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Title page

REV-ERBα Regulates CYP7A1 through Repression of Liver Receptor Homolog-1

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Running Title: Regulation of CYP7A1 by the REV-ERBα/LRH-1 axis

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Non-standard abbreviations

ChIP, chromatin immunoprecipitation; Cyp7a1/CYP7A1, mouse/human cholesterol 7α-hydrolase; EC_{50}, half maximum effective concentration; EMSA, electrophoretic mobility shift assay; FXR, farnesoid X receptor; GSK2945, N-(4-chloro-2-methylbenzyl)-N-(4-chlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine; HDAC3, histone deacetylase 3; Lrh-1/LRH-1, mouse/human liver receptor homolog 1; LXR, liver X receptor; NcoR, nuclear receptor co-repressor 1; NR, nuclear receptor; Rev-erbα/REV-ERBα, mouse/human reverse erythroblastosis virus α; RevRE, Rev-erbα/ REV-ERBα response element; Shp, small heterodimer partner.

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Abstract

The nuclear heme receptor REV-ERBα (a transcriptional repressor) is known to regulate CYP7A1 and bile acid synthesis. However, the mechanism for REV-ERBα regulation of CYP7A1 remains elusive. Here we investigate the role of LRH-1 in REV-ERBα regulation of CYP7A1 and cholesterol metabolism. We first characterized the tertiary amine GSK2945 as a highly specific Rev-erbα/REV-ERBα antagonist using cell-based assays and confirmed expression of Rev-erbα in mouse liver. GSK2945 treatment increased hepatic Cyp7a1 level and lowered plasma cholesterol in wild-type mice. Likewise, the compound increased the expression and microsomal activity of Cyp7a1 in hypercholesterolemic mice. This coincided with reduced plasma and liver cholesterol and enhanced production of bile acids. Increased levels of Cyp7a1/CYP7A1 were also found in mouse and human primary hepatocytes after GSK2945 treatment. In these experiments, we observed parallel increases in Lrh-1/LRH-1 (a known hepatic activator of Cyp7a1/CYP7A1) mRNA and protein. Luciferase reporter, mobility shift, and chromatin immunoprecipitation assays revealed that Lrh-1/LRH-1 was a direct Rev-erbα/REV-ERBα target gene. Furthermore, conditional deletion of Lrh-1 in the liver abrogated the regulatory effects of Rev-erbα on Cyp7a1 and cholesterol metabolism in mice. In conclusion, Rev-erbα regulates CYP7A1 and cholesterol metabolism through its repression of Lrh-1 receptor. Targeting REV-ERBα/Lrh-1 axis may represent a novel approach for management of cholesterol related diseases.

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Introduction

Cholesterol is a sterol molecule biosynthesized in animal cells. In addition to its importance for cell integrity, cholesterol also serves as a precursor for biosynthesis of hormones and bile acids. However, excessive cholesterol is a major risk factor for developing cardiovascular diseases (e.g., angina, heart attack, and stroke), a main cause of poor health and death (D'Agostino et al., 2008). Bile acid synthesis is the primary pathway for cholesterol catabolism. Cholesterol is converted into bile acids mainly through the multistep classic (or neutral) pathway, wherein cholesterol 7α-hydroxylase (CYP7A1) is the first and rate-limiting enzyme (Ishibashi et al., 1996). A deficiency of CYP7A1 is associated with hypercholesterolemia, leading to cardiovascular and gallstone diseases (Pullinger et al., 2002). To maintain bile acid and cholesterol homeostasis, CYP7A1 is under the control of farnesoid X receptor (FXR) (Chiang et al., 2000). In excess, bile acids (e.g., cholic acid and chenodeoxycholic acid) activate FXR receptor to decease CYP7A1 expression via small heterodimer partner (SHP)-dependent and SHP-independent mechanisms, thereby down-regulating their own biosynthesis (Inagaki et al., 2005). In addition to FXR, many other transcription factors (e.g., LXR, LRH-1, E4BP4, HNF4α, VDR, PPARα, DBP, DEC2, and REV-ERBα) also participate in the regulation of CYP7A1 (Noshiro et al., 2007; Chow et al., 2014). Of note, DBP, DEC2, and REV-ERBα may contribute to circadian rhythm of CYP7A1 (Noshiro et al., 2007).

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REV-ERBα (NR1D1) and its paralog REV-ERBβ (NR1D2), the two members of nuclear receptor (NR) 1D subfamily, are important components of the mammalian clock machinery (Preitner et al., 2002; Cho et al., 2012). REV-ERBs were initially regarded as "orphan" NRs. They are no longer "orphan" receptors after heme was identified as their endogenous ligand (Raghuram et al., 2007; Yin et al., 2007). REV-ERBs serve as clock-repressors that negatively regulate the expression of circadian and metabolic genes, thereby integrating circadian rhythms with cell metabolism (Everett and Lazar, 2014; Ercolani et al., 2015). The receptors bind to their specific response element (RevRE) (consisting of a NR half site "AGGTCA" and an A/T-rich 5' extension) and repress gene transcription by recruiting corepressor complex containing the core proteins nuclear receptor co-repressor 1 (NcoR) and histone deacetylase 3 (HDAC3) (Harding and Lazar, 1995; Duez and Staels, 2009; Yin et al., 2010; Ercolani et al., 2015). REV-ERBs have been implicated in regulation of various physiological processes (e.g., cell differentiation, adipogenesis, inflammation, lipid and glucose metabolism), thus are regarded as potential therapeutic targets for cancers and metabolic disorders such as dyslipidemia, obesity, and diabetes (Duez and Staels, 2009; Yin et al., 2010; Ercolani et al., 2015).

Although both REV-ERB paralogs are necessary in generation of circadian rhythms and in maintenance of metabolic homeostasis, REV-ERBα appears to be of greater importance (Bugge et al., 2012; Cho et al., 2012). Rev-erbα knockout in mice causes moderate disruptions to circadian rhythms and metabolic homeostasis, whereas loss of

Rev-erbβ shows no significant effects (Preitner et al., 2002; Bugge et al., 2012; Cho et al., 2012). The regulatory role of REV-ERBα in hepatic lipid homeostasis has received considerable attentions (Duez and Staels, 2008). REV-ERBα is shown to control the expression of the genes involved in lipid metabolism such as apoC-III and ELOVL3 (Raspe et al., 2002; Anzulovich et al., 2006). REV-ERBα also participates in regulation of bile acid synthesis as its absence leads to decreased bile acid accumulation in mice (Duez et al., 2008; Le Martelot et al., 2009). This regulation is attained through a modulatory effect of REV-ERBα on CYP7A1 expression. However, the exact mechanisms for REV-ERBα regulation of CYP7A1 remain elusive (Duez et al., 2008; Le Martelot et al., 2009).

Although REV-ERBα is known to be involved in bile acid synthesis, little is known about its effects on cholesterol homeostasis. In addition, the ability of REV-ERBα to be targeted by a drug is of great interest due to a critical role in circadian biology and cell metabolism (Solt et al., 2012; Trump et al., 2013). However, drug evaluation of synthetic ligands of REV-ERBα is limited by poor pharmacokinetics or the lack of nuclear receptor selectivity (Trump et al., 2013; Ercolani et al., 2015). Therefore, the objectives of this study were to investigate the role of liver receptor homolog-1 (Lrh-1) in REV-ERBα regulation of Cyp7a1 and to clarify the impact of REV-ERBα on cholesterol metabolism using a small-molecule probe. We identified the tertiary amine GSK2945 as an *in vivo* functional probe (antagonist) of Rev-erbα, and demonstrated that administration of GSK2945 to

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mice led to induction of Cyp7a1 and reduction of cholesterol via upregulation of Lrh-1. Luciferase reporter, electrophoretic mobility shift (EMSA), and chromatin immunoprecipitation (ChIP) assays supported a direct role for Rev-erbα/REV-ERBα in repression of Lrh-1/LRH-1, thereby identifying the Rev-erbα/Lrh-1 axis as a novel regulatory pathway for CYP7A1 expression and cholesterol homeostasis.

Materials and Methods

The materials, primers, antibodies, plasmids, synthesis method for GSK2945, and procedures for pharmacokinetic studies are provided in the Supplementary Materials.

