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Nuclear Receptors HNF4α and LRH-1 Cooperate in Regulating *Cyp7a1 in Vivo*[*] [S]

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Background: FGF19 inhibits bile acid synthesis by repressing transcription of *Cyp7a1* through a SHP-dependent mechanism.
Results: Eliminating HNF4α or LRH-1 in liver reduces basal *Cyp7a1* expression and disrupts its repression by FGF19 and SHP.
Conclusion: HNF4α and LRH-1 cooperate in regulating basal *Cyp7a1* transcription and its repression by FGF19.
Significance: Understanding how bile acid synthesis is repressed has implications for treating chronic diarrhea syndromes.

Fibroblast growth factor 19 (FGF19) is a postprandial enterokine induced by the nuclear bile acid receptor, FXR, in ileum. FGF19 inhibits bile acid synthesis in liver through transcriptional repression of cholesterol 7α-hydroxylase (*CYP7A1*) via a mechanism involving the nuclear receptor SHP. Here, in a series of loss-of-function studies, we show that the nuclear receptors HNF4α and LRH-1 have dual roles in regulating *Cyp7a1 in vivo*. First, they cooperate in maintaining basal *Cyp7a1* expression. Second, they enable SHP binding to the *Cyp7a1* promoter and facilitate FGF19-mediated repression of bile acid synthesis. HNF4α and LRH-1 promote active transcription histone marks on the *Cyp7a1* promoter that are reversed by FGF19 in a SHP-dependent manner. These findings demonstrate that both HNF4α and LRH-1 are important regulators of *Cyp7a1* transcription *in vivo*.

Bile acids are natural detergents that facilitate the solubilization and absorption of lipophilic nutrients in the intestine. Bile acids are synthesized in liver and stored as bile in the gallbladder. Following a meal, bile acids are released into the small intestine, where they aid digestion. Approximately 95% of the bile acids are reabsorbed in the ileum and returned to the liver via the portal circulation (1, 2).

Because of their detergent properties, bile acid concentrations are tightly regulated. The nuclear bile acid receptor, farnesoid X receptor (FXR),[3] plays a central role in this regulation. FXR is highly expressed in the liver and ileum, where it regulates numerous genes involved in maintaining bile acids at appropriate levels (3). Among the genes regulated by FXR is cholesterol 7α-hydroxylase (*CYP7A1*), which encodes the first and rate-limiting enzyme in the major bile acid synthetic pathway. FXR represses CYP7A1 through an indirect, bipartite mechanism. First, in liver, FXR induces expression of small heterodimer partner (SHP), an atypical nuclear receptor lacking a DNA binding domain. SHP binds to the CYP7A1 promoter and represses its transcription through interactions with other transcription factors (4–6). Second, in ileum, FXR induces fibroblast growth factor 19 (*FGF19*, *Fgf15* in mice), an atypical FGF that can act as a hormone. FGF15/19 represses CYP7A1 through a mechanism that requires SHP (7, 8). *Cyp7a1* and bile acid homeostasis are dysregulated in mice lacking FXR, SHP, or FGF15 (8–13).

Previous studies suggested that SHP is recruited to the *CYP7A1* promoter through interactions with liver receptor homolog-1 (LRH-1), a nuclear receptor activated by phospholipids (4, 5, 14). However, mice in which the *Lrh-1* gene was selectively disrupted in liver during development did not have defects in the negative feedback regulation of *Cyp7a1* (15, 16). Here, in a series of *in vivo* loss-of-function studies, we have examined the roles of LRH-1 and hepatocyte nuclear factor 4α (HNF4α), another nuclear receptor implicated in bile acid homeostasis (17, 18), in regulating *Cyp7a1*. Using acute conditional knock-out mouse models, we show that both LRH-1 and HNF4α are crucial transcriptional activators of the *Cyp7a1* promoter and are required for FGF19 and SHP to repress *Cyp7a1*.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—HEK293 and HepG2 cells were maintained in DMEM (Invitrogen) and MEM (Sigma), respectively. The media also contained 10% FBS and 1× penicillin/streptomycin. Transfection experiments were performed by using Lipofectamine™ 2000 (Invitrogen) on HEK293 cells and Fugene® HD (Roche) on HepG2 cells according to the manufacturer's instructions. p650-r*Cyp7a1* and p569-h*SHP* promoter-luciferase reporters were as described (5). The HNF4α and LRH-1 antibodies were from Perseus Proteomics and the TBP

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[3] The abbreviations used are: FXR, farnesoid X receptor; FGF, fibroblast growth factor; CYP7A1, cholesterol 7α-hydroxylase; SHP, small heterodimer partner; LRH-1, liver receptor homolog-1; HNF4α, hepatocyte nuclear factor 4α.

antibody from Santa Cruz Biotechnology. HA and FLAG antibody beads as well as FLAG antibody were from Sigma. Recombinant FGF19 was prepared as described (8).

