Repression of the nuclear receptor Small Heterodimer Partner (SHP) by steatotic drugs and in advanced Non-Alcoholic Fatty Liver Disease (NAFLD)

Marta Benet, Carla Guzmán, Sandra Pisonero-Vaquero, M. Victoria García-Mediavilla,

Sonia Sánchez-Campos, M. Luz Martínez-Chantar, M Teresa Donato, José Vicente Castell

and Ramiro Jover

Experimental Hepatology Unit, IIS Hospital La Fe, Valencia (MB, CG, MTD, JVC, RJ);

CIBERehd, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y

Digestivas (MB, MVGM, SSC, MLMC, MTD, JVC, RJ); Institute of Biomedicine (IBIOMED),

University of León (SPV, MVGM, SSC); CIC bioGUNE, Technology Park of Bizkaia

(MLMC); Department of Biochemistry and Molecular Biology, University of Valencia, Spain

(MTD, JVC, RJ).

Running title: *SHP Repression by C/EBPα in drug-induced steatosis*

Address correspondence to: Ramiro Jover Atienza, Unidad Mixta en Hepatología Experimental, IIS Hospital La Fe, Torre A-6.13, 46026-Valencia, Spain, Tel. +34 961 246 653; Fax. +34 961 246 620; Email: ramiro.jover@uv.es

Text Pages: 30

Tables: 0

Figures: 8

References: 62

Abstract Words: 234

Introduction Words: 743

Discussion Words: 1560

Abbreviations:

BA, bile acid; C/EBPα, CCAAT/enhancer-binding protein α; FA, fatty acid; FOXA1, forkhead box A1; GNMT, Glycine N-methyltransferase; HCC, hepatocellular carcinoma; MAT1A, methionine adenosyltransferase 1A; MCD, methionine- and choline- deficient; MEK, mitogen-activated protein kinase kinase; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PI3K, phosphatidylinositol 3 kinase; RXR, retinoid "X" receptor; ROS, reactive oxygen species; SHP, small heterodimer partner; TG, triglyceride; VLDL, very low density lipoprotein.

ABSTRACT

The small heterodimer partner (SHP, NR0B2) is an atypical nuclear receptor that lacks DNA-binding domain. It interacts with and inhibits many transcription factors, affecting key metabolic processes including bile acid, cholesterol, fatty acid and drug metabolism. Our aim was to determine the influence of steatotic drugs and non-alcoholic fatty liver disease (NAFLD) on SHP expression, and to investigate the potential mechanisms. SHP was found repressed by steatotic drugs (valproate, doxycycline, tetracycline and cyclosporin A) in cultured hepatic cells, and in the livers of different animal models of NAFLD: iatrogenic (tetracycline treated rats), genetic (glycine N-methyltransferase deficient mice) and nutritional (mice fed a methionine and choline deficient diet). Among the different transcription factors investigated, CCAAT-enhancer-binding protein α (C/EBPα) showed the strongest dominant-repressive effect on SHP expression in HepG2 and human hepatocytes. Reporter assays revealed that the inhibitory effect of C/EBPα and steatotic drugs co-localize between -340 and -509 bp of the SHP promoter and mutation of a predicted C/EBPα response element at -473 bp abolished SHP repression by both C/EBPα and drugs. Moreover, inhibition of major stress signaling pathways demonstrated that the mitogen-activated protein kinase kinase 1/2 pathway activates while the phosphatidylinositol 3 kinase pathway represses SHP in a C/EBP-dependent manner. We conclude that SHP is downregulated by several steatotic drugs and in advanced NAFLD. These conditions can activate signals that target C/EBPα and consequently repress SHP, thus favoring the progression and severity of NAFLD.

INTRODUCTION

The Small Heterodimer Partner (SHP, NR0B2) is a unique orphan nuclear receptor that contains a dimerization and ligand-binding domain, but lacks the conserved DNA-binding domain (Garruti et al., 2012; Zhang et al., 2011). SHP interacts with a wide variety of conventional nuclear receptors and transcription factors and negatively regulates their transcriptional activity (Bavner et al., 2005). SHP expression is predominantly observed in the liver (Lee et al., 1998; Seol et al., 1996), where a large number of direct and indirect SHP target genes have been identified, including bile acid (BA), cholesterol, fatty acid (FA), glucose, and drug metabolism genes (Boulias et al., 2005; Zhang et al., 2011). Moreover, the involvement of SHP in key hepatic regulatory networks denotes its relevance and central role in liver homeostasis (Garruti et al., 2012; Zhang et al., 2011). Therefore, chronic deregulation of SHP expression may lead to pathological consequences and favor liver disease. The potential negative consequences of SHP deregulation can be extrapolated from studies with liver-specific SHP transgenic and SHP deficient mice (Boulias et al., 2005; Chanda et al., 2009; Park et al., 2008; Wang et al., 2003; Zhang et al., 2010; Zhang et al., 2014; Zhang et al., 2008).

BAs are potent agonists of FXRα, which induces the expression of SHP. Induced SHP in turn leads to transcriptional repression of the rate-limiting CYP7A1 and CYP8B1 enzymes of the BA biosynthetic pathway (Chiang, 2003). In line with this, transgenic mice with sustained overexpression of SHP in the liver show depletion of the hepatic BA pool. Moreover, these mice show an indirect activation of the lipogenic factors PPARγ and SREBP-1c, which trigger triglyceride (TG) accumulation and fatty liver (Boulias et al., 2005).

SHP also represses genes with an important role in liver fibrosis (Zhang et al., 2011). Consequently, the lack of expression of SHP in SHP$^{-/-}$ mice results in increased sensitivity to liver damage and liver fibrosis induced by bile duct ligation (Park et al., 2008; Zhang et al., 2014). In agreement, progressive fibrosing steatohepatitis was reversed in wild-type mice after AMPK-mediated induction of SHP, but this was not observed in SHP$^{-/-}$ mice (Chanda et al., 2009). SHP deficiency could have even more severe consequences as spontaneous

hepatocellular carcinoma (HCC) is observed in SHP$^{-/-}$ mice due to hepatocyte hyperproliferation and increased cyclin D1 expression (Zhang et al., 2008). Moreover, SHP targets mitochondria, induces apoptosis and inhibits tumor growth (Zhang et al., 2010), but this protective role may be lost when SHP is deficient.

All these studies reinforce the notion that SHP controls major liver functions and, therefore, sustained changes in SHP expression may significantly trigger or worsen liver injury and disease progression. Induction of SHP may exacerbate diet-induced dyslipidemia and fatty liver (Boulias et al., 2005; Hartman et al., 2009). On the contrary, repression of SHP may promote fibrosis and HCC (Chanda et al., 2009; Park et al., 2008; Zhang et al., 2010; Zhang et al., 2014; Zhang et al., 2008). A key question, then, is whether hepatic SHP expression is altered in pathophysiological conditions.

It has been proven that repression of SHP by epigenetic silencing is a common denominator of HCC (He et al., 2008). However, the regulation of SHP in other conditions, such as hazardous xenobiotic exposure or fatty liver disease, is not so well characterized or is controversial. The response of SHP to endogenous factors such as BAs, FAs, cholesterol, glucose, insulin, cytokines and hormones has been well characterized (Garruti et al., 2012; Zhang et al., 2011), but the effect of drugs and xenobiotics on SHP has not been investigated. On the other hand, SHP seems to be up-regulated in genetic (leptin-deficient ob/ob mice) and dietary (high-sucrose or high-fat diet) mouse models of fatty liver (Huang et al., 2007; Trauner, 2007). However, SHP expression appears diminished in human non-alcoholic steatohepatitis (NASH) (Zhang et al., 2014).

Our aim in the present study was to determine whether SHP is regulated by steatotic dugs and NAFLD, and to investigate the potential mechanisms. We demonstrate that SHP is repressed by several steatotic drugs and that SHP is down-regulated in models of severe NAFLD. We identify C/EBPα as a SHP repressor and demonstrate that several steatotic drugs and stress pathways modulate SHP by signaling via a C/EBP response element at -473 bp in the human SHP promoter.

This study uncovers new mechanisms of transcription regulation of the human SHP gene and reveals novel pathways that target SHP expression and that could favor the severity and progression of liver disease.

