). In this manuscript, we demonstrate that Cyp2b-KD mice are age-dependent obese, show increased fat deposition, increased serum lipids, and a reduced ability to eliminate corn oil in comparison to WT mice. Female Cyp2b-KD mice treated with corn oil show greater hepatic Oil Red O staining indicative of higher lipid levels than WT mice injected with corn oil. TC-treatment was unable to ameliorate the increased liver lipids in females, which may in part be caused by lower Cyp2b induction in knockdown mice (Damiri et al., 2012).

Serum lipids are also increased by corn oil-treatment in Cyp2b-KD mice compared to their WT counterparts with TC-treatment providing little amelioration with more changes in males than females (Fig. 2). Slightly greater concentrations of triacylglycerols and VLDL were measured in the serum following TC-treatment in females. This suggests that TC is initiating elimination of lipids, most likely from the liver into the serum. However, in males TC-treatment ameliorates serum cholesterol and HDL. It is interesting that Cyp2b-KD females showed greater perturbations in lipids in the liver and males showed greater perturbations in the serum and this may be due to the gender predominant expression and induction of Cyp2b isoforms in female liver measured in multiple strains including FVB/NJ mice (Hashita et al., 2008; Hernandez et al., 2006; Hernandez et al., 2009b; Mota et al., 2011; Wiwi et al., 2004). Overall, the positive effects of TC-treatment on serum parameters in males were not reproduced in females (Fig. 2), indicating a sexually dimorphic difference in the mice possibly due to higher constitutive Cyp2b levels in the female mice. It is also possible that the greater serum effects measured in males may also have provided an environment more likely to show TC-mediated amelioration (Fig. 2).

A single high dose of corn oil was not cleared as well in Cyp2b-KD mice as it was WT mice. Interestingly, excess sunflower oil in the diet of hepatic POR-null mice (HRN) caused fatty liver presumably because of a lack of CYP activity and the inability to metabolize and eliminate fatty acids (Finn et al., 2009). Sunflower oil is 69% PUFA with linoleic acid the primary PUFA. Oleic acid (20%), and saturated fatty acids (11%) comprise the rest of the sunflower oil, similar to corn oil. Furthermore, mice treated with sunflower oil or linoleic acid show significant increases in *Cyp2b10* presumably through linoleic acid activation of CAR (Finn et al., 2009). However, the POR-null mice treated with sunflower oil had lower serum cholesterol and triacylglycerols than WT mice treated with sunflower oil, presumably due to poor depuration from the liver in the mice lacking all CYP activity (Finn et al., 2009).

Cyp2b enzymes are highly regulated by the metabolic transcription factors CAR and Foxa2 (Hashita et al., 2008; Honkakoski et al., 1998; Wei et al., 2000), and their expression is also regulated by other transcription factors such as GR, STAT5b and HNF4α (Audet-Walsh and Anderson, 2009; Nakamura et al., 2007; Wiwi et al., 2004). It has been demonstrated that

fatty liver and diabetes activate CAR and Foxa2 and increase drug metabolism due in part to increased Cyp2b9 and Cyp2b10 activity (Bochkis et al., 2013; Dong et al., 2009a; Hashita et al., 2008; Wolfrum et al., 2004). CAR activation also modulates hepatic metabolism and lowers hepatic triacylglycerols and serum glucose levels (Dong et al., 2009b), while protecting mice from diet induced obesity (Gao et al., 2009). However, others have shown that CAR activation by TC led to increased serum triacylglycerols in mice fed a high fat diet, potentially due to depuration of liver fatty acids (Maglich et al., 2009). Our studies show that the CAR activator TC partially reversed serum hyperlipidemia in male but not female Cyp2b-KD mice. The TC-induced reversal in male mice is probably due to the regulation of multiple genes, and Cyp2b's appear to play a crucial role (Fig 1,2).

Corn oil and other vegetable oils are relatively common carriers for toxicology studies and 100 μl injections are common. However, this level of lipid in one dose is quite high. For example, 100 μl in a 25 g mouse is equivalent by weight to 300 ml in a 75 kg human; an unrealistic and incredibly heavy dose of fatty acids for one meal. There are almost certainly physiological and toxicological consequences to the high dose of unsaturated fatty acids as this amount is likely to activate CAR and induce CYP (Finn et al., 2009). This could confound some toxicology studies. However, within 24 hours, WT mice appeared to acclimate and clear the high amounts of unsaturated fat, while the Cyp2b-KD mice did not fully acclimate to the corn oil treatment and in turn showed greater Oil Red O staining in female livers and higher triacylglycerols, cholesterol, LDL, HDL, and VLDL in male serum with higher cholesterol, HDL, and LDL in female serum.

