



Published in final edited form as:

Pharm Res. 2013 September ; 30(9): 2188–2198. doi:10.1007/s11095-013-1006-7.

Hepatocyte Nuclear Factor 4 Alpha and Farnesoid X Receptor Co-regulates Gene Transcription in Mouse Livers on a Genome-wide Scale

Ann M. Thomas^{1,2}, Steve N. Hart^{1,3}, Guodong Li^{1,4}, Hong Lu^{1,5}, Yaping Fang⁶, Jianwen Fang⁶, Xiao-bo Zhong^{1,7}, and Grace L. Guo^{1,8,*}

¹Department of Pharmacology, Toxicology, and Therapeutics, University of Kansas Medical Center, Kansas City, Kansas, 66160, USA

²Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, Texas, 77054, USA

³Division of Biomedical Statistics and Informatics, Mayo Clinic, Rochester, Minnesota, 55905, USA

⁴Department of Abdominal Surgery, Cancer Treatment Center, Fourth Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, 150000, China

⁵Department of Pharmacology, Upstate Medical School, Syracuse, New York, 13210, USA

⁶Applied Bioinformatics Laboratory, Structural Biology Center, University of Kansas, Lawrence, KS 66047, USA

⁷Department of Pharmaceutical Sciences, School of Pharmacy, University of Connecticut, Storrs, CT, 06269, USA

⁸Department of Pharmacology and Toxicology, School of Pharmacy, Rutgers University, Piscataway, NJ, 08854, USA

Abstract

Purpose—Farnesoid X receptor (Fxr) is a ligand-activated nuclear receptor critical for liver function. Reports indicate that functions of Fxr in the liver may overlap with those of hepatocyte nuclear factor 4 α (Hnf4 α), but studies of their precise genome-wide interaction to regulate gene transcription are lacking. Thus, we compared the genome-wide binding of Fxr and Hnf4 α in the liver of mice and characterized their cooperative activity on binding to and activating target gene transcription.

Methods and Results—ChIP-Seq of mouse livers revealed that nearly 50% binding sites of Fxr and Hnf4 α overlap. Co-immunoprecipitation assays showed a direct Fxr-Hnf4 α protein interaction dependent on Fxr activity. Hnf4 α bound to shared target sites upstream and in close proximity to Fxr. Moreover, genes co-bound by Fxr and Hnf4 α are enriched in complement and coagulation cascades and drug metabolism. Furthermore, transcriptional and binding assays suggest that Hnf4 α increases Fxr transcriptional activity; however, binding of Hnf4 α can be either Fxr-dependent or -independent at different sites.

*Corresponding Author: Grace L. Guo, Department of Pharmacology and Toxicology, School of Pharmacy Rutgers University. 170 Frelinghuysen Rd./EOHSI Bldg. - Rm322, Piscataway, NJ 08854. guo@eohsi.rutgers.edu, Tel # 848-445-8186, Fax #732-445-0119.

Conclusion—Our results showed that Fxr cooperates with Hnf4 α in the liver to modulate gene transcription. This study provides the first evidence on a genome-wide scale of both cooperative and independent interactions between Fxr and Hnf4 α in regulating gene transcription.

Keywords

Fxr; Hnf4 α ; ChIP-Seq; co-regulation; nuclear receptor interaction

INTRODUCTION

Farnesoid X receptor (FXR in humans/Fxr in rodents) is a member of the group II nuclear receptor superfamily, activated by bile acids (FXR's endogenous ligands), highly expressed in the liver and intestine, and a master regulator of the enterohepatic circulation of bile acids (1–6). Hepatocyte nuclear factor 4 alpha (HNF4 α in humans/Hnf4 α in rodents) is a highly conserved orphan nuclear receptor that is also essential for liver development, differentiation, and organism survival (7). Fxr and Hnf4 α have been shown to regulate the expression of an overlapping set of genes, including apolipoprotein C-III (ApoC-III), cholesterol 7 alpha-hydroxylase (Cyp7a1), and bile acid-CoA:amino acid N-acyltransferase (*Baat* protein) (8–12), suggesting an overlap of Fxr and Hnf4 α functions in the liver. Despite this overlap, no studies have yet determined how Fxr and Hnf4 α interact in the liver on a genome-wide scale to regulate gene transcription. However, studies have shown that HNF4 α is capable of enhancing the liver-specific functions of group II nuclear receptors. For example, HNF4 α cooperatively enhances the transcriptional activity of constitutive androstane receptor (CAR) and pregnane X receptor (PXR) at the CYP3A4 promoter (13). The effects of HNF4 α on FXR activity are largely unknown.

In addition to its role in bile acid homeostasis, Fxr also regulates other metabolic processes such as lipid homeostasis, glucose metabolism, insulin sensitivity, and gastrointestinal cancer development and therefore has become a very promising target for the treatment or prevention of cholestasis, hyperlipidemia, fatty liver, type II diabetes, liver and colon cancers (10, 14–22). Recent genome-wide binding studies have shown that Fxr displays a very high degree of tissue-specific binding, which is likely regulated by other tissue-specific co-factors (23). Motif analysis of genome-wide Fxr binding in the liver revealed a nuclear receptor half site (AGGTCA) associated with the Fxr response element, an inverted repeat separated by one nucleotide (IR-1; AGGTCA $\overline{\text{N}}$ TGACCT) (23, 24), indicating the involvement of orphan nuclear receptors in regulating tissue-specific functions of Fxr.

In hepatocytes, the orphan nuclear receptor HNF4 α localizes mainly to the nucleus, binds DNA exclusively as a homodimer, and recognizes response elements consisting of direct repeats, namely, direct repeats separated by one nucleotide (DR-1)(25). Hnf4 α regulates a myriad of liver-specific functions, including production of clotting factors, apolipoprotein synthesis, and drug metabolism (25). In addition, Hnf4 α directly regulates the transcription of Cyp7a1, the rate-limiting enzyme in bile acid synthesis, suggesting that Hnf4 α also plays a regulatory role in bile acid homeostasis (8, 12).

Due to reports of overlapping function of Fxr and Hnf4 α in liver and evidence suggesting an uncharacterized orphan nuclear receptor co-regulates the transcriptional function of Fxr, we hypothesized that Hnf4 α could be responsible for mediating Fxr function in the liver. To test our theory, this study compared the genome-wide binding of Fxr and Hnf4 α in mouse liver and characterized these two factors' cooperation in binding to target gene regions and in activating gene transcription, using chromatin immunoprecipitation (ChIP), massive parallel sequencing, quantitative polymerase chain reaction (qPCR) analysis, co-immunoprecipitation (Co-IP) assays, and luciferase assays.

MATERIALS AND METHODS

Animals

All mice were maintained at an American Animal Associations Laboratory Animal Care-accredited facility at the University of Kansas Medical Center. Animal protocols and procedures were approved by the Institutional Animal Care and Use Committee. For Hnf4 α ChIP-qPCR studies, four-month-old fasted male wild-type (WT) and whole body Fxr-knockout (Fxr KO) (5) mice ($n=4$ per group) were used. WT mice were orally gavaged with vehicle (1% methylcellulose, 1% Triton-100 in PBS) or GW4064 (75 mg/kg) twice a day for 24 h period. GW4064 is an FXR agonist (26) synthesized by the Chemical Discovery Laboratory at the University of Kansas (Lawrence, KS). Fxr KO mice were only gavaged with vehicle. Livers were collected 4 h after the second dose and prepared for Hnf4 α chromatin immunoprecipitation followed by qPCR analysis (ChIP-qPCR). Hepatocyte-specific Hnf4 α -null (Hnf4 α -HNull) mice were generated as previously described (27) and were fed the same rodent chow as the WT control mice. Livers from 45-day-old male Hnf4 α -HNull mice and from their WT control littermates ($n=4$ per group) were used for Fxr ChIP-qPCR assays. For Co-IP assays, 4-month-old C57BL/6 and Fxr KO mice ($n=3$ per group) were fed a control diet or a diet supplemented with 1% (w:w) cholic acid (CA) for 5 days. Liver whole-cell lysates were prepared and used for Co-IP analysis.

ChIP Followed by Massive Parallel Sequencing (ChIP-Seq)

ChIP-Seq analysis of Fxr and Hnf4 α in mouse liver was done to determine the degree of genome-wide overlapping in binding. Original ChIP-Seq data were obtained from mouse livers generated as previously described (23, 28). Raw Fxr and Hnf4 α ChIP-Seq data from single end sequencing on an Illumina Genome Analyzer, obtained from in-house or online databases, were re-analyzed using Model-based Analysis of ChIP-Seq (MACS) (29). Total Fxr binding sites were compared with total Hnf4 α binding sites in the liver. The binding frequency of Hnf4 α , or number of Hnf4 α binding events, relative to the distance of the Fxr binding site within shared target genes was analyzed using BEDTools (30). Histograms of Fxr and Hnf4 α binding to the *Nr0b2* (small heterodimer partner, Shp) gene were generated using the UCSC Genome Browser (University of California, Santa Cruz) (31).

Peaks identified in ChIP-Seq data that were shared by Fxr and Hnf4 α in the liver of mice were analyzed for pathway enrichment using the Functional Annotation Tool in the Database for Annotation, Visualization, and Integrated Discovery (32). *P*-value less than or equal to 0.05 were considered statistically significant.

