Inducibility of Drug-metabolizing Enzymes by Xenobiotics in Mice with Liver-specific Knockout of Ctnnb1

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Running title: Cyp induction in Ctnnb1 knockout mice

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Number of text pages: 30
Number of tables: 2
Number of figures: 6
Number of references: 29
Number of words (Abstract): 223
Number of words (Introduction): 699
Number of words (Discussion): 1413

Abbreviations: 3MC, 3-methylcholanthrene; AhR, aryl hydrocarbon receptor; BHA, butylated hydroxyanisole; CAR, constitutive androstane receptor; Cyp, cytochrome P450; Ephx1, Microsomal epoxide hydrolase; GST, glutathione S-transferase; G6P, glucose-6-phosphatase; Nqo, NAD(P)H-quinone-oxidoreductase; Nrf2, nuclear factor erythroid-related factor 2; PB, phenobarbital; PCN, pregnenolone-α-carbonitrile; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane-X-receptor; RXR, retinoid-X-receptor; TCDD, 2,3,7,8-tetrachloro-p-dibenzodioxin; TCF, T cell factor; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene; Ugt, UDP-glucuronosyl-transferase
ABSTRACT

Basal as well as xenobiotic-induced expression of the main enzymes from phase I and phase II of drug metabolism is confined to the perivenous areas of the mammalian liver lobule. Whereas signal transduction pathways that govern xenobiotic-induced expression of these enzymes via ligand-activated transcription factors such as CAR or the AhR have been intensively studied, the mechanisms regulating zone-specific basal expression of genes related to drug metabolism and preferential response of perivenous hepatocytes to xenobiotic inducers are still largely unknown. Recent publications by our and other groups point towards an important role of the Wnt/β-catenin pathway in the maintenance of the perivenous hepatocyte gene expression profile including the main hepatic detoxification enzymes, and β-catenin signaling was recently implicated in the expression of several cytochrome P450 isoenzymes. To analyze, whether the β-catenin pathway would also affect inducible expression of drug-metabolizing enzymes, mice with liver-specific knockout of the Ctnnb1 gene (encoding β-catenin) were treated with different model inducers of xenobiotic metabolism. Knockout of β-catenin led to alterations in basal expression of most drug metabolism-related genes analyzed, and resulted in strongly diminished responses to agonists of CAR-, AhR-, and Nrf2-dependent transcription. Taken together, the data presented in this study indicate that β-catenin does not only regulate basal expression of drug-metabolizing enzymes, but also determines the magnitude and hepatic localization of response to xenobiotic inducers in vivo.
In the absence of xenobiotic inducers of drug metabolism, expression of most drug-metabolizing enzymes is restricted to the perivenous hepatocyte compartment. This applies especially to many cytochrome P450 (Cyp) isoforms from families 1-3, which consist the main detoxification enzymes in mammalian liver (Jungermann and Katz, 1989; Gebhardt, 1992; Lindros, 1997; Oinonen and Lindros, 1998). Expression of these enzymes is induced to high levels by substances that bind to nuclear receptors which act as ligand-activated transcription factors, such as the constitutive androstane receptor (CAR) and the aryl hydrocarbon receptor (AhR) (for reviews see Swales and Negishi (2004) and Bock (1994). Once activated, receptors trigger the induction of distinct subsets of drug-metabolizing enzymes. In most cases, this process occurs preferentially in perivenous hepatocytes. However, the number of hepatocytes expressing the respective enzymes increases with increasing doses of enzyme inducers, and enzyme expression is extended to the adjacent, more periportal hepatocytes without altering overall zonation of the proteins (Wolf et al., 1984; Tritscher et al., 1992; Oinonen and Lindros, 1998).

Extensive research over the last decades has elucidated the mechanisms that govern xenobiotic-induced expression of mRNAs and proteins related to drug metabolism. The mechanisms which regulate zone-specific basal expression of these enzymes in perivenous hepatocytes remained largely unknown. However, expression of various Cyp isoforms has been linked to the hypothalamic-pituitary axis which seems to be involved in the repression of Cyp transcription in periportal hepatocytes and in the regulation of gender-specific Cyp isoforms (Oinonen and Lindros, 1998). Furthermore, effects of the insulin/glucagon system on Cyp expression in cultured hepatocytes were reported (Saad et al., 1994).

Recently, β-catenin has been established as a new player in the regulation of zonal gene expression in mouse liver. For reviews on β-catenin signaling, see Willert and Nusse (1998) and Lustig and Behrens (2003). In brief, the so-called ‘canonical’ Wnt/β-catenin pathway is physiologically activated by Wnt molecules which bind to receptors at the cell surface. Wnt binding leads to the inhibition of a multi-protein-complex which, in the absence of Wnt signaling, phosphorylates β-catenin thus marking the protein for proteasomal
degradation. When that phosphorylating complex is inhibited upon Wnt binding, β-catenin accumulates in the cytosol and translocates to the nucleus, where it binds to transcription factors such as T cell factor (TCF)-1 and initiates transcription of target genes.

Based on the observation that mouse hepatomas with mutationally activated β-catenin express high levels of various ‘perivenous’ marker mRNAs and proteins including a number of Cyp isoenzymes (Loeppen et al., 2005; Stahl et al., 2005; Braeuning et al., 2007a), we postulated that β-catenin-dependent signaling determines the ‘perivenous’ hepatocyte gene expression profile including the main drug-metabolizing enzymes (Hailfinger et al., 2006). Accordingly, treatment of cultured hepatocytes with inducers of β-catenin signaling was able to induce the expression of Cyp1a1, Cyp2b10, and Cyp2e1 mRNAs (Hailfinger et al., 2006). More evidence for an important contribution of β-catenin signaling to the basal expression of various Cyp enzymes comes from recent studies with transgenic mice: Sekine and colleagues described the loss of Cyp1a2, Cyp2c29, and Cyp2e1 expression in mice with liver-specific knockout of the Ctnnb1 gene (encoding β-catenin), whereas levels of Cyp1a1 and Cyp3a11 were not altered (Sekine et al., 2006). These findings were largely confirmed shortly after by Tan and co-workers, who detected down-regulation of Cyp isoforms 1a2, 2e1, 2a5, and 2d9 in the Ctnnb1 knockout mice by microarray analysis, while up-regulation was reported for Cyp1a1, 2b9, and 2b10 upon disruption of the Ctnnb1 gene (Tan et al., 2006).

