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The WNT/β-catenin pathway is a transcriptional regulator of *CYP2E1*, *CYP1A2* and aryl hydrocarbon receptor gene expression in primary human hepatocytes

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Running title: β-catenin regulates drug metabolism

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Abbreviations: AhR, aryl hydrocarbon receptor; APAP, acetaminophen; APC, adenomatous polyposis coli; CAR, constitutive androstane receptor (NR1I3, Nuclear Receptor Subfamily 1, Group I, Member 3); CTNNB1, β-catenin; CYP, cytochrome P450; DKK-3, Dickkopf-3; GLUL, glutamine synthetase; GSH, Glutathione; GSK3β, glycogen synthase kinase 3β; HNF4α, hepatocyte nuclear factor-4α; KRT19, cytokeratin 19; LGR5, Leucine-Rich repeat Containing G Protein-Coupled Receptor 5; LiCl, lithium chloride; PC, pericentral; PHH, primary human hepatocyte; PP, periportal; PXR, pregnane X receptor (NR1I2, Nuclear Receptor Subfamily 1, Group I, Member 2); siRNA, small interfering RNA; TCF/Lef, T cell factor/lymphoid-enhancing factor transcriptional activator; RT-qPCR: reverse transcription, real-time quantitative polymerase chain reaction; WNT, Wingless-type MMTV integration site family.

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ABSTRACT

The WNT/β-catenin/Adenomatous polyposis coli (CTNNB1/APC) pathway has been identified as a regulator of drug-metabolizing enzymes in rodent liver. Conversely, little is known about the role of this pathway in drug metabolism regulation in human liver. Primary human hepatocytes (PHHs) that are the most physiologically relevant culture system to study drug metabolism *in vitro* were used to investigate this issue. Therefore, the aim of this study was to assess the link between CYP expression and WNT/β-catenin pathway activity in PHHs by modulating its activity with recombinant mouse Wnt3a (the canonical activator), inhibitors of glycogen synthase kinase 3β, siRNAs to invalidate *CTNNB1* or its repressor *APC*, used separately or in combination. We found that the WNT/β-catenin pathway can be activated in PHHs, as assessed by universal β-catenin target gene expression, leucine-rich repeat containing G protein-coupled receptor 5. Moreover, WNT/β-catenin pathway activation induces the expression of *CYP2E1*, *CYP1A2* and aryl hydrocarbon receptor, but not of *CYP3A4*, hepatocyte nuclear factor-4α (*HNF4α)* and pregnane X receptor (*PXR)* in PHHs. Specifically, we show for the first time that *CYP2E1* is transcriptionally regulated by the WNT/β-catenin pathway. Moreover, CYP2E1 induction was accompanied by an increase of its metabolic activity, as indicated by the increased production of 6-OH-chlorzoxazone and by glutathione depletion following incubation with high doses of acetaminophen. In conclusion, the WNT/β-catenin pathway is functional in PHHs and its induction in PHHs represents a powerful tool to evaluate hepatotoxicity of drugs that are metabolized by CYP2E1.

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INTRODUCTION

Liver carries out many different functions thanks to the organization of hepatocytes in different functional groups, a remarkable property known as functional zonation. Hepatocytes present highly specialized metabolic functions from the portal space to the centrilobular vein and based on their localization are defined, respectively, as periportal (PP) and pericentral (PC) hepatocytes. Recently, the Wingless-type MMTV integration site family (WNT)/β-catenin-adenomatous polyposis coli (APC) pathway has been identified as the major liver "zonation-keeper" (Benhamouche et al., 2006; Colnot and Perret, 2010).

β-catenin (CTNNB1) expression and localization are tightly regulated. In the absence of WNT ligand stimulation, CTNNB1 is phosphorylated by a protein complex that include glycogen synthase kinase 3β (GSK3β) and APC, leading to its proteasomal degradation. In the presence of WNT ligands, CTNNB1 remains unphosphorylated and accumulates in the cytosol. Then, it translocates into the nucleus where it transactivates target genes by binding to transcriptional activators of the T cell factor/lymphoid-enhancing factor (TCF/Lef) family (MacDonald et al., 2009). In parallel to its transcriptional activity, CTNNB1 participates to adherens junction formation by interacting with E-cadherin and α-catenin, linking cadherins to the actin cytoskeleton (Vinken et al., 2006).

Detoxification of exogenous compounds is one of the liver major metabolic functions and is mediated by a large family of proteins, including the phase I cytochrome P450 enzymes (CYP). CYP expression is regulated by transcription factors, including hepatocyte nuclear factor-4α (HNF4α), and nuclear receptors, such as constitutive androstane receptor (CAR, NR1I3), pregnane X receptor (PXR, NR1I2) and aryl hydrocarbon receptor (AhR) of the basic Helix-Loop-Helix/Per-ARNT-Sim (bHLH/PAS) receptor family, that are the major regulators of drug disposition (Kohle and Bock, 2009).

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Histological analysis of liver sections (Braeuning et al., 2009; Hailfinger et al., 2006; Sekine et al., 2006), microarray and qPCR analysis of enriched periportal and centrilobular hepatocytes (Braeuning et al., 2006; Sekine et al., 2006) have shown that, in the mouse, Cyp2e1, Cyp1a2 and AhR are expressed in PC hepatocytes, where the Wnt/β-catenin pathway is mostly activated. Genetic manipulation of animals is a powerful tool to dissect the complexity of liver zonation. In hepatocyte-specific *Ctnnb1* knockout mice, loss of expression of several CYPs, especially Cyp2e1 and Cyp1a2, has been observed (Sekine et al., 2006). Moreover, the basal expression of most drug metabolism-related genes and the response to NR1I3 and AhR agonists are also reduced in these mice (Braeuning et al., 2009; Ganzenberg et al., 2013). Similarly, acetaminophen (APAP)-induced toxicity is abolished in *Ctnnb1-/-* mice (Sekine et al., 2006). Conversely, several CYP isoenzymes are up-regulated in liver tumors harboring *CTNNB1* activating mutations (Loeppen et al., 2005). Liver-specific *Apc* loss causes the *de novo* expression of β-catenin-positive PC genes and the suppression of β-catenin-negative PP target genes (Benhamouche et al., 2006).

Primary human hepatocytes (PHHs) are the most physiologically relevant culture system to study drug metabolism *in vitro*. However, when hepatocytes are seeded in culture, zonal organization is lost and CYP expression progressively decreases. As nothing is known about the link between CYP expression and β-catenin pathway activity in PHHs, we decided to investigate this aspect using our *in vitro* model of PHHs. We demonstrated that the β-catenin pathway can be activated in PHHs, despite the loss of the organization, to restore the expression of its target genes. We identified *CYP2E1*, *CYP1A2* and *AhR* as CTNNB1 target genes, while *CYP3A4*, *HNF4α* and *PXR* are not directly regulated by this pathway. Moreover, CYP2E1 induction is accompanied by an increase of APAP-induced toxicity in PHHs.

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MATERIALS AND METHODS

Human liver samples and preparation of primary human hepatocytes

Liver samples were obtained from liver resections performed in adult patients for medical reasons unrelated to our research program. The use of human specimens for scientific purposes was approved by the French National Ethics Committee. Written or oral informed consent was obtained from each patient of family prior to surgery. The clinical characteristics of the liver donors are presented in Table 1. PHHs were prepared and cultured as described previously (Pichard et al., 2006). PHHs were seeded in collagen-coated dishes at 1.7 x 10^5 cells/cm² in a hormonally and chemically defined medium consisting of a mixture of William's E and Ham's F-12 (1:1 in volume) and additives as described in (Ferrini et al., 1997). PHHs were cultured in a 5% CO_2 humidified atmosphere at 37°C.

Chemicals

CHIR99021 (6-((2-((4-(2,4-Dichlorophenyl)-5-(4methyl-1H-imidazol-2-yl)pyrimidin-2-yl)amino)ethyl)amino) nicotinonitrile) was from BioVision (Milpitas, CA). TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxine), rifampicin (RIF), lithium chloride (LiCl) were from Sigma-Aldrich (Saint-Louis, MO, US). mWnt3a was from Peprotech (Neuilly-Sur-Seine, France).

Cell lines

The HepaRG cell line was grown and differentiated as recommended (Parent et al., 2004). Briefly, cells were seeded at 2.6 x 10^4 cells/cm² and expanded in growth medium for 2 weeks, then differentiated by addition of 1.5% DMSO for 2 weeks. HepG2-C3 (ATCC) and HuH7 (JCRB) cells were cultured as recommended. CHIR was added to HepG2-C3 and HuH7 cells

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when they reached 80% confluence and to differentiated HepaRG cells. Cell lines were cultured in a 5% CO_2 humidified atmosphere at 37°C.

siRNA transient transfection

Adherent PHHs were transfected with 20 nM non-targeting siRNA (scrambled, siSC) or siRNAs specific for *CTNNB1*, *APC* or *AhR* (Dharmacon, Lafayette, CO) at day 1 and day 3 post-seeding using Lipofectamine RNAiMAX (Life Technology). At day 5 post-seeding, PHHs were treated for 48 h.

DNA and reporter gene expression assays

PHHs in suspension were transfected with Lipofectamine 2000 transfection reagent (Life Technology) according to the manufacturer's instructions before plating. Briefly, 2.7 x 10^4 PHHs were transfected with either 500 ng of firefly luciferase reporter plasmids SuperTOPflash (Wnt response element, WRE) or SuperFOPflash (mutated WRE) (Staal et al., 1999) and 250 ng of pTK-luc Renilla control vector (Promega, Madison, WI). PHHs were then plated in 24-well plates in DNA:liposome mix in chemically defined medium. Twenty-four hours later medium was renewed and cells were treated with 3 µM CHIR, 200 ng/mL mWnt3a or 20 mM LiCl for 24 h.

RNA isolation and RT-PCR

After extraction with Trizol reagent (Invitrogen), 500 ng of total RNA was reverse-transcribed using a random hexaprimer and the MMLV Reverse Transcriptase Kit (Invitrogen). Quantitative PCR was performed using the Roche SYBER Green reagent and a LightCycler 480 apparatus (Roche Diagnostic, Meylan, France). Amplification specificity was evaluated by determining the product melting curve. Results are expressed as indicated in the figure

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legends. The primers used were listed in Table 2. The following program was used: one step at 95°C for 10 min, 50 cycles of denaturation at 95°C for 10 sec, annealing at 65°C for 15 sec and elongation at 72°C for 15 sec.