ChIP-qPCR

ChIP-qPCR analysis was done on shared Fxr and Hnf4 α binding regions identified by ChIP-Seq analysis to validate genome-wide analysis and to determine degree of cooperative binding of these two factors. ChIP-quality antibodies for mouse Fxr and Hnf4 α were obtained from Santa Cruz Biotechnology (H-130 and C-19). Antibody specificity for Fxr has been shown in previous genome-wide binding analysis (23), and for Hnf4 α is demonstrated in Supplemental Fig. 1. For Hnf4 α ChIP-qPCR assays, we used livers from WT and Fxr KO mice treated with or without GW4064 and Fxr KO mice treated with vehicle control ($n=4$). For Fxr ChIP-qPCR assays, we used livers from WT and Hnf4 α -HNull mice ($n=4$) as previously described (23). Purified IP DNA fragments were analyzed by qPCR with primers amplifying shared Fxr and Hnf4 α binding sites: *Apoc3*, *Apoe*, *Baat*, *Nr0b2* promoter and 3' regulatory region, and *Sqstm1*. We also analyzed Fxr and Hnf4 α binding to fragments of genes involved in complement and coagulation cascades: *C2* (-50 to 0 bp upstream TSS), *C3* (-225 to -275 bp upstream TSS), *F2* (-425 to -475 bp upstream TSS), *C4b* (-17125 to -17175 bp upstream TSS), *Cfb* (-150 to -200 bp upstream TSS), *Fga* (-200 to -250 bp

upstream TSS), and *Plg* (–125 to –175 bp upstream TSS). Fxr has previously been shown to bind within the second intron of the *Fgf15* gene (1880 to 1980 bp downstream TSS) in mouse intestine but not the liver (23). This region has also been shown not to be a binding region of Hnf4 α by ChIP-Seq analysis. Therefore this region was originally used as a negative control for Fxr-Hnf4 α co-localization experiments. These above target regions were selected for ChIP-qPCR validation and analysis because they belong to pathways highly co-bound by Fxr and Hnf4 α , as revealed by ChIP-Seq analysis, and due to their physiologically significant roles in bile acid, lipid, and coagulation pathways. All primers used for ChIP-qPCR are presented in Supplemental Table I. Quantitative PCR reactions were carried out using MaximaTM SYBR Green (Fermentas Molecular Biology Tools). Data were analyzed as fold change over values from vehicle-treated WT mice.

Co-Immunoprecipitation

To investigate whether Fxr and Hnf4 α have a protein-protein interaction, we performed Co-IP assays using a kit from Invitrogen on whole-cell liver extracts from WT mice fed with or without 1% CA and from Fxr KO mice fed a control diet. Whole-cell lysates from mouse livers were prepared according to protocol and then immunoprecipitated using an antibody against Fxr (H-130, $n=3$ each group). Immunoprecipitates from each group were pooled and analyzed by standard Western blot using antibody sc-6556 to detect Hnf4 α (Santa Cruz Biotechnology).

Construction of Plasmids for Reporter Gene Luciferase Assay

Luciferase assays were done to determine the transcriptional effects of FXR and Hnf4 α on shared target regions. Specifically, the transcriptional activity of FXR/retinoid x receptor alpha (RXR α) and Hnf4 α were tested on reporter vectors containing shared Fxr-Hnf4 α binding regions within the promoter and the downstream regulator region of *Nr0b2* (Shp), the first intron of scavenger receptor class B type 1 (*Scarb1*; Sr-b1), and the downstream regulatory region of *Sqstm1* (p62). Supplemental Table II lists the location and relative Fxr and Hnf4 α binding counts in these regions. Reporter vectors of Shp promoter and downstream regulatory region were cloned as previously reported (33). An active Hnf4 α binding site located 70 bp upstream of the *Baat*(Bat) gene transcriptional start site (TSS), previously reported (9), was used as a positive control for HNF4 α transcriptional activity. For this study, this 600 bp region upstream of the Bat gene, a region shown to have high Hnf4 α activity (9), was amplified from mouse genomic DNA by PCR using pairs of primers containing XhoI and HindIII restriction enzyme sites and cloned upstream of the luciferase gene within the pGL4.23 firefly luciferase vector (Promega). The primers used to generate Bat reporter vector were Forward: 5'-CACAACTCGAGAATGGCTAAGACTATAGAT-3' and Reverse: 5'-CTGAGGAAGCTTTCTTAGTATTTCCCTCCTC-3'. A 600 bp region around Fxr and Hnf4 α binding sites within the first intron of Sr-b1 located 10.7 and 21.5 Kb downstream of the TSS, respectively, has been previously reported to be a Fxr binding site (34). These regions were amplified from mouse genomic DNA by PCR using pairs of primers containing XhoI and BglIII restriction enzyme sites. The PCR products, Sr-b1 #1 and Sr-b1 #2, were subcloned upstream of the luciferase gene into the pGL4.23 firefly luciferase vector as previously reported (34). A 2 Kb region of the p62 gene containing Fxr and Hnf4 α binding sites, located around 13.1 Kb downstream of the p62 gene TSS, has recently been determined to be an Fxr binding site (35). This region was cloned into a pGL4-TK luciferase vector (Promega) as previously reported (35). All constructs were confirmed by DNA sequencing.

Cell Culture, Transient Transfection, and Luciferase Reporter Gene Assays

Chinese hamster ovary (CHO) cells were cultured at 70–90% cell density in high-glucose Dulbecco's modified Eagle medium supplemented with 1% penicillin/streptomycin, 1% L-

proline (50 $\mu\text{g}/\text{mL}$), and 10% fetal bovine serum (Omega Scientific) and were transiently transfected by reverse transfection methods using TurboFect (Fermentas Molecular Biology Tools) with the various reporter gene constructs as well as pCMV-ICIS human FXR and/or pCMV-SPORT6 mouse Hnf4 α (Open Biosystems), PSG5 human RXR α , and pHRG-TK-*Renilla* luciferase vector (Promega, no longer available; see pGL4.74) according to protocol. Human FXR is highly homologous to mouse Fxr and has often been used to test transcriptional activity on mouse gene binding sites (33, 35, 36). After 24 h, cells were treated with 100 nM GW4064 or 0.1% dimethyl sulfoxide(control); cells from the Hnf4 α -alone groups were not treated. Firefly luciferase and *Renilla* luciferase activities were quantified 24 h post-treatment using a Dual-Glo Luciferase Assay System (Promega).

FXR/RXR α expression vectors were co-transfected with increasing amounts (3, 10, and 30 ng) of Hnf4 α expression vector with 100 nM GW4064, a FXR synthetic ligand. We used the promoter and downstream regions of Shp, the intron of Sr-b1, and the downstream region of p62 cloned into luciferase expression vectors to assess the effects of Hnf4 α on the transcriptional activity of FXR. The transcriptional activity of increasing amounts of Hnf4 α expression vector (10, 50, and 100 ng or 10 and 100 ng) on these regions, as well as on a positive control gene, Bat, was also measured by luciferase assay. The firefly luciferase activity value was normalized as a ratio over *Renilla* luciferase and expressed as firefly luciferase activity/*Renilla*. The data are presented as the average of six wells \pm SE, and the experiments were repeated at least twice.

Statistical Analysis

All data are presented as mean \pm SE. Statistical difference between the two groups was analyzed by Student's *t*-test. *P*-values \leq 0.05 were considered significant.

RESULTS

Genome-wide Fxr and Hnf4 α Binding Sites in Mouse Liver

ChIP-Seq binding data of Fxr and Hnf4 α in mouse liver from previous reports (23, 28) were re-analyzed using MACS. We found 10917 total binding sites for Hnf4 α and 7797 for Fxr, of which 3882 overlap; 50% of total Fxr binding sites co-localize with Hnf4 α (Fig 1a). Hnf4 α and Fxr do not bind to same site; rather, the frequency (y-axis) of Hnf4 α binding to shared target genes was greatest when bound upstream and in close proximity to an Fxr binding site (x-axis; location of collective Fxr binding sites are represented by "0"; Fig. 1b).

Pathway analysis of shared Fxr and Hnf4 α target genes revealed pathways involving complement and coagulation cascades had the highest number of genes targeted by Fxr and Hnf4 α (Table I). *P*-values and Bonferonni scores illustrate the degree of significance of Fxr and Hnf4 α co-localization within the pathway. *P*-values \leq 0.05 were considered statistically significant. Table II lists the shared target genes categorized by the Kyoto Encyclopedia of Genes and Genomes pathway maps as part of complement and coagulation cascades, the locations of Fxr and Hnf4 α binding sites in relation to the genes' TSS and the relative binding events (counts) of each factor.

Fig. 1c is a histogram of binding of Fxr (red) and Hnf4 α (black) to the *Nr0b2* gene in mouse liver, generated by the UCSC Genome Browser (31). Both the promoter and downstream FXR binding sites co-localized with those of HNF4 α . The binding of Hnf4 α to the *Nr0b2* promoter has previously been described (37). However, the binding of Hnf4 α to the 3' end of the *Nr0b2* gene and the co-localization with Fxr at these regions are novel findings. Sequence analysis of these regions by NUBIScan (38) showed a putative HNF4 α binding motif, DR-1, located in the promoter of the *Nr0b2* gene (within -320 to -220 bp upstream

of TSS) but not in the downstream regulatory region (data not shown), whereas a classical FXR binding motif, IR-1, has been identified in both of these regions (23, 33).