MATERIAL AND METHODS

Animals – Male mice (3 and 9 month of age) deficient in methionine adenosyltransferase 1A (MAT1A) (Lu et al., 2001; Martinez-Chantar et al., 2002) and in glycine N-methyltransferase (GNMT) (Martinez-Chantar et al., 2008), and their wild-type (WT) C57BL/6 littermates, were obtained from the animal facility of CIC bioGUNE (Derio, Bizkaia, Spain). Mice were housed at 22 °C with a 12-h light–dark cycle and allowed food (Teklad Global 18% Protein Rodent Diet 2018S, Madison, WI, USA) and water ad libitum. To induce NAFLD, male C57BL/6J mice (10–12 weeks old) were fed a MCD diet for up to 5 weeks (cat. no. 960439, ICN, Aurora, OH, USA). Control mice received the same MCD diet supplemented with DL-methionine (3 g/kg) and choline chloride (2 g/kg) (cat. no. 960441, ICN). At the end of the experiments, mice were anesthetized (sodium pentobarbital, 60 mg/kg, i.p.) and livers collected. The presence of steatohepatitis was proven histologically. Male Sprague Dawley rats (125 g) were purchased from Charles River (Barcelona, Spain), maintained for four weeks in an air-conditioned animal room (21-25 ºC, 30-70% humidity, 12-h light–dark cycle) and fed ad libitum with a standard diet (D12450B, Research Diets, Gentofte, Denmark). Rats were randomly placed into two groups (n=6) and tetracycline dissolved in methylcellulose 0.5% (vehicle) was administered by oral gavage (2g/Kg/day) during four consecutive days. Control rats were treated with vehicle. Animals were euthanized 24 h after the last administration and livers collected. The Institutional Animal Care and Use Committee that approved specifically this study was the Bioethical and Animal Welfare Committee of the CIC bioGUNE (IACUC: P-CBG-CBBA 02-10) in accordance with the guidelines of European Research Council for animal care and use.

Culture of human HepG2 and HepaRG cells - HepG2 cells (ATCC HB-8065, Rockville, MD) were cultured in Ham's F-12/Leibovitz L-15 (1:1, v/v) medium (Gibco BRL, Invitrogen,

Barcelona, Spain), supplemented with 7% newborn calf serum and 2 mM L-Glutamine. In addition, culture media were supplemented with 50 U/ml penicillin and 50 µg /ml streptomycin. HepaRG cells were obtained from Biopredic International (Rennes, France). Undifferentiated cells were cultured in growth medium (Biopredic) for two weeks, and cell differentiation was induced with differentiation medium (Biopredic) for two more weeks. At that stage, HepaRG cells reached a differentiated hepatocyte-like morphology. Cells were further maintained in HepaRG differentiation medium for the duration of the experiments.

Isolation and culture of hepatocytes - Human hepatocytes were isolated from non-steatotic liver biopsies (1–4 g) using a two-step perfusion technique (Gomez-Lechon et al., 1990). Liver samples were obtained in conformity with the rules of the Hospital's Ethics Committee. None of the donors (4 men and 1 women aged between 37 and 74) were regular consumers of alcohol or other drugs, and were not suspected of harboring any infectious disease. Rat hepatocytes were obtained from Sprague Dawley male rats (180-250 g) by perfusion of the liver with collagenase. Freshly isolated hepatocytes (150 x 10^3 viable cells/cm^2) were plated on a 6-well plate precoated with a collagen gel layer. Sandwich cultures were established by the deposition of a second layer of collagen 24-h later as described elsewhere (Gomez-Lechon et al., 1998). Cells were cultured in serum-free Williams/Ham's F-12 (1/1, v/v) medium (Gibco BRL, Invitrogen, Barcelona, Spain), supplemented with 5 mM glucose, 0.2% BSA, 0.01 µM insulin and 0.01 µM dexamethasone.

Chemicals and treatment of cultured cells - Amitriptyline, cyclosporin A, doxycycline, fluoxetine, flutamide, ketotifen, simvastatin, tamoxifen, tetracycline, tianeptine, tilorone, valproate, U0126 and LY294002 were acquired from Sigma (Madrid, Spain). Steatotic drugs and non-steatotic compounds were chosen on the basis of previous information on their steatotic, phospholipidosic, hepatotoxic or non-hepatotoxic effect (Benet et al., 2014). Stock solutions were prepared in DMSO or water, and were diluted in culture medium. Kinase inhibitors U0126 and LY294002 were prepared in DMSO and added 24 h before analysis. The

final concentration of DMSO never exceeded 0.5% (v/v), and control cultures were treated with the same amount of solvent.

Adenoviral infection - The recombinant adenoviruses encoding CAR, PXR, C/EBPα, C/EBPβ, FOXA1, PGC1α, RXRα and PPARα were developed in our laboratory as described (Guzman et al., 2013). Cultured cells were infected with recombinant adenoviruses encoding transcription factors or with an insertless adenoviral vector (Ad-CTRL) for 24 h at a multiplicity of infection (MOI) ranging from 2 to 45 plaque-forming units/cell. Thereafter, cells were washed and fresh medium was added. To activate the expressed nuclear receptors (CAR, PXR, RXRα and PPARα) selective ligand agonists were added at 24 h post-infection and at 48 h post-infection cells were harvested and analyzed. Ligands were dissolved in DMSO and added to culture media at the following concentrations: 500 nM CITCO (CAR), 1 μM GW7647 (PPARα), 50 μM Rifampicin (PXR) and 1 μM 9-cis retinoic acid (RXRα). Control cultures were treated with 0.5% DMSO.

RNA purification, reverse transcription and real-time quantitative PCR - Total RNA was extracted from cultured cells with the RNeasy Plus Mini Kit from Qiagen (Madrid, Spain). To extract RNA from tissue, frozen mouse and rat liver samples (25-50 mg) were placed in 2 ml tubes containing CK14 ceramic beads (Precellys, Saint Quentin en Yvelines, France) and 800 μl of RLT buffer (Qiagen). Then, liver tissues were homogenized twice for 10 s at 6,000 rpm at 4°C in a Precellys 24 Dual system equipped with a Criolys cooler (Precellys). Tubes were centrifuged at 3000 g for 5 min at 4°C, and total RNA was extracted from supernatants and reverse transcribed as described (Benet et al., 2014). Diluted cDNA was amplified in a LightCycler 480 Instrument (Roche Applied Science) using LightCycler DNA Master SYBR Green I (Roche Applied Sciences, Barcelona, Spain) and. In parallel, mouse and rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or human porphobilinogen deaminase (PBGD) were analyzed for normalization. In each amplification, a reference calibrator cDNA made from a pool of human livers was included. The relative mRNA was calculated with the LightCycler Relative Quantification Analysis software. The efficiency of each PCR reaction

was estimated from a serially diluted human liver cDNA curve. Based on these PCR efficiencies and the cycle thresholds (Ct) the relative concentration of target and reference (GAPDH or PBGD) cDNAs and their ratio was determined.

Specific primers for real-time quantitative PCR were as follows: human PBGD, forward, 5′- CGGAAGAAAACAGCCCAAAGA and reverse, 5′- TGAAGCCAGGAGGAAGCACAGT; human SHP, forward, 5′- CAGCAGCAGTGGAGGCAGTG and reverse, 5′- CGGGGGTTGAAGAGGATGGTC; human SREBP1c, forward, 5′- GGAGGGGTAGGGCCAACGGCCT and reverse, 5′- CATGTCTTCGAAAGTGCAATCC; mouse and rat GAPDH, forward, 5′- AGGGCTCATGACCACAGTCCAT and reverse, GCCAGTGAGCTTCCCGTTCAG; mouse SHP, forward, AGGGCACGATCCTCTTCAAC and reverse, AGGGCTCCAAGACTTCACAC; rat SHP, forward, GGCACTATCCTCTTCAACCCA and reverse, TCCAGGACTTCACACAATGCC.

Protein samples and immunoblotting analysis - Cultured cells or liver tissue were homogenized in M-PER or T-PER reagent from Pierce (Fisher Scientific, Madrid, Spain), and a protease inhibitor cocktail. Protein concentration was determined using the Protein Assay Kit from Bio-Rad Laboratories (Madrid, Spain). Proteins were resolved by 12% SDS-PAGE, transferred to Immobilion membranes (Millipore Iberica, Madrid, Spain) and incubated with anti-SHP, anti-tubulin (sc-30169 and sc-8035, Santa Cruz Biotechnology, CA) or anti-beta-actin (Sigma-Aldrich, Madrid, Spain) antibodies. Blots were developed with horseradish peroxidase-labeled IgG (DAKO, Glostrup, Denmark) using an ECL kit from Amersham Biosciences/GE Healthcare (Buckinghamshire, England). Equal loading was verified by Coomassie Brilliant Blue or Ponceau S staining.

