Activating and inhibitory functions of WNT/β-catenin in the induction of cytochromes P450 by nuclear receptors in HepaRG cells

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Running title: Role of β-catenin in the regulation of human P450 enzymes

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Number of text pages: 26

Number of Tables: 0

Number of Figures: 5

Number of references: 39

Number of words in Abstract: 223

Number of words in Introduction: 749

Number of words in Discussion: 1313

Non-standard abbreviations used:

AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; CYP, cytochrome P450; DME, drug metabolizing enzymes; PHH, primary human hepatocytes; PPARα, peroxisome proliferator-activated receptor alpha; PXR, pregnane X receptor; WNT/β-catenin, The wingless-type MMTV integration site family (WNT)/β-catenin/adenomatous polyposis coli (CTNNB1/APC) pathway.

Abstract

The WNT/β-catenin signaling pathway has been identified as an important endogenous regulator of hepatic cytochrome P450 (CYP) expression in mouse liver. In particular, it is involved in the regulation of CYP expression in response to exposure to xenobiotic agonists of the nuclear receptors CAR, AhR, and Nrf2. In order to systematically elucidate the impact of the WNT/β-catenin pathway on the regulation and inducibility of major human cytochrome P450 enzymes, HepaRG cells were treated with either the WNT/β-catenin signaling pathway agonist, WNT3a, or with siRNA directed against β-catenin, alone or in combination with a panel of activating ligands for AhR (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD), CAR (6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime, CITCO), PXR (rifampicin) and PPARα (4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid, WY14,643). Assessment of CYP gene expression and enzymatic activity following downregulation or activation of the WNT/β-catenin pathway revealed a requirement of β-catenin in the AhR-, CAR- and PXR-mediated induction of CYP1A, CYP2B6 and CYP3A4 (for CAR and PXR) and additionally of CYP2C8 (for PXR) gene expression. In contrast, activation of the WNT/β-catenin pathway prevented PPARα-mediated induction of CYP1A, CYP2C8 , CYP3A4 and CYP4A11 genes, suggesting a dominant-negative role of β-catenin in PPARα-mediated regulation of these genes. Our data indicate a significant impact of the WNT/β-catenin pathway on the regulation of cytochromes P450 enzymes in human hepatocytes and reveal a novel crosstalk between β-catenin and PPARα signaling pathways in the regulation of CYP expression.

Introduction

The WNT/β-catenin signaling pathway, traditionally well-known as a regulator of embryonic development, tissue homeostasis and carcinogenesis, has recently been identified as an important endogenous regulator of hepatic drug metabolism in mouse liver, where the activity of this particular pathway determines hepatic zonation of cytochrome P450 (CYP) expression (Loeppen et al., 2005; Hailfinger et al., 2006; Sekine et al., 2006; Braeuning and Schwarz, 2010; Braeuning et al., 2011a; Schreiber et al., 2011) and is also critically involved in the regulation of CYP transcription in response to exposure to xenobiotic agonists of a number of nuclear receptors (Braeuning et al., 2009; Ganzenberg et al., 2013; Vaas et al., 2014). Lipid- and xenobiotic-sensing nuclear receptors pregnane X-receptor (PXR, NR1I2), the constitutive androstane receptor (CAR, NR1I3), and the aryl hydrocarbon receptor (AhR), together with the liver-enriched transcription factor peroxisome proliferator-activated receptor α (PPARα, NR1C1) form networks of transcription factors that coordinately regulate hepatic expression of the majority of drug-metabolizing CYPs, phase II enzymes and transporters in response to xenobiotic exposure (Handschin and Meyer, 2005; Pascussi et al., 2008; Pelkonen et al., 2008; Thomas et al., 2013). Recent research elucidated extensive crosstalk between these receptors and other nuclear receptors and transcription factors, linking xenobiotic metabolism to the homeostasis of lipids, bile acids, glucose and other endogenous processes (Moreau et al., 2008; Gao and Xie, 2012). For WNT/β-catenin signaling in general, it has been observed that activation of the pathway in murine liver cells stimulates the expression of most CYPs and augments their inducibility by xenobiotic agonists of nuclear receptors *in vivo* and *in vitro*. Further studies link the β-catenin pathway to elevated expression of some CYPs in human hepatoblastoma (Schmidt et al., 2011) and primary human hepatocytes (PHH), underlining the relevance of these findings also for humans. In particular it was found that the WNT/β-catenin pathway activation induces the expression of CYP2E1, CYP1A2, and AhR, but not of CYP3A4, hepatocyte nuclear factor-4*α*, or PXR in PHHs (Gerbal-Chaloin et al.,

2014). However, so far there are no systematic data available on the impact of the WNT/β-catenin pathway on the regulation of basal or xenobiotic-induced CYP gene expression and activities in human cells.

Furthermore, while the interactions of signal transduction through β-catenin and the drug-sensing receptors PXR, CAR and AhR have been studied previously, a potential interaction of β-catenin with PPARα remains to be established. This nuclear receptor acts as a lipid sensor to control the expression of gene networks involved in lipid and energy homeostasis, adipocyte differentiation, and inflammatory responses (Lalloyer and Staels, 2010; Wahli and Michalik, 2012). Recently, we demonstrated that PPARα directly regulates the transcription of CYP3A4 (Klein et al., 2012; Thomas et al., 2013). Transcriptional activation of other drug biotransformation genes by PPARα has already been reported for the fatty acid ω – hydroxylase, CYP4A11 (Johnson et al., 2002; Wanders et al., 2011), several UDP-glucuronosyltransferases (Barbier et al., 2003; Senekeo-Effenberger et al., 2007) and some drug transporters (Cheng et al., 2005; Moffit et al., 2006). Our comparison of ligand-induced gene expression changes mediated by PPARα and the two prototypic xenosensors PXR and CAR further emphasizes the similar target profiles of these three nuclear receptors with respect to the regulation of drug biotransformation genes in the liver (Thomas et al., 2013). Thus, being a relatively novel player in the regulation of drug-metabolizing enzymes, PPARα activity in linking regulatory pathways of intermediary and xenobiotic metabolism warrants further studies.