Thus, it is beyond dispute that signaling through the β-catenin signaling pathway influences hepatic basal expression of different Cyps. However, there is also some evidence that β-catenin might be involved in the regulation of inducible expression of Cyps and other drug-metabolizing enzymes mediated by xenobiotic-sensing receptors: (i) the AhR is a target of β-catenin signaling (Chesire et al., 2004; Stahl et al., 2005; Hailfinger et al., 2006); (ii) co-transfection of activated β-catenin facilitates activation of an AhR-responsive luciferase reporter plasmid in mouse hepatoma cells by the AhR ligand 2,3,7,8-tetrachloro-p-dibenzodioxin (TCDD) (Loeppen et al., 2005). In this work, we analyzed the expression and hepatic localization of representative drug-metabolizing enzymes in response to different
model inducers of xenobiotic metabolism in mice with hepatocyte-specific Ctnnb1 knockout 

*in vivo.*
Methods

Animal breeding. Mice with hepatocyte-specific knockout of Ctnnb1 (encoding β-catenin) were obtained as follows: transgenic mice with loxP sites inserted in the introns flanking exons 3 and 6 of the Ctnnb1 gene (Huelsen et al., 2001) were interbred with Alb-Cre mice expressing Cre recombinase under control of the hepatocyte-specific albumin promoter (purchased from Jackson laboratory, Bar Harbor, Maine, USA). Genotyping was performed by standard PCR using the primer pairs Ctnnb1^{loxP}_{sense} 5’-ACTGCCTTTGTCTTCCCTTCTTG-3’ and Ctnnb1^{loxP}_{antisense} 5’-CAGCCAAGGAGAGCAGGTGAGG-3’; and Cre sense 5’-TCCATGACTGAACGAACCTGGCAGG-3’ and Cre antisense 5’-TTTCCTGGCATCAGCACTGGTGCG-3’. Homozygous Ctnnb1^{loxP/loxP} mice carrying a Cre allele, resulting in hepatocyte-specific knockout of the Ctnnb1 gene, are referred to as ‘Ctnnb1 knockout mice’ in the following text. Mice were kept on a 12 h dark/ light cycle and had access to food and tap water ad libitum. Mice were sacrificed between 9 and 11 a.m. to avoid circadian influences. Animals received humane care and protocols complied with institutional guidelines.

Animal experiments. Groups (n=5) of Ctnnb1 knockout or age- and sex-matched wild-type animals were treated at 8 weeks of age with different inducers of drug-metabolizing enzymes as follows: (i) a single i.p. injection of 3 mg/kg body weight 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene (TCPOBOP) (Sigma, Taufkirchen, Germany) 72 h before killing; (ii) a single i.p. injection of 90 mg/kg body weight phenobarbital (PB) (Geyer, Renningen, Germany) followed by 3 days of PB-containing (0.05 % w/v) diet (Ssniff, Soest, Germany); (iii) two i.p. injections of 50 mg/kg body weight 3-methylcholanthrene (3MC) at 48 h and 24 h before killing; (iv) a single i.p. injection of 200 mg/kg body weight pregnenolone-α-carbonitrile (PCN) 72 h before killing; (v) a single i.p. injection of 350 mg/kg body weight butylated hydroxyanisole (BHA) 72 h before killing; (vi) a single i.p. injection of 500 mg/kg body weight clofibrate (substances (iii)-(vi) all purchased from Sigma, Taufkirchen, Germany) 72 h before killing. Control groups received injections with the respective solvents, i.e. corn oil (3MC,
TCPOBOP, PCN, BHA, clofibrate) or phosphate-buffered saline (PB). Isopropanol (2 g/kg body weight) was administered by gavage in a 25 % aqueous solution.

**Isolation of hepatocytes.** Periportal and perivenous subpopulations of hepatocytes were isolated and enriched by combined digitonin/collagenase perfusion of the liver as described previously (Braeuning et al., 2006; Hailfinger et al., 2006). To obtain periportal hepatocyte subpopulations, 5 mM digitonin solution was infused through the vena cava and then immediately flushed out from the opposite direction. To obtain perivenous hepatocytes, digitonin solution was infused through the portal vein. After digitonin treatment, liver was perfused with collagenase solution. Subsequently, viable hepatocytes were separated by density gradient centrifugation. Viability of the resulting hepatocyte fractions was always ~80-90 % as determined by trypan blue staining.

**Real-time RT-PCR.** Total RNA was isolated using Trizol reagent (Invitrogen, Karlsruhe, Germany) and reverse transcribed by use of the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Real-time RT-PCR was carried out on a LightCycler instrument (Roche, Mannheim, Germany) using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche, Mannheim, Germany) and the primer pairs listed in Table 1. Relative expression ratio of the target genes in the samples was computed based on the crossing point difference of a sample versus a control and its real-time PCR efficiency according to (Pfaffl, 2001). 18s rRNA expression was used for normalization.