Mouse Animal Experiments—All animal experiments were approved by the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center. Mice were housed in a pathogen-free and a temperature-controlled environment with 12 h light/dark cycles (6 am-6 pm) and fed standard irradiated rodent chow. *Hnf4α*$^{fl/fl}$ and *Hnf4α*$^{fl/fl}$;albumin-Cre (19), *Lrh-1*$^{fl/fl}$ and *Lrh-1*$^{fl/fl}$;albumin-Cre (15) and *Shp*$^{-/-}$ (11) mice were as described. FGF19 protein was administered in a buffer (*i.e.* vehicle) containing PBS and up to 4% glycerol. Details of each experiment are provided in the figure legends.

Adenoviruses were prepared as described (8). Mice were infected with adenovirus by injection into the jugular vein. Each mouse received 1×10^{10} particles/g body weight FLAG-SHP and/or 3×10^{10} particles/g body weight Cre adenovirus in 0.15 ml of saline. Mice were killed 3–5 days after injection.

RT-qPCR—RNA was extracted from frozen liver samples using RNA-STAT60™ (Isotex Diagnostics), DNase treated, and reverse transcribed using random hexamers. Resulting cDNA was analyzed by RT-qPCR. Briefly, 25 ng of cDNA and 150 nmol of each primer were mixed with SYBR® GreenER™ PCR Master Mix (Invitrogen). Reactions were performed in triplicate in 384-well format using an ABI PRISM® 7900HT instrument (Applied Biosystems). Relative mRNA levels were calculated using the comparative Ct method normalized to cyclophilin. The primers were designed using Primer Express® Software (Applied Biosystems) and listed in the supplemental data.

Chromatin Immunoprecipitation—Frozen and crushed liver samples were crosslinked with 1% formaldehyde for 15 min in PBS at room temperature. Cross-linking was stopped by addition of glycine. After two washes with PBS, samples were homogenized with glass homogenizers in a hypotonic buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2% Nonidet P-40, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 1 mM DTT, 5% sucrose, and protease inhibitors. The homogenate was laid on a cushion buffer containing 10 mM Tris-HCl (pH 7.5), 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 10% sucrose, and spun down to obtain nuclear pellet. The pellet was washed once with PBS and lysed in SDS lysis buffer containing 0.5% SDS, 0.5% Triton X-100, 5 mM EDTA, 33 mM Tris (pH 8.1), 84 mM NaCl, and then sonicated. After centrifugation, the supernatant (chromatin) was aliquoted and used for immunoprecipitation reactions performed with a Millipore ChIP kit following the manufacturer's protocol. Antibodies for the following proteins were purchased from indicated suppliers: HNF4α and LRH-1 (Perseus Proteomics), Histone H3K4 trimethyl and RNA Polymerase II-CTD (Abcam) and Acetyl-histone H3 (Millipore). PCR purification kits from Qiagen were used to purify final DNA products. Results were analyzed by q-PCR using primers listed in the supplemental data.

For re-ChIP experiments, the above protocol was used. In the first round of ChIP, antibody bound chromatin on protein A beads was eluted with a buffer containing 1% SDS, 0.1 M $NaHCO_3$, and 5 mM DTT and diluted 10-fold with dilution buffer and used in the second round of ChIP following the Millipore protocol.

For FLAG-SHP ChIP experiments, a dual crosslinking protocol was followed. Liver samples were first crosslinked with 2 mM di(*N*-succinimidyl) glutarate (Sigma) in PBS at room temperature for 45 min. After two washes with PBS, the samples were cross-linked with formaldehyde and processed as described above. FLAG antibody beads were purchased from Sigma.

Electrophoretic Mobility Shift Assay (EMSA)—HNF4α and LRH-1 were *in vitro* translated with the TNT Quick Coupled Transcription/Translation System (Promega). Double-stranded oligonucleotides with GCTA overhangs were generated and labeled with ^{32}P dCTP by end filling. Binding reactions were performed in a total volume of 20 μl containing 75 mM KCl, 20 mM HEPES (pH 7.4), 2 mM DTT, 7.5% glycerol, 0.1% Nonidet P-40, 2 μg of poly[d(I-C)] (Sigma), 40 pmol of a non-specific single-stranded oligonucleotide (to remove nonspecific binding), and 1 μl of each *in vitro* translation protein lysate. Later, 40 fmol of ^{32}P-labeled probe was added, and the reactions incubated at room temperature for 20 min. Samples were analyzed on 5% polyacrylamide gels run in 0.25 × TBE and visualized by autoradiography.