The present study was therefore undertaken to analyze the relevance of β-catenin activation or inhibition on basal and inducible expression of a broad spectrum of CYP isoforms in the human hepatocarcinoma cell line HepaRG. When following a differentiation protocol including two weeks of cultivation in medium containing a high amount of dimethylsulfoxide, HepaRG cells differentiate into a mixed population of hepatocyte- and bile duct epithelium-like cells. Differentiated HepaRG possess several hepatocyte-specific functions and show

exceptional expression and activity of many CYPs relevant for the metabolism of drugs and xenobiotics (Aninat et al., 2006; Guillouzo et al., 2007; Klein et al., 2014). Our data suggest a significant impact of WNT/β-catenin signaling on basal as well as on inducible expression of the major CYP genes. Moreover, perturbation of the WNT/β-catenin pathway revealed the ancillary role of β-catenin in AhR-, PXR- and CAR-mediated, but an inhibitory role of β-catenin in PPARα-mediated induction of target P450 enzymes (CYP1A, CYP2,CYP3 and CYP4 isoforms). We suggest intricate interactions between WNT/β-catenin signaling, lipid homeostasis, and drug biotransformation capacity that may be of clinical relevance under diseased conditions such as obesity, hepatic steatosis and cancer.

Materials and Methods

Cell culture and treatment. Cryopreserved differentiated HepaRG cells (Biopredic, Rennes, France) were seeded in 12-well plates at a density of $0.8*10^6$ cells/well according to the supplier's instructions. HepaRG were cultured in HepaRG maintenance/metabolism medium (Biopredic) for 4 days prior to treatment. At day 4 after seeding, cells were treated with 20 ng/ml WNT3a (Sigma, Taufkirchen, Germany; dissolved in 0.1% bovine serum albumin) in HepaRG serum-free induction medium (Biopredic) and WNT3a treatment was repeated without additional medium change at days 5 and 6. Additionally, cells were exposed to known enzyme inducers at day 6 for additional 24h, alone or in combination with WNT3a treatment: 10nM 2,3,7,8-tetrachlorodibenzop-[p]-dioxin (TCDD; Ökometric, Bayreuth, Germany; dissolved in DMSO), 10µM Rifampicin (RIF; Sigma; dissolved in DMSO), 5µM CITCO (6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)-oxime; Enzo Life Sciences, Loerrach, Germany; dissolved in DMSO), or 100µM WY14,643 (4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid; gift from Dr. C. Gembardt, Ludwigshafen, Germany; dissolved in DMSO).

HuH7 cells were cultured at 37°C with 5% CO_2 concentration and passaged every 3-4 days by the Trypsin/EDTA method. HuH7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum (FCS) gold (PAA Laboratories GmbH, Pasching, Austria), 1% penicillin/ streptomycin, 1% pyruvate.

Transfections with siRNAs. For the RNAi experiments, HepaRG cells were transfected with 20 nM siRNAs using 10 pmol of Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies, Carlsbad, Germany) in 24-well plates with serum free medium. The siRNA targeting β-catenin (#146154) and a non-targeting siRNA as a negative control (Lo GC Duplex #2) were also obtained from Life Technologies. To the cells containing 100µl culture medium, 100 µl of the transfection cocktail was added per well. Upon 20 minutes of complex

formation, the liposomes were given to the cells. Following 24 hours after the transfection, cells were treated for additional 48 hours with above-mentioned agents.

Quantitative parallel real-time PCR using Fluidigm Biomark. RNA integrity and quantity were analyzed with the Agilent 2100 Bioanalyzer using the RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany). Synthesis of cDNA was performed with 500 ng RNA using Taqman Reverse Transcription Reagents (Applera GmbH, Darmstadt, Germany). Quantification of expression of 11 CYP genes was performed with Fluidigm's BioMark HD high-throughput quantitative chip platform (Fluidigm Corporation, San Francisco, USA), following the manufacturer's instructions (Spurgeon et al., 2008). All corresponding TaqMan® assays were purchased from Life Technologies (Darmstadt, Germany). For the validation of siRNA, the expression of downstream genes of WNT signaling was determined using following assays from Life Technologies: CTNNB1 (Hs00355049_m1), Axin 2 (Hs00610344_m1). The mRNA expression levels were normalized to glyceraldehyde-3-phsophate dehydrogenase (GAPDH) mRNA expression.

Measurement of P450 enzyme activities. P450 enzyme activities were determined in HepaRG cell culture supernatants using a liquid chromatography with tandem mass spectrometry-based substrate cocktail assay, as described previously (Feidt et al., 2010).

Western blot analysis. For the protein measurements, 50µg of total cell lysates were electrophoretically separated on a 10% SDS-polyacrylamide gel and subsequently transferred to a nitrocellulose membrane using a Trans-blot semi-dry Fastblot 44 transfer chamber (Biometra, Göttingen, Germany). After blocking with 5% skim milk in TBST membranes were incubated with the primary antibodies in 1% skim milk solution in TBST. For the detection of the levels of proteins of interest, we used a mouse anti-human β-catenin antibody from Becton and Dickinson (Nr. 610154; 1:500 dilution). CYP3A4 protein was detected as described earlier (Wolbold et al., 2003). For normalization, mouse anti-β-Actin (Sigma-Aldrich, A5441; 1:500) was used and goat-anti-rabbit-IRD800 (Li-COR, 926-32214;

1:10,000) and goat-anti-mouse-IRD650 (Li-COR, 926-68074; 1:10,000) were used as fluorescent-labeled secondary antibodies. Detection was performed with a Li-COR Odyssey CLx fluorescence reader (Bad Homburg, Germany).

Luciferase reporter gene assays. Human hepatocarcinoma HuH7 cells were transfected with 300 ng of reporter pGL3 basic plasmid containing 8.8 kb of CYP3A4 upstream promoter region (Tegude et al 2007) using Turbofect (Fermentas, St. Leon-Rot, Germany) as previously reported (Thomas et al., 2013). As an internal control of transfection efficiency, 25 ng of pGL3-TK-Renilla (Promega, Mannheim, Germany) was used. Cells were harvested in reporter "passive" lysis buffer (Promega, Mannheim, Germany) after 48h of incubation, and cell extracts were analyzed for firefly and renilla luciferase activities using Enspire® Multimode Plate-Reader (Perkin Elmer, Rodgau, Germany) and Luciferase Assay System from P.J.K. GmbH (Kleinblittersdorf, Germany). Firefly luciferase activities were normalized to renilla luciferase activities.

Statistical Methods. For comparisons of two groups, the Student's two-sample t-test (2-tailed) was applied. To identify statistically significant differences between more than two groups, one-way ANOVA with Student-Newman-Keuls post-test was performed. Measurements of experiments performed in triplicates were averaged prior to analyses. Statistical significance was defined as $p<0.05$. All calculations were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA). Densitometric analyses were performed using the ImageJ software available online from the National Institutes of Health, USA.