Animal studies

Male C57BL/6 mice were obtained from Beijing HFK Bioscience Co., Ltd. (Beijing, China). Mice were housed in a temperature-controlled room (22 ± 2 °C) on a standard 12/12 h light/dark cycle (07:00–19:00 h), with access to food and water ad libitum. In each set of studies, GSK2945 was administered intraperitoneally to mice at doses of 0 or 10 mg/kg twice every day (at ZT0 and ZT12) for 7 days. First, normal diet-fed mice (8-10 weeks of age, *n* = 5 per group) were treated with GSK2945, and blood and livers were harvested on day 8 at ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20. In the second study, hypercholesterolemic mice fed a Western diet (1.25% cholesterol, Trophic Animal Feed High-tech Company, Jiangsu, China) for four weeks were developed. GSK2945 was given to both normal and hypercholesterolemic mice (10-12 weeks of age, *n* = 6 per group). On day 8 (ZT12), the blood and tissues (small intestine, gallbladder, and liver) were harvested. The feces were collected over a 48-hour period (from day 6 to day 8). All animal procedures were performed with isofloran anaesthesia and analgesia with buprenorphine prior to blood and tissues harvest. All the animal procedures were approved by the Institutional Animal Care and Use Committee of Jinan University and

conform to the NIH guidelines (Guide for the care and use of laboratory animals).

Determination of bile acid pool size

Bile acid pool was extracted following published procedures (Alnouti et al., 2008). Dehydrocholic acid was used as the internal standard. Samples were analyzed using the Waters Acquity UPLC/Synapt G2 QTOF with electrospray ionization (ESI) source at the negative ion mode. Chromatographic separation was performed on a Luna® Omega C18 column (1.6 μm, 100 mm×2.1 mm, Phenomenex) at a flow rate of 0.3 ml/min. The mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient program was 25% B at 0-3 min, 25-35% B at 3-7 min, 35-40% B at 7-18 min, 40-50% B at 18-23 min, 50-90% B at 23-25 min, 90% B at 25-28 min, 90-25% B at 28-30 min. Calibration curves of reference standards were prepared to quantify the bile acids as described previously (Lee et al., 2008).

Immunohistochemical staining

Mouse livers were perfused with phosphate-buffered saline (PBS) and fixed overnight in 4% paraformaldehyde at 4°C. Four-μm-thick paraffin-embedded sections were heated at 65°C for 1 h, dewaxed in xylene and rehydrated in descending concentrations of ethanol. Antigen retrieval was achieved by boiling samples at 100°C in a citrate buffer solution (pH = 6.0) for 10 min. The sections were pre-blocked with 5% goat serum and incubated overnight with the primary anti-Rev-erbα antibody (1:50, Sigma-Aldrich, MO, USA). After

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washing with PBS, the sections were incubated with the secondary goat anti-mouse horseradish peroxidase antibody at room temperature for 1 h, followed by staining with diaminobenzidine tetrahydrochloride and counterstaining with hematoxylin. The sections were imaged with a Nikon Eclipse Ti-SR microscope (Nikon Incorporation, Tokyo, Japan).

Mouse/human primary hepatocytes

Mouse (male, CD1) and human (male, Caucasian) primary hepatocytes were obtained from XenoTech, LLC (Lenexa, KS) and plated in Biocoat™ collagen I 24-well plates (Corning, NY). After 8 h incubation at 37°C (for cell attachment), cells were overlaid with 0.25 mg/ml Matrigel in OptiCulture Media. On the next day, cells were treated with vehicle (0.5% DMSO) or 20 μM GSK2945, and harvested at 3, 6, 12, or 24 h.

Real-time PCR

All primer sequences are summarized in Supplemental Table S1-2. Total RNA extraction and quantitative reverse transcriptase PCR were performed as previously described (Zhang et al., 2015). mRNA levels were first normalized to cyclophilin b or GAPDH, and then expressed as relative mRNA expression of the control.

Western blotting

Western blotting was performed as previously described (Song et al., 2008). Total proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gels, and transferred to polyvinylidenefluoride membranes. After probing with primary and

secondary antibodies, protein bands were visualized by enhanced chemiluminescence and analyzed by the Quantity One software. Protein levels were normalized to GAPDH or β-actin.

Liver microsomal Cyp7a1 activity

Mouse liver microsomes were prepared by sequential ultracentrifugation, first at 9,000 g for 10 min and then at 100,000 g for 60 min. The microsomal Cyp7a1 activity toward cholesterol was determined using the published procedures involving the oxidation of 7α-hydroxycholesterol to 7α-hydroxy-4-cholesten-3-one by cholesterol oxidase (Chow et al., 2009).

Plasma and tissue cholesterol

Total plasma cholesterol was measured using a LabAssay Cholesterol kit (Wako Chemical, Osaka, Japan). Total cholesterol was extracted from mouse livers as previously described (Patel et al., 2011), and quantified using a Total Cholesterol assay kit (Jiancheng Bioengineering Institute, Nanjing, China).

Nuclear receptor (NR) specificity assay

The potential of GSK2945 to modulate the activities of all 48 human NRs was assessed using a Gal4 cotransfection assay system as described (Kumar et al., 2010). In Gal4 cotransfection assay, only specific ligand of test NR can bind to LBD to activate gene transcription. In brief, HEK293 cells were co-transfected with a GAL4-NR LBD construct,

pGL4.35[luc2P/9XGAL4UAS/Hygro] vector, and pRL-TK vector using the HET transfection kit (Biowit Technologies, Shenzhen, China). On the next day, GSK2945 (20 μM) or DMSO was added and incubated with the cells for 24 h. The luciferase activities were measured with the Dual Luciferase Reporter Assay kit (Promega, Madison, WI) and Glomax 20/20 Luminometer (Promega, CA, USA).

Luciferase reporter cotransfection assays

Cotransfection assays were performed in HEK293 or HepG2 cells using the HET transfection kit (Biowit Technologies, Shenzhen, China) as described (Zhao et al., 2016). Ligands were added at 16 to 20 hours post transfection. Cells harvested 6 hours later were assayed for luciferase activities. The relative luciferase activity was initially derived as the ratio of firefly over renilla luciferase activity. The relative luciferase activity values of treated cells were normalized to that of control cells.

Electrophoretic mobility shift assay (EMSA)

After transfection of HEK293 cells with Rev-erbα/REV-ERBα, nuclear extracts were prepared using a cytoplasmic/nuclear protein extraction kit (Beyotime, Shanghai, China). EMSA assays were performed using a chemiluminescent EMSA kit (Beyotime). Six-μg of nuclear extract was mixed in EMSA binding buffer. After 10-min preincubation on ice, 200-fmol of biotin-labeled probe was added and incubated for 20-min at room temperature. Reaction products were subjected to 5% polyacrylamide gel electrophoresis. After

transferring to Hybond-N^+ membrane (Amersham, Buckinghamshire, UK), the products were visualized by enhanced chemiluminesence reagent. All Oligonucleotide sequences for EMSA assays can be found in Supplemental Table S3.

Chromatin immunoprecipitation (CHIP) assay

ChIP assays were performed using a SimpleChIP® Enzymatic Chromatin IP Kit (Cell Signaling Technology, Beverly, MA) according to the manufacturer's instructions. Mouse liver was fixed in 1% formaldehyde for 20 min at room temperature, followed by digestion with micrococcal nuclease and shearing with sonication. An aliquot of sheared chromatin was immunoprecipitated with anti-Rev-erbα or normal rabbit IgG (control) by overnight incubation at 4°C. Immunoprecipitated chromatin was de-crosslinked at 65°C for 4 h and DNAs were purified using spin columns. The purified DNAs were used as a template for quantitative real-time PCR with specific primers (Supplemental Table S4).

Statistical analysis

All data are presented as mean ± SD (standard deviation). Statistical analysis on the circadian expression data was performed using a Student's t-test comparing levels of gene/protein expression of vehicle treatment vs. drug treatment at individual circadian times. The Student's t-test was also used to test for statistical differences between treatment and control groups. The level of significance was set at $p < 0.05$.

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Results

Rev-erbα protein tissue distribution

Nuclear Rev-erbα protein was found at a similar level in mouse liver and ileum (Figure 1A). The protein level of Rev-erbα was 2.1-fold higher in the brain compared to the liver or ileum (Figure 1A). By contrast, Rev-erbα protein was not detected in the kidney (Figure 1A). The presence of Rev-erbα protein within nuclei of mouse hepatocytes was further confirmed by liver immunostaining (Figure 1B).