Cyp2b isoforms metabolize linoleic acid (Bylund et al., 1998) and arachidonic acid into epoxides (Maayah et al., 2014), and in turn produce epoxyeicosatrienoic acids that are critical in kerotinocyte development (Du et al., 2005; Ladd et al., 2003). Cyp2b induction in the liver during exposure to lipids or in POR-null mice (Finn et al., 2009; Wang et al., 2005) indicates other roles for Cyp2b enzymes in the metabolism of unsaturated fatty acids. In addition, it suggests that toxicants that inhibit Cyp2b metabolism may disrupt unsaturated fatty acid metabolism.

The increased serum lipids in Cyp2b-KD mice following corn oil treatment may manifest itself as increased lipid deposition and obesity as the mice age. Therefore, age-onset obesity was followed. 35-week old Cyp2b-KD mice showed increased white adipose tissue and weighed significantly more than WT mice (Fig. 4). Both female and male Cyp2b-KD mice showed much greater levels of white adipose tissue and both female and male Cyp2b-KD mice weighed more than their WT counterparts. However, male weight differences were much greater than female weight differences. Similar to treatments with corn oil, the male Cyp2b-KD mice showed more robust effects on serum lipids, as female Cyp2b-KD mice did not demonstrate higher serum cholesterol or LDL concentrations. Obesity, increased abdominal fat deposition, and increased serum triacylglycerols are all key symptoms of prediabetic conditions and associated with metabolic syndromes and indicate that the lack or inhibition of Cyp2b's has metabolic consequences. Taken together, males with low Cyp2b levels appear to be more susceptible to age-onset obesity even though they express less Cyp2b enzymes (Hernandez et al., 2006; Kumar et al., 2017).

Foxa2 mutants also develop age-onset obesity in males (Bochkis et al., 2013) even though Foxa2 is a female predominant transcription factor that regulates the expression of *Cyp2b9* and potentially *Cyp2b13* (Bochkis et al., 2013; Hashita et al., 2008). The Foxa2 mutants show perturbed bile acid and growth hormone signaling most likely due to disruptions in the interactions of Foxa2 with Stat5b, FXR, or PXR (Bochkis et al., 2013; Nakamura et al., 2007). Interestingly, *Cyp2b9* is also the most induced gene in the liver (Leung et al., 2016) and brown fat (McGregor et al., 2013) of diet-induced obese mice. Furthermore, db/db mice also show increased *Car* and *Cyp2b10* expression (Yoshinari et al., 2006). Interestingly, the older Cyp2b-KD mice lost their RNAi-mediated Cyp2b repression and this may be lipid-mediated induction or compensation that is consistent with induction of Cyp2b members following exposure to corn oil (Finn et al., 2009), a high-fat diet (Leung et al., 2016; McGregor et al., 2013), or a compensatory mechanism due to changes in key metabolites (Hernandez et al., 2009b; Kumar et al., 2017; Mota et al., 2011). Taken together, Cyp2b9, Cyp2b10, and the transcription factors they are regulated by (Foxa2/CAR) are associated with obesity and age-onset obesity.

The endogenous role of the Cyp2b subfamily is poorly understood (Wang and Tompkins, 2008). We propose that hepatic Cyp2b's are increased in response to unsaturated fatty acids (Finn et al., 2009) and are important in the regulation, signaling, or depuration of unsaturated fatty acids in addition to their role as detoxification CYP. Thus, they either directly metabolize and depurate unsaturated fats as suggested previously (Finn et al., 2009), or they produce a key signaling molecule from unsaturated fatty acids that regulate the use of lipids. Therefore, significant inhibition of Cyp2b activity could decrease lipid metabolism, increase fatty liver, and induce obesity by increasing fat deposition in individuals eating a high-fat diet. Cyp2b substrates that act as inhibitors or competitors such as atrazine, polychlorinated biphenyls, nonylphenol, diazinon, parathion, and chlorpyrifos may be obesogens (Acevedo et al., 2005; Hodgson and Rose, 2007). In fact, there is data implicating several potential Cyp2b inhibitors such as polychlorinated biphenyls, nonylphenol, bisphenol A, chlorpyrifos, diazinon, and parathion in increased fat deposition, pre-diabetic states, and obesity (Lassiter et al., 2008; Masuno et al., 2003; Slotkin, 2011; Wada et al., 2007; Wahlang et al., 2013). Furthermore, metabolic changes in in the livers of endosulfan-treated mice are consistent with diabetes and insulin resistance (Canlet et al., 2013).