**Immunostaining.** Slices from frozen (10 µm thick) or paraffin-embedded (5 µm thick) liver samples were stained by standard methods as recently described (Hailfinger et al., 2006; Braeuning et al., 2007b) using antisera against overall-Cyp1A (1:1,000 dilution; gift of Dr. R. Wolf, Biochemical Research Centre, University of Dundee, Dundee, UK), overall-Cyp2C (1:300; gift of Dr. R. Wolf), overall-Cyp3A (1:1,000 dilution; Biotrend, Cologne, Germany), and overall-GSTm (1:500; gift of Dr. R. Wolf), in combination with appropriate horseradish peroxidase-conjugated secondary antibodies (1:20; Dako, Glostrup, Denmark) with 3-amino-9-ethylcarbazole/ H₂O₂ as substrates.
**Glucose-6-phosphatase staining.** Activity of glucose-6-phosphatase in slices from glutaraldehyde-fixed liver samples was determined by use of a method described by (Wachstein and Meisel, 1957).

**Western blotting.** Whole cell extracts were separated by SDS-PAGE (30 µg/lane) and transferred to PVDF membranes using standard methods. Protein detection was carried out as recently described (Hailfinger et al., 2006) using antibodies against overall-Cyp1A (1:5,000 dilution; gift of Dr. R. Wolf, Biochemical Research Centre, University of Dundee, Dundee, UK), overall-Cyp2C (1:1,000 dilution; gift of Dr. R. Wolf), overall-Cyp3A (1:5,000 dilution; Biotrend, Cologne, Germany), overall-GSTM (1:1,000; gift of Dr. R. Wolf), β-catenin (1:500; BD, Heidelberg, Germany), and GAPDH (1:1,000; Chemicon, Chandler’s Ford, UK) in combination with appropriate alkaline phosphatase-conjugated secondary antibodies (1:10,000; Tropix, Weiterstadt, Germany) with CDP-Star as a substrate. Chemoluminescence was monitored by use of a CCD camera system.

**Statistical analysis.** For statistical analysis of genotype differences, Student’s t-test was used. Differences were considered significant when p<0.05. Statistical probability is indicated by asterisks as follows: *p<0.05, **p<0.01, ***p<0.001. Data were also subjected to 2-way ANOVA analysis for genotype and treatment effects. These results are shown in Supplementary Table 1.
Results

Mice with hepatocyte-specific knockout of the Ctnnb1 gene (referred to as ‘Ctnnb1 knockout mice’) and age- and sex-matched controls (n=5 per group) were treated with different inducers of drug-metabolizing enzymes to elucidate the role of β-catenin signaling in xenobiotic-induced expression of drug metabolism-related genes in vivo. Due to limited availability of animals, most inducers were tested with either males or females. Induction of CAR-dependent gene expression by the differentially-acting CAR agonists TCPOBOP and phenobarbital was studied with mice from both sexes, since gender differences in response to CAR agonists have been reported in the literature (Ledda-Columbano et al., 2003). Livers were isolated and hepatic levels and zonation of various mRNAs and proteins was analyzed.

Basal expression of CAR target genes. In absence of known inducers of CAR-dependent transcription, striking alterations in the amount and hepatic localization of various drug-metabolizing enzymes from both phase I and phase II were observed in male and female Ctnnb1 knockout mice: mRNAs for the CAR targets Cyp1a2, Cyp2c, GSTm2, GSTm3, and GSTm6 were expressed at significantly lower levels in Ctnnb1 knockout mice from both sexes. Inversely, Cyp2b10 and Cyp2i2 mRNAs were more abundantly expressed in these animals with Cyp2b10 showing the more pronounced effect. Cyp3a mRNA levels were significantly increased in male Ctnnb1 knockout mice. A trend for higher expression of this isoform in the knockouts was also observed in females, but missed the criteria of significance in our analysis (Fig. 1; left bars of the diagrams). The loss of Cyp2C and GSTm expression in the livers of Ctnnb1 knockout mice was confirmed at the protein level by Western blotting. Cyp3A protein was detectable at slightly higher levels in livers from male Ctnnb1 knockout mice (Fig. 2A). The band pattern differences between the two genotypes in the Western analysis for Cyp2C may indicate differences in the expression of certain Cyp2C isoforms or thereof derived splice variants. In addition to the differences in total amount of mRNAs and proteins, immunohistochemistry revealed striking differences between the two genotypes regarding the localization of the proteins within the liver lobule (Fig. 3A): Cyp2C and GSTm were preferentially expressed in perivenous hepatocytes from wild-type mice,
while no hepatic zonation of these enzymes was detectable after knockout of the *Ctnnb1* gene (see periportal glucose-6-phosphatase staining for comparison). Interestingly, zonation of Cyp3A seemed to be inverted in the *Ctnnb1* knockouts, which displayed higher Cyp3A protein levels in the periportal hepatocyte subpopulation. To confirm these changes in zonal gene expression, enriched periportal and perivenous hepatocyte subpopulations were prepared by digitonin/collagenase perfusion and analyzed for the expression of various zonated drug-metabolizing enzymes. In contrast to what was seen in wild-type animals, *Ctnnb1* knockout mice indeed expressed higher levels of Cyp3a mRNA in periportal hepatocytes. Zonation of all other mRNAs analyzed (Cyp1a2, Cyp2b10, Cyp2c, Cyp2e1, Cyp2f2, GSTm2, GSTm3, GSTm6) was lost or at least strongly reduced in the *Ctnnb1* null mice (Table 2).