Results

Modulation of WNT/β-catenin signaling in HepaRG cells. To follow the consequences of WNT pathway activation or inhibition on basal and inducible expression of a broad spectrum of CYP isoforms in the human hepatocarcinoma cell line HepaRG, we applied two available strategies in order to either activate or inactivate the pathway, i.e. using stimulation with the canonical natural WNT ligand, WNT3a, and depletion of β-catenin using siRNA. As shown in Fig. 1A, treatment with 20ng/ml WNT3a significantly induced the expression of AXIN2 (conductin), a prototypical target of β-catenin signaling (Lustig et al., 2002), at both indicated time points, demonstrating successful activation of the pathway. Correspondingly, siRNA-mediated knock-down of β-catenin by siCAT resulted in ~ 50% decreased mRNA expression not only of CTNNB1 mRNA (CTNNB1 is the name of the gene encoding β-catenin), but also of its downstream target gene, AXIN2, as assessed 72h following siRNA transfection (Fig. 1B). Measurement of corresponding protein levels of β-catenin revealed more than 60% of protein loss 72 hours and 96 hours following siRNA transfection in comparison to the non-targeting control siRNA, siCTR, as assessed using densitometric analysis of the bands (Fig. 1B, bottom right).

Impact of WNT/β-catenin pathway modulation on basal CYP expression. Changes in mRNA expression of major P450 enzymes upon WNT/β-catenin pathway stimulation or β-catenin knock-down were investigated in three independent cultures of HepaRG cells (Fig. 2). Interestingly, expression levels of only two genes, CYP2E1 gene (2.6-fold) and CYP1A1 (1.8-fold), were significantly upregulated 72 hours following activation of β-catenin, confirming previous observations (Gerbal-Chaloin et al., 2014). Accordingly, CYP2E1, but not CYP1A1, was more than 50% downregulated by siCAT, suggesting involvement of β-catenin especially in the basal regulation of CYP2E1. In contrast, basal expression of CYP2B6 was significantly increased upon knock-down of β-catenin. However, the observed

minor decrease in CYP2B6 expression upon WNT3a treatment was not statistically significant.

Activation of WNT/β-catenin pathway increases AhR-, CAR- and PXR- but inhibits PPARα-mediated induction of CYP genes. We next asked whether the WNT/β-catenin pathway interferes with the inducibility of P450 enzymes via nuclear receptors during ligand-mediated activation by their known agonists. HepaRG cells were treated with the indicated agonists of AhR (Fig. 3A), CAR (Fig. 3B), PXR (Fig. 3C) and PPARα (Fig. 3D) in combination with WNT3a treatment. Activation of the AhR with TCDD alone resulted in a more than 4-fold activation of the AhR-target genes CYP1A1 and CYP1A2 (Fig. 3A, light-gray bars). Interestingly, paralleled activation of the WNT/β-catenin pathway led to a significantly higher increase of CYP1A1, but not of CYP1A2 expression (Fig. 3A, black bars). Also, activation of the AhR together with β-catenin restored expression of CYP2E1, which was significantly downregulated by TCDD alone. Besides of CYP1A genes, activation of CAR and PXR also resulted in the induction of CYP2B6, CYP2C19, CYP3A4 and CYP3A7 gene expression (Fig. 3B and C, light-gray bars). Induction of WNT/β-catenin signaling in combination with CAR-ligand treatment resulted in synergistic increases of CYP1A1 and CYP1A2 and to a moderate increase of CYP3A4 (Fig. 3B, black bars).The expression of the same genes, together with CYP2C8, was similarly additionally increased following activation of PXR (Fig. 3C, black bars). Surprisingly, co-treatment of HepaRG cells with WNT3a and the PPARα agonist WY14,643 antagonized the WY14,643-mediated upregulation of CYP1A1, CYP1A2, CYP2C8 and CYP3A4 and CYP4A11 gene expression (Fig. 3D, light-gray and black bars). Of note, ligand-mediated modulation of P450 gene expression in combination with WNT/β-catenin pathway activation was correspondingly reflected in the changes of their enzyme activity, as assessed by mass spectrometry-based analyses of the metabolism of a cocktail of specific substrates for the major CYPs (Supplementary Figure S1). No effects of WNT3a treatment on CYP7A1 expression were

observed, while down-regulation of CYP7A1 mRNA by PXR or PPARα activation was in line with previously published reports (Marrapodi and Chiang, 2000; Li and Chiang, 2005).

β-Catenin is required for the CYP induction via AhR, PXR and CAR and inhibits PPARα-mediated induction of CYP gene expression. Activation of the WNT/β-catenin pathway via WNT3a triggers a downstream cascade, which includes post-translational protein modifications, cytoplasm-nucleus shuttling and other secondary events which in part might not be directly related to the transcriptional activity of β-catenin. To further elucidate whether β-catenin itself interferes with the ligand-activated induction of P450 enzymes via nuclear receptors, we combined nuclear receptor agonist treatments with transfection of a siRNA (siCAT) targeting β-catenin. As shown in Fig. 4A, AhR-mediated induction of CYP1A genes was completely abolished following knockdown of β-catenin, demonstrating its requirement for TCDD-mediated upregulation. Similarly, loss of induction was also observed for CAR-mediated regulation of CYP1A1, CYP1A2, CYP2B6 and CYP3A4 (Fig. 4B), and PXR-mediated regulation of CYP1A1, CYP2B6, CYP2C8 and CYP3A4 (Fig. 4C). These results confirmed our previous observations using WNT3a treatment (cp. Fig. 3B and C) and further indicated an involvement of β-catenin in the inducibility of these genes via PXR and CAR (cp. Fig. 3B and C). In contrast, combination of WY14,643 treatment with β-catenin knockdown resulted in the significantly higher induction of CYP1A1, CYP2C8, CYP2E1 and CYP3A genes (Fig. 4D), which is in line with the WNT3a-mediated effects on PPARα-mediated induction of these CYPs (Fig. 3D). Interestingly, downregulation of β-catenin did not lead to an increased inducibility of CYP4A11 by PPARα, presumably due to already maximally induced status. Assessment of P450 enzyme activity following treatments with the agonists alone or in the combination with β-catenin knock-down confirmed the results of gene expression alterations (Supplementary Figure S2).

Activating and inhibitory roles of β-catenin in the ligand-mediated induction of CYP3A4 by PXR and PPARα. The crosstalk between the PPARα and WNT/β-catenin pathways has

not been previously studied in human hepatocytes. We thus further focused on our novel observation of the opposite influence of β-catenin on the induction of P450 enzymes by PPARα versus other receptors. We thus analyzed protein expression of CYP3A4 by Western blotting following rifampicin-mediated activation of PXR, a well-characterized regulator of CYP3A4 expression, and compared this to CYP3A4 protein expression under PPARα activation by WY14,643, either in the presence of WNT3a (Fig. 5A) or siCAT (Fig. 5B). In line with the mRNA expression profiles, co-induction of PPARα with β-catenin resulted in decreased CYP3A4 protein expression, as compared to WY14,643 treatment alone (Fig.5A, gray bars). Activation of PXR together with β-catenin, however, led to an increased fold induction of CYP3A4 protein compared to the effect of rifampicin alone, confirming the effects at the mRNA level (Fig. 5A, black bars). Correspondingly, knock-down of β-catenin led to increased CYP3A4 protein expression by activated PPARα (Fig. 5B, gray bars) and to more than 50% reduced levels of CYP3A4 protein following treatment with rifampicin (Fig. 5B, black bars). We could further confirm the opposite roles of β-catenin in the ligand-mediated CYP3A4 induction by PXR and PPARα using a luciferase reporter construct containing 8.8kb of an adjacent CYP3A4 promoter transfected in HuH7 cells (Fig. 5C and 5D). Thus we could show that β-catenin increased PXR-mediated but inhibited PPARα-mediated induction of CYP3A4 gene and protein expression.