Overall, our data indicate an influence of Cyp2b isoforms on the homeostasis of serum and hepatic lipids. Recent reports from other laboratories provide supporting data. For example, transcription factors that regulate Cyp2b such as CAR and Foxa2 are nutrient sensors involved in energy homeostasis and lipid metabolism (Bochkis et al., 2013; Finn et al., 2009; Gao et al., 2009; Wolfrum et al., 2004), and hepatic deletion of P450 oxidoreductase (POR; HRN mouse) led to a profound time and CAR-dependent induction of *Cyp2b10* associated with fatty liver (Finn et al., 2009). In conclusion, our findings indicate that Cyp2b-KD mice are unable to either utilize or metabolize unsaturated fatty acids efficiently which leads to an accumulation of central fat and the potential for metabolic disorders.

Acknowledgments

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Abbreviations:

CAR:	constitutive androstane receptor
cDNA:	complimentary DNA
Cpt1a:	carnitine palmitoyltransferase 1a
Cyp2b-KD:	Cyp2b-knockdown
Cyp2b:	Cytochrome P450 2b
DDT:	1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane
dNTP:	deoxynucleotide triphosphate
Foxa2:	forkhead box a2; also known as HNF3B, hepatocyte nuclear factor 3 beta
H&E:	hematoxylin and eosin
HDL:	High-density lipoprotein
IACUC:	Institutional Animal Care and Use Committee
LDL:	Low-density lipoprotein
i.p.:	intraperitoneal
PCBs:	polychlorinated biphenyls
PCR:	polymerase chain reaction
PUFA:	polyunsaturated fatty acid
qPCR:	Quantitative real-time PCR
shRNA:	short hairpin RNA
TCPOBOP; TC:	3,3′,5,5′-Tetrachloro-1,4-bis(pyridyloxy)benzene
T_m:	Annealing temperature
UFA:	Unsaturated fatty acid
VLDL:	very low-density lipoprotein
WT:	wildtype

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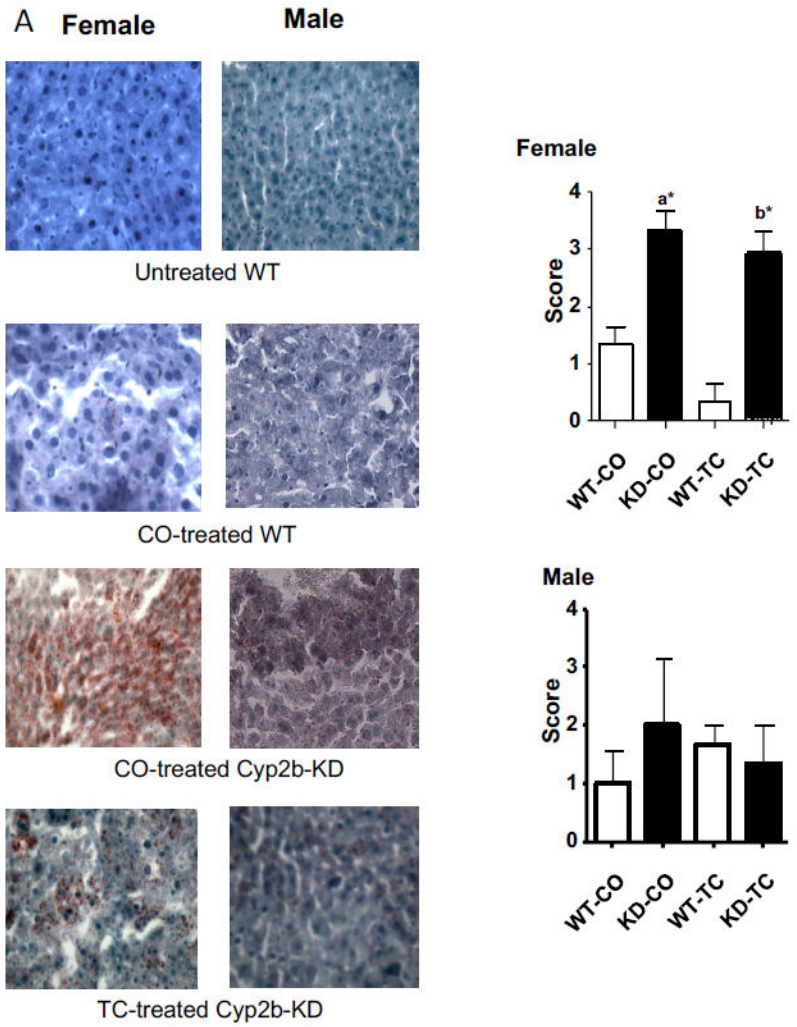

Fig. 1: Lipid deposition in livers of Cyp2b-KD and WT mice treated with corn oil.

(A) Oil Red O staining of the hepatocytes of WT and Cyp2b-KD mice treated with corn oil or TC. (B) Average Oil Red O staining scores for female and male WT and Cyp2b-KD mice (n = 3). An [a] indicates a significant difference between WT and Cyp2b-KD mice treated with corn oil. A [b] indicates a significant difference between WT and Cyp2b-KD mice treated with TC. An asterisk indicates significant difference with a $p < 0.05$ as determined by Kruskal-Wallace followed by Dunn's post-hoc test using the GraphPad Prism 4.0 software package. WT= wild-type, KD= Cyp2b-KD mice, M=male, F= female. CO = Corn oil, TC = TCOPBOP.