Plasmid Constructs - Chimeric luciferase reporter constructs with different lengths of the 5' flanking region of the human SHP gene were obtained by cloning SHP-5′ flanking sequences into the enhancerless, promoterless pGL3-Basic vector (Promega Biotech Iberica, Madrid, Spain). The -2500 to +27bp SHP-5′ flanking sequence was synthesized by GenScript Technologies (Piscataway, NJ, USA) and cloned in pUC57 plasmid. Fragments corresponding

to -2500, -1362 and -612 were excised from pUC57 plasmid by restriction enzymes to be subcloned in pGL3-Basic. Three DNA fragments spanning nucleotides -509, -340, -150 to +10 bp of the SHP 5′ flanking region were PCR-cloned from -612-SHP-pGL3 plasmid using the Expand High Fidelity PCR System (Roche Applied Science). The PCR primers used are: SHP-UP-150-MluI, 5′-CCTACGCGTACCACTTCCCCACCAT, SHP-UP-340-MluI, 5′-AATACGCGTACACCTGCTGATTGTG, SHP-UP-509-MluI, 5′-CTGACGCGTGCTTCTGGCTGACAACA and SHP-DN-HindIII, 5′-CTCAAGCTTCCAGCTCTCTGGC. The resulting amplicons were digested with the restriction and ligated into the pGL3-Basic vector previously digested with the same enzymes.

Chromatin immunoprecipitation assay - ChIP assay was done as previously reported (Benet et al., 2010). Briefly, HepG2 cells were infected with Ad-C/EBPα or with a control (insertless) adenovirus (Ad-pACC) and 48h after infection cells were treated with 1% formaldehyde for 10 min. Thereafter, cells were lysed and sonicated on ice for 8 x 15s steps at 20% output in a Branson Sonicator. DNA content was quantified, and properly diluted to maintain an equivalent amount of DNA in all samples (input DNA). For immunoprecipitation of C/EBPα-DNA complexes, 4 µg of specific antibody (sc-61, Santa Cruz Biotechnology) was added (bound DNA fraction). Mock immunoprecipitation with rabbit preimmune IgG (sc-2027, Santa Cruz Biotechnology) was performed in parallel (background DNA fraction). Samples were incubated overnight at 4°C on a 360° rotator. The immunocomplexes were affinity absorbed with protein G agarose/Salmon Sperm DNA (Millipore), and collected by centrifugation. DNA fractions were washed and the cross-links reversed using 100 µl of 10% Chelex (Bio-Rad Laboratories) (Benet et al., 2010). Amplification was real-time monitored, stopped in the exponential phase of amplification and analyzed by agarose gel electrophoresis. Amplification of a SHP 5'-flanking sequence (-329/-678 bp) among the pull of DNA was performed with specific primers flanking these region: forward, 5′- CAGACCTGGCCTTTCAGGAG and reverse, 5′-ATCAGCAGGTGTCCCCATTG. An exonic region in the RPLP0 (ribosomal protein, large,

P0) gene devoid of C/EBPα consensus binding sites, and expected not to be enriched in antibody-bound DNA fractions, was PCR-amplified as a negative control (Guzman et al., 2013).

Statistical analysis - Differences among groups were analyzed by one-way ANOVA and Tukey's pairwise comparisons. Differences between groups were evaluated by the nonparametric Mann-Whitney test, as indicated. A p value of less than 0.05 was considered to be statistically significant. Values are expressed as mean±SD.

RESULTS

Regulation of SHP by drugs. Human hepatoma HepG2 cells were incubated for 24 h with different drugs previously characterized and classified as non-hepatotoxic (NH), hepatotoxic (H), phospholipidosic (P) and steatotic. The concentrations selected were always subcytotoxic ($\leq IC_{10}$), thus avoiding acute necrotic or apoptotic side effects (Benet et al., 2014). Moreover, we have previously shown that, in these conditions, the selected steatotic drugs trigger lipid accumulation in HepG2 cells (Benet et al., 2014). We observed that most of steatotic drugs (valproate, doxycycline, tetracycline, cyclosporine A and tianeptine) negatively affected SHP expression (Figure 1A). However, this was not a common effect observed with all known steatotic compounds as tamoxifen, amiodarone and zidovudine did not repress SHP (Figure 1A and data not shown). Hepatotoxic and phospholipidosic drugs did not influence or consistently affect SHP expression.

To better support the relevance of these findings we investigated the response of SHP to steatotic drugs in HepaRG cells, a more differentiated human hepatic cell model, which is much more similar to primary human hepatocytes and liver tissue than HepG2 cells (Hart et al., 2010). Results in Figure 1B demonstrate that cyclosporine A tetracycline, doxycycline and valproate also caused a significant repression of SHP in differentiated HepaRG cells.

To find out if the repressive effect of steatotic drugs occurs *in vivo*, we treated rats with tetracycline or vehicle during four consecutive days. Results demonstrate that tetracycline

strongly repressed SHP in rat liver (Figure 1C). Finally, we incubated cultured rat hepatocytes in collagen-sandwich configuration with some selected steatotic drugs to search for potential inter-species differences between human and rat models. We found that some steatotic drugs such as cyclosporine A and tetracycline decreased SHP expression as in human hepatic cultures; however, tamoxifen only significantly repressed SHP in rat hepatocytes (Figure 1D).

Regulation of SHP in mouse models of NAFLD. Liver SHP expression was first analyzed in two genetic models of NAFLD: MAT1A$^{-/-}$ and GNMT$^{-/-}$ mice. These two models spontaneously develop fatty liver, being the GNMT$^{-/-}$ a more severe NAFLD model than the MAT1A$^{-/-}$.

The 3-month-old MAT1A$^{-/-}$ mouse does not present hepatosteatosis, but shows increased expression of lipid synthesis genes (Martinez-Chantar et al., 2002) and decreased TG output in VLDL (Cano et al., 2011), whereas the 9-month-old MAT1A$^{-/-}$ exhibits macrovesicular steatosis (25–50% of hepatocytes) and focal areas of inflammation (Lu et al., 2001). Only the 9-month-old mouse shows increased serum aminotransferases, but fibrosis is not observed (Cano et al., 2011). Intriguingly, SHP was significantly induced in 3-month-old MAT1A$^{-/-}$ mice but no differences were observed in 9-month-old deficient mice with established fatty liver (Figure 2A).

The GNMT$^{-/-}$ mouse shows elevated serum aminotransferases at both 3 and 9 months of age. The livers of the 3-month-old mutant mouse already shows steatosis and fibrosis, which are more pronounced in the livers of 9-month-old animals. Moreover, apoptosis increases and genes involved in inflammation are induced. At 9 months, the GNMT$^{-/-}$ mouse also spontaneously develops multifocal HCC (Martinez-Chantar et al., 2008; Varela-Rey et al., 2010). SHP was found repressed in the liver of GNMT$^{-/-}$ mice, particularly in 9-month-old deficient mice that exhibit more manifest liver disease (Figure 2B).

We next investigated the regulation of SHP in the methionine-choline deficient (MCD) diet model of NAFLD. Mice fed the MCD diet for more than 2 weeks exhibit elevated serum aminotransferases, inflammation and hepatic fibrosis in addition to simple steatosis (Anstee and

Goldin, 2006; Itagaki et al., 2013). Liver SHP was also found repressed in mice on MCD diet. The repression began after 1 week and was very significant at 5 weeks of diet (Figure 2C). The reduced expression at the mRNA level correlated with progressive, reduced expression at the protein level as determined by immunoblotting (Figure 2C).

Altogether, these data support that both steatotic drugs and advanced NAFLD (NASH/fibrosis) trigger SHP down-regulation in the liver, and therefore, a common mechanism may exist that causes SHP gene repression in these conditions.

Screening for SHP gene repressors: identification of C/EBPα as a SHP dominant negative factor. SHP is activated by multiple transcription factors but no SHP gene repressor has been identified. We aimed to investigate the potential repressive role of new transcription factors, whose effects on SHP gene had not been investigated yet: C/EBPα, C/EBPβ, FOXA1, CAR, PXR, PPARα, RXRα and the coactivator PGC1α. These factors are associated with liver lipid/energy metabolism and the hepatic phenotype. Previous studies from our laboratory have shown that all these factors are expressed in HepG2 at lower level than in human liver (Donato et al., 2013; Guzman et al., 2013); which discourages the use of knock-down approaches to study their role. Therefore, we decided to force their re-expression and transfected HepG2 cells with adenoviral vectors encoding these factors. The experimental conditions selected induced expression in HepG2 to levels within the wide range found in human liver (Guzman et al., 2013).

Results demonstrate that C/EBPα and FOXA1 down-regulated SHP, whereas PGC1α and RXRα caused gene activation (Figure 3A). Moreover, C/EBPα, FOXA1 and RXRα caused the same effect on SHP in cultured human hepatocytes (Figure 3B).