Dependence of Fxr and Hnf4 α for Binding to Shared Target Genes

Supplemental Table II summarizes the binding site locations and counts of Fxr and Hnf4 α to select shared target genes, including to the 5' and 3' end of *Nr0b2*, revealed by ChIP-Seq analysis. These regions were assessed for Fxr and Hnf4 α binding by ChIP-qPCR and luciferase assay. Fxr binding increased nearly 2-fold in Hnf4 α -HNull mice at sites located within the *Baat* gene promoter, the 5' and 3' regions of the *Nr0b2* gene, the downstream regulatory region of *Sqstm1* gene, and a non-shared target site within the *Fgf15* gene (Fig. 2a). This increase was only statistically significant for Fxr binding at the 5' end of *Nr0b2* and *Fgf15* (**P*-value = 0.05). *Fgf15* was thought to be an FXR target gene in mouse intestine and not liver (23), and was shown to not be bound by Hnf4 α . Therefore, this region was originally used as a negative control region for co-localization. Fxr binding to promoters of *Apoc3* and *Apoe* did not change with Hnf4 α deficiency.

We also analyzed Fxr binding to genes involved in complement and coagulation cascades (Fig. 2b) in WT versus Hnf4 α -HNull mouse liver. Fxr did bind to shared regions in genes of the complement and coagulation cascade within both WT and Hnf4 α -HNull mouse liver. Fxr binding events increased in Hnf4 α -deficient mice 1.6–2-fold at genes *Fga*, *C3*, *C4b*, *C2*, and *F2* but did not change at binding sites within *Cfb* and *Plg*.

The Hnf4 α binding pattern to shared target genes in WT mouse liver treated with or without Fxr ligand GW4064 and in Fxr KO mouse liver, varied at different target sites (Fig. 3). When compared with vehicle-treated WT liver, Hnf4 α binding events in WT mice treated with GW4064 increased at shared target sites within *Apoc3* (1.5-fold), *Apoe* (2.3-fold), *Baat* (1.8-fold), *Nr0b2* (1.6- and 1.5-fold), and *Sqstm1* (1.5-fold) but not in the negative control region (*Fgf15*) (Fig. 3a; **P*-value < 0.05). Overall, Hnf4 α binding to *Apoe*, *Baat*, *Nr0b2* 3', and *Sqstm1* regions did not decrease below baseline in Fxr KO mouse livers. There was a slight non-significant reduction in *Apoc3* binding, and binding to *Nr0b2* 3' remained elevated in Fxr KO mouse liver. Hnf4 α binding increased in mouse liver treated with GW4064 at genes *F2* (2.6-fold), *C2* (1.6-fold), *C4b* (1.7-fold), *C3* (1.4-fold), and *Cfb* (1.9-fold) (Fig. 3b; **P*-value < 0.05). As seen with the previous regions above, Hnf4 α binding to *F2*, *C3*, and *Cfb* did not decrease below baseline in FxrKO mouse liver. However, Hnf4 α binding remained elevated at regions within *C2* and *C4b* (***P*-value < 0.05). Hnf4 α binding was not regulated by Fxr activity at binding sites within *Plg* or *Fga*.

Interaction between Fxr and Hnf4 α and Dependence of FXR and Hnf4 α for Activating Target Genes

A modest Fxr-Hnf4 α protein-protein interaction was detected in WT mice fed a control diet (Fig. 4a). This interaction increased in mice fed a 1% CA diet but was nearly undetectable in Fxr KO mice (Fig. 4a).

Hnf4 α binding to Shp promoter and downstream regulatory region was detected by ChIP-Seq analysis (Fig. 2a). These binding sites were analyzed for Hnf4 α transcriptional activity using luciferase reporter assays. Transcriptional activity of Hnf4 α on the *Bat* gene promoter, which has already been characterized (9), served as a positive control. Results showed that although Hnf4 α significantly increased luciferase activities of the Shp and *Bat* promoter when compared to vector control, with 100 ng Hnf4 α having the highest activity (**P*-value = 0.05). Hnf4 α did not affect the downstream regulatory region of Shp (Fig. 4b, **top panel**).

FXR has previously been shown to transcriptionally regulate the promoter and downstream regulatory region of the *Shp* gene (33). Our results confirm that FXR significantly increases luciferase activity at both of these sites (**P*-value = 0.05; Fig. 4b, bottom panel). In addition, Hnf4 α increased transcriptional activity of FXR on *Shp*3' region nearly 2- and 1.4 fold (***P*-value = 0.05) at 3 and 10 ng. However, Hnf4 α appears to have slightly and significantly decreased transcriptional activity of FXR at the *Shp* promoter (***P*-value = 0.05).

Hnf4 α significantly reduced luciferase activity of the Sr-b1#1 (10.7 Kb regulatory region downstream of the TSS, Supplemental Table II), 2-fold for 10 ng and 1.3-fold for 100 ng of Hnf4 α expression vector (**P*-value = 0.05; Fig 5a top). Conversely, Hnf4 α significantly increased luciferase activity of the Sr-b1#2 site (21.5 Kb regulatory region downstream of the TSS, Supplemental Table II) and p62 in a dose-dependent manner (**P*-value = 0.05 at 100 ng for Sr-b1 #2 and at 10 and 100 ng for p62; Fig 5a middle and bottom). Next, the effects of Hnf4 α on FXR transcriptional activity of binding sites within Sr-b1 and p62 were tested (Fig. 5b). FXR significantly increased transcriptional activity of two binding sites within the Sr-b1 gene (#1 and #2) and downstream regulatory region of p62 (**P*-value = 0.05), which is consistent with previous reports (34, 35). Hnf4 α increased FXR-induced transcriptional activity at each of these sites. Interestingly, even though Hnf4 α alone moderately decreased luciferase activity at Sr-b1#1 (Fig. 5a, top), Hnf4 α synergistically and significantly enhanced the FXR activity nearly 20-fold in this region at 30 ng of Hnf4 α expression vector (***P*-value = 0.05 for 3 and 30 ng; Fig. 5b, top). Hnf4 α only moderately enhanced FXR's transcriptional activity at Sr-b1 #2 in a weakly additive and dose-dependent manner (***P*-values = 0.05; Fig. 5b, middle). Finally, Hnf4 α significantly enhanced FXR transcriptional activity of p62, although this effect was saturated at 3 ng of Hnf4 α expression vector and again was weakly additive (***P*-value = 0.05 for 3 and 30 ng; Fig. 5b, bottom). Collectively, these results indicate that Hnf4 α can enhance FXR activity in an additive and synergistic manner.

DISCUSSION

Our findings demonstrate that genome-wide Hnf4 α and Fxr DNA-binding sites have a very high degree of overlap in mouse liver and that these shared target genes are highly enriched within the genes involved in complement and coagulation cascades. Furthermore, within shared target regions, these two nuclear receptors bind in close proximity and exhibit a protein-protein interaction dependent on Fxr activation. Deficiency of Fxr or Hnf4 α affects the binding of either to target genes in a gene-selective manner. We conclude that Fxr and Hnf4 α likely regulate transcription of some shared target genes independently of each other, as seen with apolipoprotein genes, but cooperate to regulate transcription of other shared target genes, such as genes involved in bile acid homeostasis.

There are several potential explanations for why Fxr binding increases at select shared target genes in the absence of functional Hnf4 α , which is opposite to the original hypothesis. Hnf4 α and Fxr may compete for the same binding site to regulate transcription of the target gene, as illustrated in previous studies showing that Fxr displaces Hnf4 α binding to the Apo C-III promoter and inversely regulates transcription of this gene (10). However, in the current study, ChIP-Seq analysis on a genome-wide scale shows Hnf4 α binding not at the same location as Fxr but rather upstream of the Fxr binding site. We think that increased FXR binding is due to the increase in endogenous ligands of Fxr in the Hnf4 α mice, because loss of Hnf4 α leads to a marked increase in bile acid concentration (8, 9), which likely further activates Fxr. This is illustrated by the increase in Fxr binding within a region of *Fgf15*, which is not typically an Fxr target gene in the liver under normal conditions.

Therefore, our results suggest complex interactions among nuclear receptors via direct interaction at the chromatin level or via the modification of endogenous ligands.

Furthermore, we found that increased Hnf4 α binding to shared target genes depended on Fxr activity at some target regions, including the promoter of the *Baat* gene and the 3' regulatory region of *NrOb2*. Interestingly, baseline binding of Hnf4 α to the 3' end of the *NrOb2* gene increased in Fxr KO mice compared with WT mice. This trend was also seen at *C4b* and *C2*. This observance possibly indicates the ability of Hnf4 α to compensate for the loss of FXR at these regions. The dependence of Hnf4 α binding on Fxr activity within complement and coagulation genes also showed differential regulation. Indeed, Hnf4 α binding increased at binding sites within *F2*, *C2*, *C3*, *C4b*, and *Cfb* genes, but not at *Plg* or *Fga* genes, after Fxr activation. The increased binding at these five regions was shown to depend on the activation of Fxr at *F2*, *C3*, and *Cfb*. The mechanism responsible for the differential binding of Hnf4 α in the absence of Fxr is unknown but could be due to direct modification of Hnf4 α binding to chromatin, post-translational modification of Hnf4 α , a change in Hnf4 α ligand availability, and/or as mentioned, the compensation of Hnf4 α for Fxr loss. Further studies will be needed to clarify the underlying mechanism.