**Effects of TCPOBOP.** In the presence of the CAR activator TCPOBOP, strong induction of mRNAs encoding Cyp1a2, Cyp2b10, Cyp2c, Cyp3a, GSTm2, and GSTm3 was observed in male wild-type mice, whereas no remarkable effects were observed for GSTm6 and the 'periportal' Cyp isoform 2f2 (Fig. 1A). Cyp2b10 and Cyp3a expression was significantly higher in livers of wild-type mice after treatment with TCPOBOP, despite higher basal mRNA levels in *Ctnnb1* knockout mice. Similarly, Cyp1a2, Cyp2c, GSTm2, and GSTm3 mRNAs were also induced to significantly higher levels by TCPOBOP in mice from the wild-type group. At the protein level, induction of Cyp3A and GSTm was also clearly detectable by Western blotting, whereas only minor effects were seen for Cyp2C (Fig. 2A). Immunohistochemical analysis demonstrated an induction of Cyp2C, Cyp3A, and GSTm proteins in wild-type mice associated with the expected broadening of the perivenous hepatocyte subpopulation expressing these enzymes (Fig. 3A). Induction of the aforementioned proteins was also visible in *Ctnnb1* knockout livers, but without remarkable zonation. However, there were obvious differences in Cyp3A and GSTm (and, to a lesser extent, also Cyp2C) expression between individual cells in the response to TCPOBOP: a scattered pattern of strong and weak responders was observed (Fig. 3A).
When TCPOBOP was administered to female mice, an induction pattern of Cyp1a2, Cyp2b10, Cyp2c, Cyp3a, and GSTm2 was detected which was similar (i.e. up to higher levels in the wild-type animals) to what had been observed in males (Fig. 1B). However, genotype differences between TCPOBOP-stimulated expression of Cyp2c, Cyp3a, and GSTm2 missed our criteria of significance. GSTm3 was induced to approximately equal levels in female mice from both genotypes, whereas Cyp2f2 and GSTm6 were slightly repressed after TCPOBOP treatment. The lower fold induction of Cyp2b10 in female mice (approx. 100-fold, compared to >1,000-fold in males) was due to higher basal levels of the respective mRNA in these animals.

**Effects of phenobarbital.** An additional experiment was performed using an indirect activator of CAR signaling, phenobarbital (PB). Following treatment with PB, results were comparable to those of the TCPOBOP experiment. Significantly higher cellular levels of Cyp1a2, Cyp2c, GSTm2, GSTm3, and GSTm6 mRNAs were detected in male wild-type mice as compared to their Ctnnb1-deficient counterparts (Fig. 1C). A tendency for higher PB-induced levels of Cyp2b10 and Cyp3a was also observed in the wild-type group, but was not statistically significant. In contrast to TCPOBOP, PB was able to induce Cyp2f2 in wild-type mice, whereas Ctnnb1 null mice, which exhibited higher basal levels of this Cyp isoform, did not respond with further induction of the enzyme (Fig. 1C). Again, the results obtained with female mice largely resembled those seen with males. PB-induced expression of Cyp1a2, Cyp2b10, Cyp2c, GSTm2, and GSTm6 was significantly higher in the wild-types as compared to PB-treated Ctnnb1 knockout mice and the (non-significant) trend for higher PB-induced Cyp3a expression in wild-type mice was also seen in this experiment (Fig. 1D). As in the TCPOBOP experiment, PB induced GSTm3 up to equal levels in female mice from both genotypes, contrasting the situation in males.

In summary, activation of CAR led to the expression of higher levels of Cyp1a2, Cyp2b10, Cyp2c, GSTm2, GSTm6, and - to a lesser extent - Cyp3a, in male and female wild-type mice as compared to their littermates with hepatocyte-specific knockout of Ctnnb1.
CAR agonist-induced GSTm3 expression was also higher in wild-type males, whereas no differences regarding this particular enzyme were observed in females.

**Expression of AhR target genes - effects of 3-methylcholanthrene.** In an analogous experiment, 3-methylcholanthrene (3MC)/AhR-dependent enzyme induction was analyzed in male wild-type and *Ctnnb1* knockout mice. In absence of 3MC, mRNA for Cyp1a1 was barely detectable and did not show significant differences in expression between the two genotypes. The more constitutively expressed Cyp1a isoform Cyp1a2, two different transcript variants of Ugt1a6, Ugt1a7, and GSTa were analyzed as additional AhR target genes. Cyp1a2 and GSTa were more abundantly expressed in wild-type mice. A similar trend was also visible for the Ugts. Statistical significance, however, was only achieved in the case of one of the Ugt1a6 variants (Fig. 4).

3MC led to a strong increase in Cyp1a1 mRNA in both genotypes. Resulting Cyp1a1 mRNA levels were significantly higher in the wild-types (Fig. 4). A similar effect was also observed for Cyp1a2, but in this case the differences were not statistically significant. One of the Ugt1a6 transcripts, Ugt1a7, and GSTa were also affected by 3MC treatment, whereas the second Ugt1a6 transcript (lacking the first exon) was unsusceptible to AhR activation. 3MC-induced Ugt1a6 mRNA levels in *Ctnnb1* knockout mice remained slightly lower than in wild-type mice. GSTa, however, was induced to higher levels in the knockout mice. Ugt1a7 expression was also slightly higher in the *Ctnnb1* knockouts, but without being statistically significant (Fig. 4).

At the protein level, highest levels of Cyp1A were detected by Western blotting in 3MC-treated wild-type mice, whereas much less of the protein was detected in liver lysates from their *Ctnnb1*-deficient littermates. Resembling the findings of the mRNA expression analysis, basal Cyp1A expression was also lower in the knockout animals (Fig. 2B). Immunohistochemical staining demonstrated an almost complete loss of non-induced hepatic Cyp1A expression in the knockout mice. After injection with 3MC, increased amounts of Cyp1A protein were detectable in mice from both genotypes. In agreement with previous studies of AhR ligand-induced Cyp1A expression (see introduction section), the number of
Cyp1A-expressing cells of wild-type mice increased towards the portal vein. No clear zonation, however, was visible in the knockouts, which showed a rather uniform-like expression (Fig. 3B).