Time-course analysis demonstrated that Ad-C/EBPα causes a fast downregulation, which reaches ~50% by 8 h after adenoviral infection, both in HepG2 and cultured human hepatocytes. Ad-FOXA1 required ≥ 24 h to trigger SHP gene repression (Figure 3C).

13

Combination of these transcription factors demonstrated that C/EBPα acts on SHP as a dominant negative factor, able to substantially repress both basal and RXRα-induced SHP levels. The repressive effect of FOXA1 was less significant when combined with RXRα, and no further repression was observed when combined with C/EBPα (Figure 4).

Analysis of SREBP1c, one of the few positive targets of SHP (Boulias et al., 2005), demonstrated a very similar response: C/EBPα repressed both basal and induced SREBP1c mRNA levels, whereas FOXA1 had no significant effect on this gene (Figure 4). These results indicate that repression of SHP by C/EBPα translates into downstream SHP-dependent functions.

Altogether our results demonstrate that C/EBPα is an effective dominant repressor of SHP both in HepG2 and cultured human hepatocytes.

C/EBPα negatively regulates the human SHP gene by binding to a response element at -473 bp in the SHP promoter. We cloned 2500 bp of the 5'-flanking region of the human SHP gene and generated serially deleted LUC reporter constructs. Basal reporter analysis of the different fragments in HepG2 cells indicated that the activity of the proximal promoter between +10 and -340 bp is very low (Figure 5). Transcription increased notably and stepwise between -340 and -612 bp; and no significant further increase was observed with longer sequences up to -2500 bp. When the basal response was assayed in the non-hepatic HeLa cells (Figure 5), a very low activity with all SHP promoter fragments was detected and no major increase in activity with the sequence beyond -340 bp was achieved. Results demonstrate that most of the constitutive transcription of the SHP gene in HepG2 depends on response elements (REs) located between -340 and -612 bp, and that the transcription factors acting on these region are likely liver-enriched factors, only present in the hepatic cell line. The significant increase in reporter activity observed in HepG2 with the sequence between -509 and -612 bp can be elicited by HNF4α (not present in HeLa) as an HNF4α-RE at -580 bp in the human SHP gene has been previously reported (Shih et al., 2001).

We next investigated the response of the SHP promoter to C/EBPα. Results in Figure 6A demonstrate that C/EBPα represses SHP-LUC activity in HepG2, in consonance with the negative effects of this factor on SHP expression (mRNA and protein). The repressive effect of C/EBPα located between -340 and -509 bp.

Sequence analyses with MatInspector Software and with defined C/EBP frequency matrices from PAZAR database lead to the identification of a potential C/EBP-RE at -473 bp (GTTAGGCAAA).

To prove that this is the RE for C/EBPα in the SHP promoter, we constructed a -509 bp reporter vector with 3 bp substitution in the predicted C/EBP-RE at -473 bp (509-mut, TCAAGGCAAA, Figure 6B). We found that mutation of this element completely abolished the repressive effect of C/EBPα on SHP (Figure 6B). Indeed, the 509-mut-SHP-LUC construct showed a very similar response to that of the 340-SHP-LUC.

Chromatin immunoprecipitation (ChIP) assays were performed to assess binding of the transcription factor to the native SHP promoter. The DNA sequence responsive to C/EBPα was PCR amplified after immunoprecipitation. Infection of HepG2 cells with Ad-C/EBPα caused a noticeable increase in the amount of immunoprecipitated SHP DNA by anti-C/EBPα antibody, suggesting actual binding of this transcription factor *in vivo* (Figure 6C & D). C/EBPα is expressed at low level in human hepatoma cells (Jover et al., 1998) and consequently forced re-expression by adenoviral vector is needed to observe significant binding by ChIP assay.

We conclude that C/EBPα represses the human SHP gene via direct binding to a newly identified C/EBP-RE located at -473 bp.

Steatotic drugs modulate SHP expression by signaling via the -473bp C/EBP-RE. We have demonstrated that steatotic drugs repress SHP mRNA in cultured hepatic cells and *in vivo*. Therefore, our next step was to analyze the response of the SHP promoter to these drugs. We found that the repressive effect of valproate (1 and 2.5 mM), doxycycline (250 and 500 µM) and cyclosporine A (25 and 50 µM) localized between -340 and -509 bp of the SHP promoter

(Figure 7A); the same fragment through which C/EBPα causes SHP repression, thus suggesting that steatotic drugs may repress SHP by signaling via C/EBPα.

To evaluate this possibility we analyzed the effect of steatotic drugs on the -509 bp reporter vector with the mutated C/EBP-RE (509-mut) and found that the repressive effects of these drugs were abolished or significantly reduced (Figure 7B). Results demonstrate that steatotic drugs repress SHP at least partially by a C/EBP-dependent mechanism.

MEK1/2 and PI3K kinases modulate SHP expression by signaling via the -473bp C/EBP-RE. Because SHP repression occurs both by steatotic drugs and in advanced steatosis, it is tempting to suggest that a common mechanism may be involved. Many different pathways may be involved in metabolic and iatrogenic NAFLD, including insulin, lipid intermediate, mitochondrial dysfunction, oxidative stress and inflammatory pathways (Larrain and Rinella, 2012). To preliminary investigate the involvement of major cell stress pathways on SHP repression, we incubated HepG2 cells with well-characterized inhibitors of PI3K (LY294002), p38 (SB203580), JNK (SP600125) and MEK1/2 (U0126) kinases. Results demonstrated that inhibition of PI3K resulted in up-regulation of SHP mRNA, whereas inhibition of MEK1/2 caused SHP repression. Maximal effects were observed by 2 h incubation (Figure 8A). Inhibition of p38 and JNK did not significantly modify SHP mRNA levels in HepG2 (data not shown).

We investigated next the effects of PI3K and MEK1/2 inhibitors on the 509-SHP-reporter construct. Inhibition of PI3K induced, whereas inhibition of MEK1/2 repressed the human SHP promoter (Figure 8B), in agreement with their effects on SHP mRNA. This suggests that these pathways modulate factors that control SHP gene transcription.

MEK1/2 activate ERK1/2 kinases, whereas PI3K represses GSK3 kinase. Both ERK1/2 and GSK3 can phosphorylate C/EBP factors and modulate C/EBPα activity (Nakajima et al., 1993; Ross et al., 2004),(Ross et al., 1999). It is then feasible to think that the effects of these signaling pathways on SHP could be C/EBP-dependent. To examine this possibility we compared the effects of the MEK1/2 and PI3K inhibitors on the wild-type and the C/EBP-RE-

mut SHP promoters. Results demonstrate that the repression of the 509-SHP-LUC by U0126 (MEK/ERK inhibitor) was partially but significantly abrogated in the 509-mut-SHP-LUC, whereas the induction of the 509-SHP-LUC by LY294002 (PI3K inhibitor, GSK3 activator) was completely abolished in the mutated construct (Figure 8B). This experimental evidence supports the notion that phosphorylated C/EBP forms (by ERK1/2 and/or GSK3) activate SHP, whereas non-phosphorylated C/EBPs cause SHP repression.

DISCUSSION

One important finding of this study is that several steatotic drugs repressed SHP in human HepG2 and HepaRG cells. However, other steatotic drugs failed to significantly repress SHP in these cell models. There are several possible explanations for this discrepancy. First, not all steatotic drugs share the same mechanism of action: inhibition of β-oxidation, increased TG synthesis, alterations of mitochondria (respiratory chain, membrane potential, oxidative phosphorylation and mtDNA), inhibition of lipoprotein synthesis and secretion, etc. (Begriche et al., 2011). Secondly, only some steatotic drugs induce lipid peroxidation, lipotoxicity and ROS. Regarding the present study, some steatotic drugs with no effect on SHP induced only a low level of lipid accumulation in HepG2 (i.e. tamoxifen and amiodarone) (Benet et al., 2014), whereas others did not induce ROS (i.e. tianeptine and zidovudine) (Donato et al., 2012). Therefore, a threshold level of lipid accumulation and a particular subsequent toxic mechanism may be required for drug-induced SHP repression.

The drug concentrations selected in this *in vitro* study were above therapeutic plasma concentrations. However, the ratio of the effective concentration *in vitro* to the therapeutic peak plasma concentration (Cmax) is considered a risk index that provides an estimation of the safety margin. We found significant effects of steatotic drugs on SHP at ratios of 10- (tetracycline and valproate) and 25-fold (cyclosporine A and doxycycline) Cmax. These ratios are considered low safety margins (O'Brien et al., 2006; Xu et al., 2008), and suggest these four steatotic drugs are at risk of having negative effects on SHP under therapeutic doses *in vivo*.