Similarly, Hnf4 α transcriptional assays indicated that Hnf4 α binding detected by ChIP-Seq analysis is not directly correlated to transcriptional regulation of genes. For example, Hnf4 α showed a low level of binding to the 3' end of the *NrOb2* gene as well as to sites 10.7 Kb downstream of the Sr-b1 TSS. However, neither of these sites was transcriptionally activated by Hnf4 α . Although Hnf4 α alone did not elicit transcription of these sites, it did moderately enhance FXR's transcriptional activity in both of these regions, suggesting that Hnf4 α regulates FXR activity or other factors, possibly via modifying the chromatin structure. Furthermore, Hnf4 α alone induced transcriptional activity of the *NrOb2* promoter but seemed to have a slight, insignificant inhibitory effect on FXR's transcriptional activity in this region.

Future studies will determine whether HNF4 α interacts with FXR to stabilize localized chromatin environments surrounding gene loci. A recent study has shown that Fxr binding to the 5' and 3' end of *NrOb2* mediates a head-to-tail chromatin loop around the gene (33). This may be an essential process required for the efficient transcription of the *NrOb2* gene in response to Fxr activation. ChIP-Seq data demonstrates that Hnf4 α also co-localizes with Fxr to the 5' and 3' regions of *NrOb2*, suggesting that Hnf4 α may be important for mediating the Fxr-induced head-to-tail chromatin loop around the *NrOb2* gene.

Other studies have demonstrated that Fxr and Hnf4 α have opposite effects on gene transcription of shared target genes. Studies show that Hnf4 α binding increases the transcription of ApoC-III, a well-characterized Hnf4 α target gene (11, 39). Fxr inhibits the transcription of ApoC-III by binding to its promoter region (10). However, none of these studies have examined the transcriptional effect of Hnf4 α and Fxr on shared target genes on a genome-wide scale. Our analysis suggests these two factors can have cooperative, compensatory, or independent effects on the transcription of target genes. In addition, although not completely demonstrated here, these factors can have an antagonistic effect on gene transcription as previously reported (10).

It is interesting to note that the degree of induction in binding in experimental groups was small or lacked statistical significance. We have seen increasing numbers of genes being regulated in this way despite strong binding of transcription factors (TFs) revealed by ChIP-Seq analysis (23), and due to valid negative control comparisons, we do not believe this to be a result of false positive binding. One explanation for this observance could be the inability of antibody-TF interactions from experimental technology to enrich small fractions

of desired TF-DNA interactions from whole tissues. Furthermore, previous publications from our group have reported a similar phenomenon with Fxr (23). In this study, it was argued due to potential constitutive TF binding; ligand activation of the TF does not necessarily result in increased localization of TF to target DNA, but rather changes the recruitment of co-repressors to co-activators.

Nevertheless, this study provides the first line of evidence for interactions between Fxr and other nuclear receptors on a genome-wide scale in mouse liver. FXR and HNF4 α have been shown to be highly homologous between mouse and human (36, 40), and have similar functions between these species (41). Therefore, information gained from these mouse models will likely reveal similar FXR-HNF4 α interactions in human. It was originally thought that HNF4 α could regulate FXR activity similar to how forkhead box protein A1 (FOXA1), otherwise known as hepatocyte nuclear factor 3-alpha, directs estrogen receptor alpha genome-wide binding (42, 43). However, this study revealed a more complex Fxr-Hnf4 α interaction in mouse liver that was both Fxr-dependent and -independent, illustrated an indirect cross-talk resulting from disruption of bile acid homeostasis in Fxr and Hnf4 α deficient mice, and implicated chromatin remodeling as a mechanism of cooperative activity between these two factors. These studies help broaden our understanding of nuclear receptor function and the complicated interactions they have with other transcriptional machinery that is necessary to fine-tune target gene transcription.

CONCLUSION

In summary, our results reveal a high percentage of co-localized Fxr binding to Hnf4 α in mouse liver. We conclude that Fxr and Hnf4 α cooperate to a moderate extent to regulate gene transcription and share a direct protein interaction. They likely regulate transcription of target genes in both a dependent and independent manner and can cooperate or antagonize the activity of the other. Our findings suggest that both factors can compensate for the other's deficiency at certain sites and this compensation may be a mechanism important for maintaining cellular integrity and homeostasis. Despite a direct Fxr-Hnf4 α interaction, it is unlikely that Hnf4 α is a major determining orphan nuclear receptor responsible for directing tissue-specific binding of Fxr. Nonetheless, the Fxr-Hnf4 α interaction could play a critical role in certain diseased systems and/or within specific cellular pathways such as complement and coagulation cascades or drug metabolism and should be further investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by CPRIT Training Grants RP101502 to The University of Texas MD Anderson Cancer Center (AMT), DK031343 (GLG), DK090036 (GLG), and a Madison and Lila Self Graduate Fellowship from the University of Kansas (SNH).

ABBREVIATIONS

ApoC-III	apolipoprotein C-III
CA	cholic acid
ChIP-Seq	chromatin immunoprecipitation followed by massively parallel sequencing

ChIP-qPCR	chromatin immunoprecipitation followed by quantitative polymerase chain reaction
Co-IP	co-immunoprecipitation
Cyp7a1	cholesterol 7 alpha-hydroxylase
DR-1	direct hexanucleotide repeat separated by 1 nucleotide
FXR/Fxr	farnesoid X receptor
HNF4a/Hnf4a	hepatocyte nuclear factor 4 alpha
IR-1	inverted hexanucleotide repeat separated by 1 nucleotide
KO	knockout
RXRα	retinoid x receptor alpha
Shp	small heterodimer partner
Sr-b1	scavenger receptor class B type 1
TSS	transcriptional start site

References

1. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, et al. Bile Acids: Natural Ligands for an Orphan Nuclear Receptor. *Science*. 1999; 284:1365–1368. [PubMed: 10334993]
2. Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, et al. Identification of a Nuclear Receptor for Bile Acids. *Science*. 1999; 284:1362–1365. [PubMed: 10334992]
3. Wang H, Chen J, Hollister K, Sowers LC, Forman BM. Endogenous Bile Acids Are Ligands for the Nuclear Receptor Fxr/Bar. *Mol Cell*. 1999; 3:543–553. [PubMed: 10360171]
4. Seol W, Choi HS, Moore DD. Isolation of Proteins That Interact Specifically with the Retinoid X Receptor: Two Novel Orphan Receptors. *Mol Endocrinol*. 1995; 9:72–85. [PubMed: 7760852]
5. Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, et al. Targeted Disruption of the Nuclear Receptor Fxr/Bar Impairs Bile Acid and Lipid Homeostasis. *Cell*. 2000; 102:731–744. [PubMed: 11030617]
6. Kok T, Hulzebos CV, Wolters H, Havinga R, Agellon LB, et al. Enterohepatic Circulation of Bile Salts in Farnesoid X Receptor-Deficient Mice. *J Biol Chem*. 2003; 278:41930–41937. [PubMed: 12917447]
7. Chen WS, Manova K, Weinstein DC, Duncan SA, Plump AS, et al. Disruption of the Hnf-4 Gene, Expressed in Visceral Endoderm, Leads to Cell Death in Embryonic Ectoderm and Impaired Gastrulation of Mouse Embryos. *Genes Dev*. 1994; 8:2466–2477. [PubMed: 7958910]
8. Inoue Y, Yu AM, Yim SH, Ma X, Krausz KW, et al. Regulation of Bile Acid Biosynthesis by Hepatocyte Nuclear Factor 4alpha. *J Lipid Res*. 2006; 47:215–227. [PubMed: 16264197]
9. Inoue Y, Yu AM, Inoue J, Gonzalez FJ. Hepatocyte Nuclear Factor 4alpha Is a Central Regulator of Bile Acid Conjugation. *J Biol Chem*. 2004; 279:2480–2489. [PubMed: 14583614]
10. Claudel T, Inoue Y, Barbier O, Duran-Sandoval D, Kosykh V, et al. Farnesoid X Receptor Agonists Suppress Hepatic Apolipoprotein Ciii Expression. *Gastroenterology*. 2003; 125:544–555. [PubMed: 12891557]
11. Shih DQ, Dansky HM, Fleisher M, Assmann G, Fajans SS, et al. Genotype/Phenotype Relationships in Hnf-4alpha/Mody1: Haploinsufficiency Is Associated with Reduced Apolipoprotein (Aii), Apolipoprotein (Ciii), Lipoprotein(a), and Triglyceride Levels. *Diabetes*. 2000; 49:832–837. [PubMed: 10905494]
12. Stroup D, Chiang JY. Hnf4 and Coup-Tfii Interact to Modulate Transcription of the Cholesterol 7alpha-Hydroxylase Gene (Cyp7a1). *J Lipid Res*. 2000; 41:1–11. [PubMed: 10627496]

13. Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, et al. The Orphan Nuclear Receptor Hnf4alpha Determines Pxr- and Car-Mediated Xenobiotic Induction of Cyp3a4. *Nat Med.* 2003; 9:220–224. [PubMed: 12514743]
14. Zhang Y, Lee FY, Barrera G, Lee H, Vales C, et al. Activation of the Nuclear Receptor Fxr Improves Hyperglycemia and Hyperlipidemia in Diabetic Mice. *Proc Natl Acad Sci U S A.* 2006; 103:1006–1011. [PubMed: 16410358]
15. Watanabe M, Houten SM, Wang L, Moschetta A, Mangelsdorf DJ, et al. Bile Acids Lower Triglyceride Levels Via a Pathway Involving Fxr, Shp, and Srebp-1c. *J Clin Invest.* 2004; 113:1408–1418. [PubMed: 15146238]
16. Staels B, Kuipers F. Bile Acid Sequestrants and the Treatment of Type 2 Diabetes Mellitus. *Drugs.* 2007; 67:1383–1392. [PubMed: 17600387]
17. Cariou B, van Harmelen K, Duran-Sandoval D, van Dijk TH, Grefhorst A, et al. The Farnesoid X Receptor Modulates Adiposity and Peripheral Insulin Sensitivity in Mice. *J Biol Chem.* 2006; 281:11039–11049. [PubMed: 16446356]
18. Ma K, Saha PK, Chan L, Moore DD. Farnesoid X Receptor Is Essential for Normal Glucose Homeostasis. *J Clin Invest.* 2006; 116:1102–1109. [PubMed: 16557297]
19. Maran RR, Thomas A, Roth M, Shen Z, Esterly N, et al. Fxr Deficiency in Mice Leads to Increased Intestinal Epithelial Cell Proliferation and Tumor Development. *J Pharmacol Exp Ther.* 2008
20. Modica S, Murzilli S, Salvatore L, Schmidt DR, Moschetta A. Nuclear Bile Acid Receptor Fxr Protects against Intestinal Tumorigenesis. *Cancer Res.* 2008; 68:9589–9594. [PubMed: 19047134]
21. Kim I, Morimura K, Shah Y, Yang Q, Ward JM, et al. Spontaneous Hepatocarcinogenesis in Farnesoid X Receptor-Null Mice. *Carcinogenesis.* 2007; 28:940–946. [PubMed: 17183066]
22. Yang F, Huang X, Yi T, Yen Y, Moore DD, et al. Spontaneous Development of Liver Tumors in the Absence of the Bile Acid Receptor Farnesoid X Receptor. *Cancer Res.* 2007; 67:863–867. [PubMed: 17283114]
23. Thomas AM, Hart SN, Kong B, Fang J, Zhong XB, et al. Genome-Wide Tissue-Specific Farnesoid X Receptor Binding in Mouse Liver and Intestine. *Hepatology.* 2010; 51:1410–1419. [PubMed: 20091679]
24. Chong HK, Infante AM, Seo Y-K, Jeon T-I, Zhang Y, et al. Genome-Wide Interrogation of Hepatic Fxr Reveals an Asymmetric Ir-1 Motif and Synergy with Lrh-1. *Nucleic Acids Res.* 2010; 38:6007–6017. [PubMed: 20483916]
25. Gonzalez FJ. Regulation of Hepatocyte Nuclear Factor 4 Alpha-Mediated Transcription. *Drug Metab Pharmacokinet.* 2008; 23:2–7. [PubMed: 18305369]
26. Maloney PR, Parks DJ, Haffner CD, Fivush AM, Chandra G, et al. Identification of a Chemical Tool for the Orphan Nuclear Receptor Fxr. *J Med Chem.* 2000; 43:2971–2974. [PubMed: 10956205]
27. Hayhurst GP, Lee YH, Lambert G, Ward JM, Gonzalez FJ. Hepatocyte Nuclear Factor 4alpha (Nuclear Receptor 2a1) Is Essential for Maintenance of Hepatic Gene Expression and Lipid Homeostasis. *Mol Cell Biol.* 2001; 21:1393–1403. [PubMed: 11158324]
28. Schmidt D, Wilson MD, Ballester B, Schwalie PC, Brown GD, et al. Five-Vertebrate Chip-Seq Reveals the Evolutionary Dynamics of Transcription Factor Binding. *Science.* 2010; 328:1036–1040. [PubMed: 20378774]
29. Zhang Y, Liu T, Meyer CA, Eeckhoutte J, Johnson DS, et al. Model-Based Analysis of Chip-Seq (Macs). *Genome Biol.* 2008; 9:R137. [PubMed: 18798982]
30. Quinlan AR, Hall IM. Bedtools: A Flexible Suite of Utilities for Comparing Genomic Features. *Bioinformatics.* 2010; 26:841–842. [PubMed: 20110278]
31. Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, et al. The Human Genome Browser at Ucsf. *Genome Res.* 2002; 12:996–1006. [PubMed: 12045153]
32. Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, et al. David: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol.* 2003; 4:P3. [PubMed: 12734009]
33. Li G, Thomas AM, Hart SN, Zhong X, Wu D, et al. Farnesoid X Receptor Activation Mediates Head-to-Tail Chromatin Looping in the Nr0b2 Gene Encoding Small Heterodimer Partner. *Mol Endocrinol.* 2010; 24:1404–1412. [PubMed: 20444884]

34. Li G, Thomas AM, Williams JA, Kong B, Liu J, et al. Farnesoid X Receptor Induces Murine Scavenger Receptor Class B Type I Via Intron Binding. *PLoS One*. 2012; 7:e35895. [PubMed: 22540009]
35. Williams JA, Thomas AM, Li G, Kong B, Zhan L, et al. Tissue Specific Induction of P62/Sqstm1 by Farnesoid X Receptor. *PLoS One*. 2012; 7:e43961. [PubMed: 22952826]
36. Huber RM, Murphy K, Miao B, Link JR, Cunningham MR, et al. Generation of Multiple Farnesoid-X-Receptor Isoforms through the Use of Alternative Promoters. *Gene*. 2002; 290:35–43. [PubMed: 12062799]
37. Li T, Chiang JY. 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 Metab Dispos*. 2006; 34:756–764. [PubMed: 16455805]
38. Podvynec M, Kaufmann MR, Handschin C, Meyer UA. Nubiscan, an in Silico Approach for Prediction of Nuclear Receptor Response Elements. *Mol Endocrinol*. 2002; 16:1269–1279. [PubMed: 12040014]
39. Sladek FM, Zhong WM, Lai E, Darnell JE Jr. Liver-Enriched Transcription Factor Hnf-4 Is a Novel Member of the Steroid Hormone Receptor Superfamily. *Genes Dev*. 1990; 4:2353–2365. [PubMed: 2279702]
40. Bolotin E, Schnabl JM, Sladek FM, et al. IYDe. Hnf4a: The Transcription Factor Encyclopedia. *Genome Biol*. 2012; 13:R24. [PubMed: 22458515]
41. Modica S, Gadaleta RM, Moschetta A. Deciphering the Nuclear Bile Acid Receptor Fxr Paradigm. *Nucl Recept Signal*. 2010; 8:e005. [PubMed: 21383957]
42. Hurtado A, Holmes KA, Ross-Innes CS, Schmidt D, Carroll JS. Foxa1 Is a Key Determinant of Estrogen Receptor Function and Endocrine Response. *Nat Genet*. 2011; 43:27–33. [PubMed: 21151129]
43. Lupien M, Eeckhoutte J, Meyer CA, Wang Q, Zhang Y, et al. Foxa1 Translates Epigenetic Signatures into Enhancer-Driven Lineage-Specific Transcription. *Cell*. 2008; 132:958–970. [PubMed: 18358809]
1. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, et al. Bile Acids: Natural Ligands for an Orphan Nuclear Receptor. *Science*. 1999; 284:1365–1368. [PubMed: 10334993]
2. Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, et al. Identification of a Nuclear Receptor for Bile Acids. *Science*. 1999; 284:1362–1365. [PubMed: 10334992]
3. Wang H, Chen J, Hollister K, Sowers LC, Forman BM. Endogenous Bile Acids Are Ligands for the Nuclear Receptor Fxr/Bar. *Mol Cell*. 1999; 3:543–553. [PubMed: 10360171]
4. Seol W, Choi HS, Moore DD. Isolation of Proteins That Interact Specifically with the Retinoid X Receptor: Two Novel Orphan Receptors. *Mol Endocrinol*. 1995; 9:72–85. [PubMed: 7760852]
5. Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, et al. Targeted Disruption of the Nuclear Receptor Fxr/Bar Impairs Bile Acid and Lipid Homeostasis. *Cell*. 2000; 102:731–744. [PubMed: 11030617]
6. Kok T, Hulzebos CV, Wolters H, Havinga R, Agellon LB, et al. Enterohepatic Circulation of Bile Salts in Farnesoid X Receptor-Deficient Mice. *J Biol Chem*. 2003; 278:41930–41937. [PubMed: 12917447]
7. Chen WS, Manova K, Weinstein DC, Duncan SA, Plump AS, et al. Disruption of the Hnf-4 Gene, Expressed in Visceral Endoderm, Leads to Cell Death in Embryonic Ectoderm and Impaired Gastrulation of Mouse Embryos. *Genes Dev*. 1994; 8:2466–2477. [PubMed: 7958910]
8. Inoue Y, Yu AM, Yim SH, Ma X, Krausz KW, et al. Regulation of Bile Acid Biosynthesis by Hepatocyte Nuclear Factor 4alpha. *J Lipid Res*. 2006; 47:215–227. [PubMed: 16264197]
9. Inoue Y, Yu AM, Inoue J, Gonzalez FJ. Hepatocyte Nuclear Factor 4alpha Is a Central Regulator of Bile Acid Conjugation. *J Biol Chem*. 2004; 279:2480–2489. [PubMed: 14583614]
10. Claudel T, Inoue Y, Barbier O, Duran-Sandoval D, Kosykh V, et al. Farnesoid X Receptor Agonists Suppress Hepatic Apolipoprotein Ciii Expression. *Gastroenterology*. 2003; 125:544–555. [PubMed: 12891557]
11. Shih DQ, Dansky HM, Fleisher M, Assmann G, Fajans SS, et al. Genotype/Phenotype Relationships in Hnf-4alpha/Mody1: Haploinsufficiency Is Associated with Reduced