Identification of GSK2945 as a specific Rev-erbα/REV-ERBα antagonist

GSK2945 (Figure 2A) was synthesized by reductive amination (Supplemental Materials), and its chemical structure was verified through ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, and mass spectrometric analyses (Supplemental Figure S1). The compound displayed a superior pharmacokinetic profile with much higher systemic and liver exposures compared to SR8278, the first synthetic antagonist of REV-ERBs (Supplemental Figure S2) (Kojetin et al., 2011). The activity of GSK2945 was first assessed in HEK293 cells co-expressing a chimaeric receptor [i.e., the DNA-binding domain (DBD) of Gal4 is fused to the ligand-binding domain (LBD) of Rev-erb/REV-ERB] and a Gal4-responsive luciferase reporter. In line with previous studies (Alnouti et al., 2008; Solt et al., 2012), the agonist GSK4112 enhanced the transcriptional repression activities of Rev-erbα/REV-ERBα in the Gal4 chimaeric assay (Figure 2B). By contrast,

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GSK2945 dose-dependently inhibited the repressor activities of Rev-erbα/REV-ERBα, but showed no effects on Rev-erbβ/REV-ERBβ activities (Figure 2B) (Rev-erbα EC_{50} = 21.5 μM; REV-ERBα EC_{50} = 20.8 μM). The compound did not exhibit activities toward other types of nuclear receptors, demonstrating its exclusive action on REV-ERBα receptor (Figure 2C). In addition, GSK2945 dose-dependently enhanced the transcriptional activity in a cotransfection assay using full-length Rev-erbα and a Bmal1 (a target gene of REV-ERBs) luciferase reporter (Figure 2D) (EC_{50} = 2.05 μM). Also, the compound blocked the agonistic activity of GSK4112, resulting in induction of transcription at doses of ≥5 μM (Figure 2D) (EC_{50} = 2.47 μM). Furthermore, GSK2945 increased the mRNA expression of BMAL1 and PEPCK (i.e., known target genes of REV-ERBs) in HepG2 cells in a dose-dependent fashion (Figure 2E), a similar effect as observed when intracellular heme was reduced by succinylacetone (Supplemental Figure S3). Taken together, GSK2945 acted as an antagonist of Rev-erbα/REV-ERBα probably by blocking the action of endogenous heme.

REV-ERBα regulates hepatic Cyp7a1 and cholesterol homeostasis in normal diet-fed mice

The improved pharmacokinetic properties of GSK2945 allowed us to explore the functional effects of REV-ERBα *in vivo*. No overt toxicity was observed in mice treated with GSK2945 based on hematology and liver function tests (Supplemental Figure S4 and Table S5). GSK2945 treatment of mice resulted in significant upregulation of hepatic

Cyp7a1, the key gene involved in cholesterol catabolism (Figure 3A). The protein level of Cyp7a1 was also elevated in response to GSK2945 administration (Figure 4A). This was accompanied by significant decreases in plasma and liver cholesterol (Figure 3B). Of Cyp7a1-regulatory transcriptional factors, Lrh-1 was unregulated at both mRNA and protein levels (Figures 3C & 4B). Although E4bp4 and Insig2 mRNAs were also increased, no significant differences were noted in their protein levels (Figures 3D & 4C). GSK2945 treatment caused significant increases in mRNA and protein expression of Bmal1 and Pepck (two direct target genes of Rev-erbα) consistent with Rev-erbα antagonism (Figure 3D and Supplemental Figure S5). Expression of the cholesterolgenic regulatory genes (Srebp2 and Hmgcr) were not altered by GSK2945 (Figure 3E). Of other cholesterol-related genes, expression levels of Bsep and Abcg5/g8 (the target genes of Lrh-1) were also increased (Supplemental Figure S6).

REV-ERBα regulates Cyp7a1/CYP7A1 expression in mouse and human primary hepatocytes

Impact of Rev-erbα on Cyp7a1 and Lrh-1 was confirmed in isolated mouse primary hepatocytes. GSK2945 (20 μM, a concentration close to the EC_{50} value) treatment of cells led to significant increases in mRNA and protein (at 24-h) expression of Cyp7a1 (Figure 5A). Likewise, mRNA and protein (at 24-h) levels of CYP7A1 were increased in human primary hepatocyte after exposure to 20 μM GSK2945 (Figure 5B). Of Cyp7a1/CYP7A1-regulatory transcriptional factors, only Lrh-1/LRH-1 (a hepatic activator of

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Cyp7a1/CYP7A1) was upregulated in response to GSK2945 treatment (Figure 5). These data supported a critical role for Lrh-1/LRH-1 in REV-ERBα upregulation of Cyp7a1/CYP7A1.

REV-ERBα regulates cholesterol catabolism through upregulation of Cyp7a1 and Lrh-1 in hypercholesterolemic mice

The effects of GSK2945 on Cyp7a1 and cholesterol metabolism were further evaluated using hypercholesterolemic mice which showed markedly increased liver and plasma cholesterol (Figure 6A). GSK2945 treatment of hypercholesterolemic mice led to significant reductions in plasma cholesterol (22.6%) and liver cholesterol (29.6%) (Figure 6A). This was coincided with significantly increased Cyp7a1 mRNA (1.91-fold) and protein (1.52-fold) levels as well as microsomal activity (1.58-fold) in the liver (Figure 6B). Increased expression of Cyp7a1 may be accounted for by upregulation of Lrh-1 (Figure 5C). Owing to upregulated Lrh-1, expression of Cyp8b1 was also increased (Supplemental Figure S7). In addition, both bile acid pool size and fecal excretion of bile acids were significantly increased after GSK2945 treatment (Figure 6D and Supplemental Figure S8-9). Likewise, there was a higher amount of bile acids in the plasma (Figure 6D). These data suggested an increased bile acid production because of enhanced Cyp7a1 activity.

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Rev-erbα/REV-ERBα is a transcriptional repressor of Lrh-1/LRH-1

Luciferase reporter assays were performed to determine whether Cyp7a1 is directly regulated by Rev-erbα. In line with the literature, Rev-erbα and Rev-erbβ directly repressed Bmal1 transcription (Figure 7A). However, neither showed regulatory effects on Cyp7a1 transcription (Figure 7A). Consistently, transcription of Cyp7a1 was unaffected in the presence of GSK2945 (Figure 7A). Therefore, an indirect mechanism is necessary for regulation of Cyp7a1 by Rev-erbα receptor as indicated previously (Duez et al., 2008; Le Martelot et al., 2009). We further performed luciferase reporter assays using proximal Lrh-1 promoter (-700/+3 bp). Rev-erbα significantly decreased Lrh-1 promoter activity (Figure 7B). Addition of GSK2945 blocked the action of Rev-erbα and eliminated its repressor activity (Figure 7B). These data revealed Rev-erbα as a transcriptional repressor of Lrh-1.

Sequence analysis of mouse Lrh-1 promoter suggested two potential Rev-erb response elements (mRevRE1 and mRevRE2) (at positions -647 and -167, respectively). Accordingly, multiple truncated and/or mutated versions of Lrh-1 luciferase reporters were generated and tested for repression activities (Figure 7C). The repressor ability of Rev-erbα was unaffected when mRevRE1 site was deleted or mutated, but completely lost when the mRevRE2 site was mutated (Figure 7C). This indicated that mRevRE2 rather than mRevRE1 was responsible for the repressor activity of Rev-erbα. EMSA

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experiments with biotinylated oligonucleotides showed that Rev-erbα bound directly to the mRevRE2 site, forming a distinct DNA-protein complex (Figure 7D). Formation of this complex was markedly diminished in the presence of unlabeled competitor (Figure 7D). In a similar manner, a REV-ERBα response element (at position -79) within the human LRH-1 promoter was identified (Figure 7E-F). To confirm the interaction of Rev-erbα with Lrh-1 promoter in vivo, ChIP assays were performed using mouse liver samples at ZT8 (corresponding to a peak Rev-erbα expression). We observed significant recruitment of Rev-erbα to the mRevRE2 site (Figure 7G). Overall, these data indicated that Rev-erbα repressed transcription of Lrh-1 through its specific binding to the mRevRE2 site (i.e., the -178- to -167-bp region).

Lrh-1 is required for regulation of Cyp7a1 by Rev-erbα *in vivo*

To determine whether Lrh-1 is an actual mediator for Rev-erbα regulation of Cyp7a1, we performed *in vivo* studies using genetic mice. Conditional deletion of Lrh-1 in liver (Alb-Cre;$Lrh^{fl/fl}$) abrogated the changes in Cyp7a1 expression (Figure 8A) and plasma cholesterol (Figure 8B) as noted in wild-type mice, supporting a critical role for Lrh-1 in control of Cyp7a1 and cholesterol metabolism by Rev-erbα.

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Discussion

In this study, we established the tertiary amine GSK2945 as an *in vivo* probe (antagonist) of Rev-erbα and observed a cholesterol-lowering effect in mice after GSK2945 treatment. The cholesterol-lowing effect was associated with an elevation in expression of Cyp7a1 (the rate-limiting enzyme in the classical pathway of bile acid synthesis), that led to increased production of bile acids (Figure 6D). The enhanced bile acid synthesis was primarily responsible for reduction in body cholesterol as no significant changes were found in cholesterolgenic genes Srebp2 and Hmgcr (Figure 3E). We further observed parallel expression changes in Lrh-1 (a positive regulator of Cyp7a1), thus predicted a critical role for Lrh-1 in upregulation of Cyp7a1. Through luciferase reporter, EMSA and ChIP assays, we revealed that Rev-erbα directly repressed transcription of Lrh-1 via binding to its specific response element (Figure 7). Therefore, upregulation of Cyp7a1 by Rev-erbα works through de-repression of Lrh-1 (Figure 8C). This is the first demonstration that Rev-erbα can be targeted to alter Cyp7a1 expression and cholesterol homeostasis.