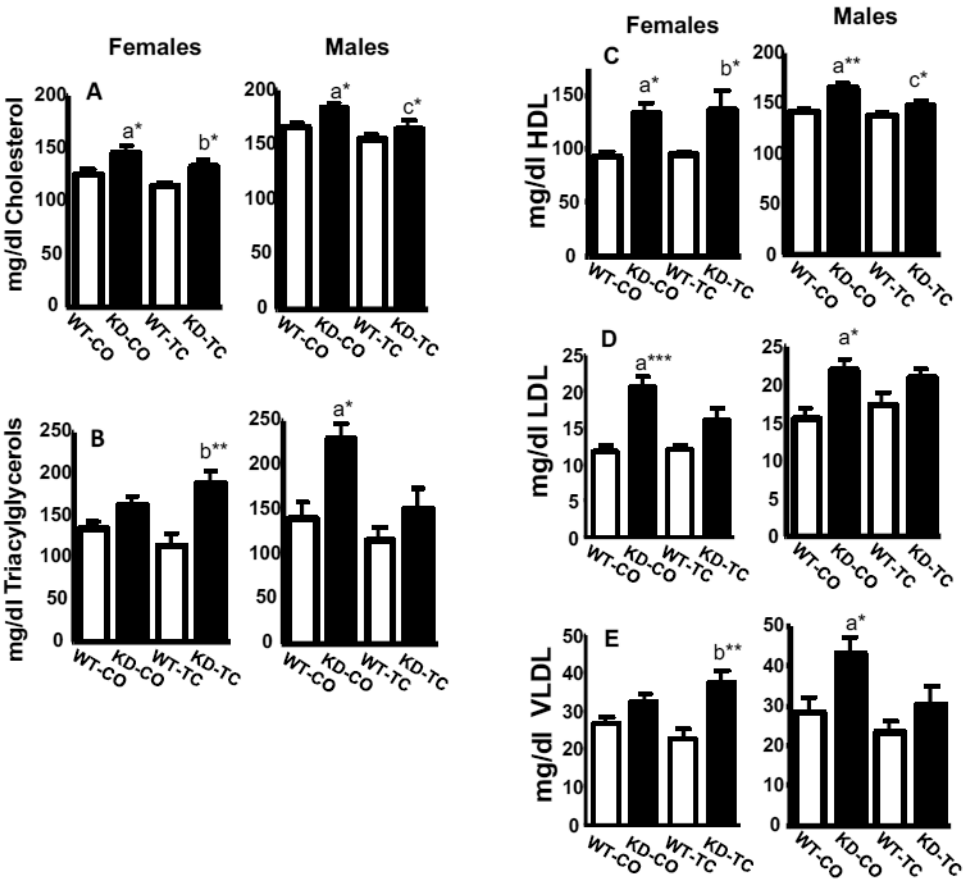

Fig. 2: Non-fasting serum cholesterol, triacylglycerols, and lipoprotein profiles in WT and Cyp2b-KD mice after corn oil and TC-treatments.

Measured (A) cholesterol, (B) triacylglycerols, (C) HDL, (D) LDL, and (E) VLDL concentrations in WT and Cyp2b-KD female and male mice following treatment with corn oil or TC. Significant differences between WT and Cyp2b-KD mice were assessed by ANOVA followed by Tukey's multiple comparison tests using GraphPad Prism 4.0 (n = 4–6). An asterisk indicates a significant difference with a $p < 0.05$, two asterisks indicate a $p < 0.01$, and three asterisks indicate a $p < 0.001$. The letter [a] indicates a significant difference between WT and Cyp2b-KD mice treated with corn oil, the letter [b] indicates a significant difference between WT and Cyp2b-KD mice treated with TC, and the letter [c] indicates a significant difference between Cyp2b-KD mice treated with corn oil and Cyp2b-KD mice treated with TC. WT = wild-type, KD= Cyp2b-KD mice, M = male, F= female, CO = corn oil, TC= TCPOBOP.

