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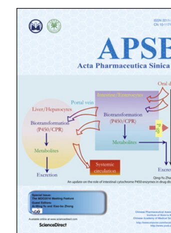

ORIGINAL ARTICLE

Characterizing drug-metabolizing enzymes and transporters that are *bona fide* CAR-target genes in mouse intestine

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Abstract Intestine is responsible for the biotransformation of many orally-exposed chemicals. The constitutive androstane receptor (CAR/Nr1i3) is known to up-regulate many genes encoding drug-metabolizing enzymes and transporters (drug-processing genes/DPGs) in liver, but less is known regarding its effect in intestine. Sixty-day-old wild-type and $Car^{-/-}$ mice were administered the CAR-ligand TCPOBOP or vehicle once daily for 4 days. In wild-type mice, *Car* mRNA was down-regulated by TCPOBOP in liver and duodenum. $Car^{-/-}$ mice had altered basal intestinal expression of many DPGs in a section-specific manner. Consistent with the liver data (Aleksunes and Klaassen, 2012), TCPOBOP up-regulated many DPGs (*Cyp2b10, Cyp3a11, Aldh1a1, Aldh1a7, Gsta1, Gsta4, Gstm1-m4, Gstt1, Ugt1a1, Ugt2b34, Ugt2b36*, and *Mrp2–4*) in specific sections of small intestine in a CAR-dependent manner. However, the mRNAs of *Nqo1* and *Papss2* were previously known to be up-regulated by TCPOBOP in liver but were not altered in intestine. Interestingly, many known CAR-target genes were highest expressed in colon where CAR is minimally expressed, suggesting that additional regulators are involved

Abbreviations: Aldh, aldehyde dehydrogenase; Asbt, solute carrier family 10, member 2 (apical sodium/bile acid cotransporter); CAR, constitutive androstane receptor; cDNA, complementary DNA; CITCO, 6-(4-chlorophenyl)imidazo [2,1-*b*](1,3)thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime; Cq, quantification cycle; Cyp, cytochrome P450; ddCq, delta delta Cq; DPGs, drug-processing genes (genes that encodes drug metabolizing enzymes or transporters); Gst, glutathione *S*-trasnferase; H3, Histone 3; hCAR, human constitutive androstane receptor; HRP, horseradish peroxidase; Mrp, multi-drug resistance-associated protein (ABC transporter family C member); Nqo1, NAD(P)H dehydrogenase quinone 1; Nrf2, nuclear factor erythroid 2-related factor 2; Oatp, organic anion transporting polypeptide (solute carrier organic anion transporter family member); Papss2, 3′-phosphoadenosine 5′-phosphosulfate synthase 2; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with 0.05% tween 20; PPARα, peroxisome proliferator activated receptor alpha; PVDF, polyvinylidene difluoride; qPCR, quantitative polymerase chain reaction; ST buffer, sucrose Tris buffer; Sult, sulfotransferase; TCPOBOP, 3,3′,5,5′-tetrachloro-1,4-bis(pyridyloxy)benzene; Ugt, UDP glucuronosyltransferase; WT, wild-type

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in regulating their expression. In conclusion, CAR regulates the basal expression of many DPGs in intestine, and although many hepatic CAR-targeted DPGs were *bona fide* CAR-targets in intestine, pharmacological activation of CAR in liver and intestine are not identical.

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1. Introduction

Oral administration is the preferred route by patients due to its convenience, price, comfort, and handling[1]. The orally-administered drugs may undergo extensive first-pass metabolism in the gastro-intestinal tract, and this may result in limited systematic bioavailability, and decreased therapeutic effects[2,3]. Absorption of orally administered drugs takes place primarily in the small intestine, followed by delivery to the liver *via* the portal blood. The small intestine is efficient in the absorption of a wide-spectrum of chemicals due to the high concentration of villi and microvilli in the order of duodenum, jejunum, and ileum[4], abundant epithelial transporters, optimal pH for absorption, high peristalsis, high blood flow, as well as contact for a long time. Alteration or failure to maintain one of these conditions may result in lower bioavailability of the drug[3,5,6]. In addition to the small intestine, the large intestine may also be important for the absorption of xenobiotics, especially oral drugs formulated for sustained release[6]. The bacteria in the large intestine contain various enzymes that metabolize xenobioitcs as well as endogenous chemicals such as bile acids and dietary constituents[7,8]. In addition, colon-specific oral drug-delivery systems have been utilized recently to administer a variety of therapeutic agents[9]. Therefore, it is important to determine the regulation of xenobiotic biotransformation in the intestine.

The drug-processing genes involved in the xenobiotic biotransformation include various phase-I and phase-II drug metabolizing enzymes, as well as uptake and efflux transporters. In general, the content of DPGs is lower in intestine than that in liver[10]. DPGs play a critical role in the absorption, metabolism, disposition, elimination and detoxification of xenobiotics and other drugs[11]. Phase-I enzymes catalyze hydrolysis, reduction, and oxidation of drugs. The cytochrome P450s (CYPs) in the first 3 families are among the most important phase-I enzymes that contribute to the biotransformation of the majority of xenobiotics, whereas the CYP4 family members are important for fatty acid metabolism. The NAD(P)H dehydrogenase, quinone 1 (*Nqo1*) is involved in reduction reactions, and it is a prototypical target gene of the oxidative stress sensor nuclear factor erythroid 2-related factor 2 (NRF2). Aldehyde dehydrogenases (ALDHs) are important phase-I enzymes for the detoxification of aldehydes, which are the metabolites of alcohols. Phase-II metabolism refers to the conjugation reactions that generally increase the water-solubility of substrates to form more polar compounds with exceptions. The three major classes of phase-II enzymes include UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), as well as glutathione *S*-transferases[12]. Whereas some drugs diffuse into the intestinal cells, there are two major classes of transporters, namely the solute carriers (SLC) and ATP-binding cassette (ABC) transporters, that are important in the disposition of many large and/or polar chemicals. Intestinal transporters mediate the translocation of chemicals in and out of enterocytes, and this process is important for drug disposition in the body[13].

DPGs can be trans-activated by various nuclear receptors following xenobiotic exposure. The constitutive androstane receptor (CAR/Nr1i3) is one of the important xenobiotic-sensing nuclear receptors that regulate the transcription of DPGs. CAR is activated by various chemicals including steroid hormones, bile acids, pharmaceuticals, as well as environmental, dietary, and occupational chemicals[14], *via* direct or indirect mechanisms. Direct activation of CAR refers to ligand-binding to the CAR protein, and the prototypical CAR ligands include TCPOBOP for the mouse CAR and CITCO for the human CAR. The indirect activation of CAR by chemicals such as phenobarbital disassociates CAR from its cytosolic repressor protein. CAR activation leads to its nuclear translocation and binding to the targeted response elements of genes, and this usually leads to the transcriptional up-regulation of DPGs. Chronic activation of CAR is known to cause liver tumor in rodents but to a much lesser extent in humans[15,16]. Pharmacological activation of CAR by TCPOBOP has also been shown to reduce obesity in mice[17]. *Car*$^{-/-}$ mice have been engineered to determine the necessity of CAR in xenobiotic metabolism and liver physiology[18]. Phenobarbital-mediated up-regulation of the prototypical CAR-target gene *Cyp2b10* does not occur in livers of *Car*$^{-/-}$ mice, and there is also decreased metabolism of the classic CYP substrate zoxazolamine, as well as a complete loss of the liver hypertrophic and hyperplastic responses to CAR-inducers.

CAR is highly expressed in liver, but it can also be detected at high amounts in the small intestine, and a lower amount in the large intestine[19–21]. Extensive studies have been done regarding the effect of CAR-activation on the hepatic DPG expression[18,22–24]. Despite the important role of the intestine in xenobiotic biotransformation, relatively less is known regarding the effect of genetic depletion of *Car* and the pharmacological activation of CAR on the basal and inducibility of DPGs in different sections of intestine. Therefore, the goal of this study was to determine whether the well-known CAR-targeted DPGs in liver are also regulated by CAR in duodenum, jejunum, ileum, and colon.

2. Materials and methods

2.1. Chemicals and reagents

The mouse CAR ligand 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (3,3′,5,5′-tetrachloro-1,4-bis(pyridyloxy)benzene, TCPOBOP) and corn oil were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Animal procedures

C57BL/6 wild-type (WT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Breeder pairs of the *Car*$^{-/-}$

mice in the C57BL/6 background were obtained from Amgen (Thousand Oaks, CA, USA). Mice were housed according to the American Animal Association Laboratory Animal Care Guidelines, and were bred under standard conditions at the University of Washington (WA, USA). All animals were given *ad libitum* access to water and irradiated Picolab Rodent Diet 20 number 5053 (PMI Nutrition International, Brentwood, MO, USA). Sixty-day-old wild type and $Car^{-/-}$ male mice were administered the CAR-ligand TCPOBOP (3 mg/kg, i.p.), or vehicle, once daily for 4 days ($n=4$–5 per group). Various sections of intestine (duodenum, jejunum, ileum, and colon) were collected on the 5th day. The tissues were frozen immediately in liquid nitrogen and then stored in a –80 °C freezer prior further analysis. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington.

2.3. RNA isolation

Total RNA was isolated from each section of frozen intestine tissues using RNA zol Bee reagent (Tel-Test Inc., Friendswood, TX, USA) directed by the manufacturer's protocol. The total RNA concentration of each sample was quantified spectrophotometrically at 260 nm using a NanoDrop 1000 Sectrophotometer (Thermo Scientific, Waltham, MA, USA). The RNA integrity of each sample was evaluated by formaldehyde agarose gel electrophoresis, and assured by appearance of 18S and 28S rRNA bands.

2.4. Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

Total RNA was reverse-transcribed to cDNA by High-Capacity cDNA Reverse Transcription Kits 1001073 (Applied Biosystems, Foster, CA, USA) in a final volume 10 μL containing 5 μL of RNA sample and 5 μL of 2 × RT master mix directed by the manufacturer's protocol. The cDNAs were diluted 10 times and amplified by PCR, using the SsoAdvanced Universal SYBR Green Supermix in a Bio-Rad CFX384 Real-Time PCR Detection System (Bio-rad, Hercules, CA, USA). Two μL of cDNA were added to 8 μL of PCR mix, containing SsoAdvanced Universal SYBR Green Supermix (2 ×), forward and reverse primers, and nuclear-free water. The primers for PCR were designed using the NCBI Primer Design Tool as shown in Table 1, and were purchased from the Integrated DNA Technologies (Coralville, IA, USA). Data were expressed as percentage of the housekeeping gene β-actin.

2.5. Western blotting analysis

Each section of intestinal homogenate was prepared using 250 mL ST buffer (250 mmol/L sucrose, 10 mmol/L Tris base, pH 7.5) with protease inhibitors. The crude membranes were prepared from each section of frozen intestine samples as described previously[25]. The protein concentrations in each section of intestines were determined by a Qubit Protein Assay Kit (Thermo Fisher Scientific, Grand Island, NY, USA) as directed by the manufacturer's instructions. The samples were subjected to polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane for 3 h on the ice. After transfer, the membranes were blocked by 5% nonfat dry milk in phosphate-buffered saline with 0.05% Tween 20 (PBST) for 1 h and incubated overnight with the following primary antibodies diluted in 1% dry milk in PBST: rabbit anti-mouse CYP2B10 polyclonal antibody (AB9916, 1:5000, EMD Millipore); or goat anti-mouse H3 polyclonal antibody (ab12079, 1:500, Abcam). After washing with 1% dry milk in PBST, the membranes were incubated for 1 h with a 1:2000 HRP-linked species-appropriate secondary antibody (Sigma Aldrich, St. Louis, MO, USA) diluted in 1% dry milk in PBST. After incubation, the membranes were washed again with 1% dry milk in PBST and then with 1% PBS, followed by incubation in Novex ECL Chemiluminescent Substrate Reagent Kit (Life Technologies, Carlsbad, CA, USA).