Expression of PXR target genes - effects of pregnenolone-α-carbonitrile. Stimulation of PXR-dependent transcription was performed in male mice by i.p. injection of PCN. Quantification of mRNAs from the PXR target genes Cyp2b10 and Cyp3a demonstrated considerable differences between the genotypes (Fig. 5A): Cyp2b10 was strongly induced by PCN in wild-type mice. PCN did not exert remarkable effects on Cyp2b10 in mice with hepatocyte-specific knockout of the Ctnnb1 gene, which possessed significantly higher basal levels of this Cyp isoform. Cyp3a mRNA levels were strongly stimulated by PCN in both groups. While basal Cyp3a expression was higher in Ctnnb1 knockout mice (see above), no significant differences were detectable between the two genotypes after treatment with PCN. Results of the mRNA quantification were confirmed by Western analysis (data not shown) and immunostaining for Cyp3A. Interestingly, a reversal of Cyp3A zonation was seen in wild-type mice, which displayed higher levels of the protein in periportal hepatocytes after PCN treatment (Fig. 3C).

Expression of PPARα target genes - effects of clofibrate. Cellular mRNA levels of the PPARα-regulated Cyp isoform 4a14 were quantified in female wild-type and Ctnnb1 knockout mice. Basal Cyp4a14 levels were reduced by ~75% in Ctnnb1 knockout mice. Induction of PPARα signaling with clofibrate led to moderately (~2-fold, as compared to the wild-type control group) elevated levels of Cyp4a14 which did not differ between both genotypes (Fig. 5B).

Expression of Nrf2 target genes - effects of BHA. Basal and BHA-induced levels of the Nrf2-dependent mRNAs encoding microsomal epoxide hydrolase (Ephx1), GSTa, and Nqo1 were analyzed (Fig. 5C): basal Ephx1 expression was slightly higher in Ctnnb1 knockout mice than in their wild-type littermates, but without being statistically significant. GSTa mRNA levels were significantly higher in the wild-types. No remarkable effect of genotype on basal Nqo1 levels was found. After BHA injection, an approx. 2.2-fold increase
in Ephx1 mRNA was observed in wild-type mice, while their knockout counterparts did not show an induction of the enzyme (Fig. 5C). Levels of GSTa mRNA were also significantly elevated by BHA in the wild-types (~2-fold), whereas mice with knockout of Ctnnb1 did not respond to BHA with a significant induction of GSTa. Comparable effects were observed for Nqo1, which was exclusively inducible by BHA in Ctnnb1 wild-type mice (Fig. 5C). In summary, Ctnnb1 null mice were not responsive to BHA-mediated induction of Nrf2-dependent transcription.

**Induction of Cyp2e1.** In absence of known inducers, Cyp2e1 was almost completely absent in livers from male and female knockout mice, whereas high levels of Cyp2e1 mRNA and protein were detectable in wild-type hepatocytes located in the perivenous zone of the liver lobule (data not shown). After intragastric administration of 2 g/kg body weight isopropanol, slightly elevated Cyp2E1 protein contents were detected by immunohistochemistry and Western blotting in wild-type animals, whereas the enzyme was still absent in Ctnnb1 knockout mice (data not shown).

**Expression of nuclear receptors involved in drug metabolism.** Levels of mRNAs encoding nuclear receptors involved in xenobiotic-mediated induction of drug-metabolizing enzymes, namely AhR, CAR, PXR, PPARα, RXRα, and Nrf2, were analyzed by real-time RT-PCR (Fig. 6). Both AhR and CAR were significantly down-regulated by ~60 % in male and in female Ctnnb1 knockout mice. No further significant differences in receptor mRNA expression were observed in males. Female Ctnnb1 knockout mice, however, expressed significantly reduced levels of PXR (~20 % lower) and also displayed a tendency towards a reduction of PPARα and RXRα mRNAs. However, the latter effects missed the criteria of significance in our analysis. Nrf2 levels were not affected by knockout of the Ctnnb1 gene.
**Discussion**

This study gives an overview of basal and inducible expression of drug-metabolizing enzymes from phase I and phase II in mice with hepatocyte-specific knockout of \textit{Ctnnb1} (encoding \(\beta\)-catenin). It should be noted that physiological activation of the \(\beta\)-catenin pathway in healthy adult liver is restricted to perivenous hepatocytes, as it has been recently demonstrated (Benhamouche et al., 2006; Sekine et al., 2007).

The significance of presented data can be subdivided into two major aspects. Firstly, the present work confirms previous reports describing a loss of basal Cyp expression (esp. Cyp1a2 and Cyp2e1) in mice with liver-specific knockout of \textit{Ctnnb1} (Sekine et al., 2006; Tan et al., 2006). It goes, however, far beyond these studies by demonstrating striking alterations in overall expression and especially in hepatic localization of several drug-metabolizing enzymes from phase I and II of xenobiotic metabolism, at the mRNA and protein levels in male and female animals. Altogether, presented data again underline the importance of \(\beta\)-catenin signaling in the maintenance of the ‘perivenous’ hepatocyte gene expression profile, as it has been suggested by previous studies from our and other groups (Hailfinger et al., 2006; Sekine et al., 2006; Tan et al., 2006; Braeuning et al., 2007a; Braeuning et al., 2007b; Sekine et al., 2007).

