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Title Page

Differential role of LXRα and LXRβ in the regulation of UDP-glucuronosyltransferase 1A1 in humanized *UGT1* mice

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Running Title Page

Running Title: *Activation of LXRα induces human UGT1A1 in hUGT1 mice*

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Tables: 2

Figures 7

Supplemental Figures: 1

Abstract: 250 words

Significance Statement: 79 words

Introduction: 677 words

Discussion: 1465 words

Abbreviations: CAR, constitutive androstane receptor; ChIP, chromatin immunoprecipitation; CYP, cytochrome P450; *hUGT1*, humanized *UGT1*; LXR, liver X receptor; NCoR1, nuclear co-repressor 1; PPARα, peroxisome proliferator-activated receptor alpha; PXR, pregnane X receptor; TSB, total serum bilirubin; SCD1, stearoyl CoA desaturase; UGT, UDP-glucuronosyltransferase

Abstract

Liver X receptors (LXRs), LXRα and LXRβ, are nuclear receptors that regulate the metabolism of cholesterol and bile acids and are activated by oxysterols. Humanized *UGT1* (*hUGT1*) mice express the 9-human *UGT1A* genes associated with the *UGT1* locus in a *Ugt1*-null background. The expression of UGT1A1 is developmentally delayed in the liver and intestines resulting in the accumulation of serum bilirubin during the neonatal period. Induction of UGT1A1 in newborn *hUGT1* mice leads to rapid reduction in total serum bilirubin (TSB) levels, a phenotype measurement that allows for an accurate prediction on UGT1A1 expression. When neonatal *hUGT1* mice were treated by oral gavage with the LXR agonist T0901317, TSB levels were dramatically reduced. To determine the LXR contribution to the induction of UGT1A1 and the lowering of TSB levels, experiments were conducted in neonatal *hUGT1/Lxrα*$^{-/-}$, *hUGT1/Lxrβ*$^{-/-}$ and *hUGT1/Lxrαβ*$^{-/-}$ mice treated with T0901317. Induction of liver UGT1A1 was dependent upon LXRα, with the induction pattern paralleling induction of LXRα specific Stearoyl-CoA Desaturase 1 (SCD1). However, the actions of T0901317 were also shown to display a lack of specificity for LXR, with the induction of liver UGT1A1 in *hUGT1/Lxrαβ*$^{-/-}$ mice, a result associated with activation of both PXR and CAR. However, the LXR agonist GW3965 was highly selective towards LXRα, showing no impact on lowering TSB values or inducing UGT1A1 in *hUGT1/Lxrα*$^{-/-}$ mice. An LXR specific enhancer site on the *UGT1A1* gene was identified, along with convincing evidence that LXRα is crucial in maintaining constitutive expression of UGT1A1 in adult *hUGT1* mice.

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Significance Statement

It has been established that activation of LXRα, and not LXRβ, is responsible for the induction of liver UGT1A1 and metabolism of serum bilirubin in neonatal *hUGT1* mice. While induction of the human *UGT1A1* gene is initiated at a newly characterized LXR enhancer site, allelic deletion of the *Lxrα* gene drastically reduces the constitutive expression of liver UGT1A1 in adult *hUGT1* mice. Combined, these findings indicate that LXRα is critical for the developmental expression of UGT1A1.

Introduction

UDP-glucuronosyltransferases (UGTs) catalyze the transformation of xeno- and endobiotics into excretable glucuronides and thus participate in biological inactivation and clearance of these compounds (Hu et al., 2014). Variations in UGT expression in addition to the induction or inhibition of these proteins can result in adverse drug effects as well as hormone imbalance (Yang et al., 2017). The *UGT1* gene family encodes nine transferases (UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10) (Ritter et al., 1992) which exhibit unique substrate specificity and are expressed in a tissue-specific as well as inducible manner (Tukey and Strassburg, 2000). Humanized *UGT1* mice (*hUGT1* mice) express the *UGT1A* genes in a tissue-specific pattern that was similar to expression patterns of the same genes in human tissues (Fujiwara et al., 2010). Interestingly, UGT1A1, which is the sole transferase responsible for the metabolism of serum bilirubin (Bosma et al., 1994), is developmentally regulated in newborn *hUGT1* mice and has been a valuable tool in examining the contribution of UGT1A1 towards the control of neonatal hyperbilirubinemia. (Fujiwara et al., 2010; Fujiwara et al., 2012; Yueh et al., 2014; Chen and Tukey, 2018). Neonatal *hUGT1* mice develop near-fatal levels of total serum bilirubin (TSB) during the first two weeks after birth (Fujiwara et al., 2010). The administration of ligands known to activate either liver or intestinal constitutive androstane receptor (CAR) (Cai et al., 2010; Fujiwara et al., 2012), the pregnane X-receptor (PXR) (Chen et al., 2005; Chen et al., 2012), or the peroxisome proliferator-activated receptor α (PPARα) (Senekeo-Effenberger et al., 2007) result in the induction of UGT1A1 and the simultaneous metabolism and elimination of circulating TSB.

The liver X receptor (LXR) was first identified in the 1990s as an orphan member of the nuclear receptor superfamily and later found to be activated by endogenous oxysterols (Peet et al., 1998a). LXR consists of two isoforms, LXRα and LXRβ. LXRα

is expressed in all tissues (Song et al., 1994), in contrast, the expression of LXRβ is restricted to the liver, intestine, kidney, spleen, macrophages, and adipose tissue (Willy et al., 1995). LXR is largely involved in the regulation of cholesterol homeostasis protecting the cells from cholesterol overload by stimulating reverse cholesterol transport (Millatt et al., 2003). Furthermore, LXR has been linked to other major physiological functions, including lipid metabolism (Ulven et al., 2005; Cha and Repa, 2007), glucose homeostasis (Grefhorst et al., 2005; Oosterveer et al., 2010), steroidogenesis, immunity, and inflammation (Zelcer and Tontonoz, 2006).

LXRα and LXRβ were cloned in 1995 (Mangelsdorf and Evans, 1995; Willy et al., 1995), and later confirmed that the ligand-binding and DNA-binding domains of the receptors were 77% homologous (Alberti et al., 2000). Following confirmation that expression of the LXRs displayed differential tissue specificity (Shinar et al., 1994; Willy et al., 1995) yet both bound to similar LXR enhancer sequences, it had been speculated and later demonstrated that activation of the LXRα/RXR and LXRβ/RXR complexes have different roles in gene expression (Annicotte et al., 2004; Hong and Tontonoz, 2008). This was first demonstrated when *LXRα*$^{-/-}$ and *LXRβ*$^{-/-}$ mice were placed on a normal or cholesterol high diet (Alberti et al., 2001). The cholesterol-enriched diet had a profound effect on *LXRα*$^{-/-}$ mice, inducing fatty liver, increased liver mass and increased cholesterol levels, eventually leading to impaired liver function. The LXRβ$^{-/-}$ mice were resistant to cholesterol-induced fatty liver formation. There are not highly selective LXRα or LXRβ ligands, making it difficult to isolate the actions of the different LXR receptors on gene expression or function. Yet, activation of LXR by non-selective LXR agonists in *LXRα*$^{-/-}$ or *LXRβ*$^{-/-}$ mice can isolate the direct actions of these receptors on gene expression. For example, the LXR agonist T0901317-induced hepatic stearoyl CoA desaturase (SCD1) expression only in wild-type or *LXRβ*$^{-/-}$ mice and not *LXRα*$^{-/-}$ mice, demonstrating that LXRα controls SCD1 expression (Zhang et

al., 2014). There is little information on the direct impact of LXR towards the induction and regulation of the *UGT1A1* gene. We have now taken a direct approach to evaluate the role of the LXR receptors on the induction of the *UGT1A1* gene by deleting LXRα, LXRβ or LXRα and LXRβ in *hUGT1* mice.

Materials and Methods

Materials.

UDP-glucuronic acid (UDPGA) and bilirubin were obtained from Sigma (St. Louis, MO). SN-38 and T0901317 were obtained from Cayman Chemical (Ann Arbor, MI). SN-38 glucuronide was from Santa Cruz Biotechnology (Santa Cruz, CA). Internal standards deuterated d10-CPT-11 and d3-SN-38 were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Rabbit anti-UGT1A1 monoclonal antibody was purchased from Abcam (Cambridge, UK). Mouse anti-GAPDH monoclonal antibody was obtained from Santa Cruz Biotechnology (Dallas, TX). Anti-mouse IgG horseradish peroxidase (HRP) conjugated antibody and anti-rabbit IgG HRP conjugated antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Human hepatoma (HepG2) and human embryonic kidney (HK293) cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured and passaged per standard protocol.

Animal Studies

Humanized *UGT1* mice were previously generated by introducing a human BAC clone encoding the entire *UGT1* locus into mice [transgenic(*TG*)*UGT1* mice] (Chen et al., 2005) and crossing *TgUGT1* mice with *Ugt1*$^{+/-}$ mice (Nguyen et al., 2008) until *TgUGT1/Ugt1*$^{-/-}$ mice (*hUGT1* mice) were created (Fujiwara et al., 2010). A breeding strategy between *hUGT1*, *Lxrα*$^{-/-}$, *Lxrβ*$^{-/-}$ and *Lxrαβ*$^{-/-}$ mice was developed that resulted in the generation of *hUGT1/Lxrα*$^{-/-}$, *hUGT1/Lxrβ*$^{-/-}$ and *hUGT1/Lxrαβ*$^{-/-}$ mice. The *Lxr*-null mice were originally developed by the David Mangelsdorf laboratory (Repa et al., 2000) and were generously obtained from Christopher Glass at UCSD. *hUGT1/Car*$^{-/-}$ mice were developed previously (Fujiwara et al., 2012) All mouse strains were housed in a pathogen-free University of California San Diego (UCSD) Animal Care Facility and

received food and water ad libitum. All animal protocols were reviewed and approved by the UCSD Animal Care and Use Committee (IUCAC). Neonatal male and female 10-day old mice were treated orally with either propylene glycol/tween 4:1 (vehicle) or 50 mg/kg T0901317 dissolved in vehicle or GW3965 (75 mg/kg) and tissues collected 48 h after treatment.