- Apolipoprotein (Aii), Apolipoprotein (Ciii), Lipoprotein(a), and Triglyceride Levels. *Diabetes*. 2000; 49:832–837. [PubMed: 10905494]
12. Stroup D, Chiang JY. Hnf4 and Coup-Tfii Interact to Modulate Transcription of the Cholesterol 7 α -Hydroxylase Gene (*Cyp7a1*). *J Lipid Res*. 2000; 41:1–11. [PubMed: 10627496]
 13. Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, et al. The Orphan Nuclear Receptor Hnf4 α Determines Pxr- and Car-Mediated Xenobiotic Induction of *Cyp3a4*. *Nat Med*. 2003; 9:220–224. [PubMed: 12514743]
 14. Zhang Y, Lee FY, Barrera G, Lee H, Vales C, et al. Activation of the Nuclear Receptor Fxr Improves Hyperglycemia and Hyperlipidemia in Diabetic Mice. *Proc Natl Acad Sci U S A*. 2006; 103:1006–1011. [PubMed: 16410358]
 15. Watanabe M, Houten SM, Wang L, Moschetta A, Mangelsdorf DJ, et al. Bile Acids Lower Triglyceride Levels Via a Pathway Involving Fxr, Shp, and Srebp-1c. *J Clin Invest*. 2004; 113:1408–1418. [PubMed: 15146238]
 16. Staels B, Kuipers F. Bile Acid Sequestrants and the Treatment of Type 2 Diabetes Mellitus. *Drugs*. 2007; 67:1383–1392. [PubMed: 17600387]
 17. Cariou B, van Harmelen K, Duran-Sandoval D, van Dijk TH, Grefhorst A, et al. The Farnesoid X Receptor Modulates Adiposity and Peripheral Insulin Sensitivity in Mice. *J Biol Chem*. 2006; 281:11039–11049. [PubMed: 16446356]
 18. Ma K, Saha PK, Chan L, Moore DD. Farnesoid X Receptor Is Essential for Normal Glucose Homeostasis. *J Clin Invest*. 2006; 116:1102–1109. [PubMed: 16557297]
 19. Maran RR, Thomas A, Roth M, Shen Z, Esterly N, et al. Fxr Deficiency in Mice Leads to Increased Intestinal Epithelial Cell Proliferation and Tumor Development. *J Pharmacol Exp Ther*. 2008
 20. Modica S, Murzilli S, Salvatore L, Schmidt DR, Moschetta A. Nuclear Bile Acid Receptor Fxr Protects against Intestinal Tumorigenesis. *Cancer Res*. 2008; 68:9589–9594. [PubMed: 19047134]
 21. Kim I, Morimura K, Shah Y, Yang Q, Ward JM, et al. Spontaneous Hepatocarcinogenesis in Farnesoid X Receptor-Null Mice. *Carcinogenesis*. 2007; 28:940–946. [PubMed: 17183066]
 22. Yang F, Huang X, Yi T, Yen Y, Moore DD, et al. Spontaneous Development of Liver Tumors in the Absence of the Bile Acid Receptor Farnesoid X Receptor. *Cancer Res*. 2007; 67:863–867. [PubMed: 17283114]
 23. Thomas AM, Hart SN, Kong B, Fang J, Zhong XB, et al. Genome-Wide Tissue-Specific Farnesoid X Receptor Binding in Mouse Liver and Intestine. *Hepatology*. 2010; 51:1410–1419. [PubMed: 20091679]
 24. Chong HK, Infante AM, Seo Y-K, Jeon T-I, Zhang Y, et al. Genome-Wide Interrogation of Hepatic Fxr Reveals an Asymmetric Ir-1 Motif and Synergy with Lrh-1. *Nucleic Acids Res*. 2010; 38:6007–6017. [PubMed: 20483916]
 25. Gonzalez FJ. Regulation of Hepatocyte Nuclear Factor 4 Alpha-Mediated Transcription. *Drug Metab Pharmacokinet*. 2008; 23:2–7. [PubMed: 18305369]
 26. Maloney PR, Parks DJ, Haffner CD, Fivush AM, Chandra G, et al. Identification of a Chemical Tool for the Orphan Nuclear Receptor Fxr. *J Med Chem*. 2000; 43:2971–2974. [PubMed: 10956205]
 27. Hayhurst GP, Lee YH, Lambert G, Ward JM, Gonzalez FJ. Hepatocyte Nuclear Factor 4 α (Nuclear Receptor 2a1) Is Essential for Maintenance of Hepatic Gene Expression and Lipid Homeostasis. *Mol Cell Biol*. 2001; 21:1393–1403. [PubMed: 11158324]
 28. Schmidt D, Wilson MD, Ballester B, Schwalie PC, Brown GD, et al. Five-Vertebrate Chip-Seq Reveals the Evolutionary Dynamics of Transcription Factor Binding. *Science*. 2010; 328:1036–1040. [PubMed: 20378774]
 29. Zhang Y, Liu T, Meyer CA, Eeckhoutte J, Johnson DS, et al. Model-Based Analysis of Chip-Seq (Macs). *Genome Biol*. 2008; 9:R137. [PubMed: 18798982]
 30. Quinlan AR, Hall IM. Bedtools: A Flexible Suite of Utilities for Comparing Genomic Features. *Bioinformatics*. 2010; 26:841–842. [PubMed: 20110278]
 31. Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, et al. The Human Genome Browser at Usc. *Genome Res*. 2002; 12:996–1006. [PubMed: 12045153]

32. Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, et al. David: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol.* 2003; 4:P3. [PubMed: 12734009]
33. Li G, Thomas AM, Hart SN, Zhong X, Wu D, et al. Farnesoid X Receptor Activation Mediates Head-to-Tail Chromatin Looping in the Nr0b2 Gene Encoding Small Heterodimer Partner. *Mol Endocrinol.* 2010; 24:1404–1412. [PubMed: 20444884]
34. Li G, Thomas AM, Williams JA, Kong B, Liu J, et al. Farnesoid X Receptor Induces Murine Scavenger Receptor Class B Type I Via Intron Binding. *PLoS One.* 2012; 7:e35895. [PubMed: 22540009]
35. Williams JA, Thomas AM, Li G, Kong B, Zhan L, et al. Tissue Specific Induction of P62/Sqstm1 by Farnesoid X Receptor. *PLoS One.* 2012; 7:e43961. [PubMed: 22952826]
36. Huber RM, Murphy K, Miao B, Link JR, Cunningham MR, et al. Generation of Multiple Farnesoid-X-Receptor Isoforms through the Use of Alternative Promoters. *Gene.* 2002; 290:35–43. [PubMed: 12062799]
37. Li T, Chiang JY. 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 Metab Dispos.* 2006; 34:756–764. [PubMed: 16455805]
38. Podvinec M, Kaufmann MR, Handschin C, Meyer UA. Nubiscan, an in Silico Approach for Prediction of Nuclear Receptor Response Elements. *Mol Endocrinol.* 2002; 16:1269–1279. [PubMed: 12040014]
39. Sladek FM, Zhong WM, Lai E, Darnell JE Jr. Liver-Enriched Transcription Factor Hnf-4 Is a Novel Member of the Steroid Hormone Receptor Superfamily. *Genes Dev.* 1990; 4:2353–2365. [PubMed: 2279702]
40. Bolotin E, Schnabl JM, Sladek FM, et al. IYDe. Hnf4a: The Transcription Factor Encyclopedia. *Genome Biol.* 2012; 13:R24. [PubMed: 22458515]
41. Modica S, Gadaleta RM, Moschetta A. Deciphering the Nuclear Bile Acid Receptor Fxr Paradigm. *Nucl Recept Signal.* 2010; 8:e005. [PubMed: 21383957]
42. Hurtado A, Holmes KA, Ross-Innes CS, Schmidt D, Carroll JS. Foxa1 Is a Key Determinant of Estrogen Receptor Function and Endocrine Response. *Nat Genet.* 2011; 43:27–33. [PubMed: 21151129]
43. Lupien M, Eeckhoutte J, Meyer CA, Wang Q, Zhang Y, et al. Foxa1 Translates Epigenetic Signatures into Enhancer-Driven Lineage-Specific Transcription. *Cell.* 2008; 132:958–970. [PubMed: 18358809]