Protein analysis

Total protein extracts were prepared using RIPA buffer supplemented with a protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA). The protein concentration was determined by the bicinchoninic acid method, according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL). Bovine serum albumin (Pierce Chemical Co.) was used as standard. Twenty µg of total proteins were separated on precast SDS-polyacrylamide gels (4-16%) (BIO-RAD, Marnes la Coquette, France), then transferred onto PVDF membranes (BIO-RAD). Membranes were incubated with rabbit polyclonal anti-CYP2E1 (Millipore, Molsheim, France) or anti-CTNNB1 (Abcam, Cambridge, UK), mouse monoclonal anti-CYP1A2 (SantaCruz) or goat polyclonal anti-Actin (SantaCruz) antibodies. Microsomes from human lymphoblastoid cells transfected with the human CYP2E1 (Gentest Corp., Woburn, MA) were used as standards.

Immunohistochemistry

Paraffin-embedded human liver tissue sections (4 µm thick) were incubated at 4°C with rabbit antibodies against CYP3A4 (Epitomics, Burlingame, CA), Cytokeratin 19 (KRT19, Epitomics), CYP2E1 (Millipore), GLUL (Abnova Corporation, Taipei, Taiwan) or mouse antibody against CYP1A2 (Santa Cruz). Immunohistochemical staining was performed using the EnVisionTM+ System (Dako, Glostrup, Denmark) according to the manufacturer's recommendations. Finally, sections were lightly counterstained with Harris hematoxylin. Slides were scanned (Montpellier RIO Imaging Facility, INM Montpellier, France) using a

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Nanozoomer Slide Scanner (Hamamatsu Photonics, Massy, France) and virtual slides were viewed using the NDP.view software (Hamamatsu Photonics, Massy, France).

Measurement of chlorzoxazone hydroxylation

After 72 h treatment with 3 µM CHIR, PHH culture medium was renewed in the presence of 15 µM chlorzoxazone (Sigma). Six hours later, extracellular medium and cells were collected and the 6-OH chlorzoxazone content was measured by LC-MS/MS using an Acquity UPLC System I-Class equipped with a Waters Acquity UPLC BEH C18 column (2.1 mm i.d. x 100 mm length, 1.7 µm particle size) coupled to a Quattro Premier mass spectrometer (all from Waters, Milford, MA) used in electrospray ion negative mode.

Measurement of glutathione (GSH) content

After 72 h incubation with 3 µM CHIR, PHH culture medium was renewed and 20 or 50 mM acetaminophen was added (Sigma). Six hours later, cells were collected and the GSH content was measured using a Glutathione Assay Kit (Cayman Chemical), according to the manufacturer's instructions. Results are expressed as the quantity of GSH lost in APAP-treated cells compared to non-treated cells.

Statistical Analysis

The values of mRNA expression were expressed as the mean ± standard error of the mean (SEM). Statistical analysis between groups was carried out by using the paired *t*-test. Analysis of the variance (ANOVA) was used to determine statistical differences and the Tukey`s multiple comparison test was performed to compare three groups. Differences were considered significant, when $p<0.05$ ($*p<0.05$, $**p<0.01$, $***p<0.001$). Data were analyzed

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by using GraphPad Prism, version 4.0, 2003 (GraphPad Software Incorporated, San Diego, CA, USA).

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RESULTS

The canonical Wnt/β-catenin pathway is functional in PHHs

When human hepatocytes are seeded in culture, zonation is lost and CYP expression progressively decreases. As *in vivo* zonation is mainly controlled by the WNT/β-catenin pathway, we first determined whether this pathway can still control gene transcription in PHHs, independently of zonal organization. To this aim, the expression of leucine-rich repeat containing G protein-coupled receptor 5 (*LGR5* or *GPR49*), a universal β-catenin target gene (Yamamoto et al., 2003), was monitored in PHHs that were cultured according to our established long-term culture conditions (Pichard et al., 2006). *LGR5* expression was progressively and strongly reduced during culture. Conversely, the expression of *APC* and *GSK3β*, which are involved in CTNNB1 proteasomal degradation, and Dickkopf-3 (DKK3), a member of the DKK family of WNT antagonists, increased over time (Figure 1A). These results suggest that CTNNB1 signaling is gradually down-regulated during PHH culture.

Then, to determine whether the WNT/β-catenin pathway could be activated in PHHs, cells were exposed, or not (untreated: UT), to recombinant mouse Wnt3a (mWnt3a, the canonical activator of this pathway) or to the GSK3β inhibitors CHIR99021 (CHIR) (Ring et al., 2003) and lithium chloride (LiCl) (Abu-Baker et al., 2013) for 48 h (day 2 to day 4 of culture) and mRNA expression of the target gene *LGR5* was analyzed by RT-qPCR. As expected, *LGR5* was induced by all these compounds, while its expression was not affected by TCDD and RIF, the prototypical AhR and PXR activators, respectively (Figure 1B). Moreover, PHH response to GSK3β inhibition or to mWnt3a showed a significant inter-individual variability (Figure 1C-D).

To demonstrate CTNNB1 ability to activate the TCF/Lef responsive element following exposure to mWnt3a, LiCl or CHIR, PHHs were transfected with TOP-luc (black

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bars) or FOP-luc plasmids (white bars) that harbor consensus or mutated TCF/Lef responsive elements, respectively. Following incubation with 3 µM CHIR for 24 h, a 3-fold increase in luciferase activity was observed in TOP-luc transfected cells compared to untreated cells (UT), whereas it remained unchanged in FOP-luc transfected cells (Figure 2A). Induction of the WNT/β-catenin pathway by the canonical CTNNB1 activator mWnt3a or LiCl also stimulated luciferase activity, although to a lesser extent than CHIR.

The involvement of the WNT/β-catenin pathway on Wnt3a- and CHIR-mediated *LGR5* up-regulation was confirmed by siRNA-mediated down-regulation of *CTNNB1* or *APC* in PHHs. APC is involved in CTNNB1 degradation and its inactivation results in CTNNB1 accumulation, thereby mimicking the activation of the WNT/β-catenin pathway (Burke et al., 2009; Colnot et al., 2004). The specific siRNAs efficiently knocked down *APC* (by 70%) and *CTNNB1* (by 90%) mRNA expression in PHHs (data not shown). Wnt3a- and CHIR-mediated *LGR5* up-regulation was abolished (Figure 2B) in *CTNNB1*-silenced PHHs, but not in control cells (scrambled siRNA, siSC). Conversely, *LGR5* basal expression was significantly increased in *APC*-silenced PHHs in comparison to siSC controls (Figure 2B), confirming that *LGR5* is a WNT/β-catenin pathway target and that CHIR-mediated *LGR5* up-regulation is dependent on CTNNB1. Similar results were obtained using hepatic cell lines in which the WNT/β-catenin pathway is functional such as HuH7 cells and differentiated HepaRG cells, or that harbor *CTNNB1* activating mutations, such as HepG2-C3 cells (de La Coste et al., 1998). Incubation with 3 µM CHIR for 48 h increased the expression of *LGR5* in HuH7 and HepaRG cells, but not in HepG2-C3 cells, confirming that the response to CHIR relies on a functional WNT/β-catenin pathway (Figure 2C).

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These findings indicate that, in PHHs, the canonical WNT/β-catenin signaling pathway is still functional and can be activated directly by the canonical activator mWnt3a or indirectly by the GSK3β inhibitor CHIR.

CYPs regulation by the WNT/β-catenin signaling pathway

CYP expression is zonated in human liver tissue. Immunohistochemistry analysis of human liver tissue serial sections showed that CYP2E1, CYP1A2 and CYP3A4 are expressed in PC hepatocytes (Figure 3A), similarly to GLUL, a well-known PC marker. Conversely, cytokeratin 19-positive ($KRT19^{+}$) biliary cells are indicative of PP areas. As observed for the CTNNB1 target gene *LGR5* (Figure 1A), CYP expression was progressively down-regulated in cultured PHHs (Figure 3B). Therefore, we asked whether this resulted from the loss of CTNNB1-mediated gene transcription.

CYP2E1

CYP2E1 is expressed in PC hepatocytes (Figure 3 for human and (Hailfinger et al., 2006; Loeppen et al., 2005; Sekine et al., 2006) for mouse liver) where the WNT/β-catenin pathway is mostly active (Benhamouche et al., 2006). To assess the effect of WNT/β-catenin pathway activation on CYP2E1 expression in PHHs, cultures were incubated with mWnt3a (the canonical CTNNB1 activator) or with GSK3β inhibitors (LiCl and CHIR). Upon induction of the WNT/β-catenin pathway, *CYPE21* mRNA expression increased in a dose-dependent manner compared to untreated cells (UT). As expected, *CYPE21* mRNA expression was not affected by TCDD or RIF (Figure 4A). Up-regulation of *CYP2E1* by mWnt3a- and CHIR was abolished in PHHs in which *CTNNB1* was silenced by siRNA down-regulation, indicating that this effect is dependent on CTNNB1 (Figure 4B). Conversely, *APC* silencing increased *CYP2E1* basal expression (Figure 4B), as observed for *LGR5* (Figure 2B),

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compared to control cells (siSC). These findings demonstrate that the canonical activator mWnt3a and the GSK3β inhibitor CHIR can regulate *CYP2E1* mRNA expression in a CTNNB1-dependent manner. As CHIR was the most efficient and easy to use activator of the WNT/β-catenin pathway, it was preferentially employed in the subsequent experiments.

Analysis of 14 different PHH cultures confirmed that CHIR strongly induced *CYP2E1* mRNA expression (mean fold change and median: 12.6 and 10.0) compared to untreated cells (Figure 5A). Like for *LGR5* (Figure 2C), and as previously described for other human CYPs (Gomez-Lechon et al., 2007), a strong inter-individual variation was observed. However, there was no correlation between the clinical characteristics of the liver donor and the level of *CYP2E1* induction in PHHs. *CYP2E1* mRNA (Figure 5B) and protein (Figure 5C) expression were followed in PHHs cultured for 4-5 days. Basal *CYP2E1* expression decreased rapidly over time; however, exposure to 3 µM CHIR from day 2 of culture significantly increased CYP2E1 mRNA and protein levels. CHIR-mediated CYP2E1 protein induction was abolished by siRNA-mediated *CTNNB1* down-regulation, whereas it was markedly increased after silencing of *APC* (Figure 5D). Moreover, CYP2E1 enzymatic activity in PHHs was confirmed by adding chlorzoxazone, a CYP2E1-specific substrate, to the cultures. A five-fold increase in the production of its metabolite 6-OH-chlorzoxazone was observed in cells incubated with CHIR for 72 h compared to untreated cells (UT) (Figure 5E). CYP2E1 also contributes to APAP metabolism by catalyzing the production of the reactive intermediate *N*-acetyl-*p*-benzoquinoneimine, which is normally rapidly detoxified by glutathione (GSH) conjugation in the liver. Therefore, in case of APAP overdose, this intermediate can lead to GSH depletion and to hepatotoxicity (James et al., 2003). Conversely, *Ctnnb1*$^{-/-}$ mice are resistant to APAP-induced toxicity (Sekine et al., 2006). Quantification of the GSH content in PHHs stimulated with CHIR for 72 h and incubated with 20 or 50 mM APAP for the last 6 h showed a strong GSH depletion compared to controls cells (UT, no CHIR stimulation) (Figure 5F).