Bilirubin Measurements

Blood was obtained from the submandibular vein and centrifuged at 16,000 x g for 2 min. Serum samples were immediately measured for total serum bilirubin (TSB) levels using a Unistat Bilirubinometer (Reichert, Inc.).

Liver Tissue Sections

Entire livers were dissected from mice, snap-frozen in liquid nitrogen, and stored at -80 °C. Frozen tissues were pulverized and the powder aliquoted for further RNA and protein extraction.

Total RNA Preparation and Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from liver tissue of treated and control mice according to the TRIzol reagent protocol as specified by the supplier (Thermo Fisher Scientific Inc., Waltham, MA). The samples were prepared for reverse transcription by using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Quantitative polymerase chain reaction (q-) PCR using newly synthesized cDNA was carried out using a CFX96 QPCR system (BioRad) by employing SsoAdvanced SYBR Green reagent (BioRad). Primers used in these studies are shown in Table 1.

Western Blot Analysis

Tissues were homogenized in RIPA lysis buffer (EMD Millipore Corporation, Temecula, CA) supplemented with protease and phosphatase inhibitor cocktail (1:100 each). Protein solutions were pooled to have a total of four samples for each genotype and condition. Western blots were performed by using NuPAGE4-12 % BisTris-polyacrylamide gels (Thermo Fisher Scientific Inc., Waltham, MA) with the protocols described by the manufacturer. Protein (30 µg) was electrophoresed at 170 V for 50 min and transferred at 20 V for 60 min to PVDF membranes (EMD Millipore Corporation, Temecula, CA). Membranes were blocked with 5 % skim milk at room temperature for one hour and incubated with primary antibodies [rabbit anti-human UGT1A1 (ab170858), mouse anti-GAPDH (sc-32233)] at 4°C overnight. Membranes were washed and exposed to HRP-conjugated secondary antibodies (anti-mouse IgG or anti-rabbit IgG) for 1 hour at room temperature. Protein was detected by Clarity Western ECL Substrate system (BioRad) and was visualized by the BioRad gel documentation system. All Western blots are cropped from the full-length blots that have been included in the Supplemental Material.

Glucuronidation Assays

Microsomal proteins were purified from tissues (50 mg), and 100 µg of liver homogenate was incubated in a previously reported glucuronidation assay buffer (Verreault et al., 2006) in the presence of bilirubin (20 µM), SN-38 (50 µM) for 30 min or imipramine (500 µM) for 2 h at 37 °C. The formation of bilirubin monoglucuronide, SN-38 glucuronide, and imipramine glucuronide was quantified by LC/ESI-MS/MS as previously reported (Lu et al., 2017). The formation of glucuronide conjugates is expressed as area under the curve [AUC] or peak area ratio.

Plasmid cloning and transient transfection assays

The various TK-pGL3 plasmids were obtained by cloning 3 copies of the corresponding dimerized oligonucleotides (Table 2) in the thymidine kinase promoter-driven luciferase reporter (TK-pGL3) vector. For transfection experiments, HepG2 cells (75 x 10^3/well) were seeded into 24-well plates and transfected with 100 ng of luciferase reporter, 30 ng of the pRL-NULL expression vector, and 10 ng of expression plasmid. All samples were complemented with a pBS-SK+ plasmid to ensure 500 ng DNA/well. Cells were transfected with the ExGen 500 reagent for 6 h at 37° C, and subsequently incubated for 24 h with either ethanol or LXR ligands at the indicated concentrations. Luciferase and renilla activities were determined as reported (Lu et al., 2017).

Electrophoretic Mobility Shift Assays (EMSA)

EMSA using *in vitro* produced LXRα and RXRα were performed as described (Johansson et al., 2003) using the radiolabeled probes (100,000 cpm) as indicated in Supplemental data-Figure 1. Oligonucleotides used in these studies are shown in Table 2.

Chromatin Immunoprecipitation (ChIP) assays

ChIP assays were performed according to the method of Forsberg et al. (Forsberg et al., 2000) in a previously modified manner (Verreault et al., 2006). Briefly, 20 x 10^6 HepG2 cells were treated with either vehicle or T0901317 (1 µM) for 4 hours. Protein-DNA cross-linking, nuclear extract preparation and sonication were performed as reported (Forsberg et al., 2000). A volume of lysate equivalent to 40 x 10^5 cells was immunoprecipitated using 4 µg of anti-LXR (Santa-Cruz, sc1202), anti-PXR (sc9690) antibodies, or with 4 µg of an anti-UGT2B (sc23479) antibody as a negative control. A

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separate volume of lysate was kept without immunoprecipitation for subsequent purification of input genomic DNA or was incubated in the presence of protein A-sepharose alone. 1/10 of the immunoprecipitated DNA was PCR amplified as described in Supplemental Figure 1.

Statistical Analysis

Statistically significant differences between vehicle- and T0901317-treated animals were evaluated using unpaired *t* test (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$) and between different genotypes we performed Tukey's multiple comparison test ($^{\#}$: $p<0.05$; $^{\#\#}$: $p<0.01$; $^{\#\#\#}$: $p<0.001$; $^{\#\#\#\#}$: $p<0.0001$).

Results

Induction of the UGT1A1 gene in neonatal hUGT1 mice

The LXR agonist T090137 has an affinity for both LXRα and LXRβ (Schultz et al., 2000) with a binding affinity that displayed an EC_{50} of approximately 20 nM. It is also capable of activating the pregnane X receptor (PXR) (Shenoy et al., 2004) and farnesoid X receptor (FXR) (Houck et al., 2004), but at a higher concentration than for LXR (EC_{50}=4-7 μM). There are five human UGT1 isoforms expressed in the liver (Strassburg et al., 1997), UGT1A1, UGT1A3, UGT1A4, UGT1A6, and UGT1A9, with UGT1A1 controlling the metabolism and clearance of serum bilirubin. To investigate the potential of LXR to induce the *UGT1A1* gene, 10-day old neonatal *hUGT1* mice were treated once with vehicle (propylene glycol/tween 80, 40:1) or with the LXR agonist T0901317 (50 mg/kg) by oral gavage. After 48 hours, serum was collected for total serum bilirubin (TSB) analysis and the livers collected for RNA extraction. Neonatal *hUGT1* mice develop severe levels of serum bilirubin. T0901317 treatment for 48 hours reduced TSB levels to normal adult levels (**Fig. 1A**). This drop in TSB levels resulted from a significant induction of liver *UGT1A1* gene expression (300-fold) as quantitated by q-PCR (**Fig. 1B**). Also, analysis of RNA expression demonstrated there was substantial induction of liver *UGT1A3* (200-fold) and *UGT1A4* (400-fold) with *UGT1A6* and *UGT1A9* genes being unaffected (**Fig. 1C**). We have shown previously that T0901317 treatment to transgenic *UGT1* mice induced the *UGT1A3* gene in liver and intestinal tissue, along with induction of UGT1A3 directed glucuronidation activity toward CDCA and LCA (Verreault et al., 2006).

The LXR receptors display gene specificity

To examine the specificity of the LXR receptors towards induction of liver UGT1A1, *hUGT1*, *hUGT1/Lxrα*$^{-/-}$, *hUGT1/Lxrβ*$^{-/-}$ and *hUGT1/Lxrα*$^{-/-}$*/Lxrβ*$^{-/-}$ (*hUGT1/Lxrαβ*$^{-/-}$) mice

were developed. To confirm the knockout conditions in these mice, q-PCR analysis was performed to verify the elimination of LXRα mRNA expression in *hUGT1/Lxrα*$^{-/-}$ and *hUGT1/Lxrαβ*$^{-/-}$ mice and the elimination of LXRβ mRNA expression in *hUGT1/Lxrβ*$^{-/-}$ and *hUGT1/Lxrαβ*$^{-/-}$ mice (**Fig. 2 A&B**). Interestingly, there was a consistent reduction in *Lxrα* gene expression in *Lxrβ*$^{-/-}$ mice treated with TO901317, yet the values were not statistically significant. Knockout of the *Lxr* genes leads to a loss of function, as demonstrated in gene expression patterns of the LXRα target genes Stearoyl CoA desaturase-1 (*Scd1*) and *Scd2* (Chu et al.; Caputo et al.; Zhang et al.). These proteins catalyze the conversion of saturated fatty acids into monounsaturated fatty acids and have previously been described to be activated by LXR agonists in either an SREBP-1c dependent or independent manner. The oral treatment of neonatal *hUGT1* and *hUGT1/Lxrβ*$^{-/-}$ mice with T0901317 resulted in transcriptional activation of *Scd1* and *Scd2* genes (**Fig. 2C&D**). Mice lacking the *Lxrα* gene did not show increases in SCD1 or SCD2 RNA (**Fig. 2C&D**).