2.6. Statistical analysis

For RT-qPCR analysis, data among multiple groups were analyzed using analysis of variance (ANOVA) followed by the Duncan's *post hoc* test ($P<0.05$) using the SPSS software (IBM Cooperation, Armonk, North Castle, NY, USA). Asterisk (*) indicates statistically significant differences between control and TCPOBOP-treated wild-type mice. Pound sign (#) indicates statistically significant differences ($P<0.05$) between control and TCPOBOP-treated $Car^{-/-}$ mice. Dollar sign ($) indicates significant differences ($P<0.05$) in the basal mRNA expression of DPGs between control WT and control $Car^{-/-}$ mice. Protein density was quantified using Image J Software (National Institutes of Health, Bethesda, MD, USA).

3. Results

3.1. DPG expressions in intestine

BioGPS[26] data were examined to determine which DPSs are highly expressed in the small and large intestine (Fig. 1). Those DPGs that were expressed in the intestine were selected (Table 2) to examine their mRNA expression in response to the CAR-ligand TCPOBOP in intestine, based on the following selection criteria: (1) the DPGs have been shown to be *bona fide* CAR-target genes in mouse liver[24], or (2) the DPGs that were not examined in liver[24] but are highly expressed in small or large intestine (http://biogps.org/)[26,27].

The relative mRNA abundance of the major DPG families in the small and large intestine are shown in Fig. 1. Data were retrieved from BioGPS as described above. For the *Cyp1* family, *Cyp1a1* appeared to be the major intestinal *Cyp1* isoform and its mRNA was much higher in small intestine than that in large intestine. *Cyp1a2* mRNA was minimally expressed in intestine (Fig. 1a), although it was shown to be the major *Cyp1* isoform in liver[28]. *Cyp1b1* was expressed at low levels and it was also small intestine-enriched. For the *Cyp2b* family, *Cyp2b10* was the only isoform that was expressed in intestine, and its mRNA was predominantly found in the small intestine but not in large intestine (Fig. 1b). For the *Cyp3a* family, the mRNAs of *Cyp3a11*, *3a13*, and *3a25* were highly expressed in small intestine; *Cyp3a13* mRNA was also detected at low levels in the large intestine, whereas other *Cyp3a* isoforms (including *Cyp3a16*, *3a41a*, and *3a44*) were not expressed in intestine (Fig. 1c). For the *Cyp4* family, *Cyp4b1* was the major intestinal isoform in both small and large intestine. *Cyp4a10* and *4a31* mRNAs were also detected at low levels in the large intestine, whereas they were minimally expressed in the small intestine. Other *Cyp4* isoforms (including *Cyp4a12a* and *4a14*) were minimally expressed in intestine (Fig. 1d). The *Nqo1* mRNA appeared to be higher than *Nqo2* mRNA in both small and large intestines (Fig. 1e). For the *Aldh*

Table 1 RT-qPCR primer sequences

Gene Symbol	Forward	Reverse
Cyp1a1	GGCCACTTTGACCCTTACAA	CAGGTAACGGAGGACAGGAA
Cyp2b10	AAGGAGAAGTCCAACCAGCA	CTCTGCAACATGGGGGTACT
Cyp3a11	ACAAACAAGCAGGGATGGAC	GGTAGAGGAGCACCAAGCTG
Cyp3a13	AAGTACTGGCCAGAGCCTGA	AATGCAGTTCCTTGGTCCAC
Cyp3a25	GCCTTGCTTCAAACCAGAAG	CATCATAGCCCCCGAAGATA
Cyp4a10	CACACCCTGATCACCAACAG	TCCTTGATGCACATTGTGGT
Cyp4b1	CTGCATGGCCCTTTATCCTA	GAAGCACTCCTTCATGCACA
Nqo1	TATCCTTCCGAGTCATCTCTAGCA	TCTGCAGCTTCCAGCTTCTTG
Aldh1a1	CTCTGTTCCCCAGGTGTTGT	CATGCAAGGGTGCCTTTATT
Aldh1a7	TGCTATTTGGCTGTCCCTGT	ACCATGTTCGCCCAGTTCTC
Aldh1b1	TTGAACGAATCCTGGGCTAC	CCGAAGACTGTGGGTTTGAT
Aldh3a2	CACCACCCAAAGTCTGTGTG	AAGATGCTCTGAGTGGCCTT
Aldh3b1	CCCAACCTGGGCAGAATCAT	GTAGCGCTCTCCCTCATCAC
Aldh9a1	AGCTGAAGACGGTGTGTGTG	CCCAAAGCCTGGATGTAAGA
Sult1a1	GGATGTAGCTGAGGCAGAGG	CAGCTCCCAGTGGCATTTAT
Sult1b1	GGTGGGAAAAGAGGGAAGAG	AAGGCCTCTTCATCCAAGGT
Sult1c2	GACCCCAGAACTGAGCAGAC	AGCTGGCATCTCATTGGCTT
Sult1d1	GCCGTCTCCTCGAATAGTGA	TTCCCACCAGCTCTTCACAT
Sult2b1	AAGGCATTCTTCAGCTCCAA	GAAGGAACTGGTCGGGTGTA
Sult5a1	CCAGTCCAAGATGGGTGACT	AGACCAGGGTTGTAGCATGG
Papss2	ACCTTGGAGACCGAAGGTTT	TTCTTGGCAACAATGAACCA
Gsta1	CGCCACCAAATATGACCTCT	TTGCCCAATCATTTCAGTCA
Gsta2	AGCCCGTGCTTCACTACTTC	CAATCTCCACCATGGGCACT
Gsta4	TGATGATGATTGCCGTGGCT	ACGAGAAAAGCCTCTCCGTG
Gstt1	CTTGCTCTACCTGGCACACA	CTTCTCCGAAGGCCCGTATG
Gstt2	ACTAAGGTCCTGGGCTGGAT	TGGATAGCTGACACCTGCTG
Gstt3	TCCAGCTGCGTACCATAGAG	ACACTCTCTGCCAAGACGAA
Gstm1	CTCCCGACTTTGACAGAAGC	TTGCTCTGGGTGATCTTGTG
Gstm2	ATGGTTTGCAGGGAACAAGGT	CTTCAGGCCCTCAAAGCGAC
Gstm3	AGAGGAGGAGAGGATCCGTG	GGGACTGCAGCAGACTATCAT
Gstm4	TATGACACTGGGTTACTGGGACATC	TCCACGCGAATCTTCTCTTCC
Gstm5	GGTTTGCAGGAGAAAAGCTG	CCTTCAGGTTTGGGAACTCA
Ugt1a1	CACCTGAAGCCTCAATACCAT	CAGTCCGTCCAAGTTCCACC
Ugt1a9	CTGGTTCAGCCAGAGGTTTC	TTGGCGACAATTAATCCACA
Ugt2b34	AGCTGCCAAAGCAGTCATTT	GCCAGGATCACATCAAACCT
Ugt2b35	GCTCAACTGCTCCAGATTCC	GGCCACCTAATCCTGACAAA
Ugt2b36	TGTGGGAAGGTGTTGGTATGG	TCCACAGCCTTTGCAAAAATAA
Oatp2a1	GGTGCCCATTCAGCCATTTG	GTGTCCACTCTGCCGTAGTC
Asbt	TGGAATGCAGAACACTCAGC	GCAAAGACGAGCTGGAAAAC
Mrp2	TCCTAGACAGCGGCAAGATT	GCTAGAGCTCCGTGTGGTTC
Mrp3	TGGTCATGCTGTCAGCTTTC	AAGGACTGAGGGGAACGAAT
Mrp4	GCAAAGCCCATGTACCATCT	ACCACGGCTAACAACTCACC

Figure 1 Expression of drug processing genes (DPGs) in small and large intestine of mice. The mRNA abundance of DPGs was retrieved from BioGPS (Biogps.org, Su et al.[26]). The abundance of DPGs was expressed as average probe intensity from the microarray data in BioGPS. DPGs that were highly expressed in at least one section of intestine and/or are known as CAR-target genes (Aleksunes and Klaassen[24]) in liver were selected for the analysis ($n=2$ per tissue). For each graph, black bar represents DPG expression in the small intestine whereas red bar represents DPG expression in the large intestine.

family, *Aldh1a1*, *1b1*, and *9a1* were the predominant isoforms and in general, they were more highly expressed in small intestine than in large intestine. *Aldh1a7*, *1l1*, *3a2*, and *6a1* were also expressed at low levels in both the small and large intestine, *Aldh16a1* and *18a1* were lowly expressed in small intestine but minimally expressed in large intestine, whereas other *Aldh* isoforms (including *Aldh1a2*, *1a3*, *1l2*, *2*, *3a1*, *3b1*, *3b2*, *4a1*, *5a1*, *7a1*, and *8a1*) were minimally expressed in small and large intestine (Fig. 1f).

For the *Gsta* family, *Gsta1* appeared to be the major intestinal isoform and its mRNA was much higher in small intestine than in large intestine. *Gsta4* mRNA was also detected at low levels in both small and large intestine. *Gsta2* and *Gsta3* were minimally expressed in intestine (Fig. 1g). For the *Gstm* family, *Gstm1, 2, and 5* were highly expressed in large intestine*;* *Gstm1, 2, 3,* and *5* were also detected at low levels in small intestine, whereas other *Gstm* isoforms (including *Gstm4*, *m6*, and *m7*) were minimally expressed in small and large intestine (Fig. 1h). For the *Gstt* family, *Gstt1*, *2*, and *3* mRNAs were the predominant isoforms in both small and large intestine, whereas *Gstt4* was minimally expressed in small and large intestine (Fig. 1i). For the *Sult* family, *Sult1b1*, *1d1*, and *2b1* appeared to be highly expressed in small intestine with the mRNA abundance in descending order; *Sult1b1* and *1d1* were also expressed at high levels in the large intestine. *Sult1a1* was only expressed in large intestine but not in small intestine, and its mRNA was highly enriched in the large intestine. *Sult1c2* and *2b1* were also lowly expressed in the large intestine. Other *Sult* isoforms (including *Sult1e1*, *2a2*, *3a1*, *4a1*, *5a1*, and *6b1*) were minimally expressed in small and large intestine (Fig. 1j). For the *Ugt* family, *Ugt1a* transcripts (note: probes were not specific to differentiate various *Ugt1a* isoforms) and *Ugt2b34* mRNA appeared to be the major *Ugt* genes expressed in small and large intestine. *Ugt1a* transcripts were more highly expressed in large intestine than small intestine. *Ugt2b5* and *2b35* were lowly expressed in both small and large intestines. *Ugt2a3* was detected at low levels only in the small intestine, whereas *Ugt2b38* and *3a2* were expressed at low levels only in large intestine. Other *Ugt* isoforms were minimally expressed in intestine (Fig. 1k).

For the Solute carrier organic anion (*Slco*) transporter family (also known as the *Oatps*), *Slco2a1*, *2b1*, *3a1*, and *4a1* were the intestine-enriched *Slco* isoforms and they were more highly expressed in small than in large intestine. Other *Slco* isoforms (including *Slco1a1, 1a4, 1a5, 1a6, 1b2, 1c1, 5a1, 6b1, 6c1*, and *6d1*) were not expressed in intestine (Fig. 1l). For the *Abcc* family (*Mrp*), *Abcc3* appeared to be the major intestinal isoform and its mRNA was approximately twice in large intestine as that in small intestine. *Abcc1* was detected at low levels in large intestine,

Table 2 Liver and Intestine regulation difference in *Car*-null control mice compared to WT control mice.