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In hepatic cell lines, basal *CYP2E1* mRNA expression was low and was up-regulated in response to CHIR exposure only in differentiated HepaRG cells, but not in HepG2-C3 and HuH7 cells (Figure 5G). This suggests that activation of the WNT/β-catenin pathway is not sufficient *per se* to induce *CYP2E1* expression and underlines the importance of a differentiated hepatic context for full regulation.

These findings demonstrate for the first time that *CYP2E1* is transcriptionally up-regulated after CTNNB1 activation in PHHs.

CYP1A2

CYP1A2 mRNA expression also was increased in PHHs after incubation with mWnt3a exposure, suggesting that CTNNB1 participates in the regulation of *CYP1A2* expression in PHHs (Figure 6A). The role the Wnt/β-catenin pathway in *CYP1A2* regulation was evaluated also by siRNA-mediated down-regulation of *CTNNB1*, *APC* and *AhR* (as a positive control because *CYP1A2* is a known AhR target gene). CYP1A2 basal expression was not affected by *CTNNB1* down-regulation (Figure 6B), whereas CYP1A2 mRNA and protein expression (Figure 6B and 6C) were strongly increased upon *APC* silencing, compared to cells transfected with scrambled siRNA (siSC). As previously described (Lee et al., 2011), *AhR* down-regulation significantly decreased basal *CYP1A2* mRNA expression (Figure 6B). Similarly, *CYP1A2* up-regulation upon incubation with TCDD (dioxin) was not affected by siRNA-mediated *CTNNB1* silencing, whereas it was increased upon *APC* down-regulation compared to control cells (siSC). As expected, *CYP1A2* induction was strongly reduced in *AhR*-silenced cells (Figure 6D), because TCDD needs to bind to AhR to act as a transcriptional regulator (Fujii-Kuriyama and Mimura, 2005). Finally, we assessed whether AhR expression was also regulated by CTNNB1 in PHHs. *AhR* mRNA expression was increased in a dose-dependent manner by incubation with mWnt3a compared to untreated

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cells (UT). A similar effect was observed following *APC* silencing (Figure 6F), while *CTNNB1* down-regulation had no effect compared to control siRNA.

CYP3A4

Differently from *CYP2E1* and *CYP1A2*, *CYP3A4* mRNA expression in PHHs was not induced by incubation with mWnt3a, the canonical activator of the CTNNB1 pathway. Conversely, and as expected, it was strongly induced by RIF, a well-known PXR activator, compared to untreated cells (UT) (Figure 7A). Thus, although *in vivo* CYP2E1 and CYP3A4 show similar zonal expression (Figure 3), basal expression of *CYP3A4* in PHHs does not seem to be regulated by the CTNNB1 pathway. Similarly, *CYP3A4* induction by RIF was not affected by *CTNNB1* down-regulation or activation via *APC* siRNA silencing (Figure 7B). *PXR* mRNA expression also was not affected by *CTNNB1* or *APC* down-regulation, or incubation with Wnt3a (not shown). Moreover, expression of *HNF4*, another factor involved in CYP3A4 regulation (Jover et al., 2001), was not affected by *CTNNB1* or *APC* silencing or stimulation with Wnt3a (not shown). These results indicate that *CYP3A4* basal expression and its PXR-mediated induction are independent of CTNNB1 and APC in our model of PHH culture.

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DISCUSSION

The aim of this work was to evaluate the impact of the WNT/β-catenin pathway, which has been described as the main zonation keeper in the liver lobule (Benhamouche et al., 2006), on CYP gene expression in PHHs. When hepatocytes are seeded in culture, zonation is lost and the expression of WNT/β-catenin target genes and CYPs is concomitantly reduced. We therefore, hypothesized that by restoring the activity of the WNT/β-catenin pathway we could restore CYP expression in PHHs, despite the loss of the zonal organization. Here, we show that the expression of the CTNNB1 target gene *LGR5* can be restored in PHHs upon activation of the WNT/β-catenin signaling pathway. Moreover, we report for the first time that *CYP2E1* expression is transcriptionally regulated by CTNNB1and that is a true marker of CTNNB1 activity in PHHs.

The activity of the WNT/β-catenin pathway has been studied in various *in vitro* cell models, such as colon cancer cell lines (Verma et al., 2003), embryonic stem cells (Kielman et al., 2002; Sato et al., 2004) or isolated mouse hepatocytes (Hailfinger et al., 2006). Here, we show that the WNT/β-catenin pathway can also be reactivated in PHHs by stimuli that trigger this signaling cascade at different levels. First, we used the canonical activator WNT. However, WNT effect depends on the expression level of positive/negative effectors of the WNT/β-catenin pathway that can vary with time in PHHs. For instance, the progressive increase of DKK3 expression, an antagonist of the WNT/β-catenin pathway (Veeck and Dahl, 2012), during PHH culture could explain the lower ability of mWnt3a to activate the WNT/β-catenin pathway in comparison to other compounds used in this work. Then, we inhibited GSK3β by using CHIR99021, which is considered one of the most specific GSK3β inhibitors (Cohen and Goedert, 2004) and has been widely used in stem cell studies (Li et al., 2012).

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Finally, we down-regulated APC. *APC* inhibits β-catenin dependent transcription by providing a scaffold for the destruction complex, by promoting its export from the nucleus and by reducing its interaction with TCF. Moreover, APC expression in cancer cells causes nuclear and cytoplasmic translocation of β-catenin to the cell membrane (Aoki and Taketo, 2007). The increase of APC mRNA expression in parallel with PHHs adhesion and polarization (Gondeau et al., 2013) could contribute to WNT/β-catenin pathway down-regulation in PHHs (Hanson and Miller, 2005).

CYP2E1 has been extensively studied because of its implication in many toxicological and carcinogenic processes (Butura et al., 2009). CYP2E1 metabolizes chemicals, including APAP, carbon tetrachloride, dimethyl sulfoxide and ethanol. We demonstrate for the first time, that CYP2E1 expression and metabolic activity can be restored through activation of the WNT/β-catenin pathway in PHHs, as indicated by the production of 6-OH chlorzoxazone and GSH depletion after exposure to APAP. This can therefore constitute a useful model to study CYP2E1 role in APAP- and other drug-induced hepatotoxicity.

CYP2E1 protein and activity are often induced by its own substrates through post-transcriptional mechanisms (Gonzalez, 2007). Post-transcriptional regulation involves also CYP2E1 mRNA stabilization (Woodcroft et al., 2002). In parallel, *CYP2E1* gene transcription is under the control of HNF1α in rat hepatocytes (Liu and Gonzalez, 1995; Woodcroft et al., 2002), or interleukin-4 in a human hepatoma cell line (Lagadic-Gossmann et al., 2000). Here, we show that *CYP2E1* can also be transcriptionally regulated by the WNT/β-catenin pathway in PHHs. Specifically, *CYP2E1* expression was significantly up-regulated in response to GSK3β inhibition (CHIR, LiCl) or CTNNB1 stimulation (mWnt3a, *APC* silencing), demonstrating that *CYP2E1* is a true CTNNB1 target gene. This is in agreement with data obtained in *Ctnnb1*$^{-/-}$ mice, where *CYP2E1* gene expression is lost (Sekine et al., 2006; Tan et

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al., 2006), while its expression is increased in mouse (Loeppen et al., 2005) and human (Schmidt et al., 2011) liver tumors harboring *Ctnnb1* activating mutations. Moreover, CYP2E1 expression is induced in primary mouse hepatocytes incubated in conditioned medium from mWnt3a-producing 3T3 fibroblasts (Hailfinger et al., 2006).

However, the direct effect of β-catenin on the CYP2E1 promoter and the presence of a functional TCF/Lef responsive element need to be demonstrated. Indeed, direct binding of TCF4 (co-activator for β-catenin) on the 5000 bp region spanning the CYP2E1 promoter could not be demonstrated (Liu et al., 2012). Recently, using Chip-seq analysis, Gougelet et al., identified a WRE motif in a 100kpb region in the upstream and intragenic regions of mouse *Cyp2e1* (Gougelet et al., 2014), but did not demonstrate its functionality. An indirect effect via HNF1α transcriptional activity was also proposed (Gonzalez, 2006).

Moreover, we demonstrated that CYP2E1 transcriptional regulation by the WNT/β-catenin pathway is dependent on the cell context. Its expression is induced in PHHs and in differentiated HepaRG cells, but not in hepatoma cell lines (HepG2-C3 and HuH7). The β-catenin pathway is constitutively active in HepG2-C3 (very high LGR5 expression) without significant expression of CYP2E1 (Figure 5G). Moreover, HNF1α expression in HepG2-C3 cells and PHHs is comparable (Funakoshi et al., 2011).

Thus, CYP2E1 is transcriptionally regulated by CTNNB1 in a direct or indirect manner, and the presence of other co-regulators is certainly needed and has to be investigated. Whether these observations obtained in PHHs can be extrapolated to the physiological condition in human liver remains to be proven, but the fact that CYP2E1 is expressed according to the β-catenin activation in the liver lobule is in favor of this statement.

CYP1A2 expression also is induced by mWnt3a in PHHs (2- to 4-fold) and is strongly up-regulated upon *APC* down-regulation (100-fold), demonstrating a strong repressive

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activity mediated by APC. *Cyp1a2* mRNA expression can be increased by mWnt3a in isolated mouse hepatocytes and its expression is reduced in *Ctnnb1*$^{-/-}$ mouse liver (Braeuning et al., 2011; Sekine et al., 2006).