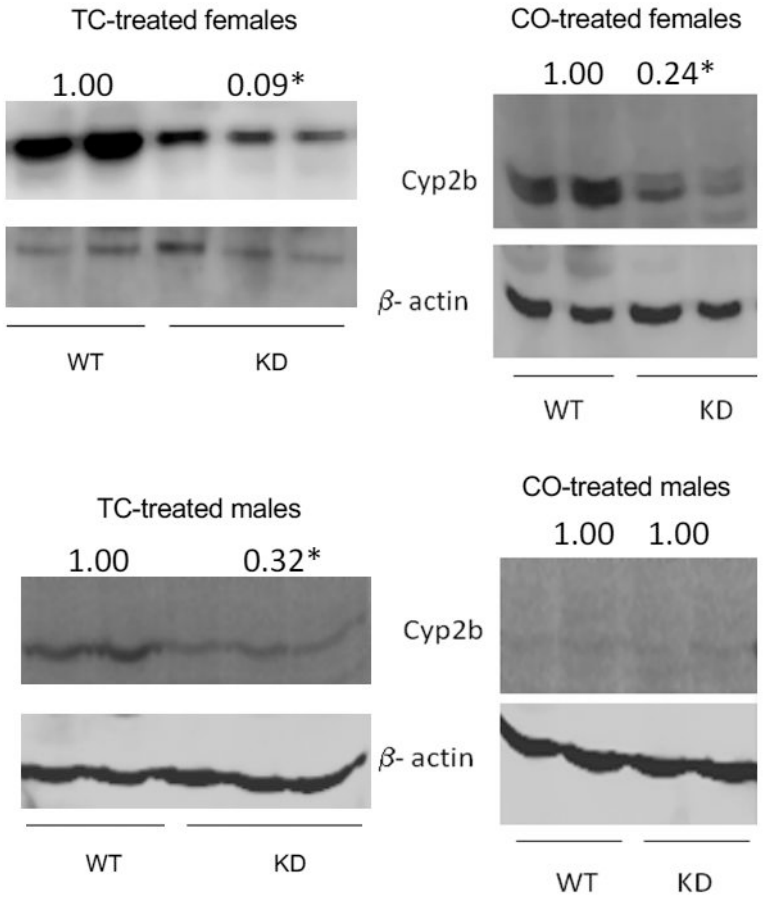

Fig. 3: Western blots from microsomes of female and male WT and Cyp2b-KD (KD) mice treated ip with either corn oil (CO) or TCPOBOP (TC).

Results are expressed as relative mean and statistical differences were determined by Student's t-tests using GraphPad Prism 4.0 with an asterisk indicating a p-value < 0.05.