Gene symbol	Full name	Categories	Liver*	Duodenum	Jejunum	Ileum	Colon
Cyp1a1	Cytochrome P450 oxidase 1a1	Phase-I		↑	↑		
Cyp2b10	Cytochrome P450 oxidase 2b10	Phase-I					↑
Cyp3a11	Cytochrome P450 oxidase 3a11	Phase-I			↑	↑	
Cyp3a13	Cytochrome P450 oxidase 3a13	Phase-I		↓			
Cyp3a25	Cytochrome P450 oxidase 3a25	Phase-I		↓	↓	↓	
Cyp4a10	Cytochrome P450 oxidase 4a10	Phase-I					
Cyp4b1	Cytochrome P450 oxidase 4b1	Phase-I		↓		↓	↓
Nqo1	NAD(P)H dehydrogenase, quinone 1	Phase-I			↑		
Aldh1a1	Aldehyde dehydrogenase 1 family member A1	Phase-I				↓	
Aldh1a7	Aldehyde dehydrogenase 1 family member A7	Phase-I					
Aldh1b1	Aldehyde dehydrogenase 1 family member B1	Phase-I			↑		↑
Aldh3a2	Aldehyde dehydrogenase 3 family member A2	Phase-I		↓		↓	
Aldh3b1	Aldehyde dehydrogenase 3 family member B1	Phase-I		↑	↑		
Aldh9a1	Aldehyde dehydrogenase 9 family member A1	Phase-I					
Sult1a1	Sulfotransferase family 1A member 1	Phase-II					
Sult1b1	Sulfotransferase family 1B member 1	Phase-II		↓			↑
Sult1c2	Sulfotransferase family 1C member 2	Phase-II				↓	
Sult1d1	Sulfotransferase family 1D member 1	Phase-II		↑	↑	↓	↓
Sult2b1	Sulfotransferase family 2B member 1	Phase-II		↓			↑
Sult5a1	Sulfotransferase family 5A member 1	Phase-II					
Papss2	3′-Phosphoadenosine 5′-phosphosulfate synthase 2	Phase-II					
Gsta1	Glutathione *S*-trasnferase alpha 1	Phase-II					
Gsta2	Glutathione *S*-trasnferase alpha 2	Phase-II					
Gsta4	Glutathione *S*-trasnferase alpha4	Phase-II	↑				
Gstm1	Glutathione *S*-trasnferase mu 1	Phase-II					
Gstm2	Glutathione *S*-trasnferase mu 2	Phase-II					
Gstm3	Glutathione *S*-trasnferase mu 3	Phase-II				↓	
Gstm4	Glutathione *S*-trasnferase mu 4	Phase-II					
Gstm5	Glutathione *S*-trasnferase mu 5	Phase-II					
Gstt1	Glutathione *S*-transferase theta 1	Phase-II				↓	
Gstt2	Glutathione *S*-transferase theta 2	Phase-II					
Gstt3	Glutathione *S*-transferase theta 3	Phase-II		↑	↑		
Ugt1a1	UDP glucuronosyltransferase 1 family, polypeptide A1	Phase-II				↓	
Ugt1a9	UDP glucuronosyltransferase 1 family, polypeptide A9	Phase-II					↓
Ugt2b34	UDP glucuronosyltransferase 2 family, polypeptide B34	Phase-II				↓	
Ugt2b35	UDP glucuronosyltransferase 2 family, polypeptide B35	Phase-II				↓	
Ugt2b36	UDP glucuronosyltransferase 2 family, polypeptide B36	Phase-II				↓	
Oatp2a1	Solute carrier organic anion transporter family member 2A1	Transporters					
Asbt	Solute carrier family10, member 2 (sodium/bile acid cotransporters)	Transporters					
Mrp2	ABC transporter C family member 2	Transporters					
Mrp3	ABC transporter C family member 3	Transporters			↑	↑	↓
Mrp4	ABC transporter C family member 4	Transporters			↑	↑	

Basal expression of genes is shown as increased or decreased relative to that in *Car*-null mice. Up-regulation suggests CAR suppresses the basal expression of the gene and down-regulation suggests CAR is necessary in maintaining the constitutive expression of the gene.
* Note: The liver data were obtained from Aleksunes and Klaassen[24], 2012.

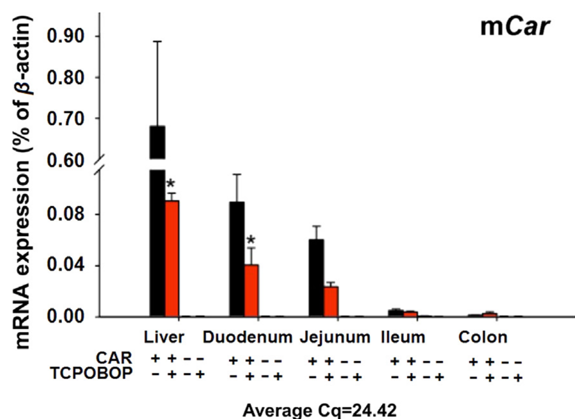

Figure 2 Messenger RNA expression of *Car* in mice liver and intestine. The *Car* mRNA in liver and various sections of the intestine was quantified using RT-qPCR as described in Section 2 (WT mice only). Data are expressed as percentage of the housekeeping gene β-actin (n=4–5 per group). Data between control and TCPOBOP-treated groups were analyzed using a Student's t-test. Asterisks (*) indicate statistically significant differences ($P<0.05$) between control and TCPOBOP-treated WT mice in the same tissue.

whereas *Abcc2* was detected at low levels in small intestine. *Abcc4*, *5, 9,* and *10* were also expressed at low levels in both small and large intestine. Other *Abcc* isoforms (including *Abcc6, c8*, and *c12*) were not detected in the intestine (Fig. 1m).

In summary, BioGPS has identified distinct DPG isoforms that are expressed in the intestine. Based on this information, as well as the previous findings regarding known CAR-targeted DPGs in liver[24], DPGs listed in Table 2 were selected for the induction studies in WT and $Car^{-/-}$ mice.

Previously, it has been shown that CAR is highly expressed in liver[24], and the basal expression of CAR was also found at relatively high levels in various sections of intestine[19,21]. To further determine the distribution of CAR in intestine as compared to liver in control and TCPOBOP-treated conditions, and to confirm the depletion of *Car* in intestine of the $Car^{-/-}$ mice, the *Car* mRNA was quantified in various sections of intestine and liver from WT and $Car^{-/-}$ mice treated with corn oil or TCPOBOP (Fig. 2). CAR was most highly expressed in liver of the WT mice, followed by duodenum and jejunum, whereas ileum and colon had very low levels of *Car* expression. TCPOBOP down-regulated the *Car* mRNA in liver and duodenum of WT mice, and tended to decrease in jejunum, although a statistically significant difference was not achieved. As expected, the *Car*

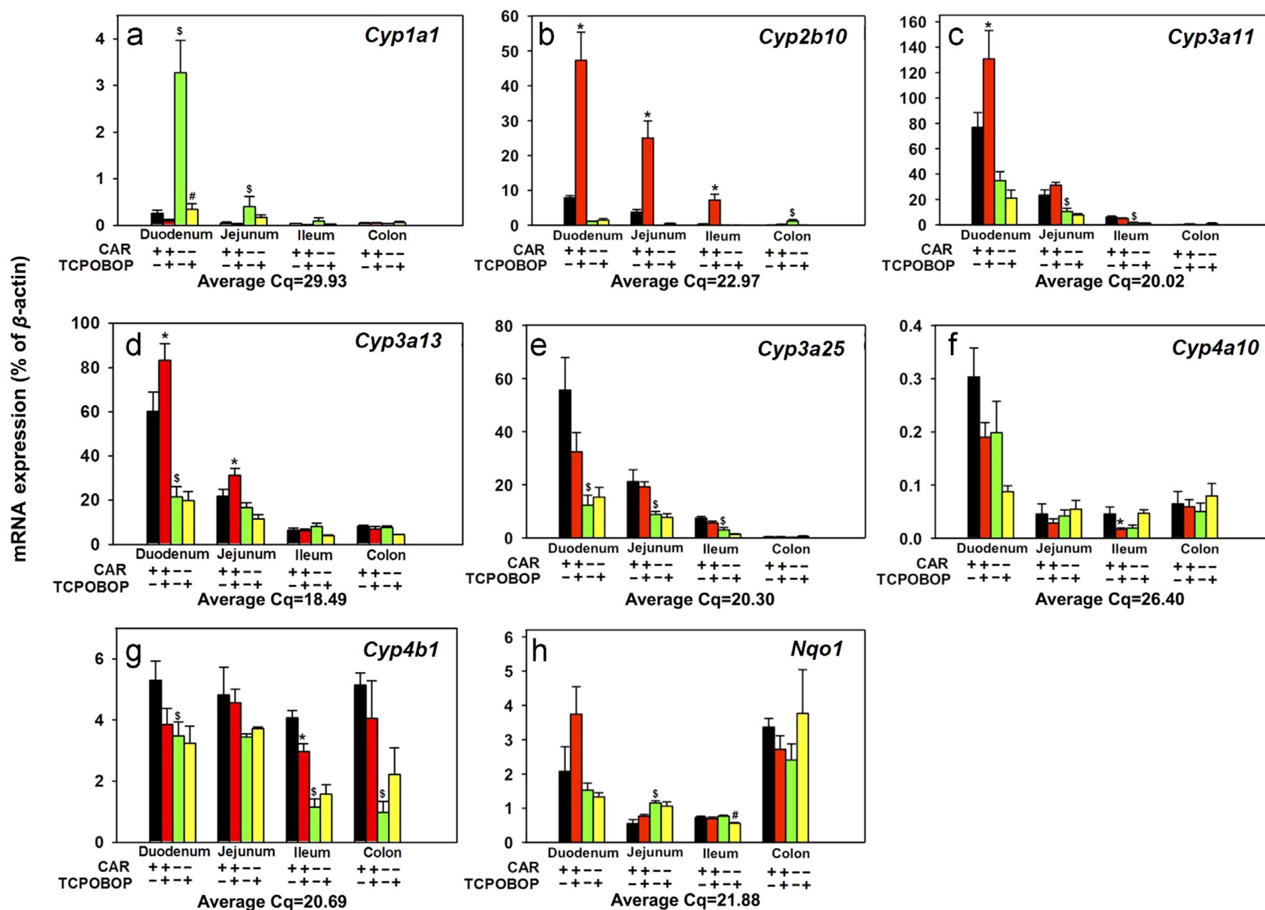

Figure 3 Messenger RNA expression of phase-I drug-metabolizing enzymes, including *Cyp1–4* and *Nqo1* in various sections of intestine (duodenum, jejunum, ileum, and colon) of WT and $Car^{-/-}$ male mice treated with vehicle (corn oil) or TCPOBOP as described in Section 2. Data are expressed as percentage of the housekeeping gene β-actin (n=4–5 per group). Data among multiple groups were analyzed using ANOVA followed by Duncan's *post hoc* test. Asterisks (*) indicate statistically significant differences ($P<0.05$) between control WT and TCPOBOP-treated WT mice at the same section of intestines. Pound signs (#) indicate statistically significant differences ($P<0.05$) between control $Car^{-/-}$ and with TCPOBOP-treated $Car^{-/-}$ mice. Dollar signs ($) indicate statistically significant differences ($P<0.05$) of the basal mRNA expression between control WT and control $Car^{-/-}$ mice.