17

These observations prompted us to study the regulation of SHP in different mouse modes of fatty liver (iatrogenic and NAFLD/metabolic) and found that SHP was repressed in tetracycline treated rat livers, and in MCD diet and GNMT$^{-/-}$ mice livers, but was not repressed in the MAT1A$^{-/-}$ model. One major difference among these NAFLD models is that the livers of GNMT$^{-/-}$ and MCD diet mice show early symptoms of severe disease with fibrosis and even HCC in the GNMT$^{-/-}$ (Anstee and Goldin, 2006; Itagaki et al., 2013; Martinez-Chantar et al., 2008; Varela-Rey et al., 2010), whereas the MAT1A$^{-/-}$ express a less severe phenotype with delayed onset and no fibrosis or HCC (Lu et al., 2001; Martinez-Chantar et al., 2002).

In a previous study, Huang et al. (Huang et al., 2007) reported that SHP is up-regulated in fatty livers of genetic (ob/ob and KKAy) and dietary (high-sucrose and high-fat) mouse models. This apparent contradiction could also be due to differences in the disease phenotype of the models. Leptin deficient (ob/ob) and KKAy mice are similar models of hyperphagia, type 2 diabetes and obesity that do not develop steatohepatitis and are resistant to fibrosis. Similarly, high-fat diet (HFD) mice develop obesity, hyperinsulinemia and hyperglycemia. However, severe fibrosis and HCC do not appear even after prolonged HFD (Imajo et al., 2013; Takahashi et al., 2012). On the contrary, GNMT$^{-/-}$ and MCD mice develop steatohepatitis with fibrosis, but without obesity and insulin resistance (Imajo et al., 2013; Takahashi et al., 2012; Varela-Rey et al., 2010). Moreover, MCD diet induces greater ROS production, mitochondrial DNA damage and apoptotic cell death than many other diet models of NAFLD (Anstee and Goldin, 2006; Gao et al., 2004).

Therefore, we can speculate that SHP is up-regulated at the onset or in less severe NAFLD, but the progression of the disease, with inflammation, liver injury and fibrosis, triggers signals that inhibit SHP expression. In agreement with this view, SHP expression is significantly decreased in human NASH cirrhotic livers (Zhang et al., 2014).

Overall, our results suggest that, independently of their iatrogenic or metabolic origin, hepatosteatosis with associated liver injury triggers SHP downregulation. The potential clinical consequence of SHP repression could be extrapolated from studies with SHP$^{-/-}$ mice, which

showed increased sensitivity to liver damage and liver fibrosis (Chanda et al., 2009; Park et al., 2008; Zhang et al., 2014), as well as hepatocyte hyperproliferation and HCC (Zhang et al., 2008). Moreover, the repression of SHP could affect the crosstalk of SHP with other nuclear receptors, particularly with PXR, a well characterized lipogenic factor (Hoekstra et al., 2009; Lee et al., 2008). SHP suppresses PXR, which is also considered a "master regulator" of drug/xenobiotic metabolism (Krausova et al., 2011). On the contrary, PXR activation by rifampicin inhibits SHP (Li and Chiang, 2006). Therefore, the repression of SHP by steatotic drugs and in advanced NAFLD could lead to higher PXR activity and enhanced lipogenesis, which would also contribute to disease severity and progression.

However, the potential negative consequences mentioned above need to be demonstrated in future studies, as the data presented here do not demonstrate a causative link between the downregulation of SHP and the severity and progression of the disease. Our study has additional limitations because the different animal models are very complex systems and presumably many genes (not only SHP) show altered expression.

Because of the critical role of SHP in liver metabolism and disease, many different studies have addressed the regulation of the SHP gene. It has been found that SHP is activated by many transcription factors in response to multiple signaling pathways (Garruti et al., 2012; Zhang et al., 2011). In the present study we demonstrate that RXRα (+ 9-cis-retinoic acid) also induces SHP. This result is in line with the induction of SHP by all-trans retinoic acid (Cai et al., 2010), and suggest that SHP can be regulated by retinoids via RXRα homodimers or RXRα-RAR heterodimers. Nevertheless, increased SHP expression by RXRα could also be mediated by other heterodimers such as RXRα-FXR, RXRα-LXRα or RXRα-PPARγ (Kim et al., 2007).

In the present study we have also uncovered two new SHP repressors: FOXA1 and C/EBPα. However, comparative analysis showed that C/EBPα was faster and more effective, and dominantly repressed SHP induction by RXRα. Further analyses demonstrated binding of C/EBPα to a negative response element at -473 bp in the human SHP gene. Mutation of this element evidenced its relevance for the regulation of SHP by C/EBPα, steatotic drugs and stress

signaling pathways. Based on these results, it is feasible to suggest that lipid-dependent insults triggered by iatrogenic or metabolic steatosis can alter or modulate signaling pathways that modify C/EBPα phosphorylation and consequently cause SHP repression.

Our results demonstrate that inhibition of MEK1/2 kinases caused a very significant repression of both SHP mRNA and SHP-LUC activity. These results suggest that conditions causing MEK/ERK inhibition will cause SHP down-regulation. ERK1/2 inhibition has already been reported in several models of NAFLD. For instances, mice on MCD diet showed a remarkable suppression of hepatic ERK1/2 activation (Wang et al., 2010a). On the opposite, an increase in the level of phosphorylated ERK1/2 improved liver steatosis (Aghazadeh and Yazdanparast, 2010). Chronic alcohol also caused a significant suppression of hepatic ERK1/2 activation and triggered fatty liver (Wang et al., 2010b). We hypothesize that one of the MEK/ERK targets could be C/EBPα or C/EBPβ. ERK can phosphorylate C/EBPα at Ser 21 (Ross et al., 2004). Moreover, phosphorylation of C/EBPβ at Thr235 by ERK2 is a key determinant of its capacity for trans-activation (Nakajima et al., 1993). Based on these studies we suggest that phosphorylated C/EBPs would activate SHP, whereas dephosphorylated forms would not activate or repress SHP.

Inhibition of PI3K induced both SHP mRNA and SHP-LUC activity in HepG2 cells. These results suggest that conditions causing PI3K activation will cause SHP repression. It has been shown that mitochondrial ROS induces hepatocyte steatosis by up-regulating the PI3K pathway (Kohli et al., 2007) and that hepatocytes exposed to MCD medium developed significant and progressive steatosis along with activation of PI3K/Akt (Sahai et al., 2006). Moreover, PI3K (p85) was significant higher in NAFLD patients (Xu et al., 2011). Activation of PI3K leads to GSK3 inhibition, and GSK3 phosphorylates C/EBPα on an evolutionarily conserved consensus site (Thr222, Thr226 and Ser230) (Ross et al., 1999). Moreover, activation of the PI3K/Akt pathway in proliferating hepatocytes and in liver tumor/hepatoma cells induced the protein phosphatase 2A-mediated dephosphorylation of C/EBPα on Ser193, leading to a failure of C/EBPα to cause growth arrest (Wang et al., 2004). Finally, insulin, a major activator of the

PI3K pathway, causes a very significant repression of SHP in primary cultured hepatocytes (Van Rooyen et al., 2011). Therefore, the inhibitory activity of PI3K on SHP could be mediated through the reciprocal repression of GSK3 and/or activation of protein phosphatases, leading both to dephosphorylation of C/EBPα, which in turn would cause SHP repression. These results again suggest that dephosphorylated C/EBPα is the SHP repressor. Adenovirus-mediated overexpression of C/EBPα would mainly generate non-phosphorylated (repressing) C/EBPα protein.

C/EBP proteins are well known recipient of extracellular signals resulting in multiple phosphorylations, acetylations and SUMOylations (Nerlov, 2008). Up to 8 different phosphorylation sites have been described in C/EBPα (Tsukada et al., 2011), and therefore the investigation of the precise phosphorylated forms involved in SHP regulation is complex and is out of the scope of this paper.

In summary, we hypothesize that the repression of SHP by C/EBPα is likely dependent on dephosphorylated C/EBPα. A similar scenario has been demonstrated for the hepatic phosphoenolpyruvate carboxykinase gene which is activated (Qiao et al., 2006) or repressed (Pedersen et al., 2007) by C/EBPα depending on its phosphorylation status. Our data also support that harmful xenobiotics or metabolic conditions causing inhibition of MEK1/2 or activation of PI3K pathways will trigger a C/EBPα-dependent repression of SHP, which could favor the progression and severity of NAFLD.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Benet, Castell, Jover.

Conducted experiments: Benet, Guzmán, Pisonero-Vaquero, García-Mediavilla, Sánchez-Campos, Martínez-Chantar.

Contributed new reagents or analytic tools: Donato, Castell, Jover.