Nuclear Lysate Preparation and Western Blotting—Frozen and crushed liver samples were homogenized using glass homogenizers in a hypotonic buffer containing 20 mM Tris (pH 7.4), 2 mM $MgCl_2$, 0.25 M sucrose, 10 mM EDTA, 10 mM EGTA, 1 mM DTT, and protease inhibitors. After centrifugation, precipitated nuclear pellet was washed once with homogenization buffer and incubated with hypertonic Buffer C containing 20 mM HEPES (pH 7.9), 2.5% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and protease inhibitors for 45 min at 4 °C with agitation. After centrifugation at 70,000 rpm for 20 min, the supernatant was used as the nuclear extract. Protein concentration was determined by Bio-Rad Bradford assay and 30 μg of proteins were used in each SDS-PAGE run. Nitrocellulose membrane was used for Western blotting. Antibody incubation was performed in TBS containing 0.05% Tween and 5% milk. For visualization of the results, either SuperSignal West Pico or ECL Western blotting substrates from Pierce were used.

Statistical Analysis—Values are expressed as mean ± S.E. Significant differences between two groups were evaluated using two-tailed, unpaired *t* test.

RESULTS

HNF4α and LRH-1 Recruit SHP to the Cyp7a1 Promoter—It was previously shown that FGF15 overexpression fails to repress *Cyp7a1* transcription in *Shp*$^{-/-}$ mice (8). Because recombinant FGF15 is relatively unstable but has strongly overlapping effects with FGF19 (20), we used FGF19 protein in our mouse studies. FGF19 treatment also failed to inhibit *Cyp7a1* transcription in *Shp*$^{-/-}$ mice sacrificed at either 8 am or 2 pm (Fig. 1*A* and supplemental Fig. S1). Thus, SHP is required for FGF19-mediated repression of *Cyp7a1*.

SHP is an unusual nuclear receptor that does not bind directly to DNA but interacts with other nuclear receptors to

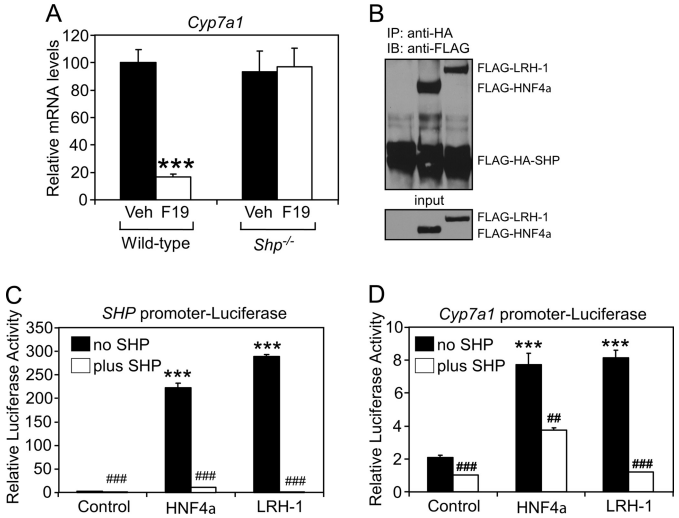

FIGURE 1. **SHP interactions with HNF4α and LRH-1.** *A*, overnight-fasted mice ($n = 6$) were injected intraperitoneally with vehicle or FGF19 protein (1 mg per kg body weight) at 8 am and sacrificed between 2–3 pm. Hepatic *Cyp7a1* mRNA levels were measured by RT-qPCR. *B*, tagged proteins were overexpressed in HEK293 cells and immunoprecipitated with HA antibody beads. *C*, HEK293 cells were transfected with a luciferase reporter under the control of human *SHP* promoter and with expression plasmids for the indicated proteins ($n = 4$). *D*, HepG2 cells were transfected with a luciferase reporter under the control of rat *Cyp7a1* promoter and with expression plasmids for the indicated proteins ($n = 4$). Values are means ± S.E. Statistical significance was determined by two-tailed *t* tests. (*) refers to differences between control and HNF4α or LRH-1 groups. (#) refers to differences between no SHP and plus SHP groups. ***, $p < 0.0005$; ##, $p < 0.005$; ###, $p < 0.0005$.