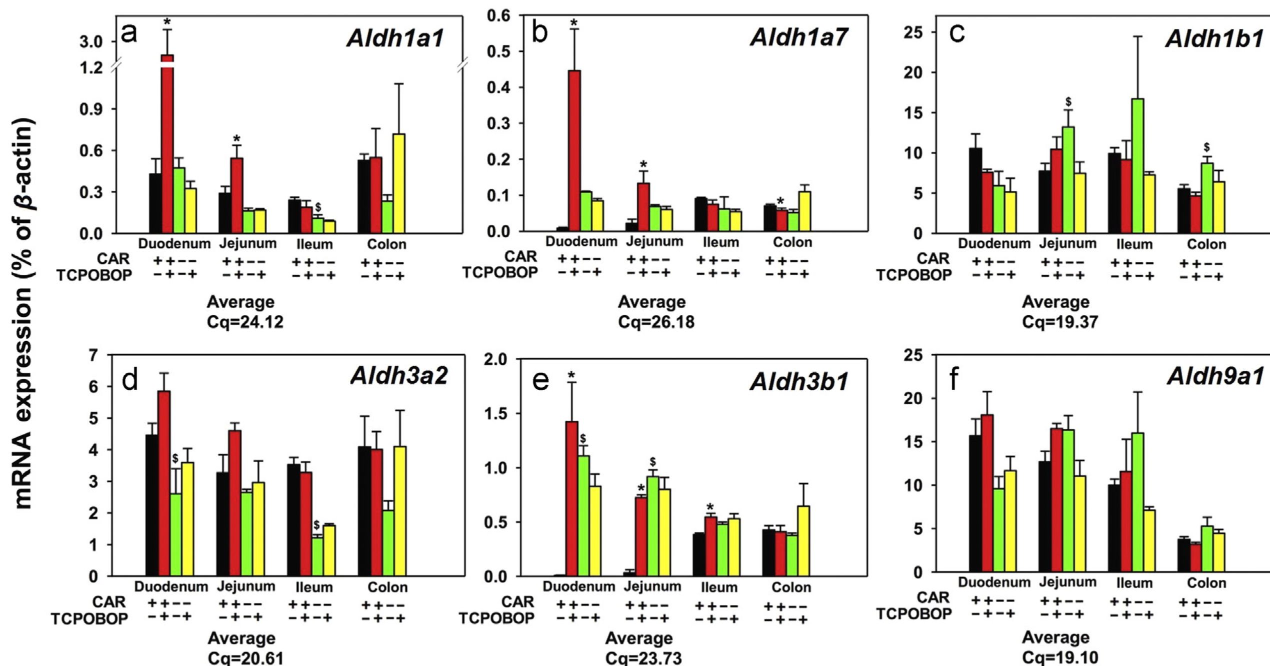

Figure 4 Messenger RNA expression of the phase-I drug-metabolizing enzymes *Aldh*s in various sections of intestine (duodenum, jejunum, ileum, and colon) of WT and $Car^{-/-}$ male mice treated with vehicle (corn oil) or TCPOBOP as described in Section 2. Data are expressed as percentage of the housekeeping gene β-actin (n=4–5 per group). Data among multiple groups were analyzed using ANOVA followed by Duncan's *post hoc* test. Asterisks (*) indicate statistically significant differences ($P<0.05$) between control WT and TCPOBOP-treated WT mice at the same section of intestines. Pound signs (#) indicate statistically significant differences ($P<0.05$) between control $Car^{-/-}$ and with TCPOBOP-treated $Car^{-/-}$ mice. Dollar signs ($) indicate statistically significant differences ($P<0.05$) of the basal mRNA expression between control WT and control $Car^{-/-}$ mice.

mRNA was not expressed in any of the tissues examined in the $Car^{-/-}$ mice.

3.2. *Regulation of phase-I drug metabolizing enzymes (Cyps and Aldh) in intestine by CAR*

The expressions of selected phase-I enzymes are shown in Figs. 3 and 4. For *Cyp*s (Fig. 3a–g), the basal expression of *Cyp1a1* was highest in duodenum, followed by jejunum and ileum, and was minimal in colon. Interestingly, in control $Car^{-/-}$ mice, the basal *Cyp1a1* mRNA increased 11.97-fold in duodenum and 6.02-fold in jejunum, suggesting that CAR suppresses the basal expression of *Cyp1a1* in duodenum and jejunum of WT mice. TCPOBOP did not alter *Cyp1a1* mRNA in any portions of the intestine in WT mice; however, it down-regulated *Cyp1a1* 89.3% in duodenum of the $Car^{-/-}$ mice, suggesting an off-target effect of TCPOBOP independent of CAR (Fig. 3a). Regarding the prototypical CAR-target gene *Cyp2b10*, as shown in Fig. 3b, the basal expression of *Cyp2b10* was the highest in duodenum followed by jejunum, but was minimally expressed in ileum and colon. In $Car^{-/-}$ mice, the basal *Cyp2b10* mRNA increased in colon (22.1-fold) but the expression was still minimal. TCPOBOP up-regulated *Cyp2b10* mRNA 4.98-fold in duodenum, 5.72-fold in jejunum, and 20.2-fold in ileum of WT mice in a CAR-dependent manner. As shown in Fig. 3c, the basal expression of *Cyp3a11* was highest in duodenum, followed by jejunum, ileum, and colon. In $Car^{-/-}$ mice, basal *Cyp3a11* mRNA decreased 55% in jejunum and 75% in ileum, suggesting that CAR is necessary for the basal expression of *Cyp3a11* in these sections. TCPOBOP up-regulated *Cyp3a11* expression 70% in duodenum in a CAR-dependent manner. However, it did not alter the *Cyp3a11* mRNAs in other sections of the intestine. As shown in Fig. 3d, the basal expression of *Cyp3a13* was highest in duodenum, followed by jejunum, and was much lower in ileum and colon. In $Car^{-/-}$ mice, the basal *Cyp3a13* mRNA expression decreased 60% in duodenum, suggesting that CAR is necessary in maintaining the constitutive expression of *Cyp3a13* in the duodenum. TCPOBOP up-regulated *Cyp3a13* mRNA expression 38% in duodenum and 44% in jejunum in a CAR-dependent manner; however, it did not alter *Cyp3a13* mRNA in ileum or colon. As shown in Fig. 3e, the basal expression of *Cyp3a25* was highest in duodenum, followed by jejunum and ileum but was minimal in colon. In $Car^{-/-}$ mice, the basal *Cyp3a25* mRNA decreased in duodenum (77.8%), jejunum (58.4%), and ileum (59.6%), suggesting that CAR is necessary for the basal expression of *Cyp3a25* in the small intestine. TCPOBOP in general did not alter the *Cyp3a25* mRNA expression in any sections of intestine. As shown in Fig. 3f, the basal expression of *Cyp4a10* was the highest in duodenum, followed by colon, jejunum, and ileum. TCPOBOP in general did not alter the expression of *Cyp4a10*, except for a down-regulation (63.5%) in ileum in a CAR-dependent manner. As shown in Fig. 3g, the basal expression of *Cyp4b1* was similarly expressed in all portions of the intestine. In $Car^{-/-}$ mice, the basal *Cyp4b1* mRNA decreased 34% in duodenum, 71% in ileum and 81% in colon, suggesting that CAR is necessary in maintaining the basal expression of *Cyp4b1* in these sections. TCPOBOP down-regulated *Cyp4b1* mRNA expression 27.4% in ileum of WT mice. As shown in Fig. 3h, the basal expression of *Nqo1* was highest in colon, followed by duodenum, ileum, and jejunum. In $Car^{-/-}$ mice, the

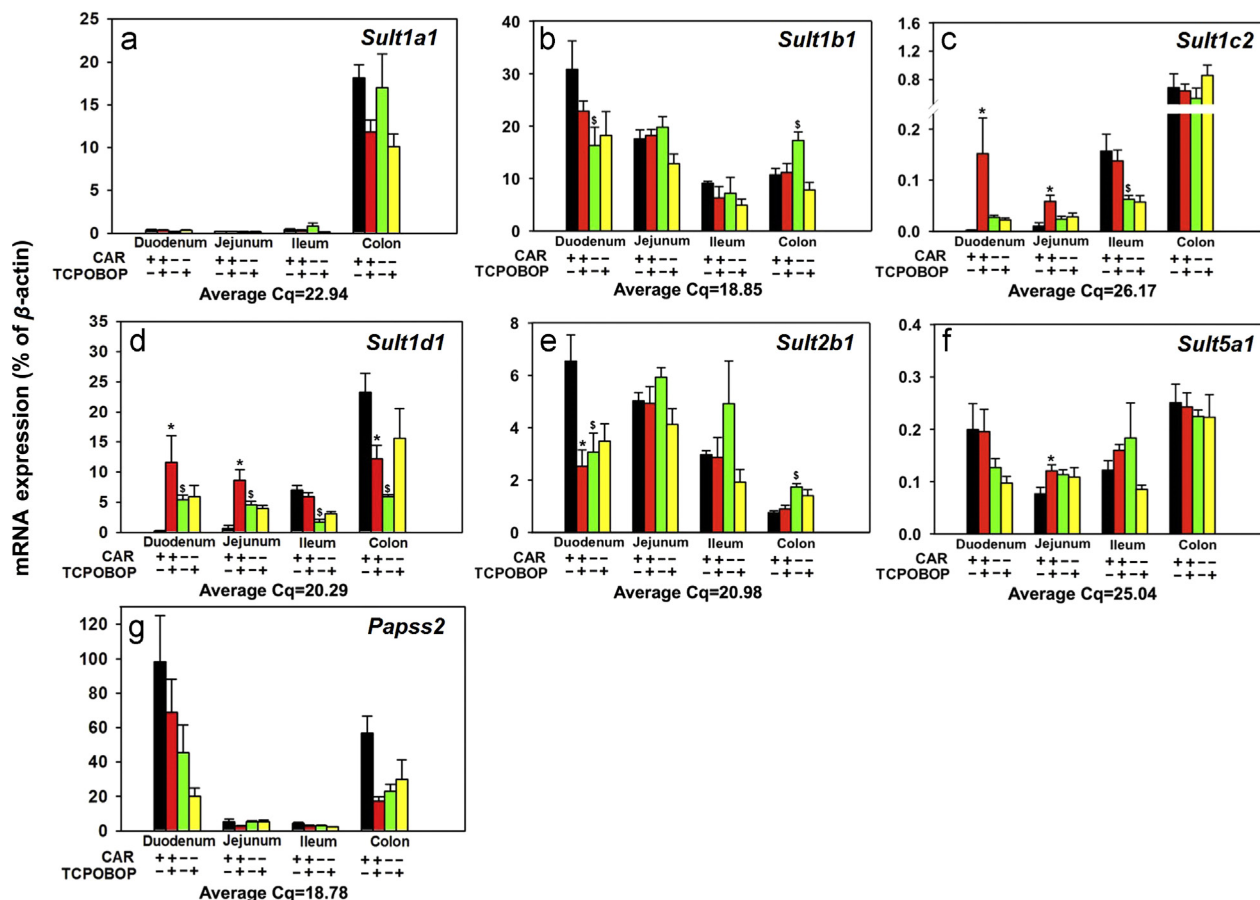

Figure 5 Messenger RNA expression of the phase-II drug-metabolizing enzymes *Sult*s in various sections of intestine (duodenum, jejunum, ileum, and colon) of WT and $Car^{-/-}$ male mice treated with vehicle (corn oil) or TCPOBOP as described in Section 2. Data are expressed as percentage of the housekeeping gene *β*-actin ($n=4$–5 per group). Data among multiple groups were analyzed using ANOVA followed by Duncan's *post hoc* test. Asterisks (*) indicate statistically significant differences ($P<0.05$) between control WT and TCPOBOP-treated WT mice at the same section of intestines. Pound signs (#) indicate statistically significant differences ($P<0.05$) between control $Car^{-/-}$ and with TCPOBOP-treated $Car^{-/-}$ mice. Dollar signs ($) indicate statistically significant differences ($P<0.05$) of the basal mRNA expression between control WT and control $Car^{-/-}$ mice.

basal *Nqo1* mRNA increased 1.09-fold in jejunum, suggesting that CAR suppresses the basal expression of *Nqo1* in WT mice intestine. TCPOBOP moderately down-regulated *Nqo1* 27.1% in ileum of the $Car^{-/-}$ mice. In summary, CAR suppresses the basal expression of *Cyp1a1* and *Nqo1*, but maintains the constitutive expression of *Cyp3a11*, *Cyp3a13*, *Cyp3a25*, and *Cyp4b1*, in distinct sections of intestine; whereas pharmacological activation of CAR by TCPOBOP up-regulates *Cyp2b10*, *Cyp3a13*, *Cyp3a13*, but down-regulates *Cyp4a10* and *Cyp4b1* in distinct sections of intestine, in a CAR-dependent manner.