Induction of UGT1A1 by LXR

Using these same tissues, the induction profile of the *UGT1A1* gene was like that of *Scd1*, with greater induction in *hUGT1* and *hUGT1/Lxrβ*$^{-/-}$ mice and significantly reduced induction in *hUGT1/Lxrα*$^{-/-}$ and *hUGT1/Lxrαβ*$^{-/-}$ mice (**Fig. 3B**). However, the induction of the *UGT1A1* gene in *hUGT1/Lxrα* and *hUGT1/Lxrαβ*$^{-/-}$ was still considerable with approximately 30% of those values detected in the liver from *hUGT1* mice. Surprisingly, the TSB values in the *hUGT1* mice and the other LXR variants were all greatly reduced when compared to vehicle-treated mice (**Fig. 3A**). The phenotype changes in serum TSB values in *hUGT1*, *hUGT1/Lxrα*$^{-/-}$, *hUGT1/Lxrβ*$^{-/-}$ and *hUGT1/Lxrαβ*$^{-/-}$ mice suggests that UGT1A1 was induced in each of these strains following T0901317 treatment. This was confirmed by Western blot analysis showing

that induction of liver UGT1A1 occurs in all strains treated, with liver abundance reflecting that of gene expression (**Fig. 3C**). Also, glucuronidation analysis using liver extracts has confirmed that bilirubin (Fig. 3D) and SN-38 (**Fig. 3E**), substrates that are conjugated by UGT1A1, show a functional induction pattern like *UGT1A1* gene expression. Since the induction levels of UGT1A1 in *hUGT1/Lxrαβ*$^{-/-}$ mice, which do not express functional LXR, are like those observed in *hUGT1/Lxrβ*$^{-/-}$ mice, this finding indicates that LXRβ plays a negligible role in the induction of UGT1A1 in neonatal mice.

T0901317 and LXR elicit cross-talk with other nuclear receptors

The dramatic induction of UGT1A1 by T0901317 in all LXR mouse lines led us to examine in greater detail this unusual property of T0901317. In the liver, the *Cyp2b10*, *Cyp3a11*, and *Cyp4a10* genes are significantly induced in *hUGT1* mice (**Fig. 4A-C**) by T0901317. It is interesting to note that in the absence of LXRα, T0901317 elicits a highly synergistic *Cyp3a11* induction response. This observation indicates that activation of PXR by T0901317, which can induce *Cyp3a11*, maybe repressed by activated LXRα. In contrast, induction of *Cyp4a10*, which can be induced by PPARα, is not induced above control values in LXRα deficient mice, indicating that induction of *Cyp4a10* by PPARα requires functional LXRα. In both of these examples, activation of either PXR or PPARα by T0901317 is interconnected with LXRα and not LXRβ. Different from these examples, however, is the induction pattern by CAR activation of the *Cyp2b10* gene in *hUGT1*, *hUGT1/Lxrα*$^{-/-}$, *hUGT1/Lxrβ*$^{-/-}$, *hUGT1/Lxrαβ*$^{-/-}$ and *hUGT1/Car*$^{-/-}$ mice (**Fig. 4C**). Western blot analysis of liver extracts from these treated mice demonstrates that the induction of CYP2B10 by T0901317 is CAR dependent since induction is significantly reduced in *hUGT1/Car*$^{-/-}$ mice (**Fig 4D**). Thus, the human *UGT1A1* gene can be induced by T0901317 through activation of PXR, PPARα, and CAR, in addition to the more dominant action of LXRα.

Induction of liver UGT1A1 by GW3965

In addition to T0901317, the synthetic LXR agonist GW3965 has been used as an alternative agent to examine LXR activated target genes. We treated 10-day old neonatal *hUGT1*, *hUGT1/Lxrβ*$^{-/-}$ and *hUGT1/Lxrα*$^{-/-}$ mice with GW3965 (75 mg/kg) and examined TSB values and *UGT1A1* gene expression patterns after two days of exposure (**Fig 5A**). TSB values were dramatically reduced in *hUGT1* and *hUGT1/Lxrβ*$^{-/-}$ mice, but the values did not change in *hUGT1/Lxrα*$^{-/-}$ mice, confirming that induction of UGT1A1 follows activation of LXRα and not LXRβThis was confirmed by two additional sets of data. First, the induction of liver *UGT1A1* gene expression did not occur in *hUGT1/Lxrα*$^{-/-}$ mice (**Fig. 5B**), and second, Western blot analysis shows induction of UGT1A1 in *hUGT1* and *hUGT1/Lxrβ*$^{-/-}$ mice only (**Fig 5C**). Based upon current knowledge of the specificity of T0901317 and GW3695 to activate both LXRα and LXRβ, the induction of liver UGT1A1 is driven selectively by activated LXRα. It can be noted that the TSB levels following oral GW3965 treatment are not as low as noted for T0901317 treatment (Fig. 1). It is possible that T0901317, which is able to activate not only LXR but also PXR and CAR, is also inducing UGT1A1 in intestinal tissue, a site that contributes towards bilirubin clearance (Chen et al., 2017).

Identification of a functional LXR response elements (LXREs) within the UGT1A1 gene promoter.

A computer-assisted analysis (Quandt et al., 1995) of the *UGT1A1* promoter gene sequence revealed the presence of 5 degenerated LXRE sequences (Willy et al., 1995) localized at positions -9903, -9855, -7632, -4076 and -888 bp of the promoter (**Supplemental Fig. 1**). Occupancy of these response elements by LXRα in living cells was analyzed through a ChIP assay performed on DNA from vehicle- or T0901317-treated HepG2 cells (**Fig 6**). Only the DNA sequences encompassing the -9855bp

LXRE and an LXRE previously identified within the human UGT1A3 promoter (positive control) (Verreault et al., 2006) were precipitated by the anti-LXRα antibody in T0901317-treated cells (**Fig. 6**, panels a & c, Lane 8), but not in untreated cells (Lane 3). None of the other LXRE-like sequences found in the *UGT1A1* gene promoter were PCR amplified in any of the precipitated DNA preparations, suggesting that they are not occupied by LXRα/β. Because T0901317 was also reported as an activator of PXR (Mitro et al., 2007), DNA was also immunoprecipitated with an anti-PXR antibody (Lanes 4 & 9). No amplifications were observed.

Transient transfection with a -9855 bp LXRE-TK-pGL3 construct revealed that co-expression of LXRα and RXRα increased activity of the LXRE in human hepatoma HepG2 cells, an effect that was dose-dependently enhanced in the presence of T0901317 and GW3965 (0.1 to 10 μM) (**Fig. 6B**). In EMSA, a clear binding was observed when a radiolabeled probe encompassing the -9855bp LXRE sequence was incubated in the presence of both LXRα and RXRα (**Fig. 6C**, Lane 4). Introducing mutated bases within the 3'-half-site of the LXRE (**Table 1**) completely abolished the formation of this binding (**Fig. 6**, lane 5). In competition experiments, LXRα/RXRα binding to the WT probe was efficiently competed by itself and the consensus LXRE (**Fig 6**, Lanes 6-8 & 12-14, respectively). In contrast, the mutated response elements failed to significantly compete for LXRα binding (lanes 9-11). Overall, these data identify the LXRE motif at position -9855 bp in the human *UGT1A1* gene promoter as a functionally active LXR response element.

The role of LXRα and expression of UGT1A1 in adult hUGT1 liver

There is a limited expression of human UGT1A1 in the liver tissue of neonatal *hUGT1* mice but in adult *hUGT1* mice there is adequate expression. When we examined the constitutive expression pattern of UGT1A1 by qPCR and Western blot analysis, there was an excellent

agreement with gene expression and protein detection (**Fig. 7**). However, it is clear from this analysis that LXRα plays a key role in basal or constitutive expression of liver UGT1A1, with greatly reduced expression in *hUGT1/Lxrα*$^{-/-}$ and *hUGT1/Lxrαβ*$^{-/-}$ mice. When the *Lxrα* allele is either null or exists in the heterozygous state (*hUGT1/Lxrα*$^{+/-}$), the expression of liver UGT1A1 is greatly reduced. This indicates that both *Lxrα* alleles are necessary to maintain the full expression of UGT1A1.

Discussion

Liver X receptors α and β have been shown to play pivotal roles in the transcriptional control of lipid metabolism (Edwards et al., 2002; Wagner et al.; Wang and Tontonoz). Activated LXRs regulate the expression of genes that are linked to functional control of cholesterol absorption, transport, efflux, excretion and conversion to bile acids (Peet et al., 1998a; Zhang et al., 2012). Under these conditions, the LXRs bind directly to these genes at LXR enhancer sequences to induce transcription (Sabol et al., 2005). Also, LXRs regulate fatty acid metabolism by controlling the lipogenic transcriptional factor sterol regulatory element-binding protein 1c (Repa et al., 2000; Wagner et al., 2003) that in turn regulates genes that encode proteins involved in fatty acid elongation and desaturation (Schultz et al., 2000). Along with these important regulatory events, LXRs also drive the incorporation of polyunsaturated fatty acids into phospholipids. Each of these regulatory events leads to the proper homeostasis of the membrane environment and lipid composition, which if disrupted can impact diseases such as atherosclerosis, diabetes, fatty liver disease, and cancer.