CYP1A2 expression and induction are under the control of nuclear receptors and transcription factors. *AhR* mRNA expression in PHHs was increased by incubation with mWnt3a and following *APC* silencing. Centrilobular expression of AhR was previously reported (Lindros et al., 1997) and AhR expression is increased in *Apc*$^{-/-}$ and decreased in *Ctnnb1*$^{-/-}$ mouse livers (Gougelet et al., 2014; Torre et al., 2011). As AhR participates in CYP1A2 basal and induced expression, the positive effect of β-catenin activation on CYP1A2 expression could therefore be direct or indirect via AhR up-regulation, as recently suggested (Gougelet et al., 2014). AhR may therefore participate in β-catenin-mediated CYP1A2 regulation and PC zonation. Moreover, several studies suggest a cross-talk between β-catenin and AhR that seems to be very complex. Specifically, β-catenin may act as a co-activator of AhR in mouse hepatocytes (Braeuning et al., 2011) and AhR participates in β-catenin degradation in mouse intestine (Kawajiri et al., 2009). Moreover, sustained activation of AhR by TCDD reduces the level of β-catenin targets genes in WB-F344 cells (Prochazkova et al., 2011).

On the other hand, our results indicate that, despite its centrilobular expression *in vivo* (Ratanasavanh et al., 1991), *CYP3A4* transcriptional regulation in PHHs is independent of β-catenin and APC expression and/or stimulation. This is in agreement with the finding that in early *Ctnnb1*$^{-/-}$ mice, *Cyp3a11* mRNA expression is not affected (Sekine et al., 2006), while *Cyp3a* is slightly induced in male mice (Braeuning et al., 2009). Conversely, *Cyp3a* expression is reduced in the liver of male *Ctnnb1*$^{loxP/loxP}$*/TTR-Cre*$^{+}$ mice, when *Ctnnb1* is invalidated in adulthood by injection of tamoxifen (Ganzenberg et al., 2013). As observed for

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CYP3A4 induction by RIF in PHHs, *Cyp3a* induction by pregnenolone-α-carbonitrile was not affected in *Ctnnb1*$^{-/-}$ mice (Braeuning et al., 2009). CYP3A4 expression in PHHs might therefore be regulated through other mechanisms. Indeed, CYP3A4 mRNA expression is up-regulated by the medium flow in PHHs (Vinci et al., 2011), and the oxygen tension in rat hepatocytes (Allen and Bhatia, 2003). Alternatively, Cyp3a localization could also be regulated in a RAS/MAPK/ERK- (Braeuning et al., 2007; Hailfinger et al., 2006), Dicer- (Sekine et al., 2009) or morphogen-dependent manners (Gebhardt and Hovhannisyan, 2010). Like for *CYP3A4*, expression of *NR1I2* is independent of β-catenin and APC in PHHs, but can be moderately induced by mWnt3a in mouse hepatocytes (Braeuning et al., 2011) and is inhibited in females *Ctnnb1*$^{-/-}$ mice (Braeuning et al., 2009).

Finally, in contrast to observations in *Ctnnb1*$^{-/-}$ mice (Sekine et al., 2006), *CTNNB1* silencing in PHHs did not seem to have an effect on *CYP2E1* and *CYP1A2* basal expression. *CTNNB1* silencing by siRNAs is a four day-long process and during this time the basal expression of *CYP2E1* and *CYP1A2* progressively decreases in culture. Therefore, *CTNNB1* invalidation has not further effect. However, *AhR* mRNA expression, which did not change during PHH culture, was also unaffected by *CTNNB1* silencing. As this gene is ubiquitously expressed in most mammalian tissues (Le Carrour et al., 2010), its basal expression is likely to be β-catenin-independent and other transcription factors might contribute to its transcriptional regulation. Similar observations were reported using rat WB-F344 progenitors cells (Prochazkova et al., 2011).

In conclusion, the Wnt/β-catenin pathway is functional in PHHs and constitutes a new regulatory network of drug metabolism in human hepatocytes. CYP2E1, CYP1A2 and AhR are transcriptionally regulated by this pathway, while CYP3A4, PXR and HNF4α are not.

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Induction of the Wnt/β-catenin pathway in PHHs represents a powerful tool to evaluate hepatotoxicity of drugs that are metabolized by CYP2E1.

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Authorship Contributions

Participated in research design: Gerbal-Chaloin and Daujat-Chavanieu

Conducted experiments: Gerbal-Chaloin, Dume, Briolotti, Klieber, Raulet and Duret,

Contributed new reagents or analytic tools: Fabre and Ramos

Performed data analysis: Gerbal-Chaloin and Daujat-Chavanieu

Wrote or contributed to the writing of the manuscript: Gerbal-Chaloin, Maurel and Daujat-Chavanieu

REFERENCES

Abu-Baker A, Laganiere J, Gaudet R, Rochefort D, Brais B, Neri C, Dion PA and Rouleau GA (2013) Lithium chloride attenuates cell death in oculopharyngeal muscular dystrophy by perturbing Wnt/beta-catenin pathway. *Cell death & disease* **4**: e821.

Allen JW and Bhatia SN (2003) Formation of steady-state oxygen gradients in vitro: application to liver zonation. *Biotechnology and bioengineering* **82**(3): 253-262.

Aoki K and Taketo MM (2007) Adenomatous polyposis coli (APC): a multi-functional tumor suppressor gene. *Journal of cell science* **120**(Pt 19): 3327-3335.

Benhamouche S, Decaens T, Godard C, Chambrey R, Rickman DS, Moinard C, Vasseur-Cognet M, Kuo CJ, Kahn A, Perret C and Colnot S (2006) Apc tumor suppressor gene is the "zonation-keeper" of mouse liver. *Dev Cell* **10**(6): 759-770.

Braeuning A, Ittrich C, Kohle C, Hailfinger S, Bonin M, Buchmann A and Schwarz M (2006) Differential gene expression in periportal and perivenous mouse hepatocytes. *Febs J* **273**(22): 5051-5061.

Braeuning A, Kohle C, Buchmann A and Schwarz M (2011) Coordinate regulation of cytochrome P450 1a1 expression in mouse liver by the aryl hydrocarbon receptor and the beta-catenin pathway. *Toxicological sciences : an official journal of the Society of Toxicology* **122**(1): 16-25.

Braeuning A, Menzel M, Kleinschnitz EM, Harada N, Tamai Y, Kohle C, Buchmann A and Schwarz M (2007) Serum components and activated Ha-ras antagonize expression of perivenous marker genes stimulated by beta-catenin signaling in mouse hepatocytes. *FEBS J* **274**(18): 4766-4777.

Braeuning A, Sanna R, Huelsken J and Schwarz M (2009) Inducibility of drug-metabolizing enzymes by xenobiotics in mice with liver-specific knockout of Ctnnb1. *Drug metabolism and disposition: the biological fate of chemicals* **37**(5): 1138-1145.

Burke ZD, Reed KR, Phesse TJ, Sansom OJ, Clarke AR and Tosh D (2009) Liver zonation occurs through a beta-catenin-dependent, c-Myc-independent mechanism. *Gastroenterology* **136**(7): 2316-2324 e2311-2313.

Butura A, Nilsson K, Morgan K, Morgan TR, French SW, Johansson I, Schuppe-Koistinen I and Ingelman-Sundberg M (2009) The impact of CYP2E1 on the development of alcoholic liver disease as studied in a transgenic mouse model. *Journal of hepatology* **50**(3): 572-583.

Cohen P and Goedert M (2004) GSK3 inhibitors: development and therapeutic potential. *Nature reviews Drug discovery* **3**(6): 479-487.

Colnot S, Decaens T, Niwa-Kawakita M, Godard C, Hamard G, Kahn A, Giovannini M and Perret C (2004) Liver-targeted disruption of Apc in mice activates beta-catenin signaling and leads to hepatocellular carcinomas. *Proceedings of the National Academy of Sciences of the United States of America* **101**(49): 17216-17221.

Colnot S and Perret C (2010) Liver Zonation, in *Molecular Pathology of Liver Diseases* (Monga SPS and Cagle PT eds) pp 7-16, Springer.

de La Coste A, Romagnolo B, Billuart P, Renard CA, Buendia MA, Soubrane O, Fabre M, Chelly J, Beldjord C, Kahn A and Perret C (1998) Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. *Proceedings of the National Academy of Sciences of the United States of America* **95**(15): 8847-8851.

Ferrini JB, Pichard L, Domergue J and Maurel P (1997) Long-term primary cultures of adult human hepatocytes. *Chem Biol Interact* **107**(1-2): 31-45.

Fujii-Kuriyama Y and Mimura J (2005) Molecular mechanisms of AhR functions in the regulation of cytochrome P450 genes. *Biochemical and biophysical research communications* **338**(1): 311-317.

Funakoshi N, Duret C, Pascussi JM, Blanc P, Maurel P, Daujat-Chavanieu M and Gerbal-Chaloin S (2011) Comparison of hepatic-like cell production from human embryonic stem cells and adult liver progenitor cells: CAR transduction activates a battery of detoxification genes. *Stem cell reviews* **7**(3): 518-531.

Ganzenberg K, Singh Y and Braeuning A (2013) The time point of beta-catenin knockout in hepatocytes determines their response to xenobiotic activation of the constitutive androstane receptor. *Toxicology* **308**: 113-121.

Gebhardt R and Hovhannisyan A (2010) Organ patterning in the adult stage: the role of Wnt/beta-catenin signaling in liver zonation and beyond. *Developmental dynamics : an official publication of the American Association of Anatomists* **239**(1): 45-55.

Gomez-Lechon MJ, Castell JV and Donato MT (2007) Hepatocytes--the choice to investigate drug metabolism and toxicity in man: in vitro variability as a reflection of in vivo. *Chem Biol Interact* **168**(1): 30-50.

Gondeau C, Briolotti P, Razafy F, Duret C, Rubbo PA, Helle F, Reme T, Ripault MP, Ducos J, Fabre JM, Ramos J, Pecheur EI, Larrey D, Maurel P and Daujat-Chavanieu M (2013) In vitro infection of primary human hepatocytes by HCV-positive sera: insights on a highly relevant model. *Gut*.

Gonzalez FJ (2006) Role of beta-catenin in the adult liver. *Hepatology* **43**(4): 650-653.

Gonzalez FJ (2007) The 2006 Bernard B. Brodie Award Lecture. Cyp2e1. *Drug metabolism and disposition: the biological fate of chemicals* **35**(1): 1-8.

Gougelet A, Torre C, Veber P, Sartor C, Bachelot L, Denechaud PD, Godard C, Moldes M, Burnol AF, Dubuquoy C, Terris B, Guillonneau F, Ye T, Schwartz M, Braeuning A, Perret C and Colnot S (2014) T-cell factor 4 and B-catenin chromatin occupancies pattern zonal liver metabolism. *Hepatology*.