Perivenous-specific expression of most mRNAs and proteins analyzed was lost or at least markedly reduced in the knockout mice. However, zonal differences were still detected for some enzymes (e.g. glucose-6-phosphatase, Cyp3a). That points towards the existence of other zonation-determining factors that act independently of \(\beta\)-catenin. The mostly uniform expression of drug-metabolizing enzymes observed throughout the liver lobules of \textit{Ctnnb1} knockout mice can be well explained by a loss of the expression-stimulating presence of \(\beta\)-catenin in perivenous hepatocytes of these animals, but the inversion of Cyp3a zonation does not fit this model. One might speculate that \(\beta\)-catenin does not only promote ‘perivenous’ gene expression, but also induces the release of a yet unknown soluble factor repressing Cyp3a expression in periportal hepatocytes of wild-type, but not of \(\beta\)-catenin-deficient mice.
It is tempting to speculate that β-catenin indirectly diminishes the levels of drug-metabolizing enzymes by transcriptional down-regulation of receptors involved in the transcriptional regulation of the respective genes, since mRNA levels of CAR and AhR were lowered in the transgenic animals. It seems, however, quite unlikely that receptor shortage should be responsible for lower basal expression of its corresponding target genes in a system that still exhibits very pronounced inducibility of these genes after receptor activation by appropriate xenobiotics. One of the possible mechanisms may be a direct effect of β-catenin/TCF transcription factor complexes on the 5'-regulatory sequences of Cyp genes, as recently put forward by Sekine and colleagues (Sekine et al., 2006). However, up to now there are no mechanistically-orientated studies available addressing this question.

Interestingly, some CAR target genes (especially Cyp2b10) were up-regulated in the knockout mice regardless of lower levels of CAR. This finding also argues against the hypothesis that a shortage of CAR is responsible for the reduced basal expression of several CAR target genes in Ctnnb1 knockout mice. β-catenin seems to play a directly or indirectly repressive role in basal Cyp2b10 expression and thus differentially acts on basal Cyp expression in a highly isoenzyme-specific manner. The loss of basal expression of various enzymes (Cyp1a2, Cyp2c, Cyp2e1, Cyp4a14, GSTa, GSTm2, GSTm3, GSTm6, Ugt1a6) is in contrast with other enzymes, which are not or only marginally altered (Cyp1a1, Cyp3a, Ugt1a7, Ephx1, Nqo1) or even significantly over-expressed in Ctnnb1 knockout mice (Cyp2b10, Cyp2f2).

Secondly - and that is the major novelty of this study - response of Ctnnb1 knockout mice to a broad spectrum of enzyme inducers was analyzed. For the first time an important role of β-catenin signaling in the regulation of inducible expression of drug-metabolizing enzymes mediated by different ligand-activated transcription factors was demonstrated. In particular, remarkable differences were observed in response to CAR, AhR, and Nrf2 activators. The situation with the xenobiotic-induced expression presents itself more consistent, as compared to basal expression: at large, xenobiotic-induced levels of the respective target enzymes were generally lower in Ctnnb1 knockout mice. On the other
hand, PXR- and PPARα-dependent gene expression was, if at all, much less affected. Diminished response to the AhR agonist 3MC in mice lacking hepatic β-catenin is in line with a previous *in vitro*-study from our group demonstrating increased response of mouse hepatoma cells to the AhR agonist TCDD after activation of β-catenin signaling (Loeppen et al., 2005).

After treatment with CAR activators, a paradoxical inversion of the original wild-type:knockout Cyp2b10 expression ratio is observed: whereas basal expression of this gene is strongly elevated after knockout of Ctnnb1, the differences are inverted after treatment with inducers. Thus, β-catenin seems to play a negative-regulatory role in basal Cyp2b10 expression. Irrespective of this repressive role, β-catenin appears to be a positive-regulatory factor in CAR-mediated Cyp2b10 expression, since cellular Cyp2b10 levels were higher in CAR agonist-treated wild-type mice as compared to their Ctnnb1 knockout littermates. This again demonstrates - despite an overall diminished response to CAR agonists was observed in the knockout mice - that the regulatory functions of β-catenin are complex and that it is not possible to establish a single model which could be applied to explain all the effects of β-catenin on each individual gene.

Hepatic localization of the induction of drug-metabolizing enzymes - a process largely taking place in perivenous hepatocytes of wild-type mice - was substantially changed in Ctnnb1 knockout animals. These mice displayed a mostly uniform-type induction of the respective proteins in all hepatocytes. In the case of treatment with TCPOBOP, weaker- and stronger-responding hepatocytes were distributed in a scattered pattern throughout the liver lobule. Thus, β-catenin does not only influence the magnitude of response, but also regulates the zone-specificity of gene expression after treatment with xenobiotic inducers.

It cannot be ruled out that diminished levels of CAR and AhR are - at least in part - responsible for the overall smaller receptor-mediated response seen in the Ctnnb1 knockout mice. However, in consideration of the fact that (i) TCPOBOP and PB induced the expression of the CAR target gene GSTm3 to equal levels in female wild-type and knockout mice, irrespective of appreciable differences observed for other CAR targets, and that (ii)
3MC-induced levels of alpha-class GSTs were higher in knockout than in the wild-type mice, while other AhR targets were induced to significantly higher levels in wild-type mice by 3MC, AhR/CAR depletion cannot bear the sole responsibility for the observed genotype differences in enzyme induction. Moreover, no alterations in cellular Nrf2 mRNA levels were detected, whereas BHA-mediated induction of Nrf2 targets was largely abrogated in Ctnnb1 knockout mice. As in the case of basal expression, these facts point towards isoenzyme-specific mechanisms by which β-catenin differentially regulates the response of individual genes to various xenobiotic stimuli.