Previous studies have consistently shown that Cyp7a1 and bile acid synthesis are under the control of Rev-erbα using engineered mice with Rev-erbα deletion or overexpression (Duez et al., 2008; Le Martelot et al., 2009). However, the exact mechanisms for this regulation remained unresolved. Duez et al believe that Rev-erbα represses Shp and E4bp4 (two potential repressors of Cyp7a1) to increase Cyp7a1 in

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mice (Duez et al., 2008). On the contrary, Le martelot et al propose that upregulation of Cyp7a1 by Rev-erbα is through downregulation of Insig2 though the authors acknowledged the lack of direct evidence (Le Martelot et al., 2009). Repressive action of Rev-erbα on Shp *in vivo* is questioned by our study and others (Le Martelot et al., 2009). This was because no change in Shp expression was observed in GSK2945-treated wild-type mice (Figure 3C) or Rev-KO (Rev-erbα knockout) mice, and increased expression of Shp was found in TgRev mice (i.e., Transgenic mice overexpressing hepatic Rev-erbα) (Le Martelot et al., 2009). We and others also argue against a major role for E4bp4 in Cyp7a1 regulation (Le Martelot et al., 2009). First, E4bp4$^{-/-}$ mice did not show increased Cyp7a1 expression compared to wild-type mice (Le Martelot et al., 2009). Second, expression of E4bp4 was increased in wild-type mice in response to Rev-erbα antagonism (Figure 3C). Further, we believed that Cyp7a1 upregulation was independent of Insig2 because expression of Insig2 was increased in GSK2945-treated mice (Figure 3C).

We propose that the Rev-erbα/Lrh-1 axis is involved in regulation of Cyp7a1, highlighting Lrh-1 as a key mediator for indirect regulation of Cyp7a1 by Rev-erbα. Small-molecule targeting of Rev-erbα de-represses Lrh-1, a hepatic activator of Cyp7a1 (Nitta et al., 1999; Noshiro et al., 2007; Out et al., 2011), to increase Cyp7a1 expression and lower cholesterol (Figure 8C). This novel mechanism of Cyp7a1 upregulation has greater potential utility in treating hypercholesterolemia compared with the VDR receptor

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activation by $1,25(OH)_2D_3$ (Chow et al., 2014). The latter is concerned with the hypercalcemic side effect of $1,25(OH)_2D_3$.The proposed mechanism also helps to explain why Rev-erbα activation by synthetic agonists (SR9009 and SR9011) results in a decrease in Cyp7a1 and why Rev-erbα knockout leads to a reduction in liver cholesterol, which were unexpected previously due to proposed positive control of Cyp7a1 by Rev-erbα (Cho et al., 2012; Ercolani et al., 2015). We observed parallel increases in expression of other Lrh-1 target genes such as Cyp8b1, Cyp27a1, Bsep, and Abcg5/g8, supporting the upregulation of Lrh-1 in response to Rev-erbα antagonism (Supplemental Figure S6-7) (Freeman et al., 2004; Lee et al., 2008; Song et al., 2008). Although Cyp8b1 participates in classical bile acid synthesis, a contributing role of Cyp8b1 upregulation to reduced cholesterol can be ruled out because Cyp8b1-mediated reaction is not a limiting step to the overall bile acid synthesis (Li and Chang, 2014). On the other hand, the increase of Cyp27a1 might contribute to reduced cholesterol because the enzyme initiates the alternative (acidic) pathway of bile acid synthesis (Li and Chang, 2014). However, the contribution, if any, would be rather limited because the acidic pathway accounts for a minor portion (< 10%) of bile acid synthesis (Li and Chiang, 2014).

We provided strong evidence that GSK2945 functions as an antagonist of Rev-erbα. First, GSK2945 dose-dependently antagonizes the repressor action of Rev-erbα in the chimaeric Gal4-LBD assay (Figure 2B). Second, GSK2945 de-represses the transcription of Bmal1 reporter and block the action of GSK4112 in a cotransfection assay with full-

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length Rev-erbα (Figure 2D). Third, GSK2945 increases the expression of Rev-erbα target genes in HepG2 and hepatocytes as well as in mice by antagonizing the action of endogenous heme (Figures 2-5). All these cell-based and in vivo data were consistent with the antagonism of Rev-erbα. It was noted that GSK2945 behaved like a REV-ERBα agonist in a previous study (Trump et al., 2013). The authors observed agonistic effects in non-standard activity assays (i.e., THP-1 interleukin 6 repression and U2OS reporter assays) (Trump et al., 2013). Although the exact reasons for this contradiction remained unknown, there was a possibility that the agonistic versus antagonistic action of GSK2945 was cell type (tissue) -dependent. This is because the activity of REV-ERB receptor is strongly affected by the cellular microenvironments such as redox state, small molecule gasses (e.g., NO and CO) and the types of cofactors (Marvin et al., 2009; Pardee et al., 2009; Trump et al., 2013; Matta-Camacho et al., 2014). Modifications of ligand-bound REV-ERB by redox conditions and gasses are likely the key determinants to ligand switching and functional effects (Kojetin et al., 2011). The high sensitivity of REV-ERB activity to the conformational changes of ligand-bound receptor complex is also evidenced by the fact that structurally related compounds [e.g., GSK2945 versus GSK4112; cobalt protoporphyrin IX versus heme] demonstrate different types of actions on REV-ERB (i.e., antagonist versus agonist) (Kojetin et al., 2011).

In summary, we characterized GSK2945 as a Rev-erbα antagonist with sufficient pharmacokinetic parameters for *in vivo* uses. GSK2945 increased the expression of

CYP7A1 and reduced total cholesterol in mice. This regulatory effect was ascribed to Rev-erbα-mediated de-repression of Lrh-1, a hepatic activator of Cyp7a1. Therefore, targeting Rev-erbα/Lrh-1 axis may represent a novel approach for management of cholesterol related diseases.

Author Contributions

Participated in research design: Zhang, Zhao, and Wu.

Conducted experiments: Zhang, Zhao, Lu, Wang, Yu, and Guo.

Contributed new reagents or analytic tools: Wen.

Performed data analysis: Zhang, Zhao, and Wu.

Wrote the manuscript: Zhang, Zhao, and Wu.

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Footnotes

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TZ and MZ contributed equally to this work.

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Legends for Figures

Figure 1 **Rev-erbα (Nr1d1) protein tissue distribution. (A)** Protein expression of Rev-erbα in mouse liver (L), kidney (K), brain (Br) and ileum (I). HEK293 cells transfected with pcDNA3.1 (-) and pcDNA3.1-Nr1d1 (+) are used as negative and positive controls, respectively. Data are presented as mean ± SD (*n* = 3).*p < 0.05 (t-test). N.D., not detected. **(B)** Liver immunostaining showing Rev-erbα protein (arrows) within nuclei of mouse hepatocytes.

Figure 2 **Identification of GSK2945 as an antagonist of Rev-erbα (Nr1d1)/REV-ERBα (NR1D1). (A)** Chemical structure of GSK2945. **(B)** Gal4 cotransfection assays with HEK293 cells demonstrating the antagonistic activity of GSK2945 versus agonistic activity of GSK4112 (also known as SR6452). **(C)** Nuclear receptor specificity assay illustrating a highly specific action of GSK2945 on REV-ERBα. The compound was tested at a concentration of 20 μM. The format of the assay was a cotransfection assay with a Gal4 DBD-nuclear receptor LBD fusion in HEK293 cells. **(D)** Cotransfection assays with full-length Rev-erbα and a Bmal1 luciferase reporter in HEK293 cells demonstrating the antagonist activity of GSK2945. **(E)** Modulation of the expression of Rev-erbα target genes by GSK2945 in HepG2 cells. Data are presented as mean ± SD (*n* = 3). *****P < 0.05 (t-test) drug versus vehicle treatment.

Figure 3 **Rev-erbα regulates the mRNA expression of cholesterol metabolism-related genes in the liver.** C57BL/6 mice were administered repeated doses of GSK2945 (10 mg/kg, i.p.) twice daily at ZT0 and ZT12 for 7 days and groups of mice (*n* = 5) were sacrificed and gene expression was assessed by qPCR. **(A)** Expression of Cyp7a1 in the livers of vehicle-treated versus GSK2945-treated mice. **(B)** Changes of blood and liver cholesterol in response to GSK2945 treatment. **(C)** Expression of Cyp7a1-regulatory transcriptional factors in the livers of vehicle-treated versus GSK2945-treated mice. **(D)** Expression of Rev-erbα target genes in the livers of vehicle-treated versus GSK2945-treated mice. **(E)** Expression of cholesterolgenic regulatory genes in the livers of vehicle-treated versus GSK2945-treated mice. Data are presented as mean ± SD (*n* = 5). Statistical analysis on the circadian expression data was performed using a Student's t-test comparing levels of gene/protein expression of vehicle treatment vs. drug treatment at individual circadian times. *****P < 0.05.