Hailfinger S, Jaworski M, Braeuning A, Buchmann A and Schwarz M (2006) Zonal gene expression in murine liver: lessons from tumors. *Hepatology* **43**(3): 407-414.

Hanson CA and Miller JR (2005) Non-traditional roles for the Adenomatous Polyposis Coli (APC) tumor suppressor protein. *Gene* **361**: 1-12.

James LP, Mayeux PR and Hinson JA (2003) Acetaminophen-induced hepatotoxicity. *Drug metabolism and disposition: the biological fate of chemicals* **31**(12): 1499-1506.

Jover R, Bort R, Gomez-Lechon MJ and Castell JV (2001) Cytochrome P450 regulation by hepatocyte nuclear factor 4 in human hepatocytes: a study using adenovirus-mediated antisense targeting. *Hepatology* **33**(3): 668-675.

Kawajiri K, Kobayashi Y, Ohtake F, Ikuta T, Matsushima Y, Mimura J, Pettersson S, Pollenz RS, Sakaki T, Hirokawa T, Akiyama T, Kurosumi M, Poellinger L, Kato S and Fujii-Kuriyama Y (2009) Aryl hydrocarbon receptor suppresses intestinal carcinogenesis in ApcMin/+ mice with natural ligands. *Proceedings of the National Academy of Sciences of the United States of America* **106**(32): 13481-13486.

Kielman MF, Rindapaa M, Gaspar C, van Poppel N, Breukel C, van Leeuwen S, Taketo MM, Roberts S, Smits R and Fodde R (2002) Apc modulates embryonic stem-cell differentiation by controlling the dosage of beta-catenin signaling. *Nature genetics* **32**(4): 594-605.

Kohle C and Bock KW (2009) Coordinate regulation of human drug-metabolizing enzymes, and conjugate transporters by the Ah receptor, pregnane X receptor and constitutive androstane receptor. *Biochem Pharmacol* **77**(4): 689-699.

Lagadic-Gossmann D, Lerche C, Rissel M, Joannard F, Galisteo M, Guillouzo A and Corcos L (2000) The induction of the human hepatic CYP2E1 gene by interleukin 4 is transcriptional and regulated by protein kinase C. *Cell biology and toxicology* **16**(4): 221-233.

Le Carrour T, Assou S, Tondeur S, Lhermitte L, Lamb N, Reme T, Pantesco V, Hamamah S, Klein B and De Vos J (2010) Amazonia!: An Online Resource to Google and Visualize Public Human whole Genome Expression Data. . *The Open Bioinformatics Journal* **4**: 5-10.

Lee YC, Oslund KL, Thai P, Velichko S, Fujisawa T, Duong T, Denison MS and Wu R (2011) 2,3,7,8-Tetrachlorodibenzo-p-dioxin-induced MUC5AC expression: aryl hydrocarbon receptor-independent/EGFR/ERK/p38-dependent SP1-based transcription. *American journal of respiratory cell and molecular biology* **45**(2): 270-276.

Li W, Jiang K and Ding S (2012) Concise review: A chemical approach to control cell fate and function. *Stem cells* **30**(1): 61-68.

Lindros KO, Oinonen T, Johansson I and Ingelman-Sundberg M (1997) Selective centrilobular expression of the aryl hydrocarbon receptor in rat liver. *The Journal of pharmacology and experimental therapeutics* **280**(1): 506-511.

Liu S, Yeh TH, Singh VP, Shiva S, Krauland L, Li H, Zhang P, Kharbanda K, Ritov V, Monga SP, Scott DK, Eagon PK and Behari J (2012) beta-catenin is essential for ethanol metabolism and protection against alcohol-mediated liver steatosis in mice. *Hepatology* **55**(3): 931-940.

Liu SY and Gonzalez FJ (1995) Role of the liver-enriched transcription factor HNF-1 alpha in expression of the CYP2E1 gene. *DNA and cell biology* **14**(4): 285-293.

Loeppen S, Koehle C, Buchmann A and Schwarz M (2005) A beta-catenin-dependent pathway regulates expression of cytochrome P450 isoforms in mouse liver tumors. *Carcinogenesis* **26**(1): 239-248.

MacDonald BT, Tamai K and He X (2009) Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* **17**(1): 9-26.

Parent R, Marion MJ, Furio L, Trepo C and Petit MA (2004) Origin and characterization of a human bipotent liver progenitor cell line. *Gastroenterology* **126**(4): 1147-1156.

Pichard L, Raulet E, Fabre G, Ferrini JB, Ourlin JC and Maurel P (2006) Human hepatocyte culture. *Methods in molecular biology* **320**: 283-293.

Prochazkova J, Kabatkova M, Bryja V, Umannova L, Bernatik O, Kozubik A, Machala M and Vondracek J (2011) The interplay of the aryl hydrocarbon receptor and beta-catenin alters both AhR-dependent transcription and Wnt/beta-catenin signaling in liver progenitors. *Toxicological sciences : an official journal of the Society of Toxicology* **122**(2): 349-360.

Ratanasavanh D, Beaune P, Morel F, Flinois JP, Guengerich FP and Guillouzo A (1991) Intralobular distribution and quantitation of cytochrome P-450 enzymes in human liver as a function of age. *Hepatology* **13**(6): 1142-1151.

Ring DB, Johnson KW, Henriksen EJ, Nuss JM, Goff D, Kinnick TR, Ma ST, Reeder JW, Samuels I, Slabiak T, Wagman AS, Hammond ME and Harrison SD (2003) Selective glycogen synthase kinase 3 inhibitors potentiate insulin activation of glucose transport and utilization in vitro and in vivo. *Diabetes* **52**(3): 588-595.

Sato N, Meijer L, Skaltsounis L, Greengard P and Brivanlou AH (2004) Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nature medicine* **10**(1): 55-63.

Schmidt A, Braeuning A, Ruck P, Seitz G, Armeanu-Ebinger S, Fuchs J, Warmann SW and Schwarz M (2011) Differential expression of glutamine synthetase and cytochrome P450 isoforms in human hepatoblastoma. *Toxicology* **281**(1-3): 7-14.

Sekine S, Lan BY, Bedolli M, Feng S and Hebrok M (2006) Liver-specific loss of beta-catenin blocks glutamine synthesis pathway activity and cytochrome p450 expression in mice. *Hepatology* **43**(4): 817-825.

Sekine S, Ogawa R, McManus MT, Kanai Y and Hebrok M (2009) Dicer is required for proper liver zonation. *The Journal of pathology* **219**(3): 365-372.

Staal FJ, Burgering BM, van de Wetering M and Clevers HC (1999) Tcf-1-mediated transcription in T lymphocytes: differential role for glycogen synthase kinase-3 in fibroblasts and T cells. *International immunology* **11**(3): 317-323.

Tan X, Behari J, Cieply B, Michalopoulos GK and Monga SP (2006) Conditional deletion of beta-catenin reveals its role in liver growth and regeneration. *Gastroenterology* **131**(5): 1561-1572.

Torre C, Benhamouche S, Mitchell C, Godard C, Veber P, Letourneur F, Cagnard N, Jacques S, Finzi L, Perret C and Colnot S (2011) The transforming growth factor-alpha and cyclin D1 genes are direct targets of beta-catenin signaling in hepatocyte proliferation. *Journal of hepatology* **55**(1): 86-95.

Veeck J and Dahl E (2012) Targeting the Wnt pathway in cancer: the emerging role of Dickkopf-3. *Biochimica et biophysica acta* **1825**(1): 18-28.

Verma UN, Surabhi RM, Schmaltieg A, Becerra C and Gaynor RB (2003) Small interfering RNAs directed against beta-catenin inhibit the in vitro and in vivo growth of colon cancer cells. *Clinical cancer research : an official journal of the American Association for Cancer Research* **9**(4): 1291-1300.

Vinci B, Duret C, Klieber S, Gerbal-Chaloin S, Sa-Cunha A, Laporte S, Suc B, Maurel P, Ahluwalia A and Daujat-Chavanieu M (2011) Modular bioreactor for primary human hepatocyte culture: medium flow stimulates expression and activity of detoxification genes. *Biotechnology journal* **6**(5): 554-564.

Vinken M, Papeleu P, Snykers S, De Rop E, Henkens T, Chipman JK, Rogiers V and Vanhaecke T (2006) Involvement of cell junctions in hepatocyte culture functionality. *Crit Rev Toxicol* **36**(4): 299-318.

Woodcroft KJ, Hafner MS and Novak RF (2002) Insulin signaling in the transcriptional and posttranscriptional regulation of CYP2E1 expression. *Hepatology* **35**(2): 263-273.

Yamamoto Y, Sakamoto M, Fujii G, Tsuiji H, Kenetaka K, Asaka M and Hirohashi S (2003) Overexpression of orphan G-protein-coupled receptor, Gpr49, in human hepatocellular carcinomas with beta-catenin mutations. *Hepatology* **37**(3): 528-533.

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FIGURES LEGENDS

Figure 1 The β-catenin pathway can be activated in PHHs. (A) RT-qPCR analysis of *LGR5*, *APC*, *GSK3β* and *DKK3* gene expression over time (maximal expression was arbitrary fixed to 100) (n=3). (B) RT-qPCR analysis of *LGR5* mRNA expression after 48 h incubation with 10 or 20 mM LiCl, 100 or 200 ng/mL mWnt3a, 1 or 3 µM CHIR, 10 nM TCDD, or 10 µM RIF (n=4). RT-qPCR analysis of *LGR5* mRNA expression in PHHs from different liver donors (PHH365, PHH359 and PHH366) incubated at day 2 after seeding with (C) 1, 3 or 10 µM CHIR (D) 100, 200 or 400 ng/ml mWnt3a for 48 h. FIH: Freshly isolated hepatocytes. UT: untreated. nd: not detected. D: Day.

Figure 2 The β-catenin pathway can be activated in PHHs. (A) Luciferase activity measurement in PHHs transfected with the TK-FOP-luc (white bars) or TK-TOP-luc (black bars) plasmids and incubated 24 h later with 3µM CHIR, 20 mM LiCl or 200 ng/mL mWnt3a for 24 h. (B) RT-qPCR analysis of *LGR5* expression in PHHs after siRNA-mediated *CTNNB1* silencing and incubation with 200 ng/mL mWnt3a or 3 µM CHIR for 24 h (n=5) or after siRNA-mediated *APC* silencing (n=3). (C) RT-qPCR analysis of *LGR5* mRNA expression in HepG2-C3A, HuH7 or differentiated HepaRG cells incubated with 3 µM CHIR for 48 h. nd: not detected.