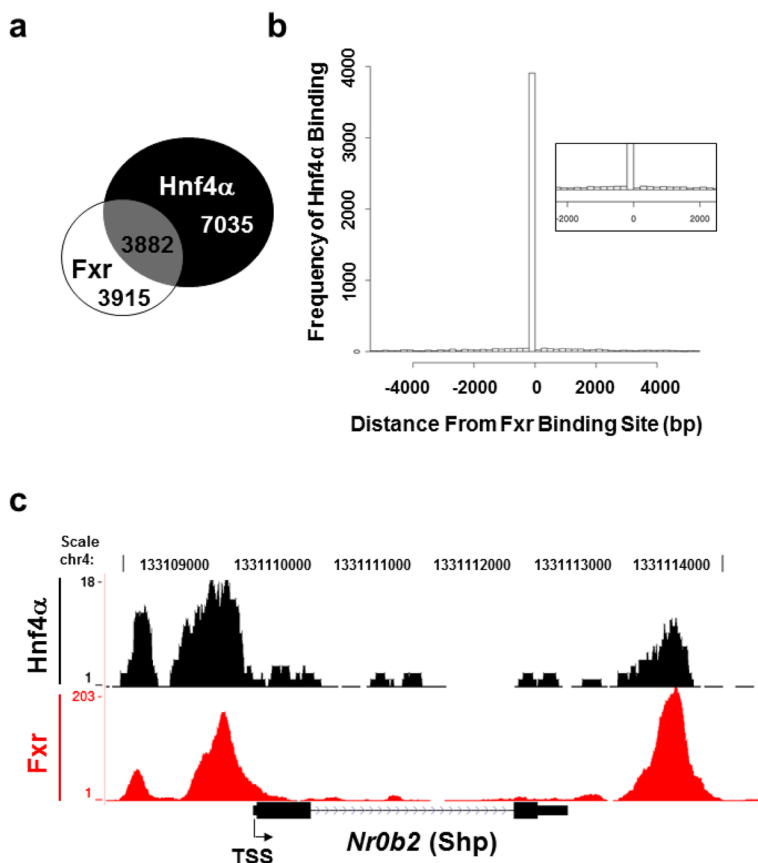
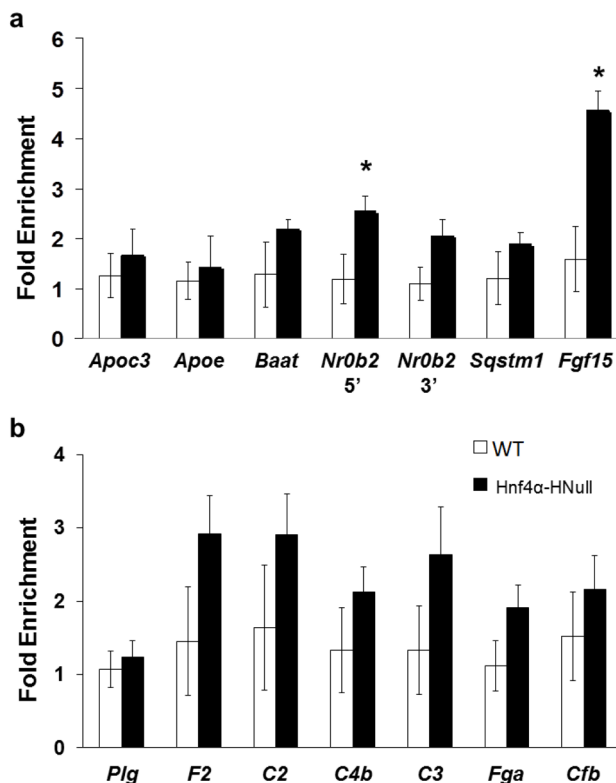


Fig. 1.

Genome-wide binding of Fxr and Hnf4 α in mouse liver. Previously reported Fxr and Hnf4 α ChIP-Seq data was reanalyzed using MACS (23, 28). (a) Venn diagram of total Hnf4 α and Fxr binding sites in mouse liver as revealed by chromatin immunoprecipitation followed by massively parallel sequencing (ChIP-Seq) analysis. We found 10917 total Hnf4 α binding sites and 7797 total Fxr binding sites in mouse liver, of which 3882 (nearly 50%) overlapped. (b) Histogram of the binding frequency, or number of binding events, of Hnf4 α (y-axis) in relation to distance from the Fxr binding site (x-axis) to the shared target genes in mouse liver. Collective Fxr binding sites are represented by “0.” (c) Histogram of Fxr (red) and Hnf4 α (black) binding to the *Nr0b2* (Shp) gene in mouse liver as determined by ChIP-Seq analysis. This histogram was generated using the UCSC Genome Browser (University of California, Santa Cruz).

**Fig. 2.**

ChIP-qPCR analysis of Fxr binding to shared target genes. QPCR analysis was performed on DNA fragments immunoprecipitated with Fxr antibody. ChIP-qPCR data are reported as fold increase (y-axis) of Fxr binding in Hnf4α-HNull (black bar) mouse liver compared to WT (white bar) mouse liver. **P*-value < 0.05. **(a)** ChIP-qPCR results of Fxr binding to shared target regions in WT and Hnf4α-HNull mouse liver. Regions within the *Apoc3* promoter, the *Apoe* promoter, the *Baat* promoter, the promoter and 3' region of *Nr0b2*, and downstream of the *Sqstm1* TSS are shared target sites of Fxr and HNF4α as revealed by ChIP-Seq analysis of mouse liver (Supplemental Table II). *Fgf15* is a target gene of Fxr (but not Hnf4α) in the intestine but not the liver and therefore was originally used as a negative control. **(b)** ChIP-qPCR results of Fxr binding to shared target regions of genes categorized within complement and coagulation cascades in WT and Hnf4α-HNull mouse liver. These binding sites are located within the promoters (within 500 bp upstream of TSS) of *Plg*, *F2*, *C2*, *C3*, *Fga*, *Cfb*, and -17125 to -17175 bp upstream of *C4b* gene TSS (Table II).

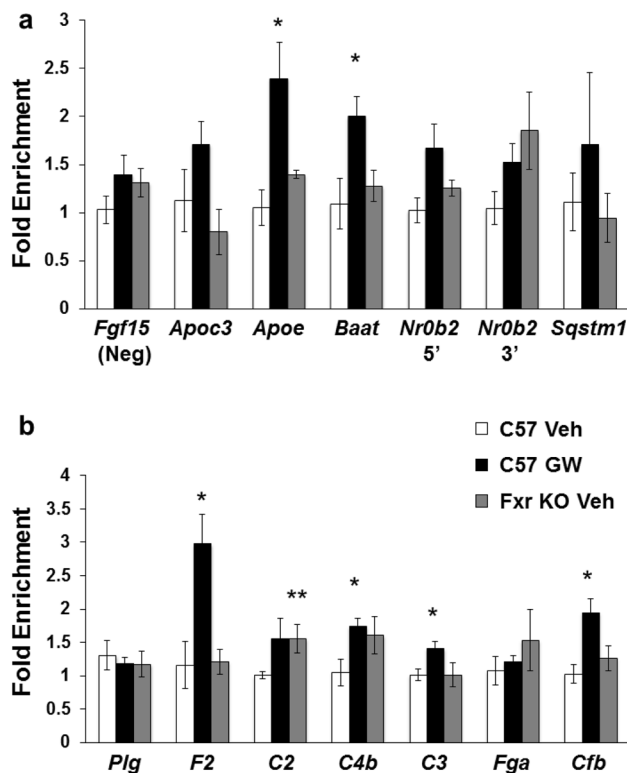


Fig. 3. ChIP-qPCR of Hnf4 α binding to shared target genes. **(a)** Fxr shares the Hnf4 α binding regions within the *Apoc3* promoter, the *Apoe* promoter, the *Baat* promoter, the promoter and 3' region of *Nr0b2*, and downstream of the *Sqstm1* TSS, revealed by ChIP-qPCR (Supplemental Table II). The *Fgf15* binding site was determined not to be an Hnf4 α binding site and therefore was used as a negative control. Hnf4 α binding to these regions was investigated in WT and Fxr KO vehicle and WT GW4064 treated mouse liver. Data are reported as fold enrichment (y-axis) of Hnf4 α binding in WT GW (black bar) or Fxr KO veh (gray bar) treated mouse liver normalized to WT veh (white bar) treated mouse liver. **(b)** ChIP-qPCR data of Hnf4 α binding to shared target regions within genes categorized as part of complement and coagulation cascade. These binding sites are located within the promoters (within 500 bp upstream of TSS) of *Plg*, *F2*, *C2*, *C3*, *Fga*, *Cfb*, and -17125 to -17175 bp upstream of *C4b* gene TSS (Table II). Data are reported as described in part (a). **P*-value 0.05 of C57 GW treated group compared to C57 veh group. ***P*-value 0.05 in Fxr KO vehicle group compared to C57 veh group.