Figure 4 **Rev-erbα regulates the protein expression of cholesterol metabolism-related genes in the liver.** C57BL/6 mice were administered repeated doses of GSK2945 (10 mg/kg, i.p.) twice daily at ZT0 and ZT12 for seven days and

groups of mice (*n* = 5) were sacrificed and protein expression was assessed by Western blotting. **(A)** Expression of Cyp7a1 in the livers of vehicle-treated versus GSK2945-treated mice. **(B)** Expression of Lrh-1 in the livers of vehicle-treated versus GSK2945-treated mice. **(C)** Expression of Cyp7a1-regulatory transcriptional factors in the livers of vehicle-treated versus GSK2945-treated mice. Data are presented as mean ± SD (*n* = 5). Statistical analysis on the circadian expression data was performed using a Student's t-test comparing levels of gene/protein expression of vehicle treatment vs. drug treatment at individual circadian times. *****P < 0.05.

Figure 5 **GSK2945 changes Cyp7a1/CYP7A1 and Lrh-1/LRH-1 expression in mouse and human primary hepatocytes. (A)** Mouse primary hepatocytes showed increased Cyp7a1 and Lrh-1 expression at 24-h after GSK2945 (20 μM) treatment. **(B)** Human primary hepatocytes showed increased Cyp7a1 and Lrh-1 expression at 24-h after GSK2945 (20 μM) treatment. *p < 0.05 (t-test) drug versus vehicle treatment at different time points.

Figure 6 **GSK2945 reduces cholesterol and enhances bile acid production by upregulating hepatic Lrh-1 and Cyp7a1 in hypercholesterolemic mice. (A)** Plasma and liver total cholesterol were reduced after GSK2945 treatment. **(B)** Hepatic Cyp7a1 expression and activity were increased after GSK2945 treatment. **(C)** Hepatic Lrh-1 mRNA and protein levels were increased after GSK2945 treatment. **(D)** GSK2945 treatment increased bile acid pool size, plasma bile acid level, and fecal bile acid excretion. # p < 0.05 (t-test) Western diet versus normal diet; *p < 0.05 (t-test) vehicle-treated versus GSK2945-treated hypercholesterolemic mice.

Figure 7 **Rev-erbα/REV-ERBα is a transcriptional repressor of Lrh-1/LRH-1. (A)** Luciferase reporter assays driven by Bmal1 or Cyp7a1 promoter in HEK293 and HepG2 cells, showing that Rev-erbα failed to directly regulate Cyp7a1. *p<0.05 (t-test) Rev-erb plasmid treatment versus control. **(B)** Luciferase reporter assays driven by Lrh-1 promoter in HEK293 and HepG2 cells, showing Rev-erbα directly repressed transcription of Lrh-1. *p<0.05 (t-test). **(C)** Luciferase reporter assays with truncated and/or mutated versions of mouse Lrh-1 promoter. The boxes denote potential RevRE sites, and  represents the construct with RevRE site mutated (sequences shown). *p<0.05 (t-test) Rev-erbα plasmid treatment versus control. **(D)** EMSA assay with biotin-labeled probes [Bmal1-RevRE probe (AAAGTAGGTTA) as control], showing Rev-erbα bound to the mRevRE2 site of Lrh-1. **(E)** Luciferase reporter assays with truncated and/or mutated versions of human LRH-1 promoter. The boxes denote potential RevRE sites, and  represents the construct with hRevRE site mutated (sequences shown).

*p<0.05 (t-test) Rev-erbα plasmid treatment versus control. **(F)** EMSA assay with biotin-labeled probes [BMAL1-RevRE probe as control], showing REV-ERBα bound to the hRevRE site of LRH-1. **(G)** ChIP assay with anti-Rev-erbα antibody (IgG as control) and mouse liver, showing Rev-erbα bound to the mRevRE2 site of Lrh-1 in vivo. Data are repressed as mean ± SD (*n* = 3). *p<0.05 (t-test). RLU, relative luciferase unit.

Figure 8 **Lrh-1 participates in Rev-erbα regulation of Cyp7a1 and cholesterol homeostasis. (A)** mRNA expression of Cyp7a1 and Lrh-1 in the livers of Alb-Cre;Lrh-1$^{fl/fl}$ mice after vehicle- or GSK2945 treatment. **(B)** Effects of hepatic Lrh-1 deletion on plasma cholesterol in response to GSK2945 treatment. Data are presented as mean ± SD (*n* = 5). *p<0.05 (t-test) drug versus vehicle treatment. **(C)** A schematic representation showing that Lrh-1 links Rev-erbα to Cyp7a1 regulation and cholesterol metabolism. Rev-erbα targeting by GSK2945 de-represses Lrh-1 to increase Cyp7a1 and enhance cholesterol catabolism, resulting in a reduced level of cholesterol in plasma and liver.

Figure 1

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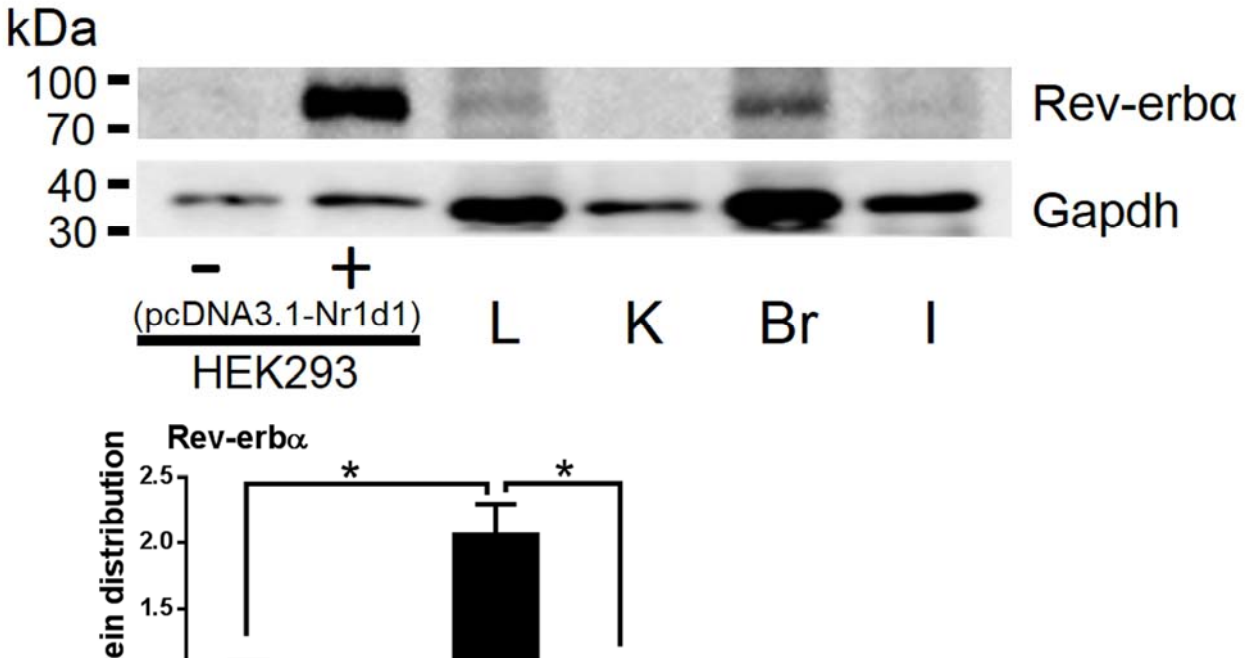
A