Figure 3 Pericentral expression of CYP2E1, CYP1A2 and CYP3A4. (A) Analysis of CYP2E1, CYP1A2, CYP3A4, GLUL and KRT19 expression in serial human liver tissue sections by immunohistochemistry. Scale bars = 1 mm. (B) RT-qPCR analysis of *CYP2E1*, *CYP1A2* and *CYP3A4* gene expression over time (expression in FIH was arbitrary fixed to 100) (n=3). CT: control without antibody, FIH: Freshly isolated hepatocytes. D: Day.

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Figure 4 *CYP2E1* is a CTNNB1 target gene (A) RT-qPCR analysis of *CYP2E1* mRNA expression following incubation with 10 or 20 mM LiCl, 100 or 200 ng/mL mWnt3a, 1 or 3 µM CHIR, 10 nM TCDD or 10 µM RIF. (B) RT-qPCR analysis of *CYP2E1* mRNA expression in PHHs after siRNA-mediated *CTNNB1* or *APC* down-regulation and incubation with CHIR 3 µM or 200ng/mL mWnt3a. ND: not determined.

Figure 5 *CYP2E1* is a CTNNB1 target gene. (A) *CYP*2E1 induction in PHHs after incubation with 3 µM CHIR for 48 h. Results are expressed as fold change relative to untreated cells (UT) (n=14). CYP2E1 mRNA (B) and protein (C) expression in PHHs before and after addition (or not) of 3 µM CHIR at day 2. One pmol of recombinant CYP2E1 (rCYP2E1) was used as control. (D) CYP2E1 protein expression in siRNA-transfected PHHs after incubation or not with 3 µM CHIR for 48 h. (E) CYP2E1-mediated 6-OH chlorzoxazone production in PHHs (n=2). (F) GSH content depletion in PHHs incubated or not with 3 µM CHIR for 72 h and incubated with 20 or 50 mM APAP for 6 h. (G) RT-qPCR analysis of *CYP2E1* mRNA expression in HepG2-C3A, HuH7 or differentiated HepaRG cells after incubation with 3 µM CHIR for 48 h. Results are expressed as percentage of the *CYP2E1* mRNA expression level in PHHs at day 1 post-seeding. FIH: freshly isolated hepatocytes. UT: Untreated.

Figure 6 CYP1A2 regulation in PHHs. (A) RT-qPCR analysis of *CYP1A2* expression in PHHs after incubation, or not, with 100 or 200 ng/mL mWnt3a (n=4). (B) *CYP1A2* mRNA expression after *CTNNB1*, *APC* or *AhR* silencing. (C) CYP1A2 protein expression in PHHs after siRNA-mediated *APC* silencing. (D) *CYP1A2* mRNA expression after *CTNNB1*, *APC* or *AhR* silencing and incubation with 10 nM TCDD for 24 hours. Results are expressed as fold change relative to untreated hepatocytes (UT) (E) RT-qPCR analysis of *AhR* expression in

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PHHs after incubation, or not, with 100 or 200 ng/mL mWnt3a. (F) *AhR* mRNA expression after *CTNNB1*, *APC* or *AhR* silencing. siSC: Scrambled siRNA. UT: Untreated.

Figure 7 CYP3A4 regulation in PHHs. (A) RT-qPCR analysis of *CYP3A4* expression in PHHs after incubation, or not, with 100 or 200 ng/mL mWnt3a or 10 µM RIF (n=4). (B) *CYP3A4* mRNA expression after *CTNNB1* or *APC* silencing and treatment with 10 µM RIF for 24 hours. UT: Untreated.

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TABLES

Table 1 Clinical characteristics of the liver donors

Liver Identification	Sex	Age	Pathology
PHH351	M	75	Metastasis of urothelial cancer
PHH353	M	60	Metastasis of colorectal cancer
PHH354	F	53	Metastasis of neuroendocrine tumor
PHH356	M	42	Organ Donor
PHH358	F	45	Metastasis of endocrine tumor
PHH359	M	58	Hepatocellular carcinoma
PHH361	F	67	Cholangiocarcinoma
PHH365	M	70	Abscess of engrafted liver
PHH366	M	62	Metastasis of colorectal cancer
PHH367	M	59	Cholangiocarcinoma
PHH374	F	41	Adenoma
PHH376	M	77	Hepatocellular carcinoma
PHH377	M	58	Cholangiocarcinoma
PHH378	M	72	Cholangiocarcinoma
PHH381	M	70	Metastasis of colorectal cancer
PHH382	M	70	Metastasis of GIST
PHH383	F	37	Angioma

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Table 2 Primer pair sequences

Gene Name	**Forward primers**	**Reverse primers**
AhR	ATCAGTGCCAGCCAGAACCTC	AGGTCTGGCTTCTGACGGATG
APC	CACACTTCCAACTTCTCGCAACG	AGGCTGCATGAGAGCACTTGTG
CYP1A2	CATCCCCCACAGCACAACAA	TCCCACTTGGCCAGGACTTC
CYP2E1	GACTGTGGCCGACCTGTT	ACTACGACTGTGCCCTTGG
CYP3A4	GCCTGGTGCTCCTCTATCTA	GGTGTTGACCATCATAAAG
DKK3	GTGCATCATCGACGAGGACTGT	TGGTCTCCACAGCACTCACTGT
GSK3β	CCGACTAACACCACTGGAAGCT	AGGATGGTAGCCAGAGGTGGA
LGR5	CCTGCTTGACTTTGAGGAAGACC	CCAGCCATCAAGCAGGTGTTCA
PXR	GGACCAGCTGCAGGAGCAAT	CATGAGGGGCGTAGCAAAGG

Figure 1

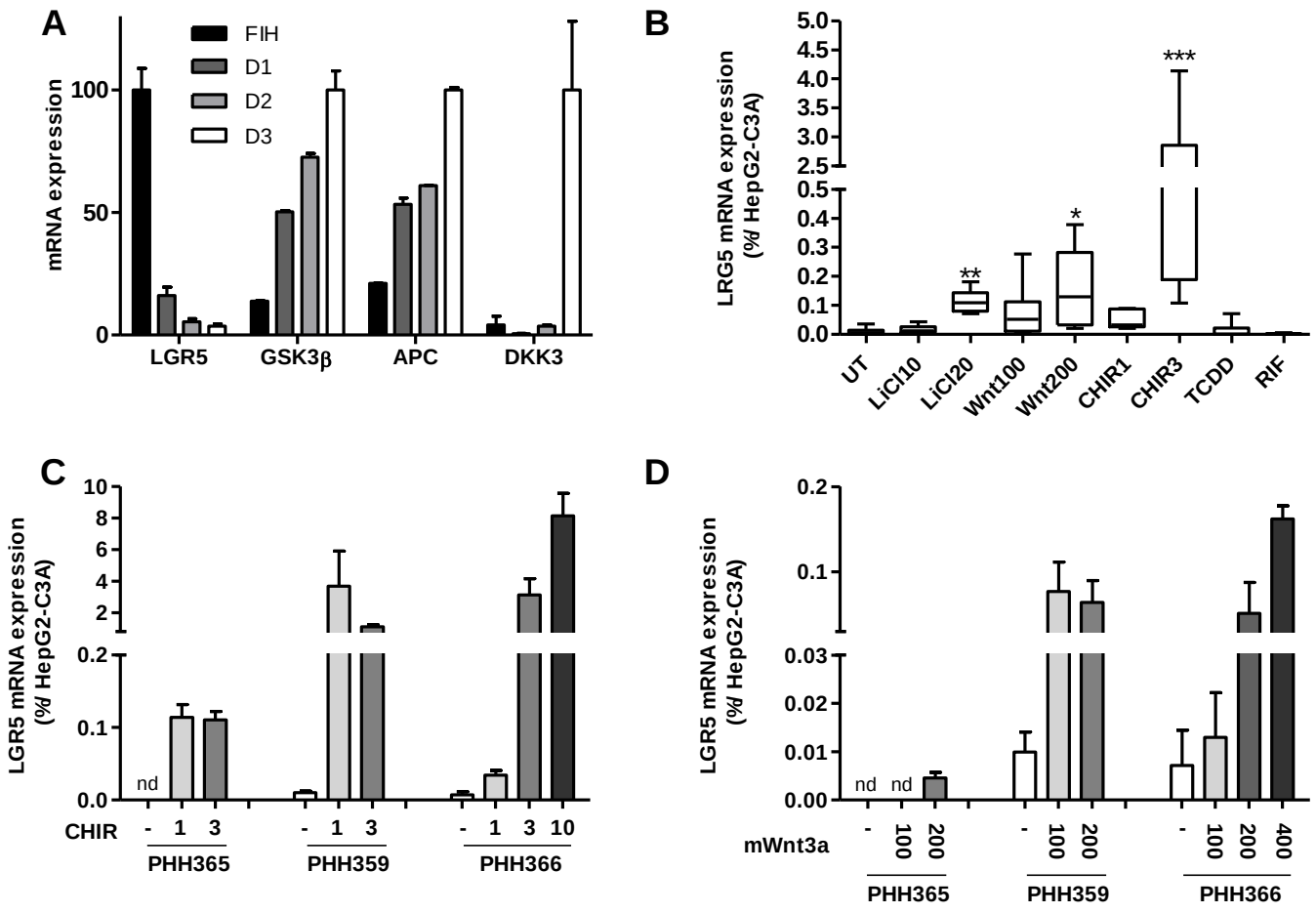

A
FIH
D1
D2
D3
mRNA expression
100
50
0
LGR5
GSK3β
APC
DKK3
B
LRG5 mRNA expression
(% HepG2-C3A)
5.0
4.5
4.0
3.5
3.0
2.5
0.5
0.4
0.3
0.2
0.1
0.0
**
*
***
UT
LiCl10
LiCl20
Wnt100
Wnt200
CHIR1
CHIR3
TCDD
RIF
C
LGR5 mRNA expression
(% HepG2-C3A)
10
8
6
4
2
0.2
0.1
0.0
nd
CHIR
- 1 3
- 1 3
- 1 3 10
PHH365
PHH359
PHH366
D
LGR5 mRNA expression
(% HepG2-C3A)
0.2
0.1
0.03
0.02
0.01
0.00
nd nd
mWnt3a
- 100 200
- 100 200
- 100 200 400
PHH365
PHH359
PHH366