Performed data analysis: Benet, Jover.

Wrote or contributed to the writing of the manuscript: Benet, Jover.

REFERENCES

Aghazadeh S and Yazdanparast R (2010) Inhibition of JNK along with activation of ERK1/2 MAPK pathways improve steatohepatitis among the rats. *Clinical nutrition* **29**(3): 381-385.

Anstee QM and Goldin RD (2006) Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. *International journal of experimental pathology* **87**(1): 1-16.

Bavner A, Sanyal S, Gustafsson JA and Treuter E (2005) Transcriptional corepression by SHP: molecular mechanisms and physiological consequences. *Trends in endocrinology and metabolism* **16**(10): 478-488.

Begriche K, Massart J, Robin MA, Borgne-Sanchez A and Fromenty B (2011) Drug-induced toxicity on mitochondria and lipid metabolism: mechanistic diversity and deleterious consequences for the liver. *Journal of hepatology* **54**(4): 773-794.

Benet M, Lahoz A, Guzman C, Castell JV and Jover R (2010) CCAAT/enhancer-binding protein alpha (C/EBPalpha) and hepatocyte nuclear factor 4alpha (HNF4alpha) synergistically cooperate with constitutive androstane receptor to transactivate the human cytochrome P450 2B6 (CYP2B6) gene: application to the development of a metabolically competent human hepatic cell model. *The Journal of biological chemistry* **285**(37): 28457-28471.

Benet M, Moya M, Donato MT, Lahoz A, Hervas D, Guzman C, Gomez-Lechon MJ, Castell JV and Jover R (2014) A simple transcriptomic signature able to predict drug-induced hepatic steatosis. *Archives of toxicology* **88**(4): 967-982.

Boulias K, Katrakili N, Bamberg K, Underhill P, Greenfield A and Talianidis I (2005) Regulation of hepatic metabolic pathways by the orphan nuclear receptor SHP. *The EMBO journal* **24**(14): 2624-2633.

Cai SY, He H, Nguyen T, Mennone A and Boyer JL (2010) Retinoic acid represses CYP7A1 expression in human hepatocytes and HepG2 cells by FXR/RXR-dependent and independent mechanisms. *Journal of lipid research* **51**(8): 2265-2274.

Cano A, Buque X, Martinez-Una M, Aurrekoetxea I, Menor A, Garcia-Rodriguez JL, Lu SC, Martinez-Chantar ML, Mato JM, Ochoa B and Aspichueta P (2011) Methionine adenosyltransferase 1A gene deletion disrupts hepatic very low-density lipoprotein assembly in mice. *Hepatology* **54**(6): 1975-1986.

Chanda D, Lee CH, Kim YH, Noh JR, Kim DK, Park JH, Hwang JH, Lee MR, Jeong KH, Lee IK, Kweon GR, Shong M, Oh GT, Chiang JY and Choi HS (2009) Fenofibrate differentially regulates plasminogen activator inhibitor-1 gene expression via adenosine monophosphate-activated protein kinase-dependent induction of orphan nuclear receptor small heterodimer partner. *Hepatology* **50**(3): 880-892.

Chiang JY (2003) Bile acid regulation of hepatic physiology: III. Bile acids and nuclear receptors. *American journal of physiology Gastrointestinal and liver physiology* **284**(3): G349-356.

Donato MT, Jover R and Gomez-Lechon MJ (2013) Hepatic cell lines for drug hepatotoxicity testing: limitations and strategies to upgrade their metabolic competence by gene engineering. *Current drug metabolism* **14**(9): 946-968.

Donato MT, Tolosa L, Jimenez N, Castell JV and Gomez-Lechon MJ (2012) High-content imaging technology for the evaluation of drug-induced steatosis using a multiparametric cell-based assay. *Journal of biomolecular screening* **17**(3): 394-400.

Gao D, Wei C, Chen L, Huang J, Yang S and Diehl AM (2004) Oxidative DNA damage and DNA repair enzyme expression are inversely related in murine models of fatty liver disease. *American journal of physiology Gastrointestinal and liver physiology* **287**(5): G1070-1077.

Garruti G, Wang HH, Bonfrate L, de Bari O, Wang DQ and Portincasa P (2012) A pleiotropic role for the orphan nuclear receptor small heterodimer partner in lipid homeostasis and metabolic pathways. *Journal of lipids* **2012**: 304292.

Gomez-Lechon MJ, Jover R, Donato T, Ponsoda X, Rodriguez C, Stenzel KG, Klocke R, Paul D, Guillen I, Bort R and Castell JV (1998) Long-term expression of differentiated functions in hepatocytes cultured in three-dimensional collagen matrix. *Journal of cellular physiology* **177**(4): 553-562.

Gomez-Lechon MJ, Lopez P, Donato T, Montoya A, Larrauri A, Gimenez P, Trullenque R, Fabra R and Castell JV (1990) Culture of human hepatocytes from small surgical liver biopsies. Biochemical characterization and comparison with in vivo. *In vitro cellular & developmental biology* **26**(1): 67-74.

Guzman C, Benet M, Pisonero-Vaquero S, Moya M, Garcia-Mediavilla MV, Martinez-Chantar ML, Gonzalez-Gallego J, Castell JV, Sanchez-Campos S and Jover R (2013) The human liver fatty acid binding protein (FABP1) gene is activated by FOXA1 and PPARalpha; and repressed by C/EBPalpha: Implications in FABP1 down-regulation in nonalcoholic fatty liver disease. *Biochimica et biophysica acta* **1831**(4): 803-818.

Hart SN, Li Y, Nakamoto K, Subileau EA, Steen D and Zhong XB (2010) A comparison of whole genome gene expression profiles of HepaRG cells and HepG2 cells to primary human hepatocytes and human liver tissues. *Drug metabolism and disposition* **38**(6): 988-994.

Hartman HB, Lai K and Evans MJ (2009) Loss of small heterodimer partner expression in the liver protects against dyslipidemia. *Journal of lipid research* **50**(2): 193-203.

He N, Park K, Zhang Y, Huang J, Lu S and Wang L (2008) Epigenetic inhibition of nuclear receptor small heterodimer partner is associated with and regulates hepatocellular carcinoma growth. *Gastroenterology* **134**(3): 793-802.

Hoekstra M, Lammers B, Out R, Li Z, Van Eck M and Van Berkel TJ (2009) Activation of the nuclear receptor PXR decreases plasma LDL-cholesterol levels and induces hepatic steatosis in LDL receptor knockout mice. *Molecular pharmaceutics* **6**(1): 182-189.

Huang J, Iqbal J, Saha PK, Liu J, Chan L, Hussain MM, Moore DD and Wang L (2007) Molecular characterization of the role of orphan receptor small heterodimer partner in development of fatty liver. *Hepatology* **46**(1): 147-157.

Imajo K, Yoneda M, Kessoku T, Ogawa Y, Maeda S, Sumida Y, Hyogo H, Eguchi Y, Wada K and Nakajima A (2013) Rodent models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *International journal of molecular sciences* **14**(11): 21833-21857.

Itagaki H, Shimizu K, Morikawa S, Ogawa K and Ezaki T (2013) Morphological and functional characterization of non-alcoholic fatty liver disease induced by a methionine-choline-deficient diet in C57BL/6 mice. *International journal of clinical and experimental pathology* **6**(12): 2683-2696.

Jover R, Bort R, Gomez-Lechon MJ and Castell JV (1998) Re-expression of C/EBP alpha induces CYP2B6, CYP2C9 and CYP2D6 genes in HepG2 cells. *FEBS letters* **431**(2): 227-230.

Kim HI, Koh YK, Kim TH, Kwon SK, Im SS, Choi HS, Kim KS and Ahn YH (2007) Transcriptional activation of SHP by PPAR-gamma in liver. *BBRC* **360**(2): 301-306.

Kohli R, Pan X, Malladi P, Wainwright MS and Whitington PF (2007) Mitochondrial reactive oxygen species signal hepatocyte steatosis by regulating the phosphatidylinositol 3-kinase cell survival pathway. *The Journal of biological chemistry* **282**(29): 21327-21336.

Krausova L, Stejskalova L, Wang H, Vrzal R, Dvorak Z, Mani S and Pavek P (2011) Metformin suppresses pregnane X receptor (PXR)-regulated transactivation of CYP3A4 gene. *Biochemical pharmacology* **82**(11): 1771-1780.

Larrain S and Rinella ME (2012) A myriad of pathways to NASH. *Clinics in liver disease* **16**(3): 525-548.