While there is approximately 78% amino acid similarity between LXRα and LXRβ, the genes encoding these proteins in mice exist on different chromosomes, with *Lxrα* residing on chromosome 2 and *Lxrβ* on chromosome 7. The evolutionary split of these genes indicates that their expression may differ in selective tissues coupled with changes in receptor function. The LXRs are activated by naturally occurring oxysterols and by synthetic nonsteroidal compounds such as T0901317 and GW3965. However, LXRα and LXRβ are expressed differently with regards to abundance and tissue specificity. Annicotte et al. (Annicotte et al., 2004) employed selectively in situ hybridization in embryonic and adult tissue to demonstrate that LXRα is highly expressed in metabolically active tissues such as the liver, intestine, and adipose tissue while LXRβ is ubiquitously expressed. While there is a commonality in the ability

of ligands to activate the LXRs, there are several key examples that the LXRs elicit unique functional roles. For example, mice lacking LXRα accumulate massive amounts of cholesterol in the liver when fed a high-cholesterol diet (Peet et al., 1998b). This results from the inability of cytochrome P450 7A1, a key LXRα target gene, to metabolize cholesterol to bile acids. This does not occur when cholesterol is fed to LXRβ-deficient mice. Numerous other examples have been reported favoring a selective advantage in controlling physiological functions or gene regulation for one of the receptors over the other (Alberti et al.; Jakobsson et al.; Zhang et al.; Whitfield et al., 2016; Endo-Umeda et al.).

In the absence of ligand, the LXR/RXR complexes bind to transcriptional co-repressors, such as the nuclear co-repressor 1 (NCoR1), and repress target gene expression (Chen and Evans, 1995; Horlein et al., 1995; Li et al., 2013). Upon ligand binding, LXRs dissociate from NCoR1 and assemble with coactivators, leading to modulation of histone acetylation or chromatin remodeling, facilitating transcriptional activation. When NCoR1 is selectively deleted in target tissues, LXR becomes activated in the absence of ligand and stimulates transcription of target genes (Li et al., 2013). In neonatal *hUGT1* mice that are deficient in intestinal NCoR1, the *UGT1A1* gene was derepressed leading to the metabolism of serum bilirubin (Chen et al., 2017). Gene expression data indicated that LXR target genes were in part activated in *hUGT1/Ncor1*$^{-/-}$ mice, leading us to examine in greater detail the role of LXRα and LXRβ in the potential induction of the *UGT1A1* gene. Using neonatal *hUGT1* mice deficient in LXRα, LXRβ or both LXRα and LXRβ that were orally treated with synthetic LXR agonists T0901317 or GW3695, induction of hepatic UGT1A1 was dominated by LXRα. However, the induction patterns of UGT1A1 and TSB values in these mice were quite different when we compared the impact of the two agents. The oral administration of GW3695 led to hepatic induction of UGT1A1 in *hUGT1* and *hUGT1/Lxrβ*$^{-/-}$ mice only,

which matched the reduction in TSB values. The absence of UGT1A1 induction in *hUGT1/Lxrα*$^{-/-}$ and *hUGT1/Lxrαβ*$^{-/-}$ mice establishes that activated LXRα underlies the induction process.

The most well-characterized synthetic LXR ligand to date is T0901317, but this agent elicited a different pattern of induction. While the general pattern of UGT1A1 induction in these mice by T0901317 was like that of GW3695, a reduction in TSB was observed in all treatment groups. Also, there was considerable *UGT1A1* gene and protein expression detected in *hUGT1/Lxrα*$^{-/-}$ and *hUGT1/Lxrαβ*$^{-/-}$ mice, which showed little induction when these mice were treated with GW3695. We now believe that the induction of hepatic *UGT1A1* in *hUGT1/Lxrα*$^{-/-}$ and *hUGT1/Lxrαβ*$^{-/-}$ mice by T0901317 results from activation of additional nuclear receptors, such as PXR and CAR, and not LXR. It has been reported previously that T0901317 can activate PXR and effectively induce PXR target genes such as *Cyp3a11* in mice (Shenoy et al., 2004). PXR activation in neonatal *hUGT1* mice leads to the induction of UGT1A1 and a reduction in TSB levels (Chen et al., 2012; Fujiwara et al., 2012), and in this study dramatically induces liver CYP3A11 RNA in all four mouse lines. It is interesting to note that T0901317 induced *Cyp3a11* gene induction in *hUGT1/LXRα*$^{-/-}$ and *hUGT1/LXRαβ*$^{-/-}$ mice 2-3-fold greater than in *hUGT1* and *hUGT1/Lxrβ*$^{-/-}$ mice. This superinduction of the *Cyp3a11* gene by T0901317 in *hUGT1/Lxrα*$^{-/-}$ and *hUGT1/Lxrαβ*$^{-/-}$ mice indicates that LXRα expression serves to inhibit full activation of the PXR receptor or has inhibitory action associated with the *Cyp3a11* gene. Also, T0901317 induced hepatic CYP2B10 in a fashion that was independent of the expression of LXRα and LXRβ. The induction of CYP2B10 was driven by activated CAR, since no induction of UGT1A1 occurred in *hUGT1/Car*$^{-/-}$ mice. It has been suggested that there is functional cross-talk between LXRα and CAR with activation of CAR leading to the inhibition of LXR target genes(Zhai et al., 2010). If this mechanism is in play following T0901317

exposure which activates both LXRα and CAR, induction of LXRα target genes may be attenuated as a result of the inhibitory actions of CAR. The use of T0901317 as an LXR agonist to examine the physiological impacts and gene expression patterns following LXR activation can be complicated knowing that it serves as an agonist for PXR, FXR (Houck et al., 2004) and CAR while inducing cross-talk between LXRα, CAR and PXR.

With the treatment of *hUGT1* neonatal mice with T0901317 or GW3965 we present convincing evidence the LXRα is directly activating the *UGT1A1* gene in the liver, an event that leads to the metabolism and clearance of serum bilirubin. This analysis is based upon the induction patterns of UGT1A1 in *hUGT1* mice that are deficient either LXRα or LXRβ in hepatic tissue. However, it does not account for the relative abundance of LXRα and LXRβ in this tissue or the relative binding affinities of the ligands to the receptors. Few examples document the abundance of these receptors in different tissues. Using LXRα and LXRβ cDNAs to generate antisense RNA for in situ hybridization experiments, Annicotte (Annicotte et al., 2004) examined the developmental expression of the receptors in postcoitum at different days as well as in adult tissue. The conclusions from this experiment indicated that LXRα and LXRβ were both expressed in liver tissue but the abundance of LXRβ RNA dropped as the pregnancy came closer to term, with LXRβ RNA being reduced in adult liver. However, LXRβ is expressed in liver tissue. Since GW3695 has a greater binding affinity towards LXRβ, we can be confident that both LXRα and LXRβ are being activated in liver tissue following oral administration. With the identification of the LXRE binding sequence flanking the human *UGT1A1* gene and the complete absence of UGT1A1 induction by GW3695 in *hUGT1/Lxrα*$^{-/-}$ mice, our findings strongly implicate LXRα as a regulator of liver UGT1A1 gene expression.

The initial discovery that LXRs could be activated by oxysterols was the first of many clues suggesting that LXRs controlled cholesterol homeostasis, eventually implicating the production of bile acids and controlling lipogenesis (Wang and Tontonoz, 2018). Our findings have confirmed that LXRα is a potent regulator of the *UGT1A1* gene during development and could serve as a therapeutic target in events that require the regulation of accumulating serum bilirubin. It remains unclear the physiological advantage of regulating the *UGT1A1* gene along with other genes of the *UGT1* locus by LXRα since there is no perceived link between oxysterols and the function of UGT1A1. One might consider, however, that oxysterols play an important role in maintaining adequate LXR driven constitutive expression of the *UGT1A1* gene, which is essential in facilitating metabolism and elimination of the major heme metabolic product, bilirubin. Regardless, these findings suggest that events linking abnormal cholesterol and lipid homeostasis to the onset of lipogenic diseases will impact on the control and regulation of UGT1A1 and the functional properties of this important protein in endogenous and exogenous drug metabolism.

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Author Contributions

Participated in research design: All Authors

Conducted experiments: Hansmann, Yoda, Menillo, Verreault, Chen

Performed data analysis: Hansmann, Menillo, Yoda, Tukey

Wrote or contributed to the writing of the manuscript: Hansmann, Barbier, Tukey

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Footnotes

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Figure Legends

Figure 1. Induction of UGT1A mRNA expression by T0901317 in neonatal *hUGT1*. Ten-day old neonatal *hUGT1* mice were treated with 50 mg/kg T0901317 or vehicle (propylene glycol/tween 80 4:1) by oral gavage. After 48 hours, blood was collected for TSB analysis (**A**) and the mice sacrificed to collect the livers. Tissues were pulverized under liquid nitrogen and used for the preparation of total RNA using the TRIzol protocol. Gene expression of *UGT1A1* (**B**), *UGT1A3*, *UGT1A4*, *UGT1A6* and *UGT1A9* (**C**) transcript levels were determined by q PCR and expressed relative to *m*CPH. Values are the means ±SEM (n>4). Statistically significant differences are outlined in Material and Methods.

Figure 2. Gene expression of *Lxrα* and *Lxrβ* and LXR downstream target genes *Scd1* and *Scd2* in LXR deficient mice. Following the treatment of neonatal *hUGT1*, *hUGT1/Lxrα*$^{-/-}$ (*Lxrα*$^{-/-}$), *hUGT1/Lxrβ*$^{-/-}$ (*Lxrβ*$^{-/-}$), and *hUGT1/Lxrαβ*$^{-/-}$ (*Lxrαβ*$^{-/-}$) mice with 50 mg/kg T0901317 for 48 hours (see Fig.1), *Lxrα* (**A**) and *Lxrβ* (**B**) *Scd1* (**C**) and *Scd2* (**D**) gene expression was determined by real time PCR and expressed relative to mouse *Cph* gene expression. Values are the means ±SEM (n>4). Statistically significant differences are outlined in Materials and Methods.