Discussion

Although the involvement of WNT/β-catenin pathway in the regulation of several P450 enzymes has been well characterized and described for mice, less evidences exist so far about β-catenin-mediated modulation of CYP gene expression in humans. One previous study links the expression of some CYP isoforms in hepatoblastomas, pediatric human liver tumors, to the mutational activation of β-catenin signaling (Schmidt et al., 2011). In another very recent publication, a group of authors from France demonstrated the regulation of basal CYP2E1 and CYP1A2 expression by β-catenin in human primary hepatocytes (Gerbal-Chaloin et al., 2014). Thus we were interested to provide a systematic analysis of P450 gene expression modulation by β-catenin in HepaRG cells, not only at the basal level but also including CYP induction via nuclear receptor agonists. The latter phenomenon has, to the best of our knowledge, not been previously analyzed in human cells. HepaRG cells were chosen because of their high relevance as an *in vitro* model for human hepatocytes, which shows less inter-individual variability compared to cultures of primary human hepatocytes (Klein et al., 2014). By two different, complementary approaches of modulating the WNT pathway, namely by using the canonical ligand WNT3a as well as by specific siRNA-mediated β-catenin downregulation, our data provide evidence for an intricate crosstalk between the WNT/β-catenin pathway and various receptors involved in the regulation of P450 gene expression. In particular, we could demonstrate that β-catenin is required for the full induction of a number of downstream CYP genes by AhR, PXR and CAR. In contrast, β-catenin prevents the induction of CYP1A1, CYP2C8, CYP3A4 and CYP4A11 by PPARα. We could further show that the differential modulation of PXR- or PPARα-mediated CYP3A4 induction by β-catenin occurs within an 8.8kb upstream region of the CYP3A4 promoter.

Our data on the influence of β-catenin on basal CYP expression are in accordance with a recent study which demonstrated basal regulation of CYP1A1 and CYP2E1 by WNT/β-catenin (Gerbal-Chaloin et al., 2014). A similar connection between β-catenin and the murine

Cyp2e1 (Braeuning et al., 2009; Gougelet et al., 2014) and Cyp1a1/2 mRNAs (Braeuning et al., 2009, 2011b; Vaas et al., 2014) has also been reported. Interestingly, the basal level of the mouse homolog of the human CYP2B6 gene, Cyp2b10, was upregulated in *Ctnnb1* knock-out mice (Braeuning et al., 2009; Ganzenberg et al., 2013), thereby confirming our findings on increased basal expression of CYP2B6 following siRNA-mediated β-catenin knockdown. The lack of major effects of β-catenin on the expression of CYP3A enzymes is also in line with previous results (Gerbal-Chaloin et al., 2014).

Synergistic activation of target gene expression by β-catenin and the nuclear receptors AhR and CAR has been previously studied in mice as well as in murine cell lines (Braeuning et al., 2009, 2011a; Ganzenberg et al., 2013; Vaas et al., 2014). Data presented in this paper show, for the first time, synergistic effects of the WNT/β-catenin pathway and the receptors AhR, CAR and PXR in human liver cells. By showing a synergism of AhR or CAR with β-catenin, the present data are in line with previous data obtained in murine liver cells. Cooperative effects of β-catenin and PXR, as now observed in human liver cells, were not detectable in *Ctnnb1* knock-out mice treated with the PXR inducer pregnenolone-α-carbonitrile (PCN) (Braeuning et al., 2009). This indicates that the regulation of some aspects of drug metabolism differs between rodent and human cells, thus necessitating studies with cells of human origin.

An important novel aspect presented in this study is the crosstalk between β-catenin and PPARα in the regulation of different CYPs, especially CYP3A4, the most important CYP isoforms in humans. The role of PPARα in the regulation of drug-metabolizing enzymes from CYP families 1-3 has long been underestimated. However, there are a number of previous reports which demonstrate involvement of PPARα in the regulation of CYP enzymes mediating detoxification of exogenous compounds, including CYP3A4, not only *in vitro* (Schröder et al., 2011; Klein et al., 2012; Thomas et al., 2013, 2014), but also *in vivo* (de Keyser et al., 2013; Tsamandouras et al., 2014). Contrasting the situation with the other receptors, β-catenin antagonizes the effects of the activated PPARα on the expression of target

genes. This effect occurs at the transcriptional level at the CYP3A4 promoter and is especially interesting, as the expression of the same gene is synergistically influenced by β-catenin and PXR.

Classically, active β-catenin binds to transcription factors of the TCF (T-cell factor) family to initiate the transcription of target genes, acting as a transcriptional co-activator of the TCF proteins. Synergistic interactions between binding sites for TCF/β-catenin and binding sites for nuclear receptors, e.g. by easier access of the ligand-activated nuclear receptor to a gene promoter which has already bound TCF/β-catenin and therefore changed its chromatin structure, may play a role in the observed interactions between β-catenin and the receptors AhR, CAR, and PXR. Of note, an interaction between a TCF/β-catenin binding site and an AhR binding site in the human *CYP1A1* promoter has been reported (Braeuning et al., 2011b). However, so far no unequivocal consensus TCF binding sites were identified within the CYP3A4 promoter, which means that other possible mechanisms should be taken into account. Transcription-inducing effects of β-catenin on certain genes might also be mediated by β-catenin acting as a co-activator of other transcription factors than TCF family members. For example, it has been published that β-catenin physically interacts with the AhR and modulates the activity of the ligand-activated AhR at its DNA binding sites (Braeuning et al., 2011b). This effect is thought to be exerted either by β-catenin itself, acting as transcriptional co-activator of the AhR, or by effects of β-catenin on the recruitment of other proteins involved in the transcription process. The AhR is also a direct transcriptional target of β-catenin in human cells (Chesire et al., 2004; Gerbal-Chaloin et al., 2014). Moreover, recent data point towards a crosstalk of β-catenin and hepatocyte nuclear factor 4α (HNF4α) in the regulation of expression of liver-specific genes *in vitro* and *in vivo*, suggesting a further possible mechanism of CYP modulation (Colletti et al., 2009; Gougelet et al., 2014). HNF4α plays an important role in the regulation of various CYPs which are also regulated by different xeno-sensing nuclear receptors.