Lee HK, Lee YK, Park SH, Kim YS, Lee JW, Kwon HB, Soh J, Moore DD and Choi HS (1998) Structure and expression of the orphan nuclear receptor SHP gene. *The Journal of biological chemistry* **273**(23): 14398-14402.

Lee JH, Zhou J and Xie W (2008) PXR and LXR in hepatic steatosis: a new dog and an old dog with new tricks. *Molecular pharmaceutics* **5**(1): 60-66.

Li T and Chiang JY (2006) Rifampicin induction of CYP3A4 requires pregnane X receptor cross talk with hepatocyte nuclear factor 4alpha and coactivators, and suppression of small heterodimer partner gene expression. *Drug metabolism and disposition* **34**(5): 756-764.

Lu SC, Alvarez L, Huang ZZ, Chen L, An W, Corrales FJ, Avila MA, Kanel G and Mato JM (2001) Methionine adenosyltransferase 1A knockout mice are predisposed to liver injury and exhibit increased expression of genes involved in proliferation. *Proceedings of the national academy of sciences USA* **98**(10): 5560-5565.

Martinez-Chantar ML, Corrales FJ, Martinez-Cruz LA, Garcia-Trevijano ER, Huang ZZ, Chen L, Kanel G, Avila MA, Mato JM and Lu SC (2002) Spontaneous oxidative stress and liver tumors in mice lacking methionine adenosyltransferase 1A. *FASEB journal* **16**(10): 1292-1294.

Martinez-Chantar ML, Vazquez-Chantada M, Ariz U, Martinez N, Varela M, Luka Z, Capdevila A, Rodriguez J, Aransay AM, Matthiesen R, Yang H, Calvisi DF, Esteller M, Fraga M, Lu SC, Wagner C and Mato JM (2008) Loss of the glycine N-methyltransferase gene leads to steatosis and hepatocellular carcinoma in mice. *Hepatology* **47**(4): 1191-1199.

Nakajima T, Kinoshita S, Sasagawa T, Sasaki K, Naruto M, Kishimoto T and Akira S (1993) Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. *Proceedings of the national academy of sciences USA* **90**(6): 2207-2211.

Nerlov C (2008) C/EBPs: recipients of extracellular signals through proteome modulation. *Current opinion in cell biology* **20**(2): 180-185.

O'Brien PJ, Irwin W, Diaz D, Howard-Cofield E, Krejsa CM, Slaughter MR, Gao B, Kaludercic N, Angeline A, Bernardi P, Brain P and Hougham C (2006) High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening. *Archives of toxicology* **80**(9): 580-604.

Park YJ, Qatanani M, Chua SS, LaRey JL, Johnson SA, Watanabe M, Moore DD and Lee YK (2008) Loss of orphan receptor small heterodimer partner sensitizes mice to liver injury from obstructive cholestasis. *Hepatology* **47**(5): 1578-1586.

Pedersen TA, Bereshchenko O, Garcia-Silva S, Ermakova O, Kurz E, Mandrup S, Porse BT and Nerlov C (2007) Distinct C/EBPalpha motifs regulate lipogenic and gluconeogenic gene expression in vivo. *The EMBO journal* **26**(4): 1081-1093.

Qiao L, MacDougald OA and Shao J (2006) CCAAT/enhancer-binding protein alpha mediates induction of hepatic phosphoenolpyruvate carboxykinase by p38 mitogen-activated protein kinase. *The Journal of biological chemistry* **281**(34): 24390-24397.

Ross SE, Erickson RL, Hemati N and MacDougald OA (1999) Glycogen synthase kinase 3 is an insulin-regulated C/EBPalpha kinase. *Molecular and cellular biology* **19**(12): 8433-8441.

Ross SE, Radomska HS, Wu B, Zhang P, Winnay JN, Bajnok L, Wright WS, Schaufele F, Tenen DG and MacDougald OA (2004) Phosphorylation of C/EBPalpha inhibits granulopoiesis. *Molecular and cellular biology* **24**(2): 675-686.

Sahai A, Pan X, Paul R, Malladi P, Kohli R and Whitington PF (2006) Roles of phosphatidylinositol 3-kinase and osteopontin in steatosis and aminotransferase release by hepatocytes treated with methionine-choline-deficient medium. *American journal of physiology Gastrointestinal and liver physiology* **291**(1): G55-62.

Seol W, Choi HS and Moore DD (1996) An orphan nuclear hormone receptor that lacks a DNA binding domain and heterodimerizes with other receptors. *Science* **272**(5266): 1336-1339.

Shih DQ, Screenan S, Munoz KN, Philipson L, Pontoglio M, Yaniv M, Polonsky KS and Stoffel M (2001) Loss of HNF-1alpha function in mice leads to abnormal expression of genes involved in pancreatic islet development and metabolism. *Diabetes* **50**(11): 2472-2480.

Takahashi Y, Soejima Y and Fukusato T (2012) Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *World journal of gastroenterology* **18**(19): 2300-2308.

Trauner M (2007) A little orphan runs to fat: the orphan receptor small heterodimer partner as a key player in the regulation of hepatic lipid metabolism. *Hepatology* **46**(1): 1-5.

Tsukada J, Yoshida Y, Kominato Y and Auron PE (2011) The CCAAT/enhancer (C/EBP) family of basic-leucine zipper (bZIP) transcription factors is a multifaceted highly-regulated system for gene regulation. *Cytokine* **54**(1): 6-19.

Van Rooyen DM, Larter CZ, Haigh WG, Yeh MM, Ioannou G, Kuver R, Lee SP, Teoh NC and Farrell GC (2011) Hepatic free cholesterol accumulates in obese, diabetic mice and causes nonalcoholic steatohepatitis. *Gastroenterology* **141**(4): 1393-1403, 1403.

Varela-Rey M, Martinez-Lopez N, Fernandez-Ramos D, Embade N, Calvisi DF, Woodhoo A, Rodriguez J, Fraga MF, Julve J, Rodriguez-Millan E, Frades I, Torres L, Luka Z, Wagner C, Esteller M, Lu SC, Martinez-Chantar ML and Mato JM (2010) Fatty liver and fibrosis in glycine N-methyltransferase knockout mice is prevented by nicotinamide. *Hepatology* **52**(1): 105-114.

Wang GL, Iakova P, Wilde M, Awad S and Timchenko NA (2004) Liver tumors escape negative control of proliferation via PI3K/Akt-mediated block of C/EBP alpha growth inhibitory activity. *Genes & development* **18**(8): 912-925.

Wang L, Han Y, Kim CS, Lee YK and Moore DD (2003) Resistance of SHP-null mice to bile acid-induced liver damage. *The Journal of biological chemistry* **278**(45): 44475-44481.

Wang Z, Yao T and Song Z (2010a) Extracellular signal-regulated kinases 1/2 suppression aggravates transforming growth factor-beta1 hepatotoxicity: a potential mechanism for liver injury in methionine-choline deficient-diet-fed mice. *Experimental biology and medicine* **235**(11): 1347-1355.

Wang Z, Yao T and Song Z (2010b) Involvement and mechanism of DGAT2 upregulation in the pathogenesis of alcoholic fatty liver disease. *Journal of lipid research* **51**(11): 3158-3165.

Xu D, Huang XD, Yuan JP, Wu J, Fan Y, Luo HS and Yang YH (2011) Impaired activation of phosphatidylinositol 3-kinase by leptin is a novel mechanism of hepatic leptin resistance in NAFLD. *Hepato-gastroenterology* **58**(110-111): 1703-1707.

Xu JJ, Henstock PV, Dunn MC, Smith AR, Chabot JR and de Graaf D (2008) Cellular imaging predictions of clinical drug-induced liver injury. *Toxicological sciences* **105**(1): 97-105.

Zhang Y, Hagedorn CH and Wang L (2011) Role of nuclear receptor SHP in metabolism and cancer. *Biochimica et biophysica acta* **1812**(8): 893-908.

Zhang Y, Soto J, Park K, Viswanath G, Kuwada S, Abel ED and Wang L (2010) Nuclear receptor SHP, a death receptor that targets mitochondria, induces apoptosis and inhibits tumor growth. *Molecular and cellular biology* **30**(6): 1341-1356.

Zhang Y, Xu N, Xu J, Kong B, Copple B, Guo GL and Wang L (2014) E2F1 is a novel fibrogenic gene that regulates cholestatic liver fibrosis through the Egr-1/SHP/EID1 network. *Hepatology* **60**(3):919-930.

Zhang Y, Xu P, Park K, Choi Y, Moore DD and Wang L (2008) Orphan receptor small heterodimer partner suppresses tumorigenesis by modulating cyclin D1 expression and cellular proliferation. *Hepatology* **48**(1): 289-298.