Figure 3. The loss of LXRα and/or LXRβ impacts T0901317-dependent modulation of UGT1A1 expression and activity.

Ten-day old neonatal *hUGT1*, *hUGT1/Lxrα*$^{-/-}$ (*Lxrα*$^{-/-}$), *hUGT1/Lxrβ*$^{-/-}$ (*Lxrβ*$^{-/-}$) and *hUGT1/Lxrαβ*$^{-/-}$ (*Lxrαβ*$^{-/-}$) mice were treated with 50 mg/kg T0901317 or vehicle (propylene glycol/ Tween 80 4:1) by oral gavage. After 48 hours, serum was collected, and the mice were sacrificed to collect liver tissue. The liver was pulverized under liquid nitrogen and used for the preparation of total RNA, whole-cell extract and glucuronidation assays. (**A**) Before and forty-eight hours after the treatment TSB levels

were taken. (**B**) *UGT1A1* gene expression was determined by real time PCR and expressed relative to *Cph* gene expression. (**C**) Whole liver tissue extracts were used to perform Western blot analysis to examine UGT1A1 expression. The bands have been cropped from the full-length blots but not enhanced in any way. Densitometric values of UGT1A1 protein bands obtained using the GAPDH signal as normalizing control are shown. (**D**) 100 µg of liver homogenate was incubated in the presence of bilirubin (20 µM) or (**E**) SN-38 (50 µM) for 30 min at 37 °C. The formation of bilirubin monoglucuronide and SN-38 glucuronide was quantified by LC/ESI-MS/MS. The formation of glucuronide conjugates is expressed as area under the curve [AUC] or peak area ratio. Values are the means ±SEM (n>4). Statistically significant differences were performed as outlined in Materials and Methods.

Figure 4. The impact of the LXR agonist T0901317 and activation of additional nuclear receptors.

Ten-day old neonatal *hUGT1*, *hUGT1/Lxrα*$^{-/-}$ (*Lxrα*$^{-/-}$), *hUGT1/Lxrβ*$^{-/-}$ (*Lxrβ*$^{-/-}$) and *hUGT1/Lxrαβ*$^{-/-}$ (*Lxrαβ*$^{-/-}$) mice were treated with 50 mg/kg T0901317 or vehicle (propylene glycol/ Tween 80 4:1) by oral gavage. RNA was isolated from liver tissue after 48 hours and gene expression was evaluated by real-time PCR for *Cyp3a11* (**A**), *Cyp4a10* (**B**) and *Cyp2b10* (**C**). Samples of liver tissue were used for analysis of CYP2B10 expression by Western blot analysis (**D**). Statistically significant differences were performed as outlined in Materials and Methods.

Figure 5. Induction of UGT1A1 by the LXR agonist GW3965.

Ten-day old neonatal *hUGT1*, *hUGT1/Lxrα*$^{-/-}$ (*Lxrα*$^{-/-}$), *hUGT1/Lxrβ*$^{-/-}$ (*Lxrβ*$^{-/-}$) and *hUGT1/Lxrαβ*$^{-/-}$ (*Lxrαβ*$^{-/-}$) mice were treated with 75 mg/kg T0901317 or vehicle (propylene glycol/ Tween 80 4:1) by oral gavage. After 48 hours, serum was collected

along with liver tissue. TSB values (**A**) were determined along with UGT1A1 gene expression (**B**). Liver tissue was prepared as outlined and used in Western Blot analysis to examine UGT1A1 expression (**C**).

Figure 6. Ligand-activated LXRα activates a positive LXRE at position -9855 in the UGT1A1 promoter.

(**A**) Soluble chromatin was prepared from HepG2 cells treated with vehicle (ethanol-lanes 1-5), T0901317 (1 µM-lanes 6-10) for 4 hours and immunoprecipitated with antibodies directed against LXRα (lanes 3&8), PXR (lanes 4&9) or with an anti-UGT2B antibody as a negative control (lanes 5&10). The final DNA extracts were amplified using pairs of primers covering the 5 degenerated DR4 motifs identified within the *UGT1A1* gene promoter (b-f) or a previously reported LXRE from the human *UGT1A3* gene promoter (Verreault et al., 2006) as a positive control (a). 1/50 (input) or 1/10 (precipitated DNA) of PCR products were separated on an ethidium-bromide-stained 2 % agarose gel.

(**B**) HepG2 cells were transfected with the indicated LXRE-driven luciferase (Luc) reporter plasmids (TK-pGL3 constructs: 100 ng) in the absence or presence of LXRα and RXRα plasmids (10 ng) and a pRL-NULL expression plasmid (30 ng). Cells were treated for 24 h with vehicle (ethanol) or increasing concentrations of T0901317 or GW3695 (0.1; 0.5; 1; 2.5; 5 and 10 µM) as indicated. Values are expressed as fold induction over the control (TKpGL3, set at 1), normalized to internal renilla activity. (C) EMSA were performed with the end-labeled -9855 bp LXRE wild type (wt) or mutated (mt) probes, in the presence of *in vitro* produced RXRα, LXRα or both, as indicated. Competition of the wild type probe was performed by adding 5-, 25-, or 50-fold molar excess of the indicated cold competitor probe in EMSA with RXRα and LXRα. n.s.: nonspecific binding.

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Figure 7. Contribution of LXR towards the expression of UGT1A1 in adult liver.

Adult livers from untreated *hUGT1*, *hUGT1/Lxrα*$^{-/-}$ (*Lxrα*$^{-/-}$), *hUGT1/Lxrα*$^{+/-}$ (*Lxrα*$^{+/-}$), *hUGT1/Lxrβ*$^{-/-}$ (*Lxrβ*$^{-/-}$) and *hUGT1/Lxrβ*$^{+/-}$ (*Lxr*$^{+/-}$) mice were collected to examine *UGT1A1* gene expression by qPCR (**A**) and protein expression by Western blot analysis (**B**).

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Table 1. *Sequence of primers used in qPCR analysis*.

Genes		Oligonucleotide sequence (5'-3')
Human UGT1A1	Forward	CCATCATGCCCAATATGGTT
	Reverse	CCACAATTCCATGTTCTCCA
Human UGT1A3	Forward	AGGTGACTGTCCAGGACCTA
	Reverse	CAAATTCCTGGGATAGTGGATTTT
Human UGT1A4	Forward	CAACGGGAAGCCACTATCTC
	Reverse	TGAGACCATTGATCCCAAAGA
Human UGT1A6	Forward	ACCGGGGTCATGAGATTGTA
	Reverse	TGGTCATACGGCACTGGATA
Human UGT1A9	Forward	GAACATTTATTATGCCACCG
	Reverse	CAACAACCAAATTGATGTGTG
Scd-1	Forward	GCTCTACACCTGCCTCTTCG
	Reverse	CAGCCGAGCCTTGTAAGTTC
Scd-2	Forward	TCCTGCAAGCTCTACACCTG
	Reverse	TGCCTTGTATGTTCTGTGGC
LXRα	Forward	TACAACCGGGAAGACTTTGC
	Reverse	CAGAGAAGATGCTGATGGCA
LXRβ	Forward	CTTGGTGGTGTCTTCTTGA
	Reverse	TGTGGTAGGCTGAGGTGTA
Cyp2b10	Forward	AAAGTCCCGTGGCAACTTCC
	Reverse	CATCCCAAAGTCTCTCATGG
Cyp3a11	Forward	TTCTGTCTTCACAAACCGGC
	Reverse	GGGGGACAGCAAAGCTCTAT
Cyp4a10	Forward	GATGGACGCTCTTTACCCAA
	Reverse	AAGGGTCAAACACCTCTGGA
Cph	Forward	ATGGTCAACCCCACCGTGT
	Reverse	TTCTTGCTGTCTTTGGAACTTTGTC