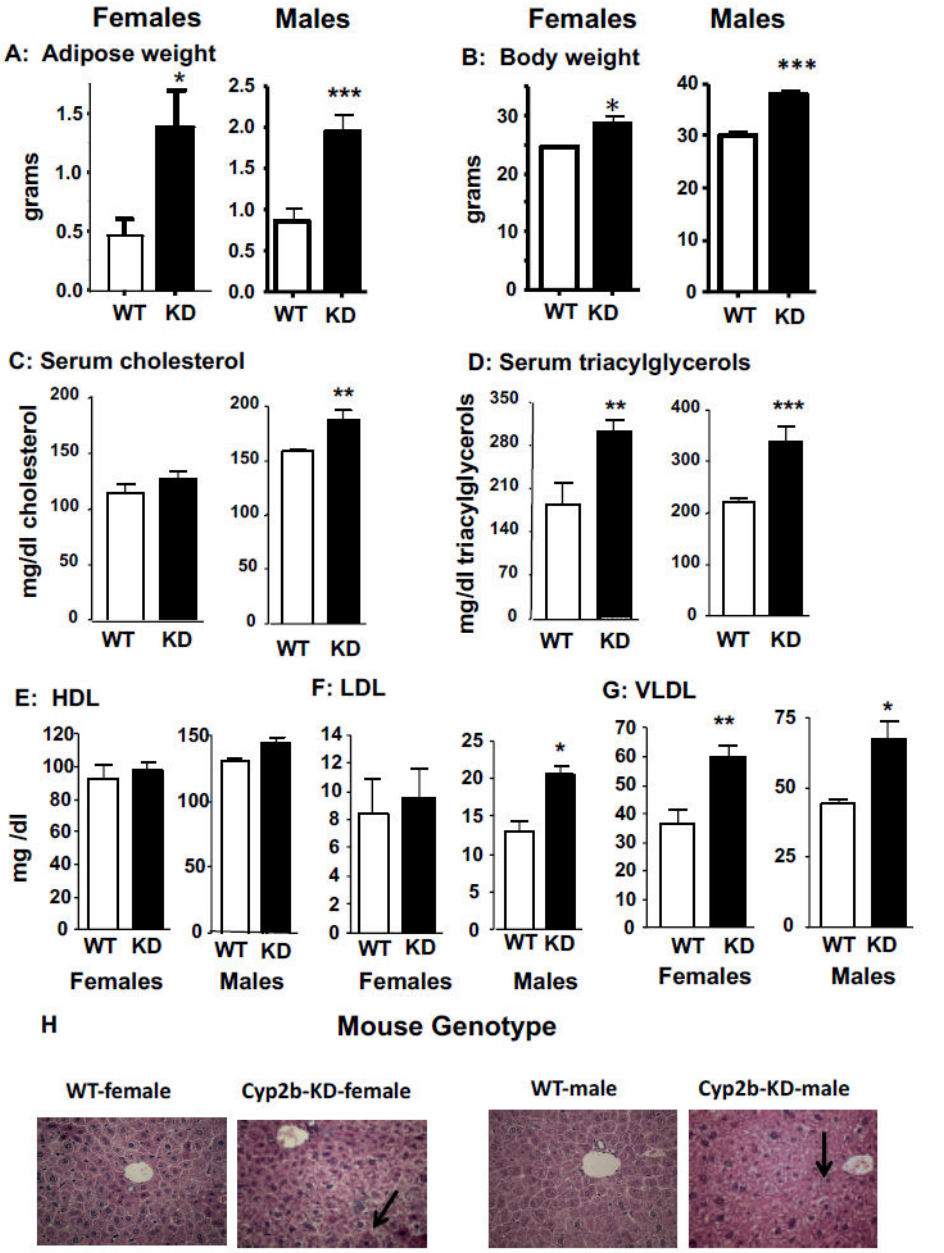

Fig. 4: Weight, serum, and hepatic differences between 35-week old WT and Cyp2b-KD mice.

(A) Adipose weight, (B) body weight (C), serum cholesterol, (D) triacylglycerols, (E) HDL, (F) LDL, and (G) VLDL concentrations, and (H) histopathology observations in female and male WT mice compared to female and male Cyp2b-KD mice at 35-weeks of age show minor periportal hypertrophy in Cyp2b-KD mice. Significant differences between WT and Cyp2b-KD mice were assessed by Student's t-test (n = 9–11). An asterisk indicates a significant difference with a $p < 0.05$, two asterisks indicate a $p < 0.01$, and three asterisks indicate a $p < 0.001$. WT = wild-type mice, KD = Cyp2b-KD mice, M = male, F= female.

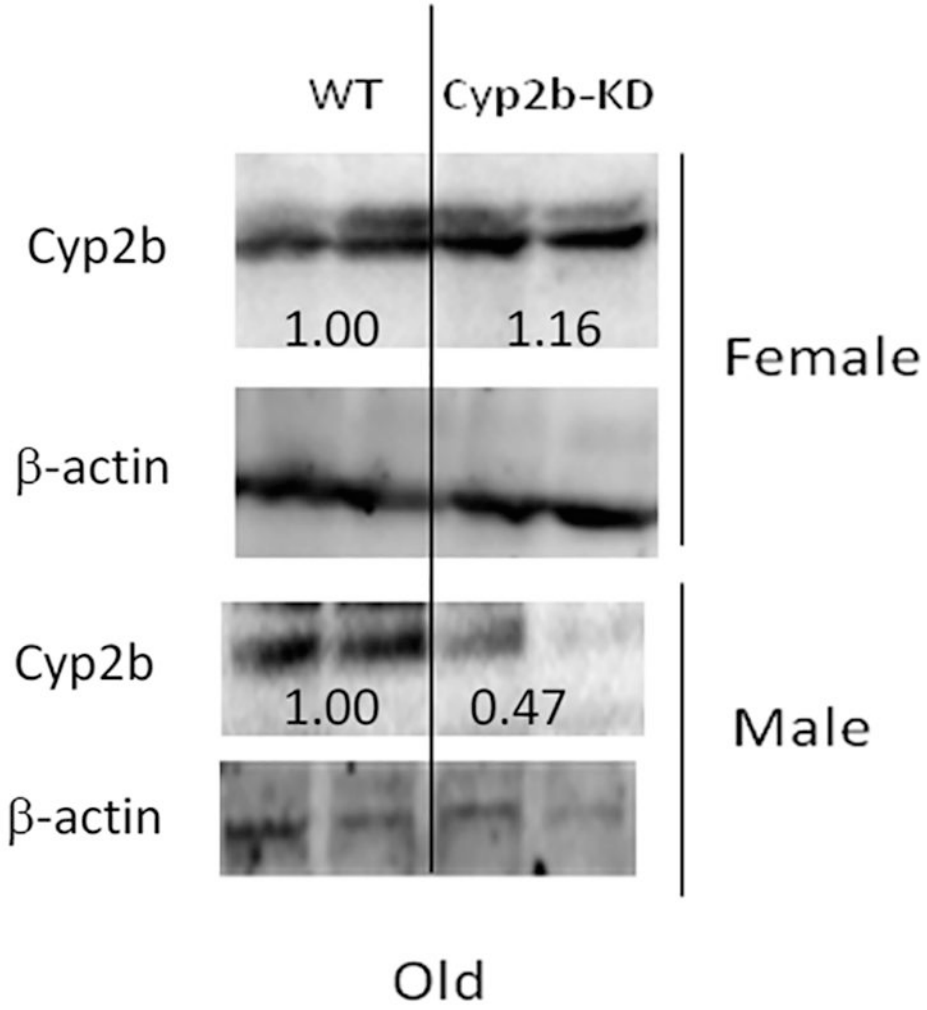

Fig. 5: Western blots from microsomes of female and male WT and Cyp2b-KD mice after 35-weeks (old) of age.