Figure 2

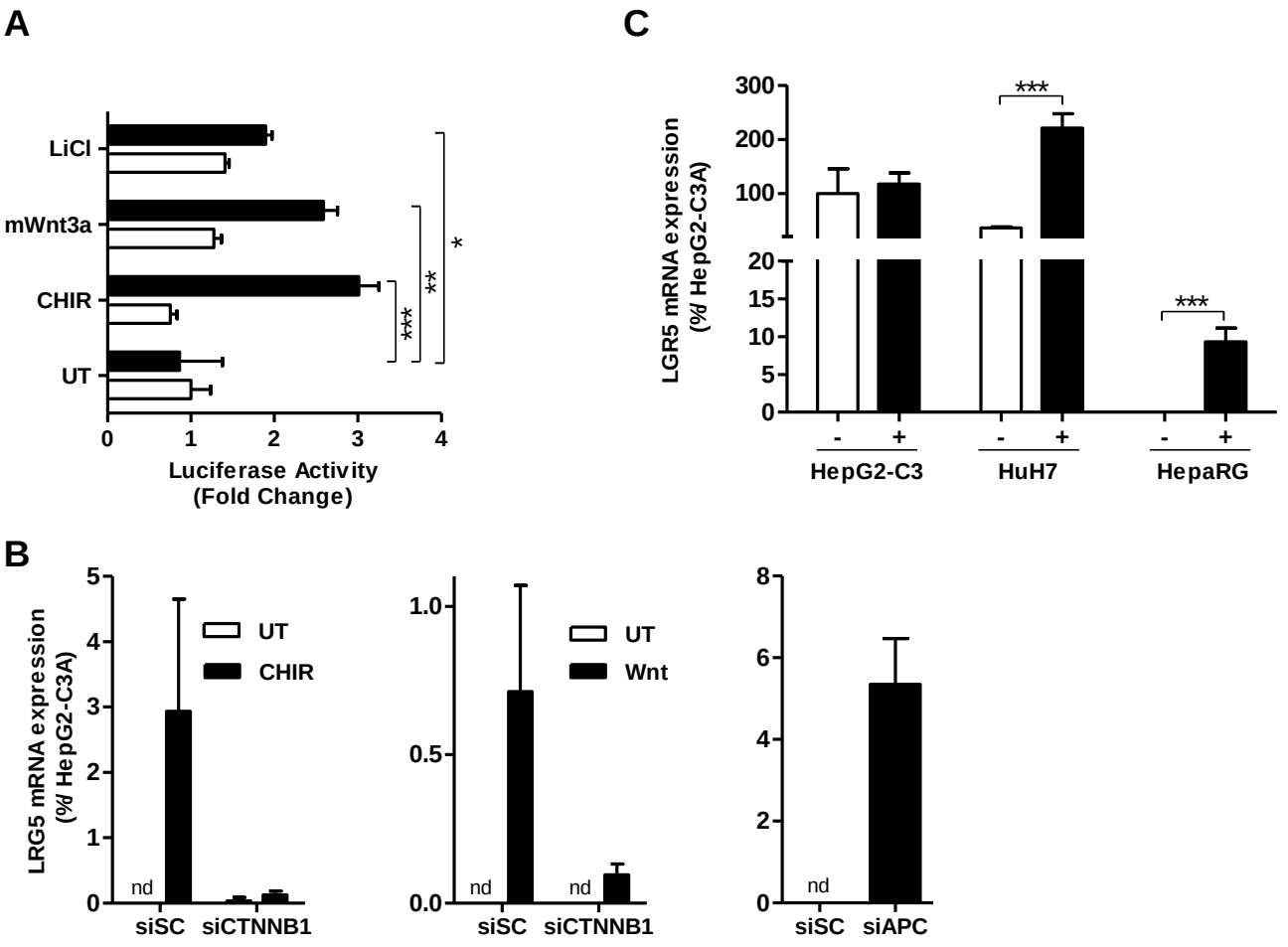
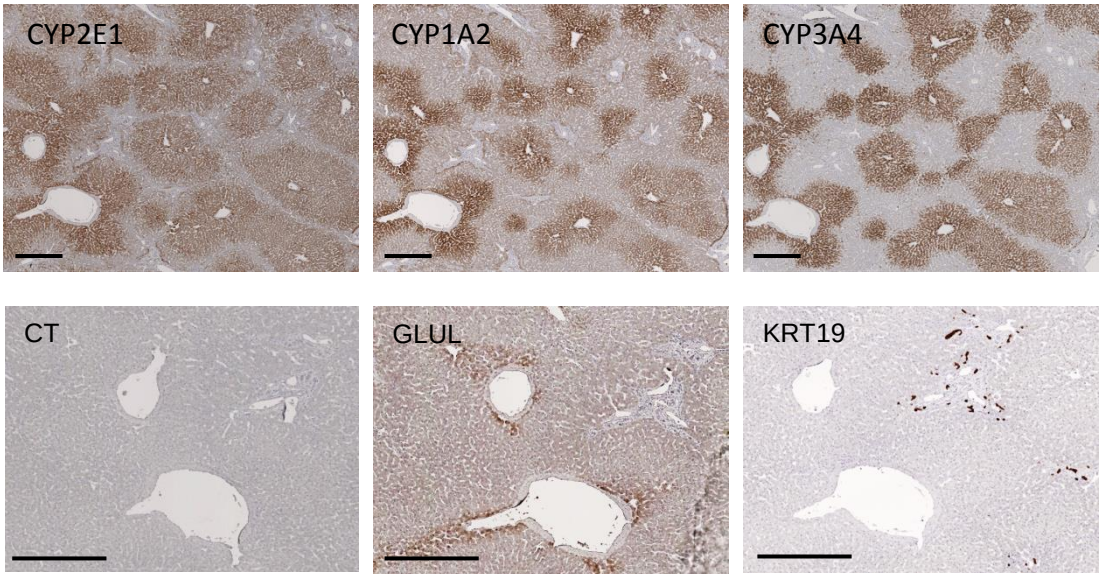

A
LiCl
mWnt3a
CHIR
UT
0
1
2
3
4
Luciferase Activity
(Fold Change)
***
**
*
C
LGR5 mRNA expression
(% HepG2-C3A)
300
200
100
20
15
10
5
0
***
***
- + - + - +
HepG2-C3
HuH7
HepaRG
B
LRG5 mRNA expression
(% HepG2-C3A)
5
4
3
2
1
0
UT
CHIR
nd
siSC
siCTNNB1
1.0
0.5
0.0
UT
Wnt
nd
nd
siSC
siCTNNB1
8
6
4
2
0
nd
siSC
siAPC

Figure 3

A


CYP2E1
CYP1A2
CYP3A4
CT
GLUL
KRT19

B

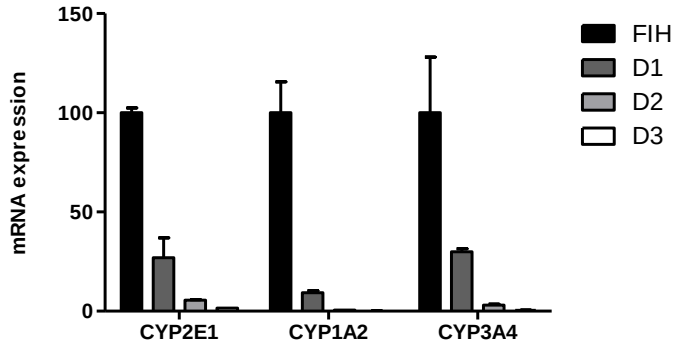

150
100
50
0
mRNA expression
CYP2E1
CYP1A2
CYP3A4
FIH
D1
D2
D3

Figure 4

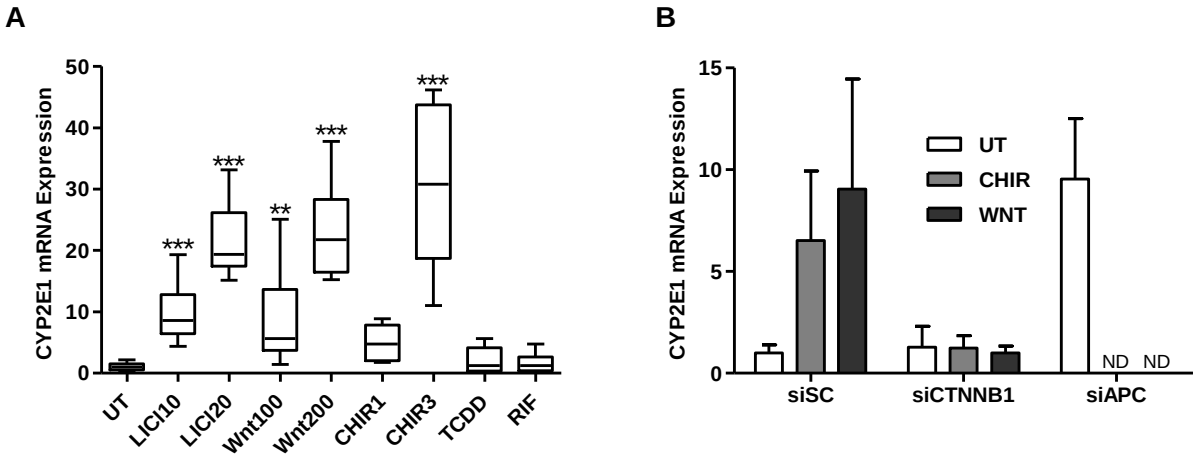

A
CYP2E1 mRNA Expression
50
40
30
20
10
0
***
***
**
***
***
UT
LICl10
LICl20
Wnt100
Wnt200
CHIR1
CHIR3
TCDD
RIF
B
CYP2E1 mRNA Expression
15
10
5
0
UT
CHIR
WNT
ND ND
siSC
siCTNNB1
siAPC