Regarding the regulation of the aldehyde dehydrogenases (*Aldhs*), as shown in Fig. 4a, the basal expression of *Aldh1a1* was highest in colon and duodenum, followed by jejunum, and ileum. In $Car^{-/-}$ mice, the basal *Aldh1a1* mRNA moderately decreased 54% in ileum. TCPOBOP up-regulated *Aldh1a1* mRNA 5.37-fold in duodenum and 87% in jejunum in a CAR-dependent manner. However, TCPOBOP did not alter the *Aldh1a1* mRNA expression in ileum and colon. As shown in Fig. 4b, the basal expression of *Aldh1a7* was highest in ileum, followed by colon, jejunum, and minimal in duodenum. The absence of CAR leads to an apparent increase in *Aldh1a7* in duodenum and jejunum, although a statistical significance was not achieved. TCPOBOP up-regulated *Aldh1a7* mRNA 51.4-fold in duodenum and 5.00-fold in jejunum in a CAR-dependent manner, but it slightly decreased *Aldh1a7* mRNA in colon. As shown in Fig. 4c, the basal expression of *Aldh1b1* was relatively similar in all portions of the intestine. In $Car^{-/-}$ mice, the basal expression of *Aldh1b1* mRNA was 70.9% higher in jejunum and 57.4% in colon than in WT mice, and TCPOBOP did not alter *Aldh1b1* mRNA in any intestinal sections. As shown in Fig. 4d, the basal expression of *Aldh3a2* was relatively similar in all portions of intestine. In $Car^{-/-}$ mice, basal *Aldh3a2* mRNA decreased 41.6% in duodenum and 65.6% in ileum. TCPOBOP did not have any effect on the *Aldh3a2* mRNA expression in intestine. As shown in Fig. 4e, the basal expression of *Aldh3b1* expression was highest in colon and ileum, and was minimal in duodenum and jejunum. Interestingly, In $Car^{-/-}$ mice, the basal *Aldh3b1* mRNA markedly increased 124.7-fold in duodenum and 26.8-fold in jejunum, which suggest that CAR suppresses the basal expression of *Aldh3b1* in WT mice intestine. TCPOBOP up-regulated *Aldh3b1* expression 160.4-fold in duodenum, 20.9-fold in jejunum, and 41.9% in ileum in a CAR-dependent manner. As shown in Fig. 4f, the basal expression of *Aldh9a1* decreased from duodenum to colon. TCPOBOP did not

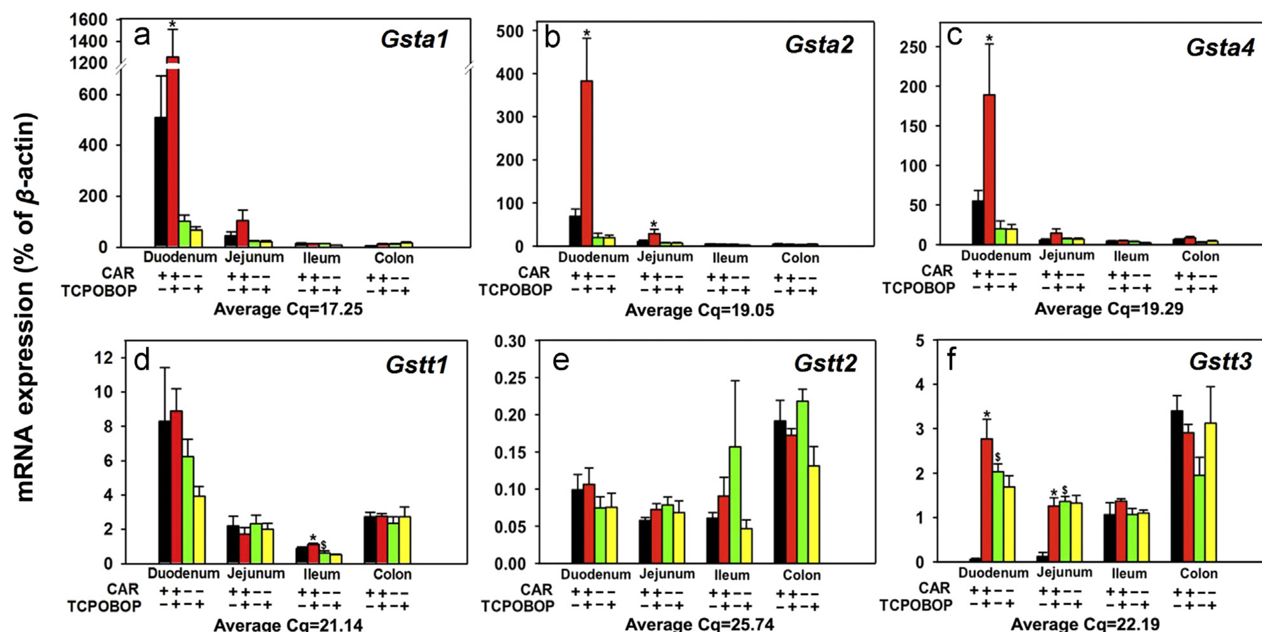

Figure 6 Messenger RNA expression of the phase-II drug-metabolizing enzymes *Gsta*s and *Gstt*s in various sections of intestine (duodenum, jejunum, ileum, and colon) of WT and $Car^{-/-}$ male mice treated with vehicle (corn oil) or TCPOBOP as described in Section 2. Data are expressed as percentage of the housekeeping gene β-actin (n=4–5 per group). Data among multiple groups were analyzed using ANOVA followed by Duncan's *post hoc* test. Asterisks (*) indicate statistically significant differences ($P<0.05$) between control WT and TCPOBOP-treated WT mice at the same section of intestines. Pound signs (#) indicate statistically significant differences ($P<0.05$) between control $Car^{-/-}$ and with TCPOBOP-treated $Car^{-/-}$ mice. Dollar signs ($) indicate statistically significant differences ($P<0.05$) of the basal mRNA expression between control WT and control $Car^{-/-}$ mice.

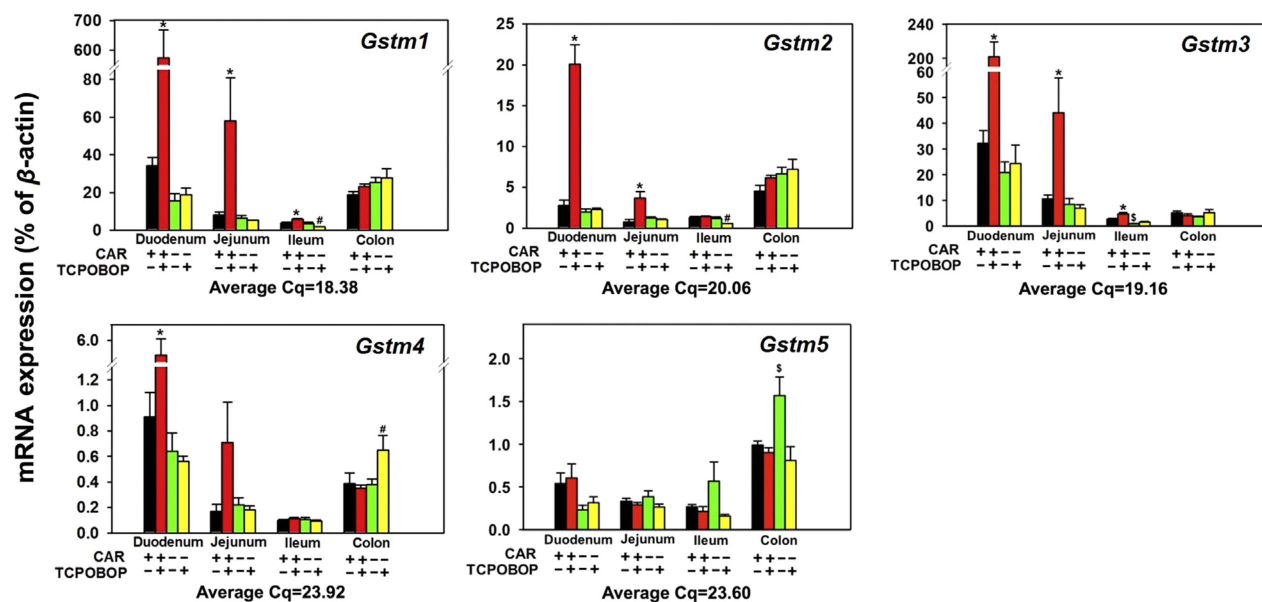

Figure 7 Messenger RNA expression of the phase-II drug-metabolizing enzymes *Gstm*s in various sections of intestine (duodenum, jejunum, ileum, and colon) of WT and $Car^{-/-}$ male mice treated with vehicle (corn oil) or TCPOBOP as described in Section 2. Data are expressed as percentage of the housekeeping gene β-actin (n=4–5 per group). Data among multiple groups were analyzed using ANOVA followed by Duncan's *post hoc* test. Asterisks (*) indicate statistically significant differences ($P<0.05$) between control WT and TCPOBOP-treated WT mice at the same section of intestines. Pound signs (#) indicate statistically significant differences ($P<0.05$) between control $Car^{-/-}$ and with TCPOBOP-treated $Car^{-/-}$ mice. Dollar signs ($) indicate statistically significant differences ($P<0.05$) of the basal mRNA expression between control WT and control $Car^{-/-}$ mice.