Results are expressed as relative mean. Student's t-tests were performed using GraphPad Prism 4.0. No significant differences were detected.

Table 1:

PCR primer sequences used in qPCR

Gene	Forward primer	Reverse primer	Tm (°C)	Reference
Car	GGAGCGGCTGTGGAAATATTGCAT	TCCATCTTGTAGCAAAGAGGCCCA	56.5	This manuscript
Cpt1a	TTGATCAAGTGCCGGACGAGT	GTCCATCATGGCCAGCACAAAGTT	55.5	This manuscript
Cyp2a4	AGCAGGCTACCTTCGACTGG	GCTGCTGAAGGCTATGCCAT	63.6	(Wiwi et al., 2004)
Cyp2b9	CTGAGACCACAAGCGCCAC	CTTGACCATGAGCAGGACTCC	64.3	(Wiwi et al., 2004)
Cyp2b10	CTGAATCCGCTCCTCCACACTC	TGAGCCAACCTTCAAGGAATAT	61.4	(Hernandez et al., 2006)
Cyp2b13	GAACTGAGACTACCAGCACCACTCCT	TGAGCATGAGCAGGAAACCACT	61.5	(Wiwi et al., 2004)
Cyp2c29	GGCCTCAAAGCCCTACTGTCA	AACGCCAAAACCTTTAATC	53.6	(Luo et al., 1998)
Cyp3a11	CTTTCCTTCACCCTGCATTCC	CTCATCCTGCAGTTTTTTCTGGAT	64.2	(Wiwi et al., 2004)
Foxa2	TCAACGACTGCTTTCTCAAGGTGC	TTCTCGAACATGTTGCCCGAGTCT	57.8	(Wiwi et al., 2004)
18s rRNA	CGCCGCTAGAGGTGAAATTC	CCAGTCGGCATCGTTTATGG	51	(Hernandez et al., 2006)

T_m: Annealing Temperature

Table 2:

qPCR measured expression of Cyp and metabolic transcription factors in corn oil (CO)- and TC-treated, WT and Cyp2b-KD (KD) female mice.

Females	Treated (8–12) weeks			
	WT-CO	KD-CO	WT -TC	KD-TC
Cyp2b9	1.0 ± 0.05	2.23 ± 0.35[a,**]	1.07 ± 0.02	0.13 ± 0.03[d,***]
Cyp2b10	1.0 ± 0.03	2.14 ± 0.70	51.0 ± 8.39[c,**]	135.0 ± 25.2[d,***]
Cyp2b13	1.0 ± 0.28	2.31 ± 0.59	0.59 ± 0.08	1.74 ± 0.27
Cyp2a4	1.0 ± 0.38	0.96 ± 0.22	1.54 ± 0.34	1.05 ± 0.41
Cyp2c29	1.0 ± 0.10	4.63 ± 1.56	45.8 ± 9.93	126.0 ±32.9
Cyp3a11	1.0 ± 0.22	1.74 ± 0.49	7.19 ± 2.08[c,***]	20.6 ± 1.83[b,d,***]
Car	1.0 ± 0.24	3.1 ± 1.03	0.46 ± 0.08	2.66 ± 0.55
Foxa2	1.0 ± 0.40	4.2 ± 1.17	0.53 ± 0.23	3.0 ± 0.75
Cpt1a	1.0 ± 0.19	3.9 ± 0.99	0.41 ± 0.23	1.3 ± 0.28

Data are presented as mean ± SEM normalized to WT-CO treated mice. Significant changes in gene expression were determined by ANOVA followed by Tukey's multiple comparison tests using the GraphPad Prism 4.0 software package (n = 5–6).

*P value <0.05

**< 0.01

***< 0.001

a: WT-CO vs KD-CO

b: WT-TC vs KD-TC

c: WT-CO vs WT-TC

d: KD-CO vs KD-TC

Table 3:

qPCR measured expression of Cyp and metabolic transcription factors in corn oil (CO)- and TC-treated, WT and Cyp2b-KD (KD) male mice.