FOOTNOTES

LEGENDS FOR FIGURES

Figure 1. **Regulation of SHP by drugs**. **A)** HepG2 cells **B)** HepaRG cells and **D)** cultured rat hepatocytes were incubated with steatotic and non-steatotic control compounds for 24 h, and SHP mRNA was measured and normalized to GAPDH. NH, Non-Hepatotoxic; H, Hepatotoxic and P, Phospholipidosic. Drug abbreviations: AMIT, Amitriptyline; KETO, Ketotifen; SIMV, Simvastatine; FLUT, Flutamide; FLUO, Fluoxetine; TILO, Tilorone; TAMO, Tamoxifen; TIAN, Tianeptine; CYCA, Cyclosporine A; TETR, Tetracycline; DOXY, Doxycycline; VALP, Valproate; CITR, citrate. Results are expressed as fold change vs DMSO treated cells and represent the mean ± SD of 3–5 independent experiments. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs control cells (one-way ANOVA and Tukey´s test). **C)** Liver SHP expression on rats treated with tetracycline (n=5) or vehicle (n=5) was determined by Q-RT-PCR and normalized to GAPDH. Results are represented in box plots with the median (black line) and the mean (cross), and expressed as fold change vs a control rat liver pool. **$p<0.01$ (Mann-Whitney test).

Figure 2. **Liver SHP expression is decreased in mouse models of advanced NAFLD**. Total RNA from 3- and 9-month old MAT1A$^{-/-}$ **(A)** and GNMT$^{-/-}$ **(B)** male mouse livers was purified and the mRNA level of SHP was determined by real time Q-RT-PCR and normalized to GAPDH mRNA. **C)** SHP expression level was analyzed in mice livers fed with a MCD or control diet up to 5 weeks by both Q-RT-PCR and Western blot (SHP MW: ~28 kDa). Data represent the mean ± SD (n = 5–6) expressed as fold change vs a control mouse liver pool. *$p<0.05$ and **$p<0.01$ by the nonparametric Mann-Whitney test.

Figure 3. **C/EBPα and FOXA1 repress SHP, whereas RXRα (+9-cis-RA) induces SHP transcription**. **A)** HepG2 cells and **B)** cultured human hepatocytes were infected with adenoviruses encoding 8 transcription factors for 48h (pACC: insertless control adenovirus). Selective ligand agonists for CAR, PXR, RXRα and PPARα (see Material and Methods) were added during the last 24 h. SHP mRNA level was determined by Q-RT-PCR and normalized to PBGD. Data represents mean ± SD (n=3-5) expressed as fold change vs control non infected cells. **A)** *$p<0.05$ and **$p<0.01$ vs control cells (one-way ANOVA and Tukey´s test) and **B)**

*p<0.05 and **p<0.01 vs control cells (Mann-Whitney test). **C)** Time-dependent alterations in SHP mRNA after transduction of HepG2 and human hepatocytes cells with Ad-FOXA1 and Ad-C/EBPα. Data represent the mean of two experiments expressed vs control adenovirus infected cells.

Figure 4. **SHP Response to combined transcription factors**. HepG2 cells were infected with Ad-C/EBPα, Ad-FOXA1 and Ad-RXRα either individually or combined. Twenty-four h later, 1 μM 9 cis-retinoic acid or solvent (DMSO) was incorporated, and at 48 h post-infection, cells were harvested and processed for Q-RT-PCR and Western blot. Data represent the mean ± SD (n=3) expressed as fold change vs control non infected cells. *p<0.05 and **p<0.01 vs Ad-RXRα infected cells (one-way ANOVA and Tukey´s test).

Figure 5. **Basal activity of SHP promoter deletion constructs in HepG2 and HeLa cells**. HepG2 **(A)** or HeLa **(B)** cells were transfected with sequential deletion fragments of SHP-Firefly-LUC vectors and the normalization pRL-SV40 plasmid. Luciferase activities were determined 48 h post-transfection. Bars represent the mean ± SD of 4-5 independent experiments, expressed as firefly/Renilla luciferase activity ratios. Statistical differences were evaluated by one-way ANOVA and Tuckey´s pairwise comparisons, ***p<0.001.

Figure 6. Transactivation of SHP reporter constructs by C/EBPα. **A)** Repressing effect of C/EBPα on SHP promoter**.** HepG2 cells were infected with Ad-control or Ad-C/EBPα. Next day cells were transfected with the different SHP promoter vectors and the normalization pRL-SV40 plasmid. At 48 h post-transfection cells were lysed, and both firefly and Renilla reniformis luciferase activities determined. Bars represent the mean ± SD of 3-5 independent experiments, expressed as fold-change vs Ad-Control.*p<0.05, **p<0.01 and ***p<0.001 (one-way ANOVA and Tuckey´s test). **B)** Response of the SHP promoter with mutated C/EBP-RE to C/EBPα. Cells were infected with Ad-control or Ad-CEBPα and next day were transfected with wild type (509) or C/EBP-RE mutated (509-mut) promoter reporter vectors. Luciferase activities were measured 48 h post-transfection. Bars represent the mean ± SD of 3-5 independent experiments, expressed as fold-change vs Ad-Control. *p<0.05 (Mann-Whitney

test). **C)** ChIP assay demonstrates binding of C/EBPα to the SHP promoter. Formaldehyde cross-linked chromatin from adenovirus-infected HepG2 cells was incubated with antibody against C/EBPα or with preimmune IgG. Immunoprecipitated, purified DNA was PCR amplified with primers specific for SHP 5'-flanking region (-329/-678 bp, 34 cycles) or for an exonic region of the human RPLP0 (negative binding control, 39 cycles). Aliquots (10 µl) of PCR amplifications from a representative experiment were subjected to electrophoresis on 2 % agarose gel and stained with SYBR Green Safe. M, 100-bp DNA ladder. **D)** Recovery of DNA in the immunoprecipitates is represented as the percentage of input DNA (100%).

Figure 7. Steatotic drugs modulate negatively SHP by a C/EBPα-dependent mechanism. **A)** HepG2 cells were transfected with 3 different SHP-promoter vectors (-150, -340 and -509). Next day, cells were treated with steatotic drugs or vehicle (DMSO). Twenty-four hours latter cells extracts were prepared and assayed for firefly and Renilla luciferase activities. **B)** Response of the SHP promoter with mutated C/EBP-RE to steatotic drugs. Cells transfected with reporter vectors with mutated CEBP-RE (509-mut) or wild type (509) SHP promoters were lysed at 48 hours post-transfection. Steatotic drugs were added 24 h before processing. Bars represent the mean ± SD (n=3-5), expressed as fold-change vs DMSO. *p<0.05, **p<0.01 and **p<0.01 (one-way ANOVA and Tuckey´s test).

Figure 8. SHP mRNA expression and promoter activity after inhibition of MEK1/2 and PI3K pathways. A) Time-course analysis of SHP mRNA level in HepG2 cells after treatment with 20 μM LY294002 (PI3K inhibitor), 10 μM U0126 (MEK1/2 inhibitor) or vehicle. **B)** HepG2 cells were transfected with the -509 and 509-mut reporter vectors and next day were exposed to kinase inhibitors for 24 h. Data represent firefly activities normalized to Renilla luciferase. Bars represent the mean ± SD of 3-4 independent experiments. *p<0.05, (Mann-Whitney test).
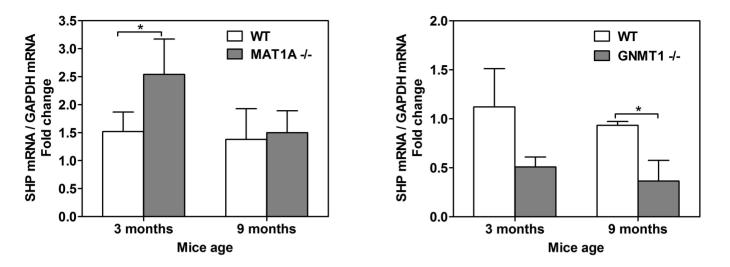
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A

Firefly LUC / Renilla LUC (Fold-change)

Ad-CTRL
Ad-C/EBPα

-150 -340 -509 -612 -1362 -2500

B

509   G T T A G G C A A A
         * * *
509-MUT T C A A G G C A A A

Firefly LUC / Renilla LUC (Fold-change)

509
509-MUT

Ad-CTRL   Ad-C/EBPα

C

Ad-CTRL   Ad-C/EBPα

SHP

RPLP0

Input IgG Anti-C/EBPα   Input IgG Anti-C/EBPα

D

Recovery (% Input DNA)

IgG
Anti-CEBPα

SHP

Ad-CTRL   Ad-CEBPα

Figure 6

Figure 7

Figure 8