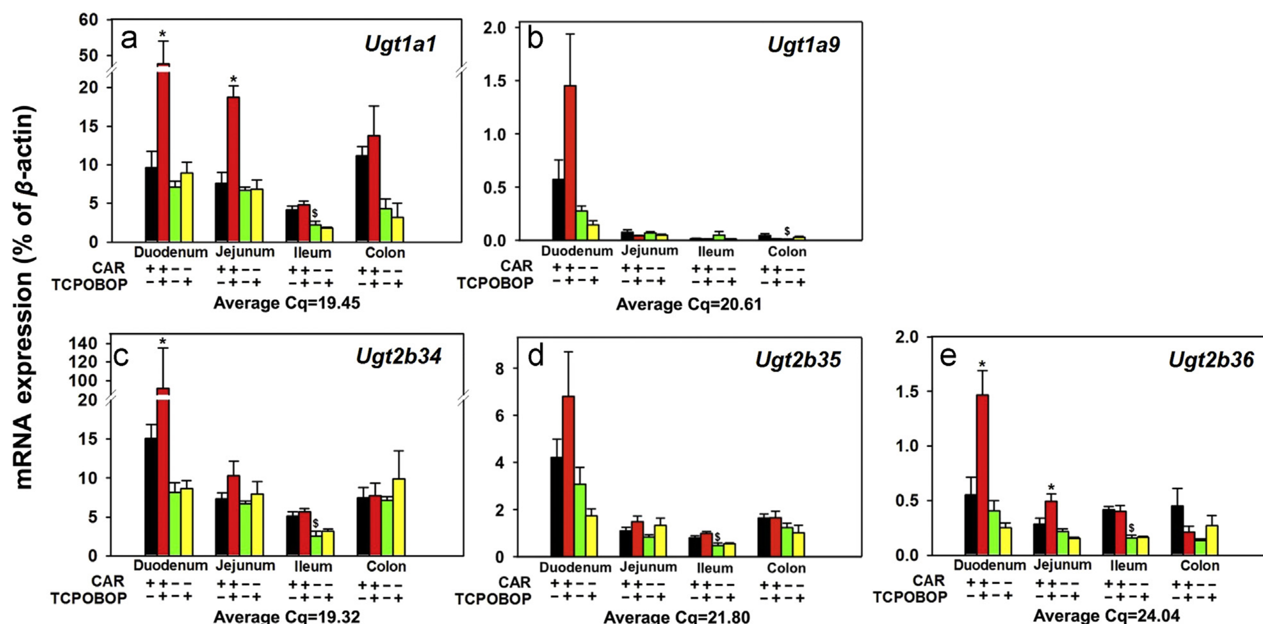

Figure 8 Messenger RNA expression of the phase-II drug-metabolizing enzymes *Ugt*s in various sections of intestine (duodenum, jejunum, ileum, and colon) of WT and $Car^{-/-}$ male mice treated with vehicle (corn oil) or TCPOBOP as described in Section 2. Data are expressed as percentage of the housekeeping gene β-actin ($n=4$–5 per group). Data among multiple groups were analyzed using ANOVA followed by Duncan's *post hoc* test. Asterisks (*) indicate statistically significant differences ($P<0.05$) between control WT and TCPOBOP-treated WT mice at the same section of intestines. Pound signs (#) indicate statistically significant differences ($P<0.05$) between control $Car^{-/-}$ and with TCPOBOP-treated $Car^{-/-}$ mice. Dollar signs ($) indicate statistically significant differences ($P<0.05$) of the basal mRNA expression between control WT and control $Car^{-/-}$ mice.

alter the *Aldh9a1* mRNA expression in any portions of intestine. In summary, CAR suppresses the basal expression of *Aldh3b1* but maintains the constitutive expression of *Aldh1a1*, *Aldh1b1*, and *Aldh3a2* in distinct sections of intestine, whereas pharmacological activation of CAR by TCOBOP up-regulates *Aldh1a7* and *Aldh3b1* in a CAR-dependent manner in distinct sections of small intestine.

3.3. *Regulation of phase-II drug metabolizing enzymes (Sult, Gsta, Gstt, Gstm, Ugt) in intestine by CAR*

Figs. 5–8 illustrate the regulation of various phase-II enzymes by CAR in intestine. Regarding the regulation of *Sult*, as shown in Fig. 5, the basal *Sult1a1* mRNA expression was predominantly expressed in colon, and was very lowly expressed in all portions of the small intestine. TCPOBOP did not alter the expression of *Sult1a1* mRNA in any portions of intestine (Fig. 5a). As shown in Fig. 5b, the basal expression of *Sult1b1* mRNA was highest in duodenum, followed by jejunum, colon, and ileum. In $Car^{-/-}$ mice, there was a decrease in the basal *Sultb1* mRNA in duodenum (47.1%), but an increase in the basal *Sult1b1* mRNA in colon (61.2%). TCPOBOP did not alter the *Sult1b1* mRNA expression in any portions of intestine. As shown in Fig. 5c, the basal mRNA expression of *Sult1c2* is highest in colon, followed by ileum and jejunum, and was minimally expressed in duodenum. In $Car^{-/-}$ mice, the basal *Sult1c2* mRNA expression was 60% lower in ileum, suggesting that CAR is necessary in maintaining constitutive expression of *Sult1c2* in ileum of WT mice. TCPOBOP up-regulated *Sult1c2* mRNA 73.6-fold in duodenum and 4.8-fold in jejunum in a CAR-dependent manner. However, TCPOBOP did not alter *Sult1c2* mRNA expression in ileum or colon. As shown in Fig. 5d, the basal mRNA expression of *Sult1d1* increased from duodenum to colon. In $Car^{-/-}$ mice, the basal *Sult1d1* mRNA increased 23.9-fold and 6.21-fold in duodenum and jejunum; however, it decreased 75.8% in ileum and 74.4% in colon. TCPOBOP up-regulated *Sult1d1* mRNA 52.4-fold in duodenum and 12.5-fold in jejunum in a CAR-dependent manner, whereas it had no effect on the *Sult1d1* mRNA in ileum, and down-regulated 47.5% in colon of WT mice. As shown in Fig. 5e, the basal expression of *Sult2b1* mRNA decreased from duodenum, to colon. In $Car^{-/-}$ mice, the basal *Sult2b1* mRNA decreased in duodenum (53.1%) but increased in colon (1.31-fold). TCPOBOP down-regulated *Sult2b1* mRNA 61.4% in duodenum, but had no effect in other portions of the intestine. As shown in Fig. 5f, the basal expression of *Sult5a1* was highest in colon and duodenum, and lower in ileum, and jejunum. TCPOBOP had minimal effect on *Sult5a1* mRNA expression, except for a moderate increase (57.4%) in jejunum in a CAR-dependent manner. As shown in Fig. 5g, the basal mRNA expression of *Papss2* was highest in duodenum and colon, but was much lower in jejunum and ileum. TCPOBOP did not have any effect on *Papss2* mRNA expression in any portions of intestine of either genotype.

Regarding the *Gsta*, as shown in Fig. 6a–c, the basal mRNAs of multiple *Gsta* family members (*Gsta1*, *2*, and *4*) followed a similar expression pattern, which was highest expression in duodenum, followed by jejunum, and minimal expression in ileum and colon. In duodenum, TCPOBOP up-regulated the mRNAs of *Gsta1* (1.46-fold), *Gsta2* (4.56-fold), and *Gsta4* (2.45-fold) in a CAR-dependent manner (Fig. 6a–c). In jejunum, TCPOBOP also up-regulated *Gsta2* mRNA 1.72-fold in a CAR-dependent manner. However, TCPOBOP had no effect on the *Gsta* expression in other portions of intestine in either WT or $Car^{-/-}$ mice (Fig. 6a–c). Regarding the *Gstt*, as shown in Fig. 6d, *Gstt1* mRNA basal

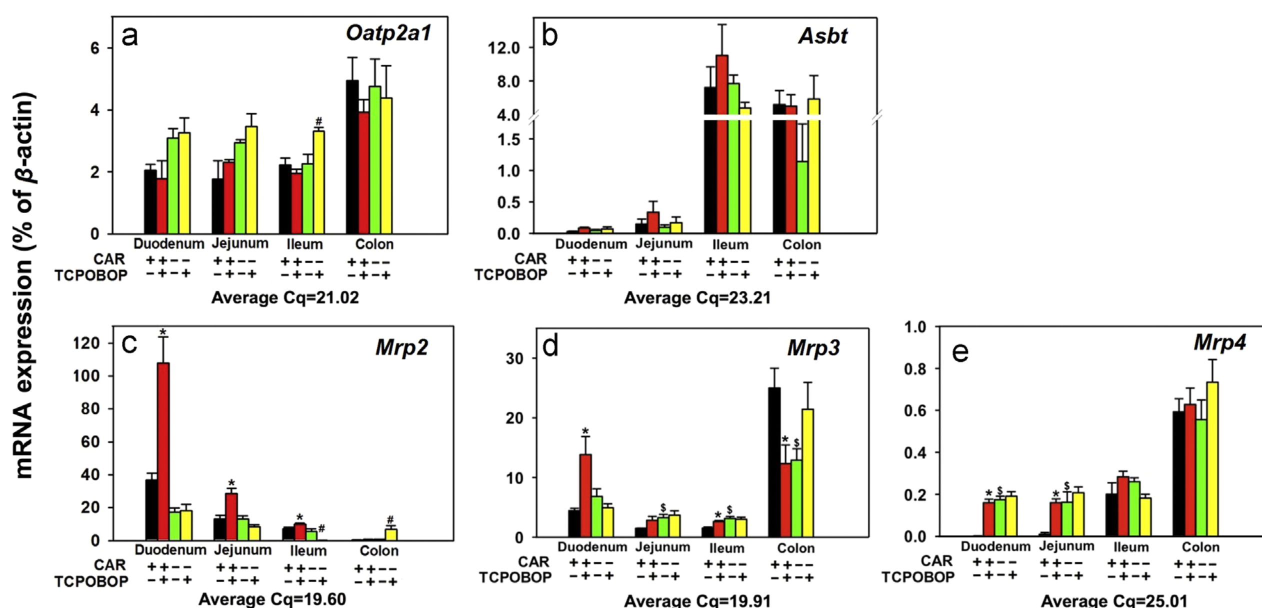

Figure 9 Messenger RNA expression of the transporters in various sections of intestine (duodenum, jejunum, ileum, and colon) of WT and $Car^{-/-}$ male mice treated with vehicle (corn oil) or TCPOBOP as described in the Section of materials and methods. Data are expressed as percentage of the housekeeping gene β-actin ($n=4$–5 per group). Data among multiple groups were analyzed using ANOVA followed by Duncan's *post hoc* test. Asterisks (*) indicate statistically significant differences ($P<0.05$) between control WT and TCPOBOP-treated WT mice at the same section of intestines. Pound signs (#) indicate statistically significant differences ($P<0.05$) between control $Car^{-/-}$ and with TCPOBOP-treated $Car^{-/-}$ mice. Dollar signs ($) indicate statistically significant differences ($P<0.05$) of the basal mRNA expression between control WT and control $Car^{-/-}$ mice.

expression was highest in duodenum, followed by colon, jejunum, and ileum, and was not readily altered by TCPOBOP in any portions of intestine, except for a moderate increase by TCPOBOP in ileum of WT mice. CAR deficiency moderately decreased the basal *Gstt1* mRNA in ileum (27.8%). As shown in Fig. 6e, the basal expression of *Gstt2* mRNA was highest in colon, followed by duodenum, ileum, and jejunum, and was not altered by TCPOBOP in any intestinal sections. As shown in Fig. 6f, the basal expression of *Gstt3* was low in duodenum and jejunum, but was much higher in ileum and highest in colon. CAR deficiency resulted in a 34.5-fold up-regulation of *Gstt3* mRNA in duodenum and a 10.2-fold in jejunum of WT mice. Interestingly, TCPOBOP also up-regulated *Gstt3* mRNA in these two sections (47.4-fold and 9.34-fold, respectively) in a CAR-dependent manner, whereas it did not have any effect in ileum and colon. Regarding the *Gstm* family members (Fig. 7a–e), the basal mRNA expression of *Gstm1* (Fig. 7a), *Gstm4* (Fig. 7d), and *Gstm5* (Fig. 7e) was highest in duodenum, followed by colon, jejunum, and ileum; the basal mRNA expression of *Gstm2* was highest in colon, followed by duodenum, ileum, and jejunum; whereas the basal mRNA expression of *Gstm3* was highest in duodenum, followed by jejunum, colon, and ileum (Fig. 6d–g). In $Car^{-/-}$ mice, the basal *Gstm3* mRNA decreased 67.0% in ileum, but *Gstm5* mRNA increased 58.4% in colon. TCPOBOP up-regulated all *Gstm* genes, except for *Gstm5*, in duodenum (15.8-fold, 6.28-fold, 5.27-fold, and 5.07-fold, respectively) and jejunum (6.20-fold, 3.65-fold, 3.17-fold, and 3.16-fold, respectively), in a CAR-dependent manner. In ileum, TCPOBOP also moderately up-regulated certain *Gstm* genes, such as *Gstm1* (58%) and *Gstm3* (74.7%) in a CAR dependent manner. TCPOBOP in general had minimal effect on the *Gstm* genes in colon of either genotype (Fig. 7a–e). TCPOBOP moderately down-regulated the *Gstm1* and *Gstm2* mRNAs in ileum, and up-regulated *Gstm4* in colon of $Car^{-/-}$ mice, likely due to off-target effect of the chemical.