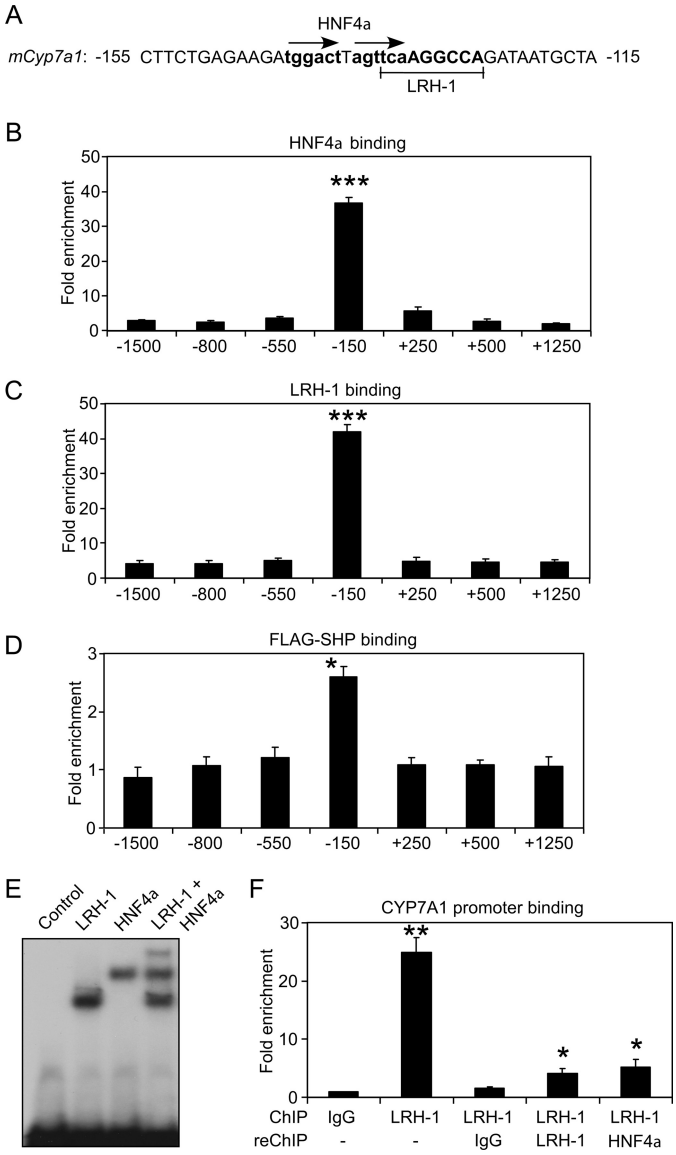

FIGURE 2. **HNF4α, LRH-1, and SHP binding to the *Cyp7a1* promoter.** *A*, putative HNF4α and LRH-1 binding sites on the *Cyp7a1* promoter are shown. *B* and *C*, antibodies against HNF4α and LRH-1 were used in ChIP experiments to test binding of these proteins to different locations on the *Cyp7a1* promoter and proximal gene body ($n = 3$). Liver samples are from the experiments shown in Fig. 4, *A* and *B*. *D*, FLAG-SHP was overexpressed in mouse liver via adenoviral expression. ChIP was performed with FLAG antibody beads ($n = 3$). Liver samples are from the experiment shown in Fig. 5, *A–D*. *E*, EMSA experiments were performed with *in vitro* translated proteins and a probe with the sequence shown in *A*. *F*, LRH-1 bound chromatin was immunoprecipitated and used for a second round of ChIP with indicated antibodies ($n = 3$). Values are means ± S.E. Statistical significance was determined by two-tailed *t* tests. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$ relative to the IgG group.

repress their transcriptional activity (6). The *Cyp7a1* promoter contains conserved putative DNA binding sites for two nuclear receptors, HNF4α and LRH-1, which interact with SHP (5, 21–24). Consistent with these earlier studies, FLAG-HA-tagged SHP co-immunoprecipitated FLAG-tagged HNF4α and LRH-1 (Fig. 1*B*), and SHP overexpression repressed HNF4α and LRH-1 transcriptional activity on the *SHP* and *Cyp7a1* promoters in luciferase reporter assays (Fig. 1, *C* and *D*). These data support the hypothesis that SHP represses *Cyp7a1* through interactions with HNF4α and LRH-1.