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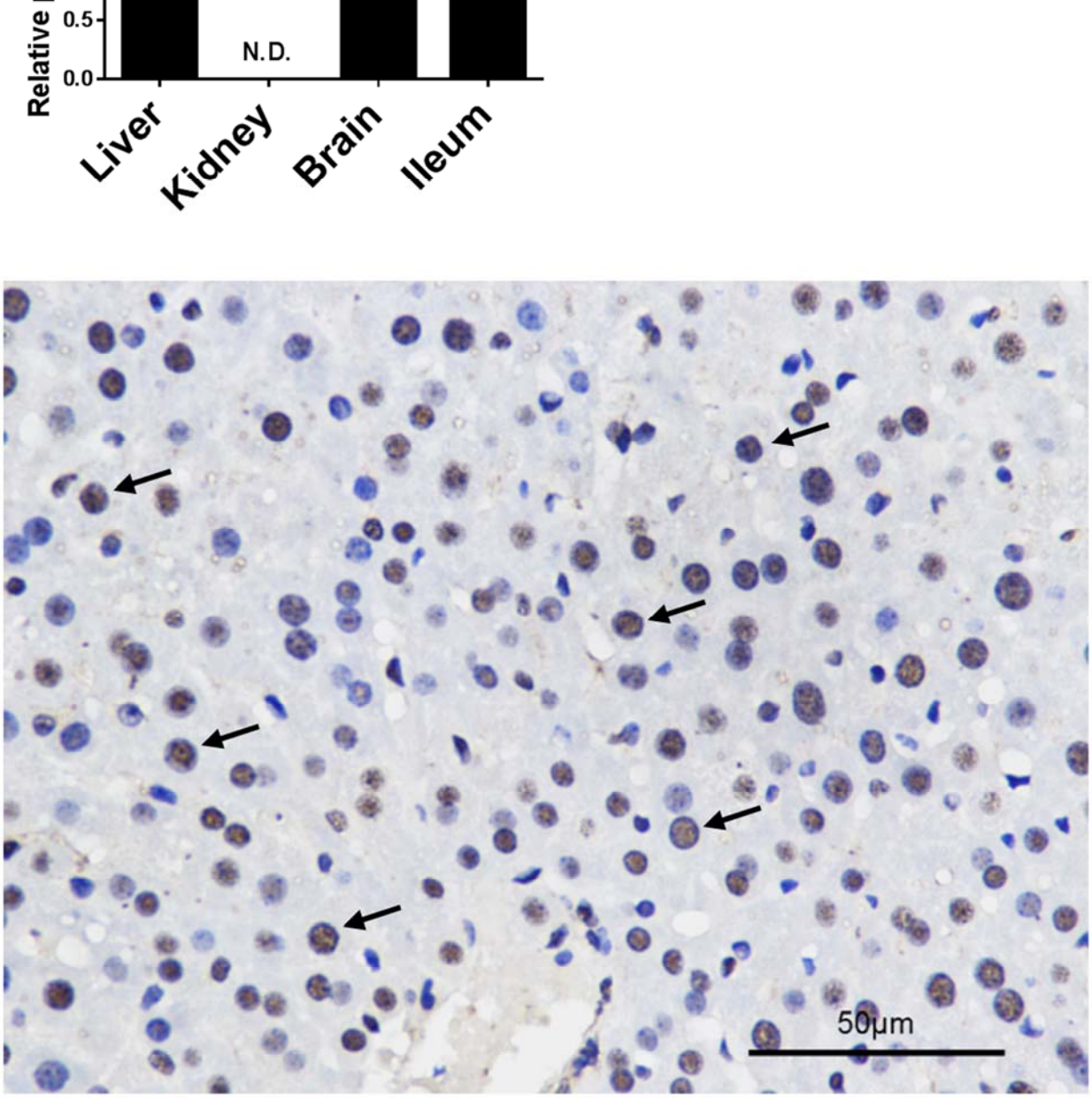

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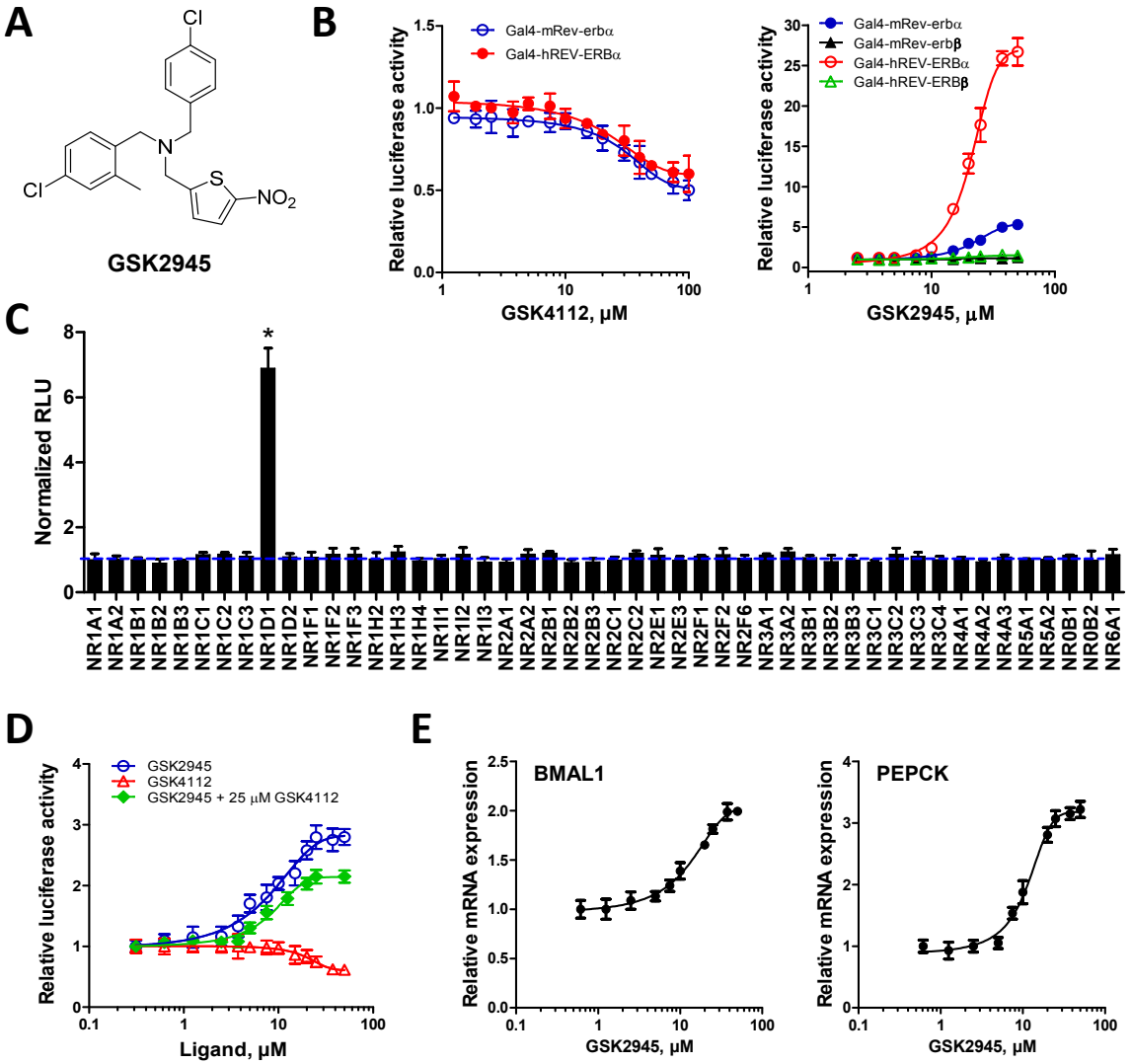