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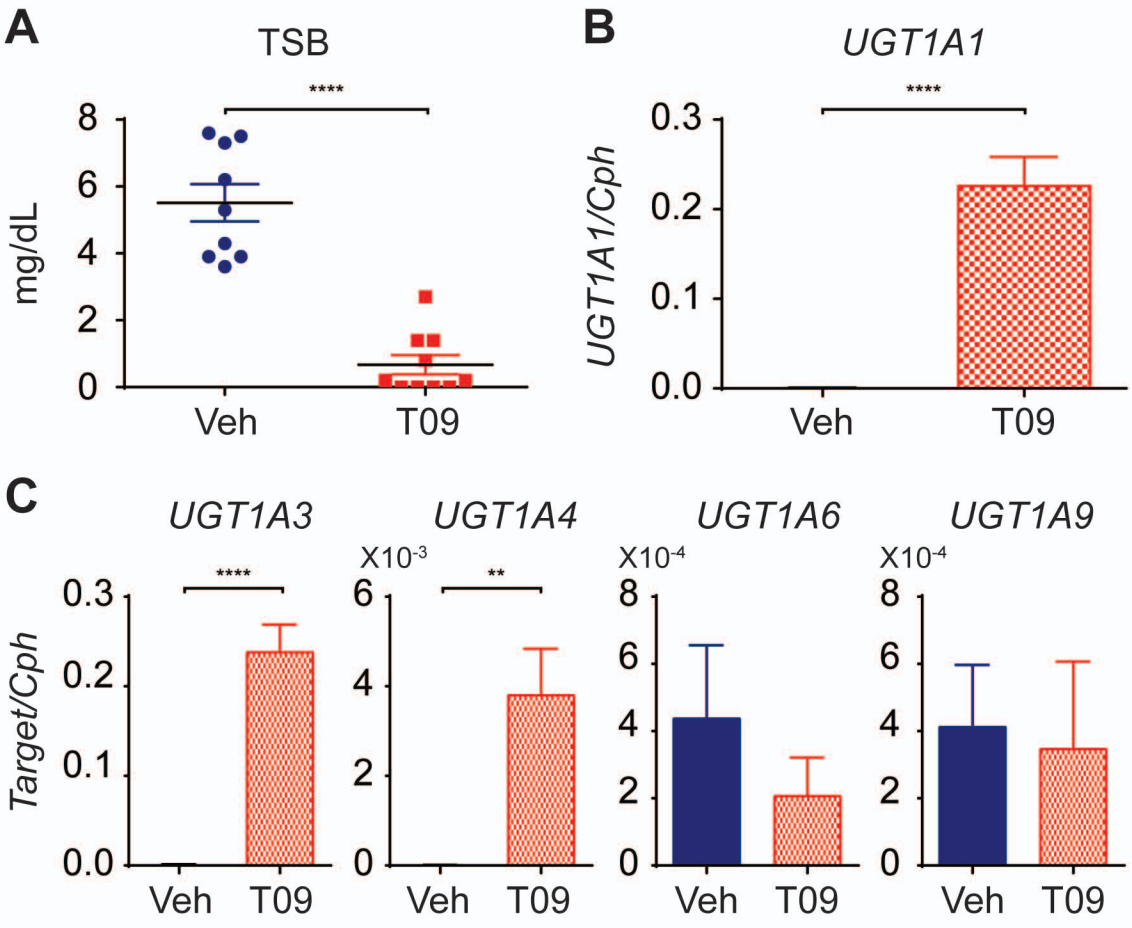
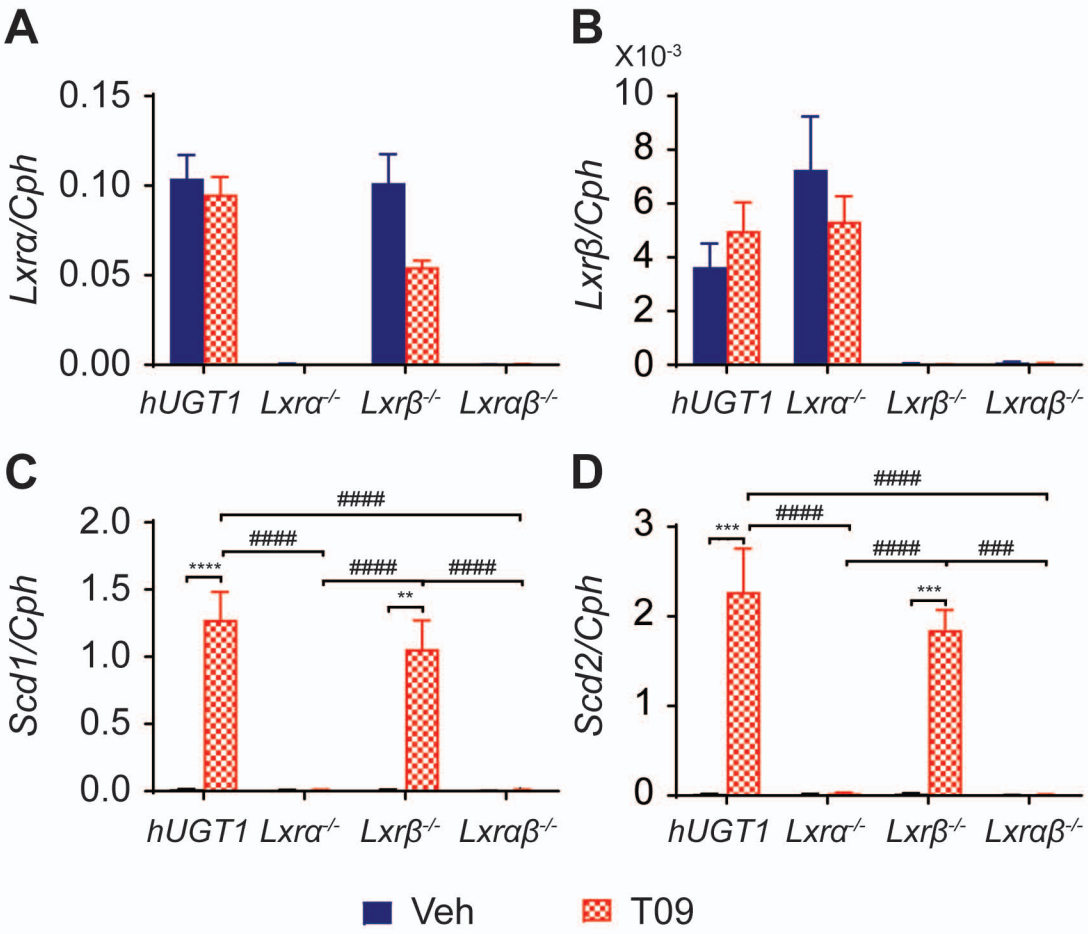
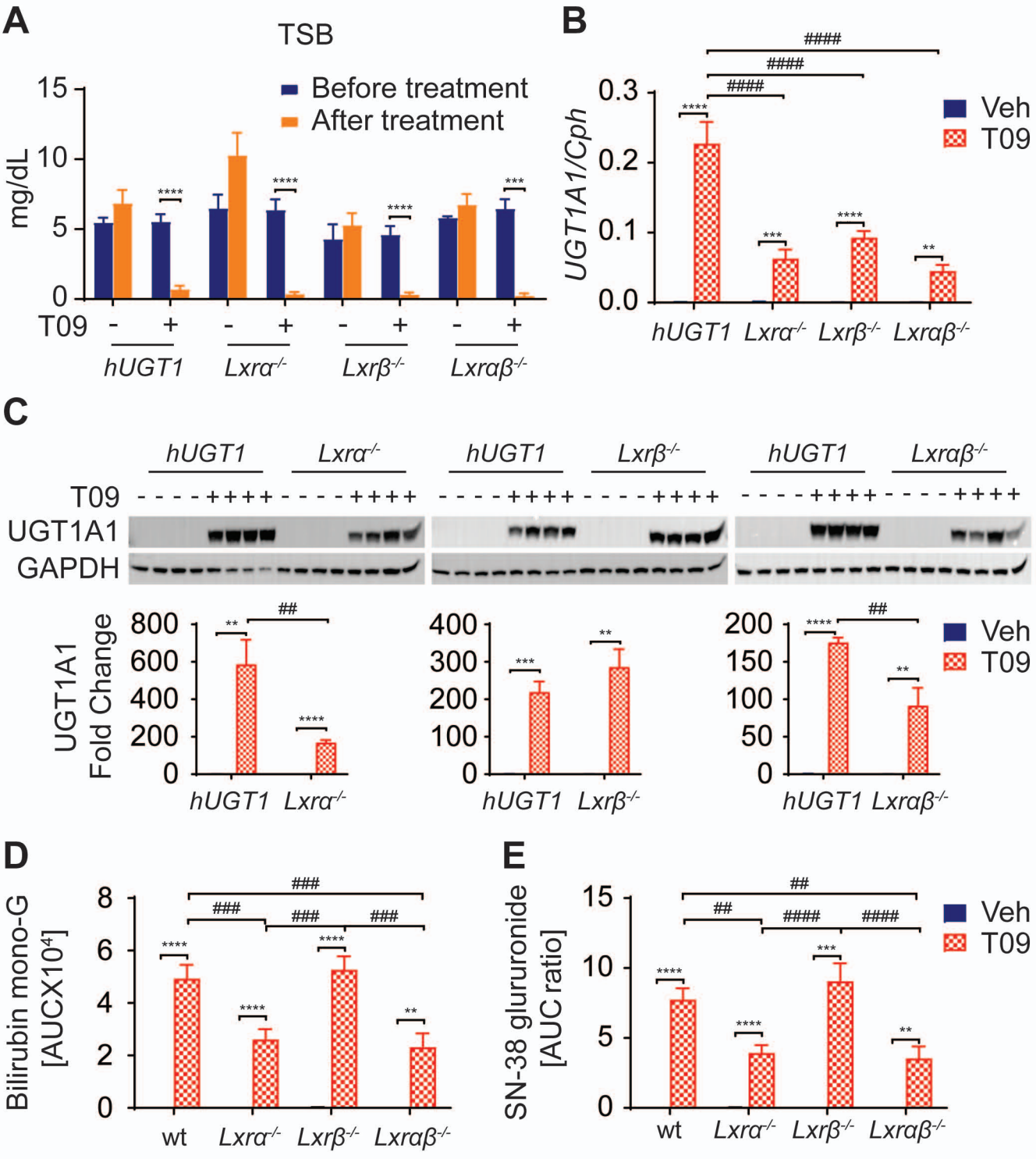
Table 2. *Sequence of oligonucleotides used in LXR binding element analysis*.