Figure 5

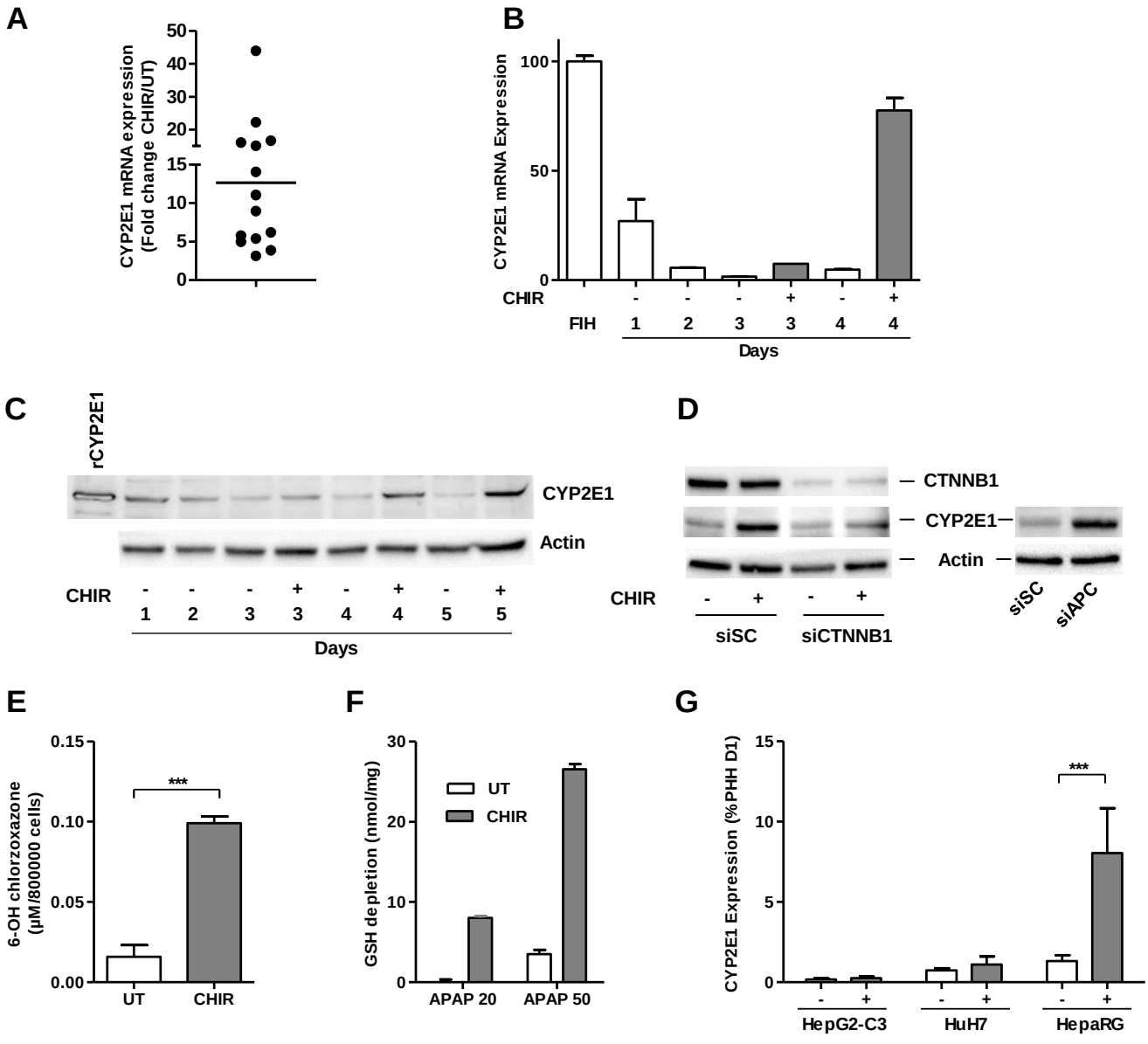

A
CYP2E1 mRNA expression (Fold change CHIR/UT)
50
40
30
20
15
10
5
0
B
CYP2E1 mRNA Expression
100
50
0
CHIR - - - + - +
FIH 1 2 3 3 4 4
Days
C
rCYP2E1
CYP2E1
Actin
CHIR - - - + - + - +
1 2 3 3 4 4 5 5
Days
D
CTNNB1
CYP2E1
Actin
CHIR - + - +
siSC siCTNNB1
siSC siAPC
E
6-OH chlorzoxazone (µM/800000 cells)
0.15
0.10
0.05
0.00
***
UT CHIR
F
GSH depletion (nmol/mg)
30
20
10
0
UT
CHIR
APAP 20 APAP 50
G
CYP2E1 Expression (%PHH D1)
15
10
5
0
***
- + - + - +
HepG2-C3 HuH7 HepaRG

Figure 6

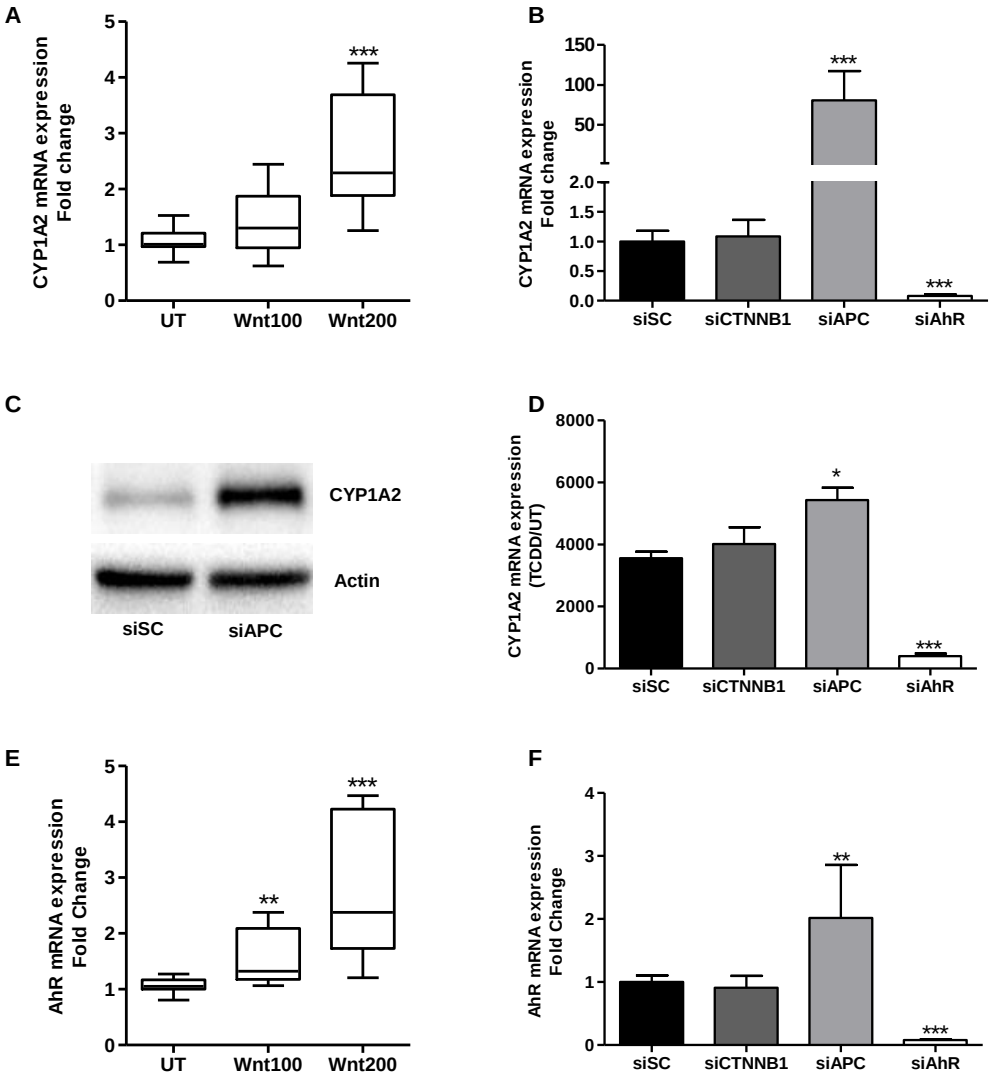
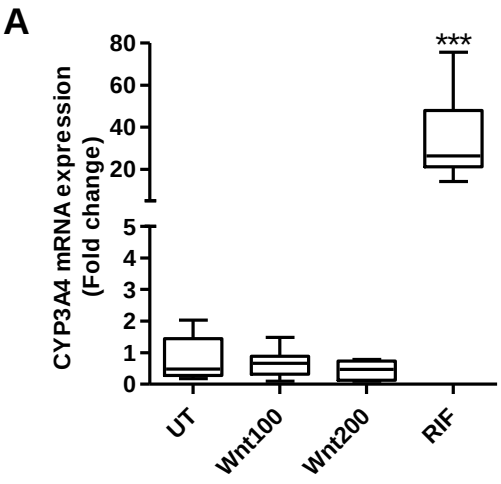
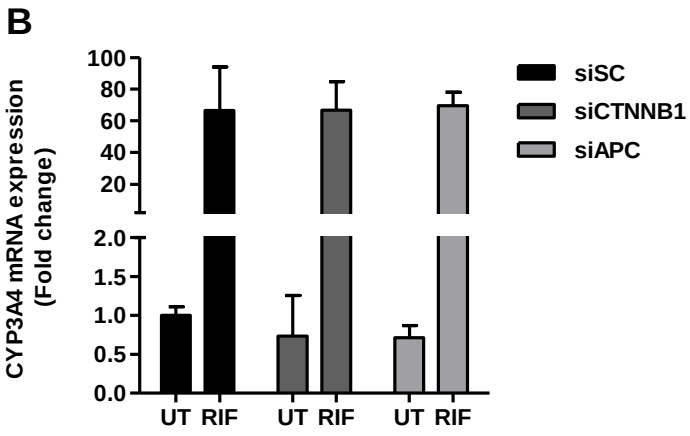

A
CYP1A2 mRNA expression
Fold change
5
4
3
2
1
0
***
UT
Wnt100
Wnt200
B
CYP1A2 mRNA expression
Fold change
150
100
50
2.0
1.5
1.0
0.5
0.0
***
***
siSC
siCTNNB1
siAPC
siAhR
C
CYP1A2
Actin
siSC
siAPC
D
CYP1A2 mRNA expression
(TCDD/UT)
8000
6000
4000
2000
0
*
***
siSC
siCTNNB1
siAPC
siAhR
E
AhR mRNA expression
Fold Change
5
4
3
2
1
0
**
***
UT
Wnt100
Wnt200
F
AhR mRNA expression
Fold Change
4
3
2
1
0
**
***
siSC
siCTNNB1
siAPC
siAhR

Figure 7


A
CYP3A4 mRNA expression
(Fold change)
80
60
40
20
5
4
3
2
1
0
***
UT
Wnt100
Wnt200
RIF


B
CYP3A4 mRNA expression
(Fold change)
100
80
60
40
20
2.0
1.5
1.0
0.5
0.0
UT RIF UT RIF UT RIF
siSC
siCTNNB1
siAPC