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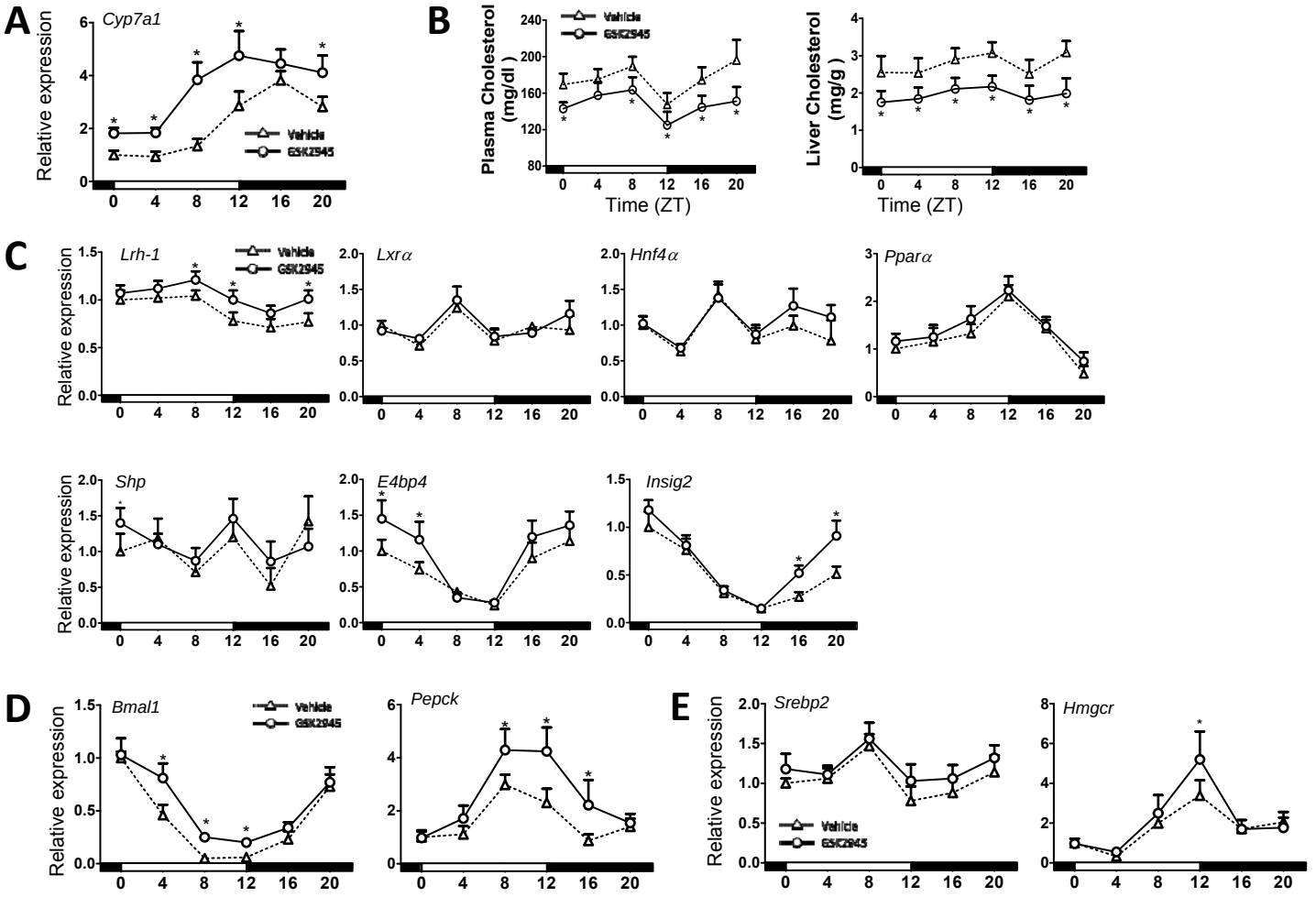

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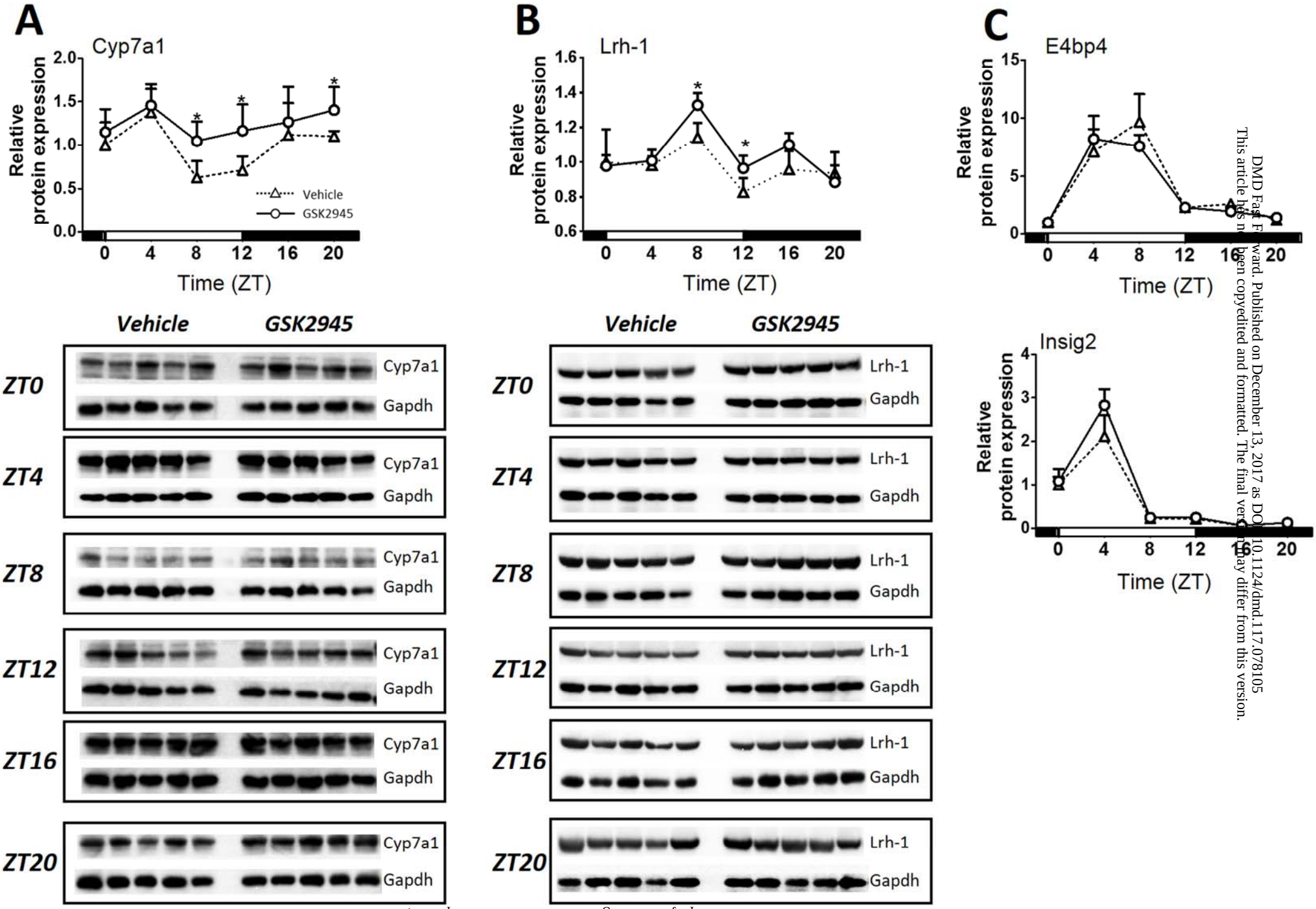

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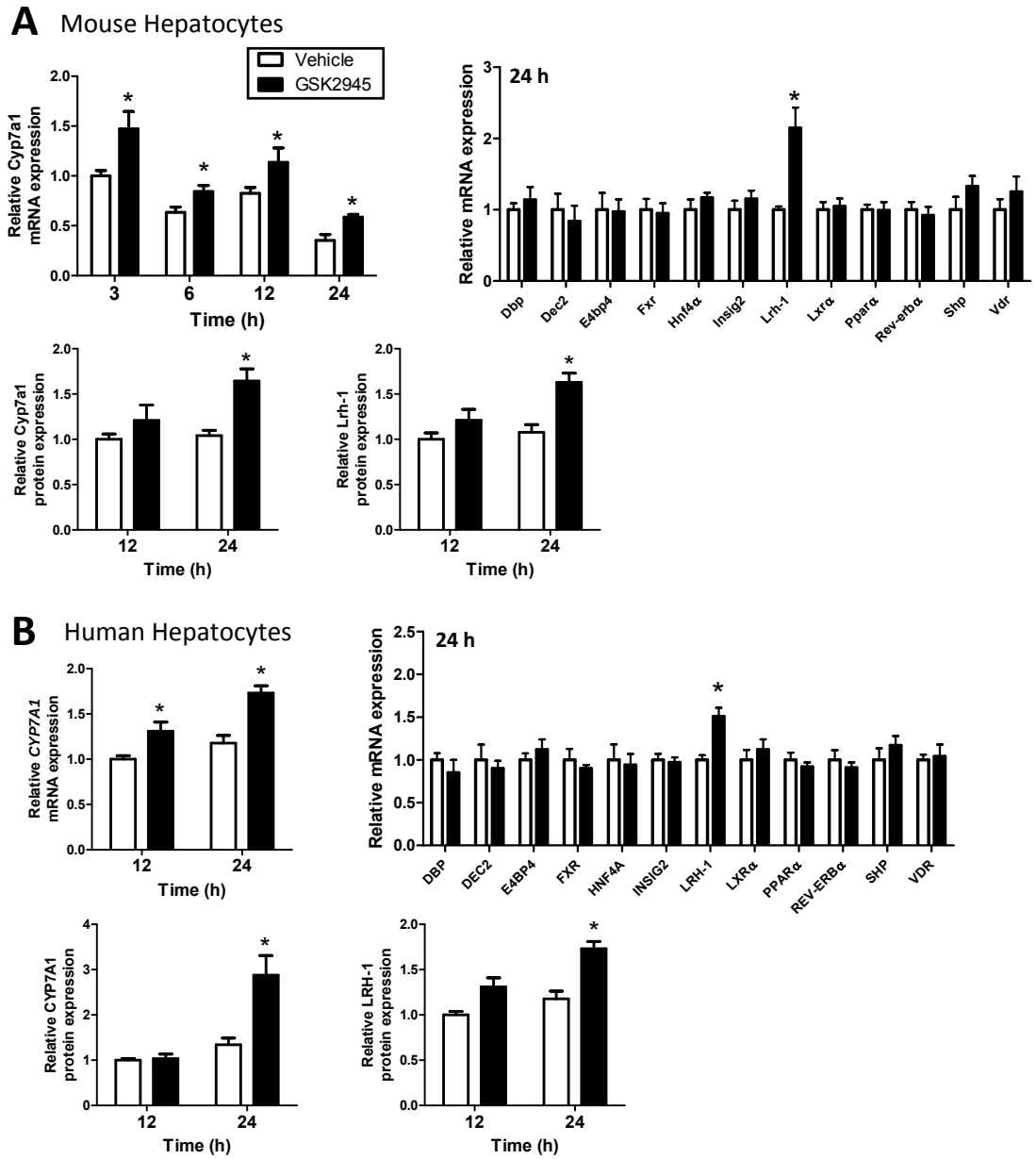