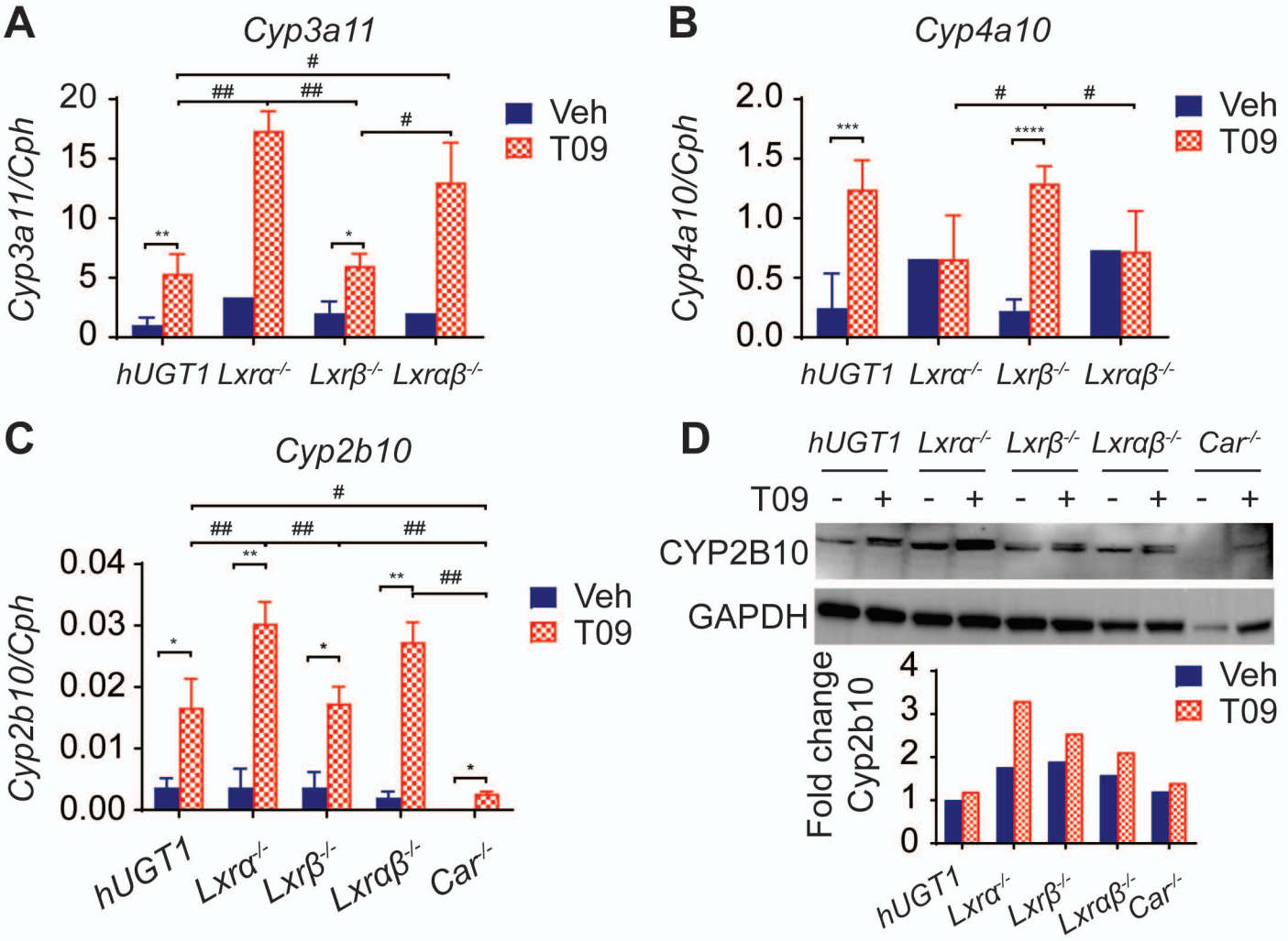
EMSA	**Sequence (5'-3')**	
DR4 -9903wt	ATAGAGAGG**TGACCA**CAGG**AGACCT**AAGCACT	
DR4 -9855wt	TAAAG**AGGTTG**ACTC**AGTTCA**GTGGAAG	
DR4 -7632wt	GAGGAGGAA**GGATCA**CTTG**AGCCCA**GAAATTCAT	
DR4 -4076wt	TCTGAAG**GGATTA**GTTT**AGGACA**ACCCTC	
DR4 -888wt	TAACCGTG**TGACCT**GTAG**TAAGCA**AAGGGCC	
DR4 -9855mt	TAAAG**AGGTTG**ACTC**A<u>T</u>TT<u>T</u>A**GTGGAAG	
LXRE consensus	CCCAG**GGTTTA**AATA**AGTTCA**TCACA	
ChIP		
UGT1A3 LXRE	Forward	5'-TCAGCTGCTGCCTGATAAACATG
	Reverse	5'- GACCGATCATGACTATCTTGAAA
UGT1A1 -9903bp	Forward	5'-CATTGAGACTTGACCCATCTGG
	Reverse	5'-TTAACACTTCTACTTCCTGC
UGT1A1 -9855bp LXRE	Forward	5'-CACAGGAGACCTAAGCACTCGC
	Reverse	5'-CTGGAAACTGAACTCAGTGTG
UGT1A1 -7632bp	Forward	5'-GACAACATAGTGGGTCAACATCAT
	Reverse	5'-GGTCTCTCTCTGTAGCTGTTGC
UGT1A1 -4076bp	Forward	5'-ATTACGGAAATAGTTTTGAC
	Reverse	5'-GTAATCAAGAGATGACTAGAGGT
UGT1A1 -888bp LXRE	Forward	5'-GAGCCCTGAGTGGCTGAGGTG
	Reverse	5'-GAACCTGAAAGAGCCAGTCCTGT
DR4 Multicopy		
LXRE consensus (x3)	5'-TGA**TGAACT**TATT**TAAACC**CTGGGGATCTG	
DR4 -9903 (x3)	5'-ATAGAGAGG**TGACCA**CAGG**AGACCT**AAGCACT	
DR4 -9855 (x3)	5'-TAAAG**AGGTTG**ACTC**AGTTCA**GTGGAAG	
DR4 -4076 (x3)	5'-TCTGAAG**GGATTA**GTTT**AGGACA**ACCCTC	
DR4 -888 (x3)	5'-ACCGTG**TGACCT**GTAG**TAAGCA**AAGGGCCTA	

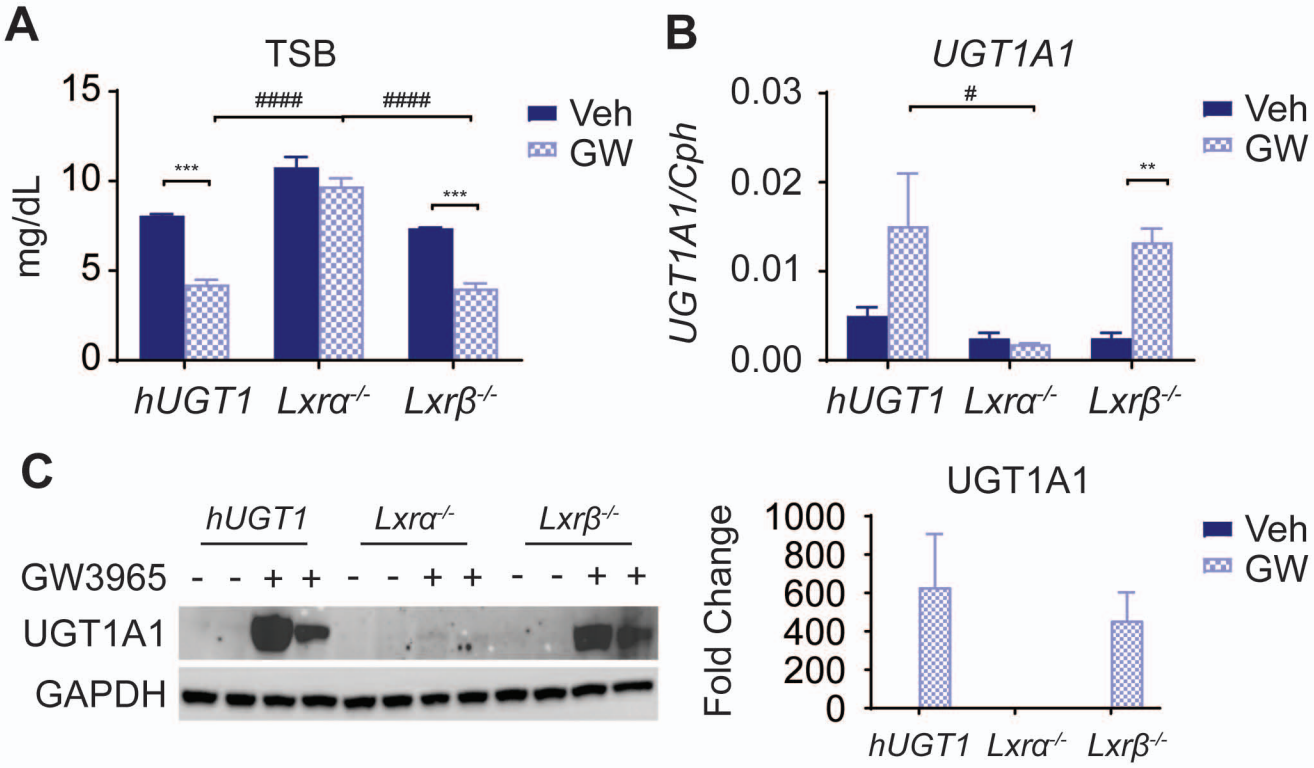
Bold nucleotides represent potential LXR/RXR binding sequences and <u>underlined</u> nucleotides correspond to mutated bases.