Regarding the regulation of the *Ugt* family, as shown in Fig. 8a, the basal mRNA expression of *Ugt1a1* was expressed at comparable levels in various sections of the intestine. In $Car^{-/-}$ mice, there was a moderate decrease in the basal *Ugt1a1* mRNA only in ileum (46.3%). TCPOBOP up-regulated *Ugt1a1* mRNA 3.95-fold in duodenum and 1.46-fold in jejunum in a CAR-dependent manner. However, TCPOBOP had no effect on the *Ugt1a1* mRNA in ileum or colon. As shown in Fig. 8b, the basal expression of *Ugt1a9* was highest in duodenum, and was lowly expressed in the other sections of the intestine. The basal *Ugt1a9* mRNA was further decreased in colon of the $Car^{-/-}$ mice. TCPOBOP in general did not alter the *Ugt1a9* mRNA expression in any sections of intestine, although it tended to increase the *Ugt1a9* mRNA in duodenum (a statistical significance was not achieved). As shown in Fig. 8c and d, the basal mRNAs of *Ugt2b34* and *Ugt2b35* were both highest in duodenum, followed by colon, jejunum, and ileum. In $Car^{-/-}$ mice, the basal *Ugt2b34* and *2b35* mRNAs decreased in ileum (50.5% and 40.7%, respectively), suggesting that CAR is necessary in maintaining constitutive expression of both *Ugt2b34* and *2b35*. TCPOBOP up-regulated *Ugt2b34* mRNA 5.09-fold in duodenum in a CAR-dependent manner; however, it did not alter the *Ugt2b34* mRNA in other sections of intestine. TCPOBOP had a similar effect on the *Ugt2b35*, except that the mRNA increased in duodenum was not statistically significant. As shown in Fig. 8e, the basal mRNA expression of *Ugt2b36* was comparable in various sections of intestine. In $Car^{-/-}$ mice, the basal *Ugt2b36* mRNA was down-regulated 61.4% in ileum. TCPOBOP up-regulated *Ugt2b36* mRNA 1.65-fold in duodenum and 73.1% in jejunum in a CAR-dependent manner, however, it did not alter the *Ugt2b36* mRNA in ileum or colon.

In summary, CAR regulates the basal expression of many phase-II enzymes in distinct sections of intestine, whereas pharmacological activation of CAR up-regulates *Sult1c2*, *Sult1d1*, *Sult5a1*, *Gsta1*, *Gsta2*, *Gsta4*, *Gstt3*, *Gstm1-4*, *Ugt1a1*, *Ugt2b34*, *Ugt2b36*, and tends to up-regulate *Ugt1a9* and *Ugt2b35*, in distinct sections of intestine in a CAR-dependent manner. In contrast, pharmacological activation of CAR down-regulates *Sult1d1* in colon and *Sult2b1* in duodenum in a CAR-dependent manner; however, because *Car* is lowly expressed in colon (Fig. 2), the TCPOBOP-mediated effects in colon may be due to the involvement of other regulatory factors.

3.4. Regulation of the transporters in intestine by CAR

Fig. 9 shows the mRNA expression of uptake and efflux transporters in various sections of intestine of WT and $Car^{-/-}$ mice treated with corn oil or TCPOBOP. Regarding the uptake transporters (Fig. 9a–c), the basal mRNA expression of *Oatp2a1* was higher in colon than in the three sections of the small intestine, and TCPOBOP did not alter the *Oatp2a1* mRNA expression in any sections of intestine, except for a moderate increase in ileum of $Car^{-/-}$ mice (46.5%, Fig. 9a). The basal mRNA expression of *Asbt* was highest in ileum and colon, but was minimally expressed in duodenum and jejunum, and TCPOBOP had no effect on the *Asbt* mRNA expression in any sections of intestine (Fig. 9b).

Regarding the efflux transporters *Mrp2–4* (Fig. 9c–e), the basal mRNA expression of *Mrp2* was highest in duodenum, followed by jejunum, and ileum, but was minimally expressed in colon. TCPOBOP up-regulated *Mrp2* 1.93-fold in duodenum, 1.16-fold in jejunum, and 40.3% in ileum in a CAR-dependent manner. In $Car^{-/-}$ mice, TCPOBOP down-regulated *Mrp2* mRNA 96.5% in ileum, but up-regulated *Mrp2* mRNA 9.03-fold in colon, which may be due to off-target effect of the chemical (Fig. 9c). The basal mRNA expression of *Mrp3* was highest in colon, followed by duodenum, jejunum, and ileum. In $Car^{-/-}$ mice, the basal *Mrp3* mRNA expression was up-regulated 1.18-fold in jejunum and 1.05-fold in ileum, but was down-regulated 48.4% in colon. TCPOBOP up-regulated *Mrp3* mRNA 2.11-fold in duodenum and 71.9% in ileum in a CAR-dependent manner (it also tended to increase *Mrp3* mRNA in jejunum although a statistically significant difference was not achieved). In contrast, TCPOBOP down-regulated *Mrp3* mRNA 50.7% in colon of WT mice. Considering that *Car* is minimally expressed in colon (Fig. 2), the TCPOBOP-mediated down-regulation of *Mrp3* may be due to off-target effect of the chemical (Fig. 9d). The basal mRNA expression of *Mrp4* was highest in colon followed by ileum, but was minimally expressed in duodenum and jejunum. Interestingly, in $Car^{-/-}$ mice, there was a marked increase in the basal *Mrp4* mRNA in both duodenum and jejunum (81.1-fold and 15.6-fold, respectively), suggesting CAR suppresses *Mrp4* basal expression in these two sections. Conversely, pharmacological activation of CAR by TCPOBOP also increased *Mrp4* mRNA in duodenum and jejunum (74.2-fold and 15.4-fold, respectively), in a CAR-dependent manner. However, TCPOBOP did not alter the *Mrp4* mRNA in ileum and colon (Fig. 9e).

In summary, CAR suppresses the basal expression of *Mrp3* in jejunum and ileum, as well as *Mrp4* in duodenum and jejunum, whereas pharmacological activation of CAR by TCPOBOP has minimal effect on the uptake transporters but markedly increases the efflux transporters *Mrp2–4* in small intestine, but decreases the *Mrp3* mRNA in colon, in a CAR-dependent manner. Even though *Mrp3* and *Mrp4* are well known CAR-target genes in liver[24] and intestine (Fig. 9c–e), their basal expressions are highest in colon where *Car* is lowly expressed, suggesting that other regulatory factors are involved in the basal expression of these transporters.

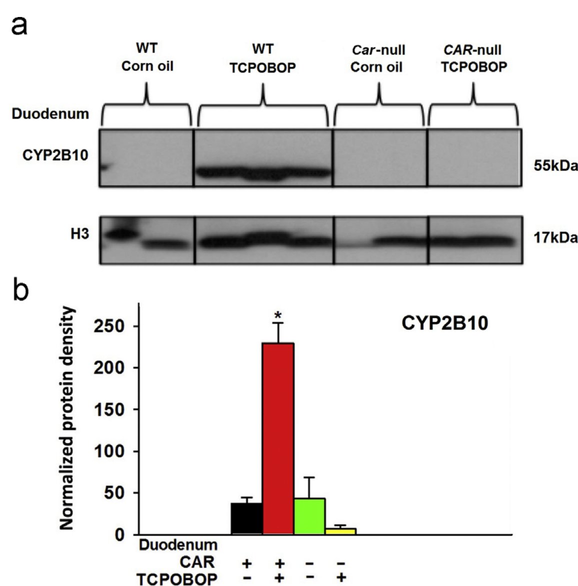

Figure 10 Western blot analysis of CYP2B10 protein and H3 in duodenum (small intestine) of wild-type and $Car^{-/-}$ mice treated with vehicle (corn oil) or TCPOBOP. Asterisks (*) indicate statistically significant differences ($P < 0.05$) between control WT and TCPOBOP-treated WT mice in duodenum.

3.5. Regulation of CYP2B10 protein in duodenum by CAR

Because duodenum has the highest *Car* and *Cyp2b10* mRNA expression (Figs. 2 and 3b), the protein for the prototypical CAR-target gene *Cyp2b10* was quantified in duodenum of WT and $Car^{-/-}$ mice by Western blotting analysis (Fig. 10). Following TCPOBOP treatment, consistent with the mRNA data, CYP2B10 protein was also increased (5.11-fold) in the duodenum of WT mice (Fig. 10b). However, such TCPOBOP-mediated induction in the CYP2B10 protein expression was completely abolished in the duodenum of $Car^{-/-}$ mice, suggesting that TCPOBOP-mediated up-regulation of CYP2B10 protein in duodenum is CAR-dependent.

4. Discussion

In conclusion, the present study has demonstrated that in addition to its important roles in liver[24], CAR is also critical in both maintaining the basal expression of certain DPGs and the pharmacological regulation of certain DPGs in a section-specific manner of the intestine. A systematic comparison between liver (previous studies) and intestine (present study) has shown that CAR activation in liver and intestine produces overlapping but not identical results. The present study has also compared the section-specific CAR-mediated effect on the DPG expression, and has demonstrated that in general, duodenum appears to be the most responsive section following exposure to the CAR-ligand TCPOBOP, likely because CAR is highest expressed in duodenum as compared to other sections of the intestine. TCPOBOP not only has inducible but also suppressive effect on the DPG expression in intestine. In addition, the CAR-independent off-target effect of TCPOBOP has also been

Table 3 Liver and Intestine regulation difference in WT TCPOBOP-treated mice compared to WT control mice.