Figure 6

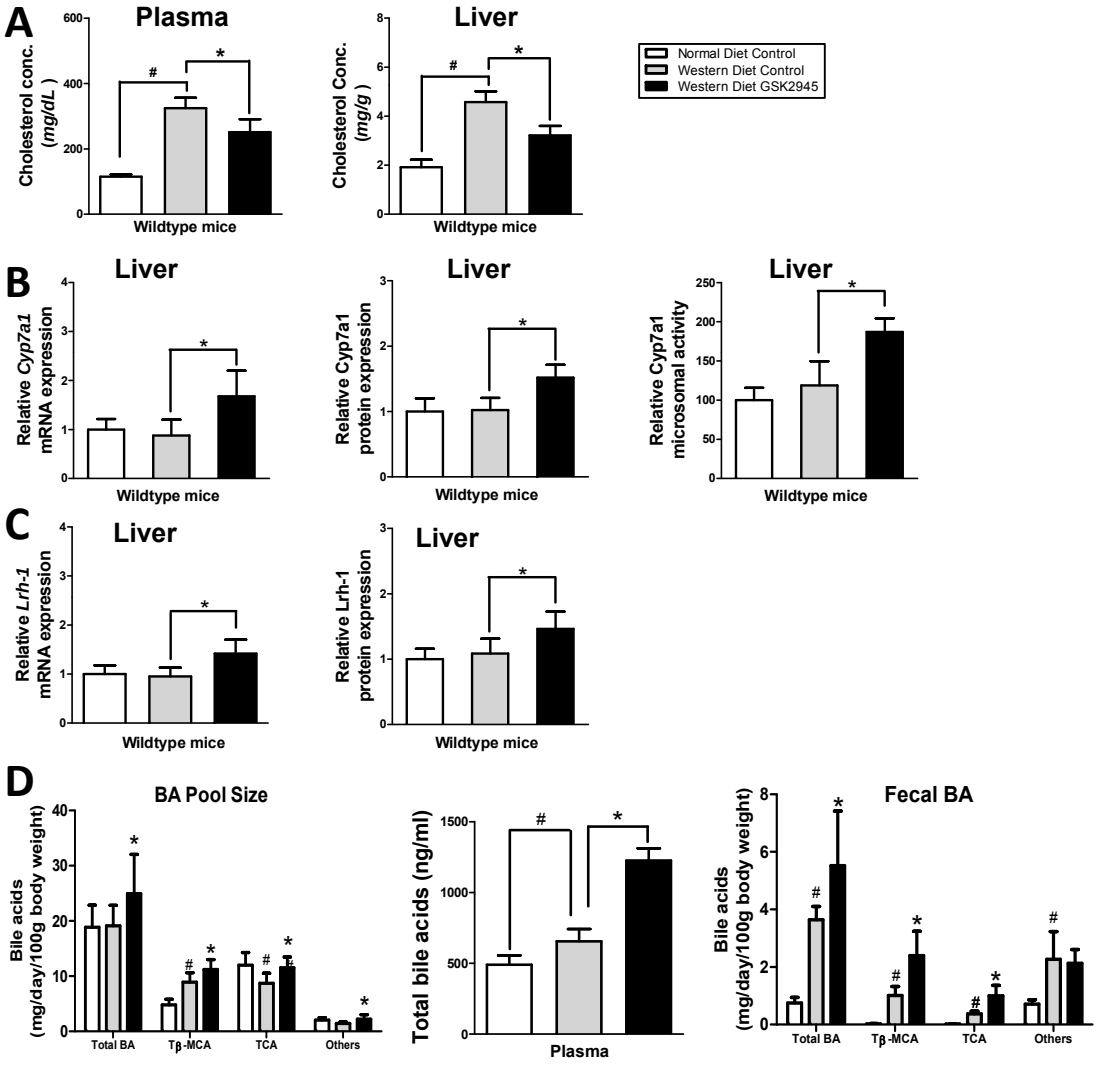

Figure 7

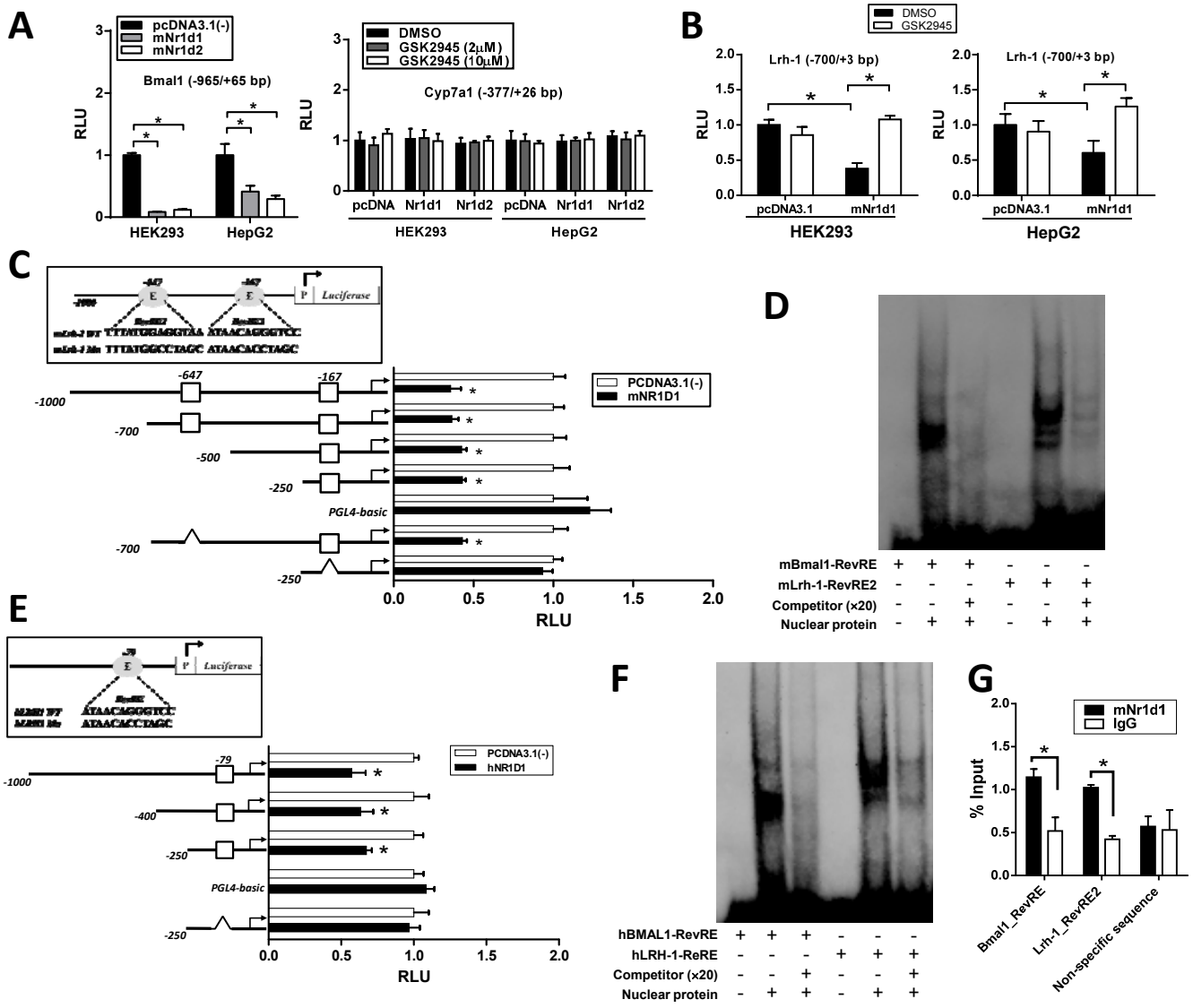

Figure 8