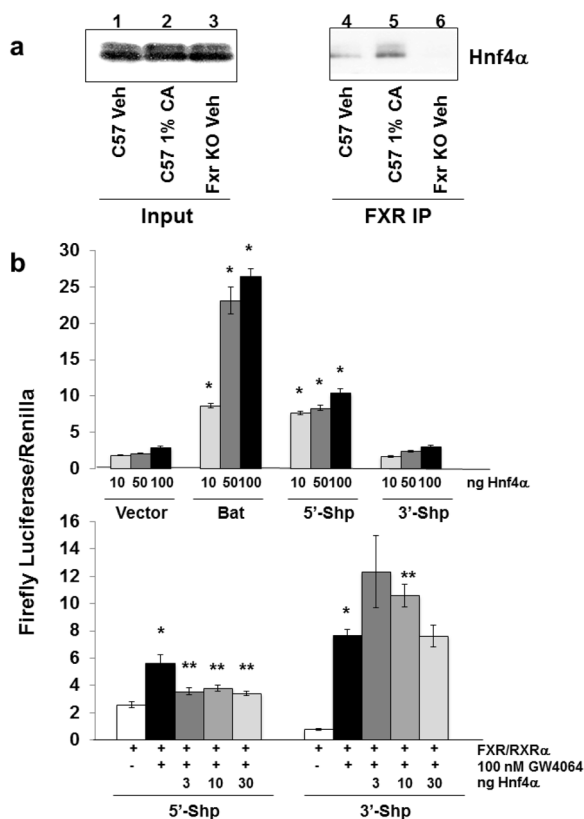


Fig. 4. Co-IP of Fxr and Hnf4 α and luciferase assays of Shp regulatory regions. **(a)** Co-IP of WT mice fed control or 1% CA diet and Fxr KO mice fed control diet. Whole cell liver lysates were prepared and immunoprecipitated using an antibody against Fxr. Liver lysates (input) and IP fractions were pooled and analyzed by Western blot analysis using antibody against Hnf4 α . Lanes 1–3 show the levels of Hnf4 α within 15 μ g of pooled whole-cell lysates from WT control diet (Lane 1), WT 1% CA diet (Lane 2), and Fxr KO control diet (Lane 3) groups. Lane 4–6 show levels of Hnf4 α detection within WT control diet (Lane 4), WT 1% CA diet (Lane 5), and Fxr KO control diet (Lane 6) liver lysates immunoprecipitated with an Fxr antibody. **(b)** Luciferase expression assays showing the effects of increasing amounts of mouse Hnf4 α expression vector (10, 50, 100 ng) on transcriptionally activating regulatory regions within the Bat gene promoter, the 5' and 3' regulatory regions of the Shp gene, or the luciferase vector control (top panel). Bottom panel shows the effects of increasing mouse Hnf4 α expression vector amounts (3, 10, and 30 ng) on FXR-induced transcription of the 5' and 3' regulatory regions of the Shp gene after activation of FXR with 100 nM of GW4064. Results are reported as a ratio of firefly luciferase activity over *Renilla* luciferase activity (y-axis). **P*-value ≤ 0.05 of HNF4 α transfected groups when compared to vector or FXR/RXR α GW treated groups compared to veh control. ***P*-value ≤ 0.05 of Hnf4 α transfected GW treated groups compared to FXR/RXR α alone GW treated groups.

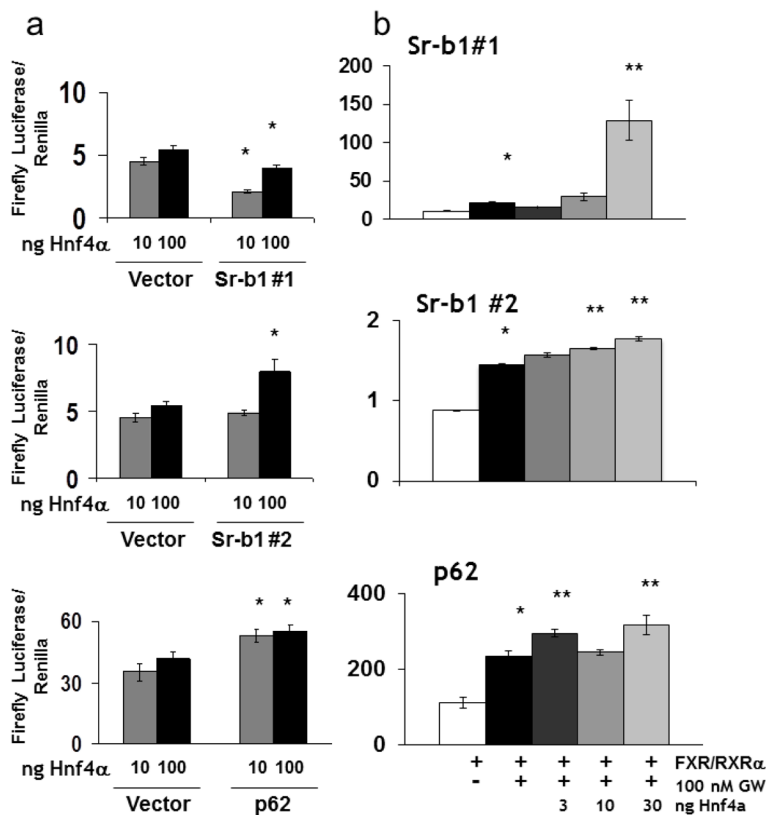


Fig. 5. Transcriptional effects of Hnf4α alone and with FXR at shared target genes by luciferase assays. **(a)** Luciferase expression assays showing the effects of increasing amounts Hnf4α expression vector (10 and 100 ng) on transcriptionally activating regulatory regions within 10.6 Kb (#1; top) and 21.5 Kb (#2; middle) downstream of the Sr-b1 TSS, in the downstream regulatory region of p62 (bottom), and in the luciferase vector controls. **(b)** Effects of increasing amounts of Hnf4α expression vector (3, 10, and 30 ng) on FXR-induced transcription of regulatory regions within the Sr-b1 gene (#1 (top) and #2 (middle)) and p62 (bottom) after activation of FXR with 100 nM of GW4064. Results of luciferase assays (both **[a]** and **[b]**) are reported as a ratio of firefly luciferase activity over *Renilla* luciferase activity (y-axis). **P*-value = 0.05 of Hnf4α transfected groups when compared to vector or FXR/RXRα GW treated groups compared to veh control. ***P*-value = 0.05 of Hnf4α transfected GW treated groups compared to FXR/RXR alone GW treated groups.

Table IPathways enriched by both FXR and Hnf4 α binding in mouse livers

Pathway	Genes*	% bound by FXR and HNF4 α	P-Value	Bonferroni
Complement and coagulation cascades	17	2.24	1.38E-07	2.25E-05
Drug metabolism	16	2.11	8.33E-07	1.36E-04
PPAR signaling pathway	16	2.11	1.67E-06	2.73E-04
Metabolism of xenobiotics by cytochrome P450	13	1.71	2.97E-05	4.83E-03
Insulin signaling pathway	12	1.58	4.55E-02	0.999
Glycine, serine and threonine metabolism	9	1.18	6.15E-05	9.97E-03
Steroid hormone biosynthesis	9	1.18	7.58E-04	0.116
Adipocytokine signaling pathway	9	1.18	9.80E-03	0.799
Pyruvate metabolism	8	1.05	2.07E-03	0.287
Fatty acid metabolism	8	1.05	3.59E-03	0.443
Drug metabolism	8	1.05	5.19E-03	0.572
Retinol metabolism	8	1.05	3.20E-02	0.995
Cysteine and methionine metabolism	7	0.92	3.10E-03	0.397
Linoleic acid metabolism	7	0.92	1.61E-02	0.929
Biosynthesis of unsaturated fatty acids	6	0.79	6.32E-03	0.644
Starch and sucrose metabolism	6	0.79	2.13E-02	0.97
ABC transporters	6	0.79	4.99E-02	1
Selenoamino acid metabolism	5	0.66	1.80E-02	0.948
Alanine, aspartate and glutamate metabolism	5	0.66	4.36E-02	0.999
Primary bile acid biosynthesis	4	0.53	2.74E-02	0.989

Note:

* indicates the number of genes bound by FXR and Hnf4 α in this pathway

Table IIList of target genes within complement and coagulation cascades bound by both FXR and Hnf4 α .

Official Gene Symbol	Binding Site Location from TSS	FXR Total Counts*	HNF4 α Total Counts*
<i>Plg</i>	-144, 1992, 6986, 9608	99, 155, 127, 95	46, 39, 31, 33
<i>Fga</i>	-225, -5561	150, 105	40, 53
<i>Cpb2</i>	-9013, 9	52, 34	38, 19
<i>Serpina1e</i>	-4534, 3769	76, 35	62, 27
<i>Fgg</i>	-345, -4534, 1904, 2607	70, 197, 20, 28	9, 47, 26, 25
<i>F2</i>	-436	129	67
<i>Mbl1</i>	-44, 9558	192, 156	24, 64
<i>Cfh</i>	21	109	21
<i>Knz2</i>	-125, -10481	69, 40	39, 17
<i>Serpine1</i>	-507	115	33
<i>Cfb</i>	-183	217	78
<i>Serpinf2</i>	-66	41	22
<i>C4b</i>	-17142	93	83
<i>C2</i>	-4	68	46
<i>C3</i>	-236, -2276, -2788, -5187	884, 75, 69, 175	46, 15, 10, 55
<i>Proc</i>	-1366	77	53
<i>Knz1</i>	-124, -1946	96, 24	35, 21

* Counts are the number of binding events recorded for FXR and HnfF4 α at each location.