Gene symbol	Full name	Categories	Liver*	Duodenum	Jejunum	Ileum	Colon
Cyp1a1	Cytochrome P450 oxidase 1a1	Phase-I	N/A	-	-	-	-
Cyp2b10	Cytochrome P450 oxidase 2b10	Phase-I	↑	↑	↑	↑	-
Cyp3a11	Cytochrome P450 oxidase 3a11	Phase-I	↑	↑	-	-	-
Cyp3a13	Cytochrome P450 oxidase 3a13	Phase-I	N/A	↑	↑	-	-
Cyp3a25	Cytochrome P450 oxidase 3a25	Phase-I	N/A	-	-	-	-
Cyp4a10	Cytochrome P450 oxidase 4a10	Phase-I	N/A	-	-	↓	-
Cyp4b1	Cytochrome P450 oxidase 4b1	Phase-I	N/A	-	-	↓	-
Nqo1	NAD(P)H dehydrogenase, quinone 1	Phase-I	↑	-	-	-	-
Aldh1a1	Aldehyde dehydrogenase 1 family member A1	Phase-I	↑	↑	↑	-	-
Aldh1a7	Aldehyde dehydrogenase 1 family member A7	Phase-I	↑	↑	↑	-	↓
Aldh1b1	Aldehyde dehydrogenase 1 family member B1	Phase-I	-	-	-	-	-
Aldh3a2	Aldehyde dehydrogenase 3 family member A2	Phase-I	-	-	-	-	-
Aldh3b1	Aldehyde dehydrogenase 3 family member B1	Phase-I	N/A	↑	↑	↑	-
Aldh9a1	Aldehyde dehydrogenase 9 family member A1	Phase-I	-	-	-	-	-
Sult1a1	Sulfotransferase family 1A member 1	Phase-II	N/A	-	-	-	-
Sult1b1	Sulfotransferase family 1B member 1	Phase-II	N/A	-	-	-	-
Sult1c2	Sulfotransferase family 1C member 2	Phase-II	N/A	↑	↑	-	-
Sult1d1	Sulfotransferase family 1D member 1	Phase-II	N/A	↑	↑	-	↓
Sult2b1	Sulfotransferase family 2B member 1	Phase-II	N/A	↓	-	-	-
Sult5a1	Sulfotransferase family 5A member 1	Phase-II	↑	-	↑	-	-
Papss2	3'-Phosphoadenosine 5'-phosphosulfate synthase 2	Phase-II	↑	-	-	-	-
Gsta1	Glutathione *S*-trasnferase alpha 1	Phase-II	↑	↑	-	-	-
Gsta2	Glutathione *S*-trasnferase alpha 2	Phase-II	N/A	↑	↑	-	-
Gsta4	Glutathione *S*-trasnferase alpha4	Phase-II	↑	↑	-	-	-
Gstm1	Glutathione *S*-trasnferase mu 1	Phase-II	↑	↑	↑	↑	-
Gstm2	Glutathione *S*-trasnferase mu 2	Phase-II	↑	↑	↑	-	-
Gstm3	Glutathione *S*-trasnferase mu 3	Phase-II	↑	↑	↑	↑	-
Gstm4	Glutathione *S*-trasnferase mu 4	Phase-II	↑	↑	-	-	-
Gstm5	Glutathione *S*-trasnferase mu 5	Phase-II	N/A	-	-	-	-
Gstt1	Glutathione *S*-transferase theta 1	Phase-II	↑	-	-	↑	-
Gstt2	Glutathione *S*-transferase theta 2	Phase-II	-	-	-	-	-
Gstt3	Glutathione *S*-transferase theta 3	Phase-II	N/A	↑	↑	-	-
Ugt1a1	UDP glucuronosyltransferase 1 family, polypeptide A1	Phase-II	↑	↑	↑	-	-
Ugt1a9	UDP glucuronosyltransferase 1 family, polypeptide A9	Phase-II	↑	-	-	-	-
Ugt2b34	UDP glucuronosyltransferase 2 family, polypeptide B34	Phase-II	↑	↑	-	-	-
Ugt2b35	UDP glucuronosyltransferase 2 family, polypeptide B35	Phase-II	↑	-	-	-	-
Ugt2b36	UDP glucuronosyltransferase 2 family, polypeptide B36	Phase-II	↑	↑	↑	-	-
Oatp2a1	Solute carrier organic anion transporter family member 2A1	Transporters	N/A	-	-	-	-
Asbt	Solute carrier family10, member 2 (sodium/bile acid cotransporters)	Transporters	N/A	-	-	-	-
Mrp2	ABC transporter C family member 2	Transporters	↑	↑	↑	↑	-
Mrp3	ABC transporter C family member 3	Transporters	↑	↑	-	↑	↓
Mrp4	ABC transporter C family member 4	Transporters	↑	↑	↑	-	-

Basal expression of genes is shown as increased or decreased relative to that in wild type mice. (–) denotes none change. N/A: not available.
* Note: The liver data were obtained from Aleksunes and Klaassen[24], 2012.

observed in the present study, evidenced by TCPOBOP-mediated changes in DPG expression in $Car^{-/-}$ mice, and TCPOBOP-mediated changes in DPG expression in WT colon where CAR is minimally expressed. Many *bona fide* CAR-target genes in small intestine were highest expressed in colon where CAR is minimally expressed, suggesting that additional regulatory factors are involved in the basal expression of these genes.

A systematic comparison of the CAR effect on DPG expression in between liver and various sections of intestine is shown in Table 2 (CAR-mediated basal expression of DPGs) and Table 3 (effect of pharmacological activation of CAR on the expression of DPGs). In general, the basal CAR expression is more important for the constitutive expression of DPGs in intestine rather than in liver[24] (Table 2). Regarding the effect of the pharmacological activation of CAR, consistent with liver data[24], TCPOBOP up-regulated many DPGs (*Cyp2b10*, *Cyp3a11*, *Aldh1a1*, *Aldh1a7*, *Gsta1*, *Gsta4*, *Gstm1-m4*, *Ugt1a1*, *Ugt2b34*, *Ugt2b36*, and *Mrp2–4*) in certain portions of the small intestine in a CAR-dependent manner, with duodenum generally being the most inducible section. In contrast, *Nqo1*, *Papss2*, *Ugt1a9*, and *Ugt2b35* were up-regulated by TCPOBOP in liver but were not changed in intestine, therefore the pharmacological activation of CAR in liver and intestine are not identical. Such tissue-specific effects may be due to tissue-specific chromatin epigenetic environment, such as different signatures for DNA methylation (suppressive signal for gene transcription) and/or histone modification patterns, which prevent the CAR-mediated trans-activation of certain DPGs in intestine. The epigenetic signatures within the enhancers and promoters of certain DPGs will need to be examined in future studies. Using the epithelial cells scraped from the whole small intestine, another study in the literature has also shown liver- *vs.* small intestine–specific regulation of some DPGs by TCPOBOP[29]. Our finding is consistent with that study regarding the regulation of *Cyp2b10*, *Gsta1*, *Gstm2*, *Mrp2*, and *Mrp3*, and in addition, the present study has investigated the intestinal-specific CAR-mediated regulation of many other genes, including *Cyp3a13*, *3a25*, *Cyp4a10*, *Cyp4b1*, *Nqo1*, *Gsta2*, *Gsta4*, *Gstt2*, *Gstt3*, *Gstm3*, *Gstm4*, *Mrp4*, and this has added new information to the existing knowledge. Certain discrepancies are also observed between the present study and the Maglich et al.[29] study, in that *Aldh1a1*, *Aldh1a7*, and *Cyp3a11* were not changed in the whole small intestine by TCPOBOP in the previous study, but were up-regulated by TCPOBOP in the present study in a CAR-dependent manner. In addition, the basal *Cyp1a1* expression was decreased in the previous study but increased in the present study. Such discrepancy may be due to difference doses of TCPOBOP (a single dose of TCPOBOP at 0.3 mg/kg in corn oil with 5% DMSO of the previous study *vs.* 3 mg/kg of TCPOBOP in corn oil once daily for 4-days in the present study), or different sample preparation procedures (epithelial cells scraped from whole small intestine in the previous study *vs.* various sections of small intestine in the present study).

Previous studies have demonstrated that the basal expression of *Car* is high in liver and small but is lower in the large intestine[19,21,30], whereas the present study has confirmed the basal tissue distribution of CAR, and is among the first to show that pharmacological activation of CAR by TCPOBOP actually down-regulates the *Car* expression in liver, duodenum, and jejunum (Fig. 2), and this is likely due to a negative feedback mechanism to prevent excessive CAR-signaling through decreasing the CAR synthesis. Regarding the regulation of DPGs by CAR, a previous study in the literature has demonstrated the CAR-dependent up-regulation of *Cyp2b10* in duodenum[30]. The present study on *Cyp2b10* in duodenum is consistent with that study, and our study has also examined the expression of *Cyp2b10* and other DPGs in other sections of intestine. Another previous study has performed a preliminary survey in WT male mice regarding the regulation of a few *Ugt*s by TCPOBOP in duodenum, jejunum, ileum, and colon using pooled samples (*i.e.*, 1 pooled sample from $n=5$ biological replicates)[31]. The apparent TCPOBOP-mediated increase in the mRNAs of *Ugt1a1* and *Ugt2b35* in that previous study is consistent with the present study, but *Ugt2b34* mRNA is only up-regulated by TCPOBOP in the present study. Such differences are likely due to different method of detection (branched DNA amplification technology *vs.* RT-qPCR), and/or pooled *vs.* individual samples. Our finding on CAR-dependent up-regulation of *Ugt1a1* mRNA by TCPOBOP in duodenum is also consistent with a previous study using Northern blot of *Ugt1a1* in duodenum of WT and $Car^{-/-}$ mice[29]. The expression of *Car* is gender-divergent (*i.e.* higher in females than in males) in liver but not in any sections of intestine[19]. Therefore, the present study has only tested the effect of CAR activation in intestines of male mice.

Although many orally administered drug absorption and delivery are known to take place mostly in small intestine and liver, the present study has shown that many *bona fide* CAR-target DPGs in liver and small intestine are highest expressed in colon, where *Car* is lowly expressed. Examples of these genes include *Aldh3b1*, *Sult1c2*, *Sult1d1*, *Gstt3*, *Mrp3* and *Mrp4*. Functionally speaking, the high expression of *Aldh3b1* may be important in detoxifying the microbial aldehyde produced from ethanol by the intestinal bacteria, and this may be critical in reducing the risk of colon cancer derived from microbial aldehyde[32]. The high expression of certain phase-II enzymes such as *Sults* and *Gst* in colon correlate with its critical function to conjugate and thus detoxify various substances in large intestine. GSTs are also involved in the metabolism of endogenous and exogenous carcinogenic substances, which are implicated in the risks of colorectal cancer[33,34]. The colon-specific efflux transporters Mrp3 and Mrp4 may also favor the elimination of various potentially toxic chemicals into feces. Another functional significant of colon-specific expression of certain DPGs is that it is associated with the critical roles of DPGs in metabolizing colon-targeted drugs or prodrugs. In addition to the microbial enzymes capability to bio-activate and/or detoxify xenobiotics, the colon tissue derived host enzymes may also contribute to the biotransformation of certain chemicals.

One critical question that arises is in regard of the potential species differences in the CAR-mediated regulation of intestinal DPGs. CAR is highly expressed in liver and small intestine of both mice and humans[21,35]. The species differences of CAR and its tumorigenesis potential have been well characterized in liver, in that pharmacological activation of mouse CAR leads hepatomegaly followed by hepatocarcinogenesis in a CAR-dependent manner[36]. Although human CAR activation is not a risk to cause liver tumor in human, it may cause liver hypertrophy without hyperplasia in response to the human CAR activators phenobarbital and chlordane, suggesting that hCAR is able to induce hypertrophic responses in response to xenobiotic stress[37]. However, both the mouse and human CAR proteins appear to share high similarities in regulating the genes involved in xenobiotic biotransformation in liver. For example, the mouse CAR activation by TCPOBOP up-regulates the expression of *Cyp1a2*, *Cyp2b10*, *Cyp3a11*, and *Ugt1a1* in a CAR-dependent manner in liver[24], whereas the human CAR activation by the human CAR activators also up-regulates the expression of the human orthologs *CYP1A2*, *CYP2B6*, and *UGT1A1*[29]. In human intestine-derived Caco2 cells phenobarbital up-regulates *CYP2B6* and *CYP3A4*[38,39], and our

finding regarding the CAR-mediated up-regulation of the mouse orthologs *Cyp2b10* and *Cyp3a11* in duodenum is consistent with the previous studies. However, relatively less is known regarding the intestinal effect of pharmacological activation or genetic depletion of *Car* in the regulation of many other DPGs *in vivo*, thus the present study has filled this critical knowledge gap. Identification of the xenobiotic responses to CAR activation in mouse and human intestines is critical for understanding certain adverse drug reactions for orally exposed chemicals.

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