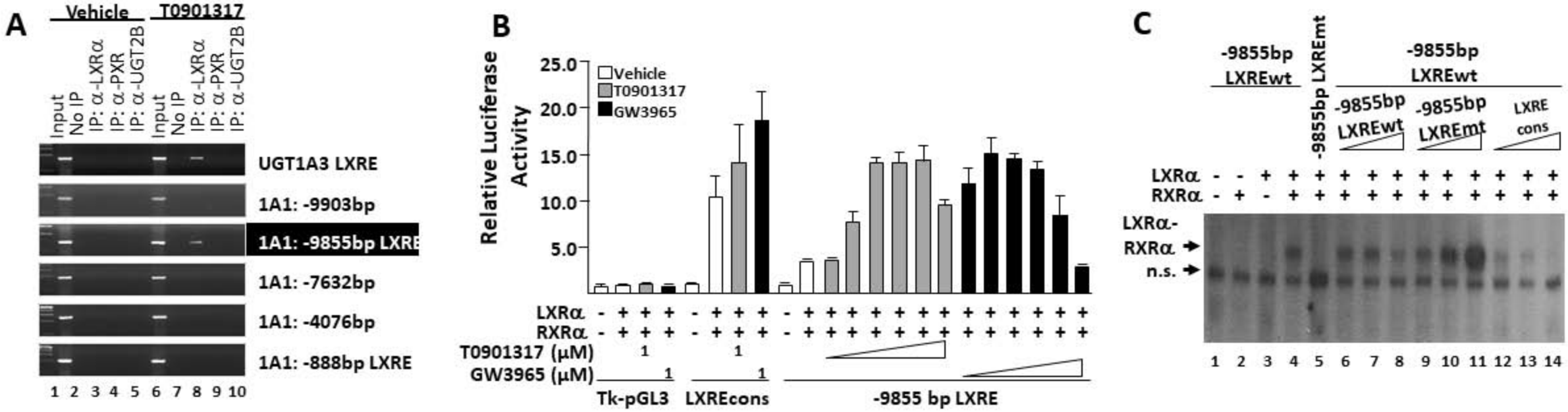


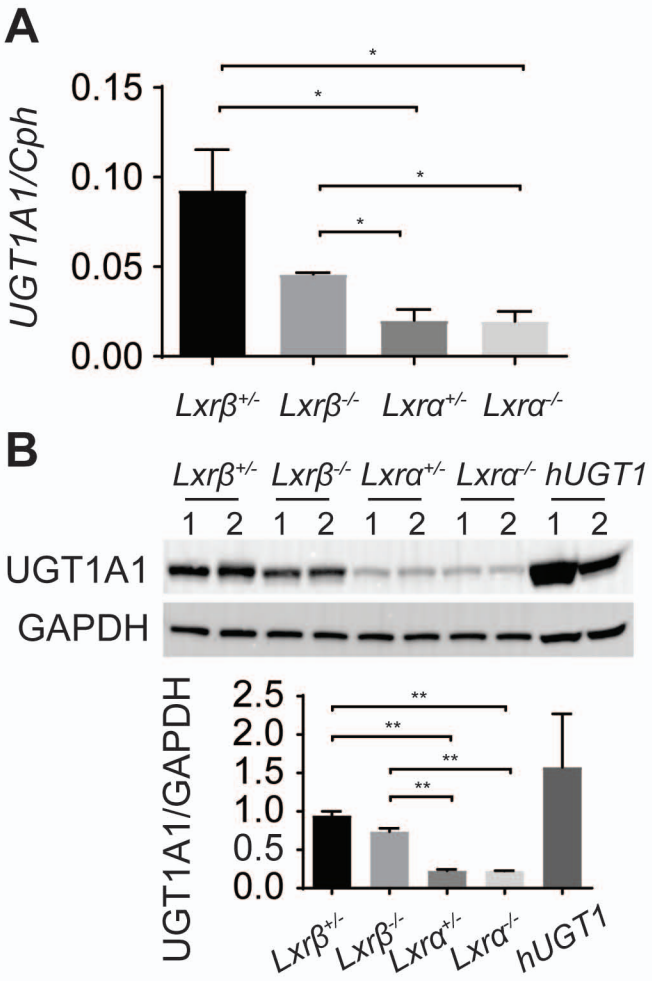









A
Vehicle
T0901317
Input
No IP
IP: α-LXRα
IP: α-PXR
IP: α-UGT2B
UGT1A3 LXRE
1A1: -9903bp
1A1: -9855bp LXRE
1A1: -7632bp
1A1: -4076bp
1A1: -888bp LXRE
1 2 3 4 5 6 7 8 9 10
B
Relative Luciferase Activity
25.0
20.0
15.0
10.0
5.0
Vehicle
T0901317
GW3965
LXRα
RXRα
T0901317 (μM)
GW3965 (μM)
Tk-pGL3
LXREcons
-9855 bp LXRE
C
-9855bp LXREwt
-9855bp LXREmt
-9855bp LXREwt
-9855bp LXREwt
-9855bp LXREmt
LXRE cons
LXRα
RXRα
LXRα-RXRα
n.s.
1 2 3 4 5 6 7 8 9 10 11 12 13 14



Supplemental Data

Drug Metabolism and Disposition

Differential role of LXRα and LXRβ in the regulation of UDP-glucuronosyltransferase 1A1 in humanized *UGT1* mice

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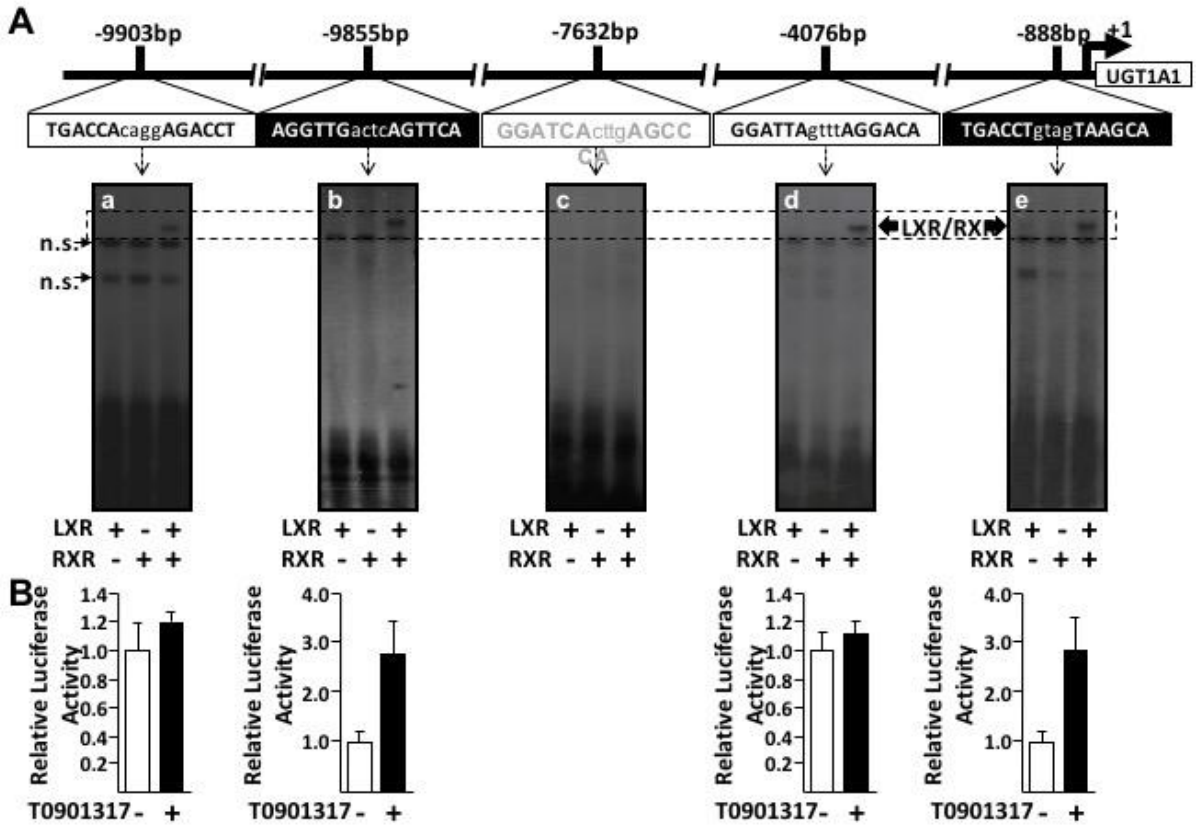

Figure 1.

(**A**) *In silico* analysis of the 10kb promoter sequence of the human *UGT1A1* gene (Genebank number AF297093) revealed the presence of 5 degenerated DR4 motifs localized at positions -9903 (**a**), -9855 (**b**), -7632 (**c**), -4076 (**d**) and -888 (**e**) bp of the promoter. These elements were assayed for LXRα/RXRα interaction in EMSA by using end-labeled DNA probes, in the presence of *in vitro* produced RXRα, LXRα or both RXRα and LXRα proteins as indicated. n.s.: non-specific binding.

(**B**) HK293 cells were transfected with 100ng of empty firefly luciferase (Luc) reporter construct (TKpGL3) or containing 3 copies of the 4 LXRα-interacting DNA motifs identified in **A**, in the presence of the expression plasmids for LXRα and RXRα (10ng) and the renilla luciferase (pRL-NULL) expression plasmid (30ng). Cells were subsequently treated with ethanol (vehicle) or T0901317 (1µM) for 24H. Values are

expressed as fold induction over the control (vehicle-treated cells) set at 1, normalized to internal renilla activity. Values represent the means ± SD.