It is more difficult to find mechanistic explanations for the observed inhibitory action of β-catenin on PPARα-dependent effects. An interference via glycogen synthase 3β (GSK) seems possible, as GSK, a key component in canonical WNT signaling, might be also involved in the regulation of PPARα (Pawlak et al., 2014). However, this scenario would only apply to effects of WNT3a, not for siRNA-mediated silencing of β-catenin, since the latter approach interferes with the WNT pathway downstream of GSK. Moreover, it is not known to which extent the overall activity of GSK is modulated by WNT signaling, since a major part of the regulation during WNT3a-mediated activation of the pathway seems to be related to the dissociation of the GSK-containing multi-protein complex rather than to direct inhibition of the GSK enzyme. The interaction between β-catenin and the AhR has been studied in detail and therefore might provide hints for mechanisms possibly also relevant for the interaction of β-catenin with PPARα: β-catenin acts as an ubiquitin ligase for the AhR in murine intestinal cells (Kawajiri et al., 2009). Taken together, these findings indicate that β-catenin can exert much more diverse cellular effects than dimerization with TCF proteins. Further studies will reveal in-depth mechanistic details of the novel β-catenin/PPARα interaction. In summary, our present findings show that β-catenin is an important modulator of basal, but also of nuclear receptor-mediated CYP expression triggered by xenobiotics in human liver cells. The identification of the antagonistic connection of β-catenin and PPARα adds a new layer of crosstalk to the complex network of β-catenin and nuclear receptors and substantiates the role of the WNT/β-catenin signaling pathway as a master regulator of drug and xenobiotic metabolism also in the human liver.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Igor Liebermann, Britta Klumpp and Kyoko Momoi (Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart) for expert technical

assistance. We are deeply indebted to Christophe Chesne and Biopredic for the help and support with HepaRG cells.

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Footnotes

This study was supported by the German Federal Ministry of Education and Research, Virtual Liver Network Grant [0315755] and by the Robert Bosch Foundation, Stuttgart, Germany.

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Figure legends

Figure 1: Validation of WNT3a-mediated activation and siRNA-mediated inhibition of WNT/β-catenin activity. A. Activation of WNT/β-catenin signaling pathways in HepaRG cells by treatment with WNT3a for 24 and 72 hours, as monitored by mRNA levels of the model β-catenin target gene AXIN2. **B.** Specific knock-down of the β-catenin transcript in HepaRG cells using specific siRNA targeting the β-catenin transcript (siCAT). Levels of mRNA expression of the β-catenin gene, CTNNB1, and of its downstream model target gene, AXIN2, were determined by using specific TaqMan assays in comparison to non-targeting siRNA (siCTR). Mean values of at least three independent experiments +SD are depicted; statistical significance ($p<0.05$) is indicated by asterisks.

Figure 2. Impact of WNT/β-catenin signaling on basal CYP450 expression. For β-catenin activation, HepaRG cells were treated for 72 h with 20ng/ml WNT3a (white bars) or with siRNA directed against β-catenin (dotted bars). The levels of the indicated CYPs were measured by qRT-PCR. Vehicle controls (0.1% bovine serum albumin and non-targeting control siRNA, respectively) were set to 1. Data represent means +SD of three independent experiments measured in triplicates. Statistical significance ($p<0.05$, paired t-test) is indicated by asterisks.

Figure 3. Expression profiling of cytochromes P450 during ligand-activated induction of nuclear receptors in combination with WNT pathway activation. Expression levels of indicated CYPs were analyzed by real-time qRT-PCR following treatment of HepaRG cells for 72 hours with 20ng/ml of WNT3a alone (white bars on every panel) or with 10nM of the AhR agonist TCDD alone (**A**), 5μM of the CAR agonist CITCO (**B**), 10μM of the PXR agonist rifampicin (**C**), or 100μM of the PPARα agonist WY14,643 (**D**), as indicated for each treatment with light-gray bars on every panel. Combined treatment with WNT3a together with

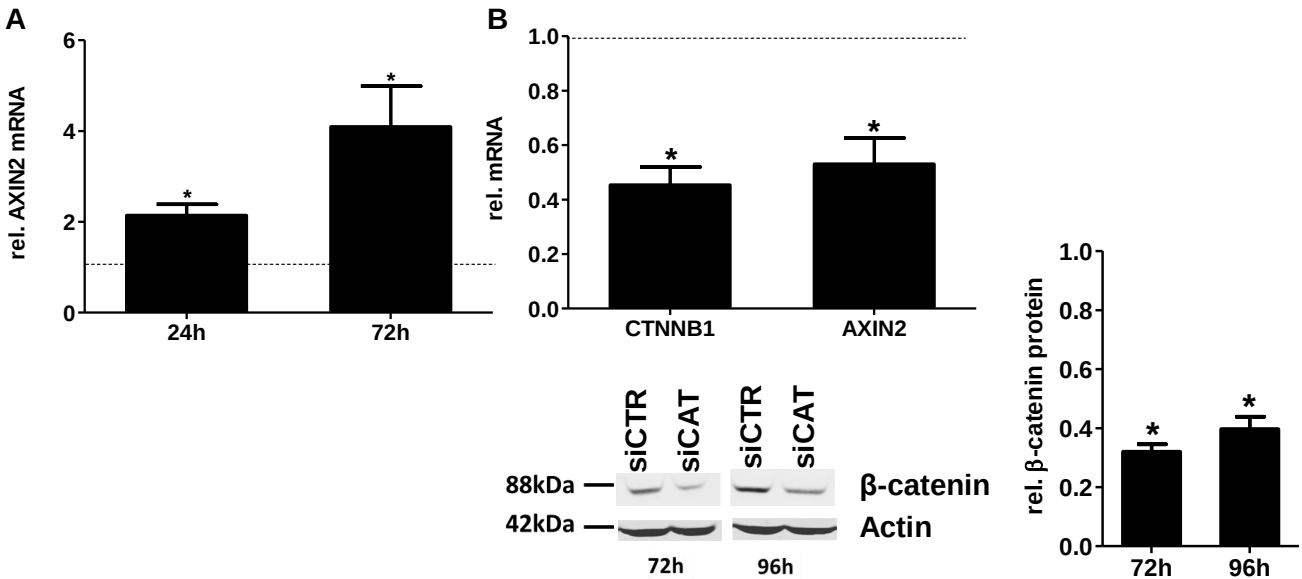
each agonist is shown with black bars on every panel. The bars indicate means + SD relative to vehicle control set at 1. Asterisks indicate significant changes in the expression of target genes ($p<0.05$, paired t-test).