Males	Treated (8–12) weeks			
	WT-CO	KD-CO	WT -TC	KD-TC
Cyp2b9	1.0 ± 0.92	19.0 ± 7.05	91.1 ± 29.8	13.8 ± 6.61
Cyp2b10	1.0 ± 0.37	6.31 ± 2.33	514 ± 171[a,*]	552 ± 138[b,*]
Cyp2b13	1.0 ± 0.98	3.37 ± 2.53	5.34 ± 4.52	0.136 ± 0.07
Cyp2a4	1.0 ± 0.20	1.19 ± 0.42	1.5 ± 0.65	0.37± 0.11
Cyp2c29	1.0 ± 0.54	0.043 ± 0.023	13.4 ± 5.1	21.7 ± 8.5
Cyp3a11	1.0 ± 0.42	0.46 ± 0.15	2.9 ± 1.36	2.88 ± 0.45
Car	1.0 ± 0.47	1.74 ± 0.39	4.41 ± 1.74	1.34 ± 0.46
Foxa2	1.0 ± 0.51	5.79 ± 2.2	9.67 ± 7.03	2.60 ± 0.77
Cpt1a	1.0 ± 0.34	1.33 ± 0.32	0.59 ± 0.16	1.84 ± 0.55

Data are presented as mean + SEM normalized to WT-CO treated mice. Significant changes in gene expression were determined by ANOVA followed by Tukey's multiple comparison tests using the GraphPad Prizm 4.0 software package (n = 5–6).

*P value <0.05

**< 0.01

***< 0.001

*a:*WT-CO vs WT-TC

*b:*KD-CO vs KD-TC

Table 4:

qPCR measured expression of Cyp and metabolic transcription factors in young (9-week old) and old (35-week old) WT and Cyp2b-KD (KD) female mice.

Females	Untreated (8–12) weeks		Old 35-weeks	
	WT	KD	WT	KD
Cyp2b9	1.0 ± 0.18	1.33 ± 0.24	1.0 ± 0.30	0.98 ± 0.18
Cyp2b10	1.0 ± 0.29	0.33 ± 0.09 *	1.0 ± 0.24	0.61 ± 0.11
Cyp2b13	1.0 ± 0.07	0.46 ± 0.09 *	1.0 ± 0.49	3.11 ± 1.12
Cyp2a4	1.0 ± 0.03	0.81 ± 0.15	1.0 ± 0.53	1.10 ± 0.15
Cyp2c29	1.0 ± 0.29	1.05 ± 0.34	1.0 ± 0.27	0.74 ± 0.12
Cyp3a11	1.0 ± 0.29	1.03 ± 0.22	1.0 ± 0.79	1.17 ± 0.25
Car	1.0 ± 0.30	0.30 ± 0.30	1.0 ± 0.27	0.85 ± 0.09
Foxa2	1.0 ± 0.21	0.42 ± 0.08	1.0 ± 0.36	1.34 ± 0.55
Cpt1a	1.0 ± 0.16	0.85 ± 0.23	1.0 ± 0.31	0.84 ± 0.15

Data are presented as mean ± SEM normalized to untreated mice at 9- or 35-weeks of age. Significant changes in gene expression were determined by Student's t-tests using the GraphPad Prism 4.0 software package (n = 5–6).

* P value <0.05

** < 0.01

*** < 0.001

Table 5:

qPCR measured expression of Cyp and metabolic transcription factors in young (9-week old) and old (35-week old) WT and Cyp2b-KD (KD) male mice.

Males	Untreated (9) weeks		Old 35-weeks	
	WT	KD	WT	KD
Cyp2b9	1.0 ± 0.28	0.29 ± 0.31**	1.0 ± 0.43	8.76 ± 2.89
Cyp2b10	1.0 ± 0.31	0.33 ± 0.08	1.0 ± 0.26	2.12 ± 0.99
Cyp2b13	1.0 ± 0.30	0.21 ± 0.03***	1.0 ± 0.26	8.75 ± 5.43
Cyp2a4	1.0 ± 0.19	0.48 ± 0.12*	1.0 ± 0.53	0.20 ± 0.03
Cyp2c29	1.0 ± 0.32	1.19 ± 0.41	1.0 ± 0.28	1.79 ± 0.5
Cyp3a11	1.0 ± 0.18	1.43 ± 0.64	1.0 ± 0.11	1.49 ± 0.48
Car	1.0 ± 0.18	0.89 ± 0.24	1.0 ± 0.22	1.44 ± 0.54
Foxa2	1.0 ± 0.23	0.60 ± 0.12	1.0 ± 0.23	0.83 ± 0.21
Cpt1a	1.0 ± 0.32	0.85 ± 0.29	1.0 ± 0.32	0.65± 0.41

Data are presented mean ± SEM normalized to untreated 9-week old mice. Significant changes in gene expression were determined by Student's t-tests using the GraphPad Prism 4.0 software package (n = 5–6).

* P value <0.05

** < 0.01

*** < 0.001