In our experiments, we observed an inversion of Cyp3A zonation in wild-type mice after treatment with PCN. This was unexpected, since PXR-dependent enzyme induction was reported to preferentially occur in perivenous hepatocytes of Sprague-Dawley rats (Wolf et al., 1984). This discrepancy might be explained by species differences in enzyme induction patterns. However, preferential induction of a ‘perivenous’ Cyp isoform in periportal hepatocytes has been reported before: 3MC leads to the expected perivenous-dominant induction pattern of Cyp1A, whereas administration of administration of another AhR agonist, β-naphtoflavone, induces Cyp1A expression in periportal hepatocytes (Wolf et al., 1984; Foster et al., 1986; Bars and Elcombe, 1991; Oinonen et al., 1994). The mechanisms that govern this particular type of enzyme induction are still unknown. Notably, β-naphtoflavone induction of isolated hepatocytes from different zones of the liver could not reproduce the zonal differences observed in vivo (Oinonen et al., 1994). It is therefore likely that regionally acting factors, possibly some not yet identified zonal-specific expressed AhR-associated proteins modulate periportal- and perivenous-specific responses to different AhR agonists, rather than inherent features of certain acinar hepatocyte subpopulations.

In summary, present data clearly show that β-catenin participates in the coordinate regulation of xenobiotic-induced expression of phase I and phase II enzymes of drug metabolism, where - on the whole - β-catenin supports enzyme induction via AhR, CAR and Nrf2. β-catenin is also involved in the regulation of basal expression of many drug-metabolizing enzymes. In this particular context, β-catenin seems to have both positive- and
negative-regulatory functions, depending on the individual gene. Moreover, the nearby complete loss of zonation of basal and drug-induced in the Ctnnb1 knockout mice provides further evidence that β-catenin functions as a central regulator of perivenous drug metabolism in mouse liver.
Acknowledgments

We greatly acknowledge the excellent technical assistance by Johanna Mahr, Silvia Vetter, and Elke Zabinsky. We also thank Dr. R. Wolf (Dundee, UK) for gift of Cyp and GST antisera.
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a rat tumor promotion model: quantification and immunolocalization of CYP1A1 and CYP1A2 in the liver. Cancer Res 52:3436-3442.


Footnotes

This study was supported by the Deutsche Krebshilfe [Grant 106356].

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Legends for figures

Fig. 1. Basal and inducible expression of CAR target mRNAs in livers from wild-type and *Ctnnb1* knockout mice, as determined by real-time RT-PCR. Gene expression in response to treatment with TCPOBOP in male (A) and female (B) mice, and in response to phenobarbital treatment in males (C) and females (D) is shown relative to vehicle controls. Mean ± S.D. (n=5 per group) are given relative to the values obtained with the wild-type controls. Significant differences between genotypes are indicated as follows: *p<0.05, **p<0.01, ***p<0.001 (Student’s t-test). Abbreviations: Cyp, cytochrome P450; GST, glutathione S-transferase; PB, phenobarbital; TCP, 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene; wt, wild-type; ko, *Ctnnb1* knockout.

Fig. 2. Western analysis of protein expression in livers from male wild-type and *Ctnnb1* knockout mice. (A) Expression of CAR targets Cyp2C, Cyp3A, and GSTm in TCPOBOP-treated animals and the respective controls. Successful knockout of *Ctnnb1* is demonstrated by Western analysis for β-catenin protein. (B) Expression of AhR-dependent Cyp1A in 3MC-treated animals. In all experiments, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as a loading control. Abbreviations: 3MC, 3-methylcholanthrene; β-cat, β-catenin; Cyp, cytochrome P450; GST, glutathione S-transferase; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene; wt, wild-type; ko, *Ctnnb1* knockout.

Fig. 3. Immunohistochemical staining of serial liver sections from male wild-type and *Ctnnb1* knockout mice treated with TCPOBOP (A), 3MC (B), PCN (C) or with corn oil as a vehicle control. For orientation, preferential periportal activity of glucose-6-phosphatase is shown. Abbreviations: c, central vein; p, portal vein; 3MC, 3-methylcholanthrene; Cyp, cytochrome P450; G6P, glucose-6-phosphatase; GST, glutathione S-transferase; PCN, pregnenolone-α-carbonitrile; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene; wt, wild-type; ko, *Ctnnb1* knockout.
Fig. 4. Basal and 3MC-induced expression of AhR target mRNAs in livers from male wild-type and Ctnnb1 knockout mice, as determined by real-time RT-PCR. Mean ± S.D. (n=5 per group) are given relative to the values obtained with the wild-type controls. Significant differences between genotypes are indicated as follows: *p<0.05, **p<0.01, ***p<0.001 (Student’s t-test). Abbreviations: 3MC, 3-methylcholanthrene; Cyp, cytochrome P450; GST, glutathione S-transferase; Ugt, UDP-glucuronosyl-transferase; wt, wild-type; ko, Ctnnb1 knockout.

Fig. 5. Real-time RT-PCR analysis of basal and inducible expression of (A) PXR target mRNAs in livers from male wild-type and Ctnnb1 knockout mice, treated with PCN, (B) PPARα-dependent Cyp4a14 in clofibrate-treated females, and (C) Nrf2 target mRNAs in male wild-type and Ctnnb1 knockout mice in response to treatment with BHA. Mean ± S.D. (n=5 per group) are given relative to the values obtained with the wild-type controls. Significant differences between genotypes are indicated as follows: *p<0.05, **p<0.01, ***p<0.001 (Student’s t-test). Abbreviations: BHA, butylated hydroxyanisole; Clo, clofibrate; Cyp, cytochrome P450; Ephx1, microsomal expoxide hydrolase; GST, glutathione S-transferase; Nqo, NAD(P)H-quinone-oxidoreductase; PCN, pregnenolone-α-carbonitrile; wt, wild-type; ko, Ctnnb1 knockout.