Figure 4. Expression profiling of P450 enzymes during ligand-activated induction of nuclear receptors in combination with β-catenin knock-down. Expression levels of indicated CYPs were analyzed in HepaRG cells by real-time qRT-PCR following β-catenin knock-down alone (white bars on every panel, siCAT), or with 10nM of the AhR agonist TCDD alone (**A**), 5μM of the CAR agonist CITCO (**B**), 10μM of the PXR agonist rifampicin (**C**), or 100μM of the PPARα agonist WY14,643 (**D**), as indicated for each treatment with light-gray bars on every panel. Combined treatment of β-catenin siRNA together with each agonist is shown with black bars on every panel. The bars indicate means + SD relative to non-coding siRNA, siCTR, set at 1. Asterisks indicate significant changes in the expression of target genes ($p<0.05$, paired t-test).

Figure 5. Activating and inhibitory roles of β-catenin in the ligand-mediated induction of CYP3A4 by PXR and PPARα. Protein expression levels of CYP3A4 were analyzed by Western blotting following activation of PPARα with 100μM of WY14,643 (gray bars on both panels) or 10μM of the PXR agonist rifampicin (black bars on both panels), in the presence of WNT3a (**A**) or siCAT (**B**). Western blot analysis with a previously characterized antibody against CYP3A4 (Wolbold et al., 2003) was performed in three biological replicates; one representative is shown. Corresponding densitometric analyses of three replicates are shown under each blot, relative to PBS control (**A**) or non-coding siRNA, siCTR (**B**), set to 1. The error bars indicate standard deviations between three independent experiments, statistical significance is indicated by asterisks ($p<0.05$). For further analyses at the level of gene transcription, a luciferase reporter gene construct containing 8.8 kb of the CYP3A4 promoter

was transfected into HuH7 cells. Cells were treated with 10µM rifampicin (**C**) or 100µM WY14,643 (**D**) for 48h (vehicle control: DMSO), in the presence or absence of non-targeting siRNA (siCTR), β-catenin targeting siRNA (siCAT), or WNT3a. Firefly luciferase activities were normalized to renilla luciferase activities. Data are means +SD of three independent experiments, each performed in triplicates. Statistical significance is indicated by asterisks ($p<0.05$).

Figure 1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rel. AXIN2 mRNA
6
4
2
0
*
*
24h
72h
B
rel. mRNA
1.0
0.8
0.6
0.4
0.2
0.0
*
*
CTNNB1
AXIN2
siCTR
siCAT
siCTR
siCAT
88kDa
42kDa
β-catenin
Actin
72h
96h
rel. β-catenin protein
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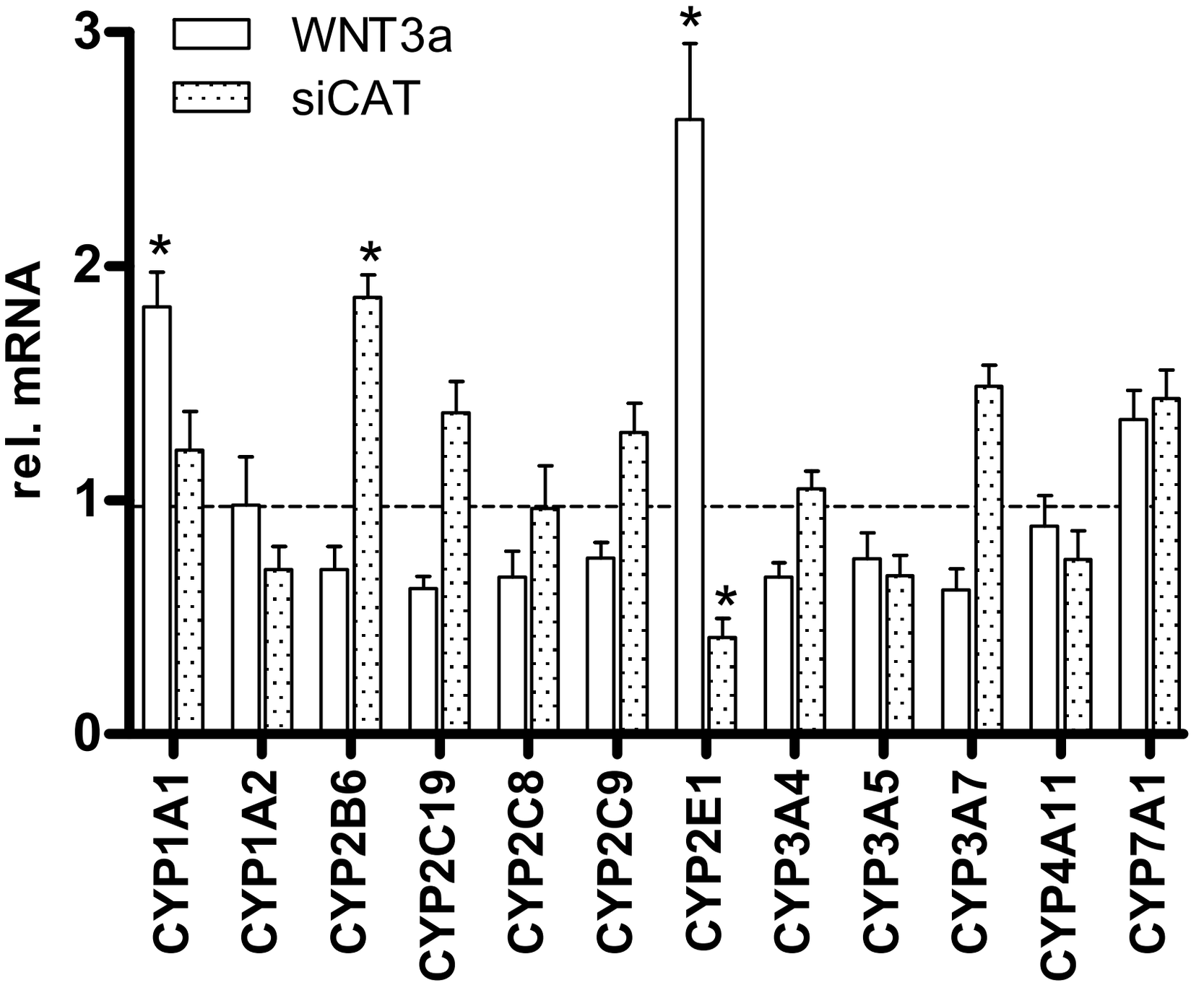
0.8
0.6
0.4
0.2
0.0
*
*
72h
96h