Fig. 6. Expression of nuclear receptors related to drug metabolism in livers from male and female Ctnnb1 knockout and wild-type mice, as determined by real-time RT-PCR. Mean ± S.D. (n=5 per group) are given relative to the values obtained with the wild-type controls. Significant differences between genotypes are indicated as follows: *p<0.05, **p<0.01, ***p<0.001 (Student’s t-test). Abbreviations: AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; Nrf2, nuclear factor erythroid-related factor 2; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane-X-receptor; RXR, retinoid-X-receptor.
**Tables**

**TABLE 1**

Real-time RT-PCR primers used in this study.

<table>
<thead>
<tr>
<th>gene</th>
<th>forward primer (5’-3’)</th>
<th>reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AhR</td>
<td>GTCAAATCCTTCTAAGCGACACA</td>
<td>AACCAGCACAAGCCATTCA’</td>
</tr>
<tr>
<td>CAR</td>
<td>AAACAACAGTCTCGGCTCCAAA</td>
<td>AGCATTTCTATTGCCACTCCC</td>
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<tr>
<td>Cyp1a1</td>
<td>TGTCCTCCGTTACCTGCTTA</td>
<td>GTGTCAAAACCAGCTCCAAA</td>
</tr>
<tr>
<td>Cyp1a2</td>
<td>GAGCGCTGTATATCTACATAAAACCA</td>
<td>GGGTGGAACATGATAGACACTATTGT</td>
</tr>
<tr>
<td>Cyp2b10</td>
<td>TACTCCTATTCCATGTCTCCAAA</td>
<td>TCCAAAGTCTCTTTTCACATGT</td>
</tr>
<tr>
<td>Cyp2c</td>
<td>CTCCCTCTGGCCCCCACC</td>
<td>GGACACAGCTCAGTGA</td>
</tr>
<tr>
<td>Cyp2e1</td>
<td>TCCCTAAGTATCCTCCGTGA</td>
<td>GTAATCGAAGCTTTTTGTA</td>
</tr>
<tr>
<td>Cyp2f2</td>
<td>AAAGAAGCATCGAGGAGC</td>
<td>CAAAGACAGACAGCACAGAT</td>
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<tr>
<td>Cyp3a</td>
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<td>GGAATCATCAGTTGACCCCT</td>
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<td>Cyp4a14</td>
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<td>TCCATTGTCCCAAGAGTCTAATACT</td>
</tr>
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<td>AGGCTTTCTGGGCTGCCAGGG</td>
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<td>GSTm3</td>
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<td>Nrf2</td>
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<tr>
<td>PPARα</td>
<td>TGAAAGATTCGGAAACTGCA</td>
<td>AGCGTCTTCTCGGCA</td>
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<td>PXR</td>
<td>CATGGAGTGCTAGTCAGGCATAT</td>
<td>GGGGTTGTGGGCTATCAGAT</td>
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</table>
RXRa  ACATTGGGCTCGGACTGG  CTTGTGTCTCGGAGGTGTAGGT
Ugt1a6_a  AGAGAGTACAGGAACAAATGTTTATC
Ugt1a6_b  AGAGAGTACAGGAACAGCATGCTTACAT  CAACGATGCCATGCTCC
Ugt1a7  AAGAACTCGGAACCTTTTGGCC
18s rRNA  CGGCTACCACATCCAAGGAA  GCTGGAATTACCGGGCT
### TABLE 2

Hepatic zonation of mRNAs encoding drug-metabolizing enzymes in *Ctnnb1* wild-type and knockout mice.

<table>
<thead>
<tr>
<th>gene</th>
<th>zonation of gene expression (perivenous hepatocytes, % of periportal)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Ctnnb1</em> wild-type</td>
</tr>
<tr>
<td>Cyp1a2</td>
<td>821±72***</td>
</tr>
<tr>
<td>Cyp2b10</td>
<td>319±82*</td>
</tr>
<tr>
<td>Cyp2c</td>
<td>964±44***</td>
</tr>
<tr>
<td>Cyp2e1</td>
<td>1736±151***</td>
</tr>
<tr>
<td>Cyp2f2</td>
<td>4±1**</td>
</tr>
<tr>
<td>Cyp3a</td>
<td>169±36</td>
</tr>
<tr>
<td>GSTm2</td>
<td>906±117**</td>
</tr>
<tr>
<td>GSTm3</td>
<td>1579±350**</td>
</tr>
<tr>
<td>GSTm6</td>
<td>162±22</td>
</tr>
</tbody>
</table>

Mean (n=3 (wt); n=4 (ko)) ±SEM are given. Significant differences in zonal gene expression between wild-type and *Ctnnb1* knockout mice are indicated by asterisks: *p<0.05, **p<0.01, ***p<0.001 (Student’s t-test).
Figure 1
Figure 4

The figure shows bar charts for various gene expression levels under different conditions.

- **Cyp1a1**
  - WT: 2000, KO: 1000
  - 3MC: -

- **Cyp1a2**
  - WT: 20, KO: 8
  - 3MC: -

- **Ugt1a6_a**
  - WT: 1.5, KO: 1
  - 3MC: -

- **Ugt1a6_b**
  - WT: 1.5, KO: 1
  - 3MC: -

- **Ugt1a7**
  - WT: 2, KO: 1
  - 3MC: -

- **GSTa**
  - WT: 4, KO: 1
  - 3MC: -

Each chart indicates relative expression levels with error bars representing standard deviation.
Figure 5

A

Cyp2b10

**

Cyp3a

Cyp4a14

relative expression

PCN

wt  ko  wt  ko

wt  ko  wt  ko

wt  ko  wt  ko

B

Clo

relative expression

C

Ephx1

GSTa

Nqo1

relative expression

BHA

wt  ko  wt  ko

wt  ko  wt  ko

wt  ko  wt  ko

**

**

**
Figure 6