Figure 2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siCAT
rel. mRNA
3
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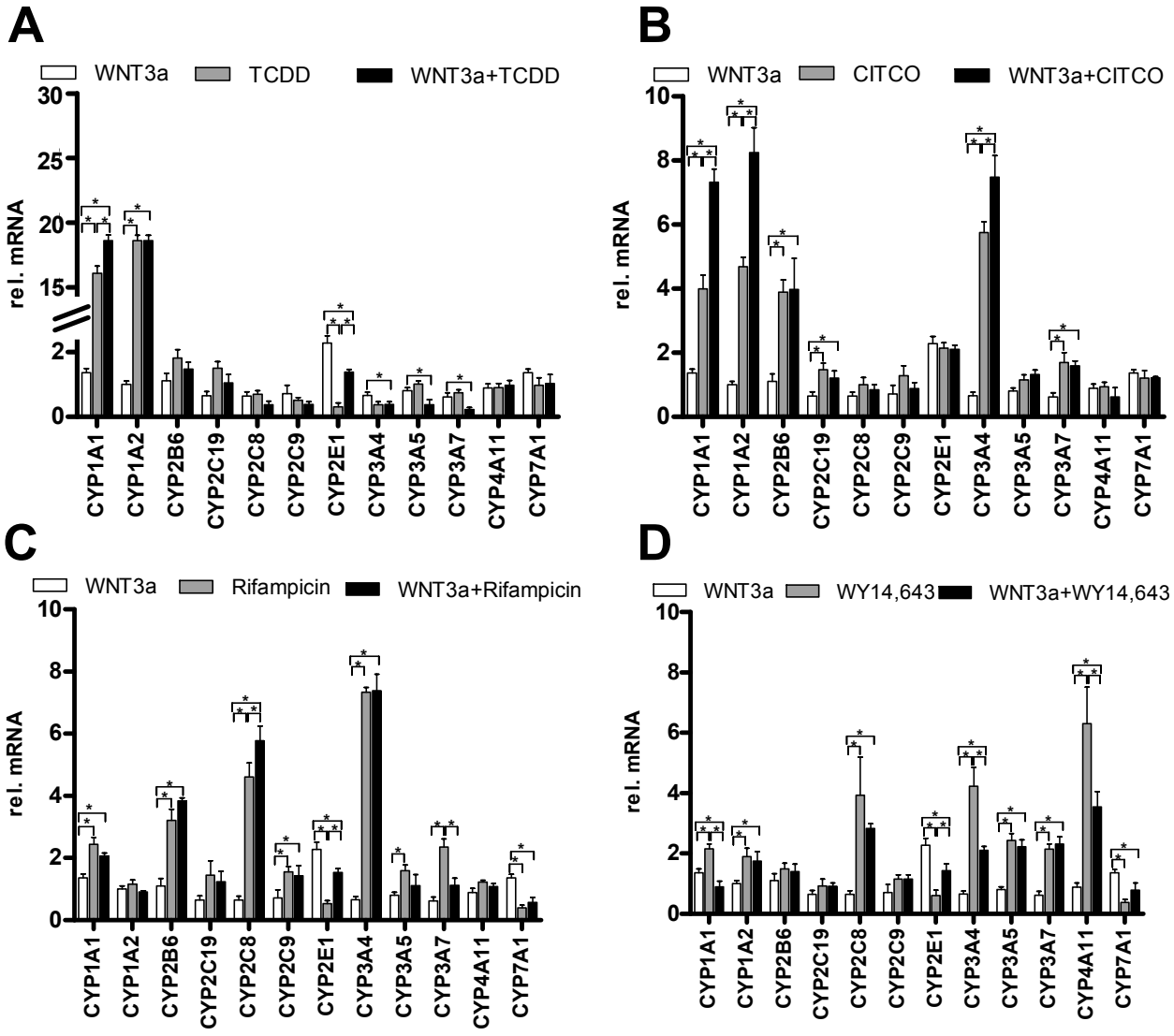
CYP7A1

Figure 3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TCDD
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CYP2C19
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CYP3A4
CYP3A5
CYP3A7
CYP4A11
CYP7A1
B
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CITCO
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rel. mRNA
10
8
6
4
2
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CYP1A1
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CYP3A5
CYP3A7
CYP4A11
CYP7A1
C
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Rifampicin
WNT3a+Rifampicin
rel. mRNA
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CYP1A1
CYP1A2
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CYP2C9
CYP2E1
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CYP3A5
CYP3A7
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CYP7A1
D
WNT3a
WY14,643
WNT3a+WY14,643
rel. mRNA
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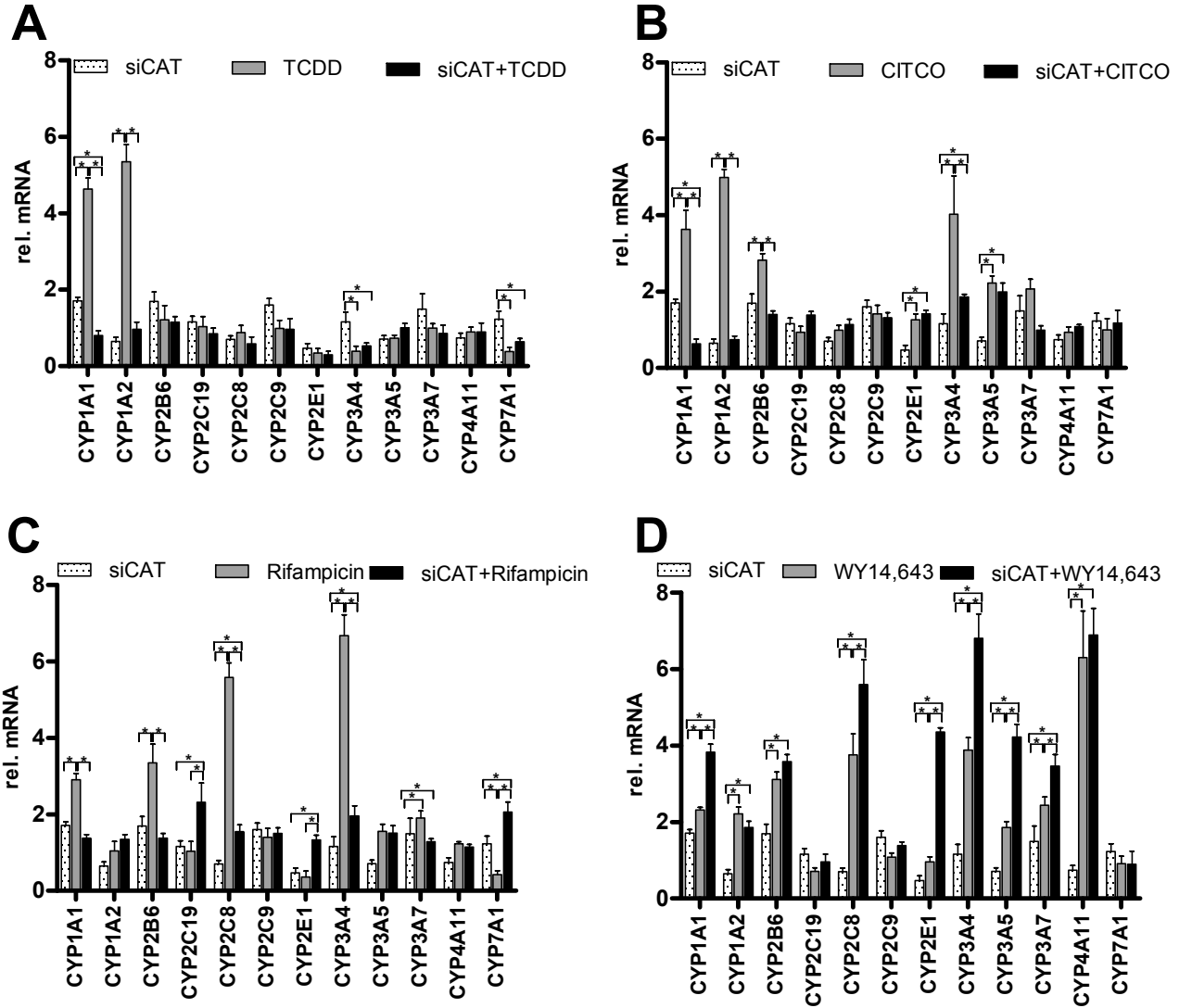
Figure 4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TCDD
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CYP2C19
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CYP2C9
CYP2E1
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CYP3A5
CYP3A7
CYP4A11
CYP7A1
B
siCAT
CITCO
siCAT+CITCO
rel. mRNA
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D
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WY14,643
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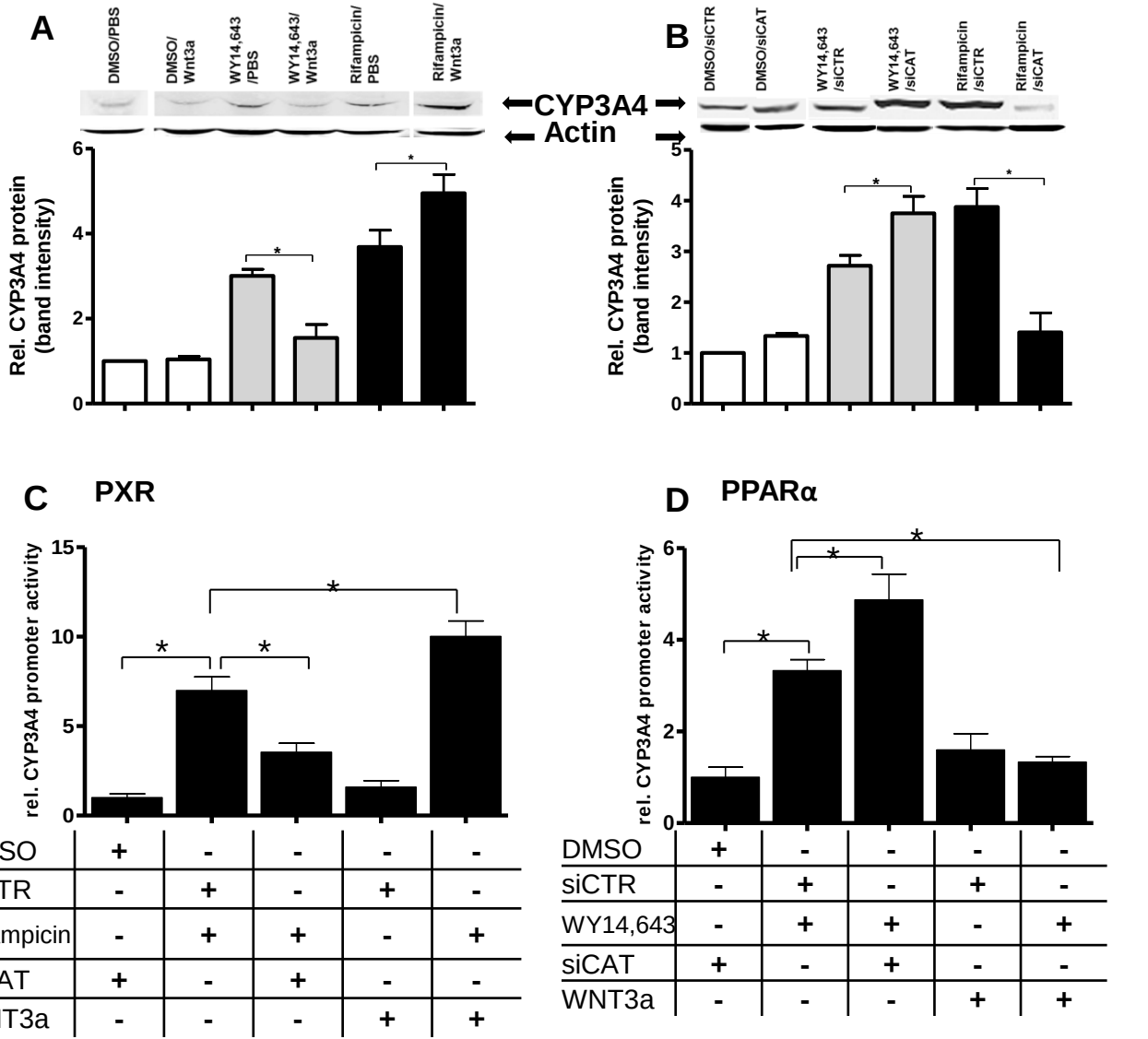
rel. mRNA

Figure 5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DMSO/ Wnt3a
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WY14,643/ Wnt3a
Rifampicin/ PBS
Rifampicin/ Wnt3a
CYP3A4
Actin
Rel. CYP3A4 protein (band intensity)
B
DMSO/siCTR
DMSO/siCAT
WY14,643 /siCTR
WY14,643 /siCAT
Rifampicin /siCTR
Rifampicin /siCAT
Rel. CYP3A4 protein (band intensity)
C
PXR
rel. CYP3A4 promoter activity
DMSO + - - - -
siCTR - + - + -
Rifampicin - + + - +
siCAT + - + - -
WNT3a - - - + +
D
PPARα
rel. CYP3A4 promoter activity
DMSO + - - - -
siCTR - + - + -
WY14,643 - + + - +
siCAT + - + - -
WNT3a - - - + +