We next tested whether SHP, HNF4α, and LRH-1 bind to the *Cyp7a1* promoter in mouse liver. Putative overlapping HNF4α and LRH-1 binding sites are located ~150 bp upstream of the *Cyp7a1* transcription start region (Fig. 2*A*). In chromatin immunoprecipitation (ChIP) experiments done with liver extracts, both HNF4α and LRH-1 bound to this region (Fig. 2, *B* and *C*). Since adequate antibodies against endogenous SHP are not available, we overexpressed FLAG-tagged SHP in liver via adenoviral expression. ChIP experiments were performed with anti-FLAG beads. FLAG-SHP co-localized with HNF4α and LRH-1 on the *Cyp7a1* promoter (Fig. 2*D*), suggesting that SHP interacts with these two factors *in vivo*.

Given the overlap in the HNF4α and LRH-1 binding sites (Fig. 2*A*), we examined whether both factors bind simultaneously to the *Cyp7a1* promoter. In electrophoretic mobility shift assays (EMSAs), HNF4α and LRH-1 each resulted in a shifted complex (Fig. 2*E*) (5, 21, 23, 24). When both proteins were mixed, a third, more slowly migrating complex appeared (Fig. 2*E*). This third complex disappeared if either the HNF4α or LRH-1 binding sites were mutated (supplemental Fig. S2). Notably, there was no evidence of cooperative binding. In re-ChIP assays in which liver chromatin was first immunoprecipitated with an LRH-1 antibody and then subjected to a second round of ChIP, comparable results were obtained with LRH-1 and HNF4α antibodies (Fig. 2*F*). Taken together, the EMSA and re-ChIP data demonstrate that HNF4α and LRH-1 can interact with the *Cyp7a1* promoter simultaneously but do not do so cooperatively.

To determine whether HNF4α and LRH-1 contribute to SHP binding to the *Cyp7a1* promoter *in vivo*, we used conditional

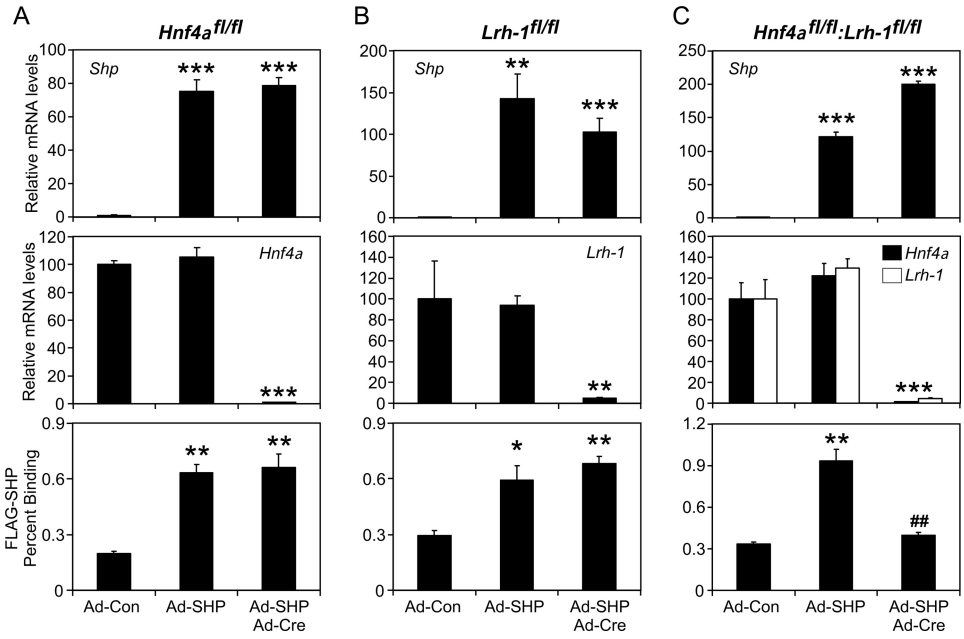

FIGURE 3. **SHP requires HNF4α or LRH-1 for binding to the *Cyp7a1* promoter.** *Hnf4α*$^{fl/fl}$ (*A*), *Lrh-1*$^{fl/fl}$ (*B*), and *Hnf4α*$^{fl/fl}$:*Lrh-1*$^{fl/fl}$ (*C*) mice ($n = 3–6$) were infected with control, Cre and/or FLAG-SHP adenoviruses. Hepatic *Shp*, *Hnf4α*, and *Lrh-1* mRNA levels were tested by RT-qPCR. FLAG-SHP binding to the *Cyp7a1* promoter was tested by ChIP ($n = 3$). Values are means ± S.E. Statistical significance was determined by two-tailed *t* tests. (*) refers to differences compared with Ad-Con group. (#) refers to differences between Ad-SHP and Ad-SHP/Ad-Cre groups. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$; ##, $p < 0.005$.

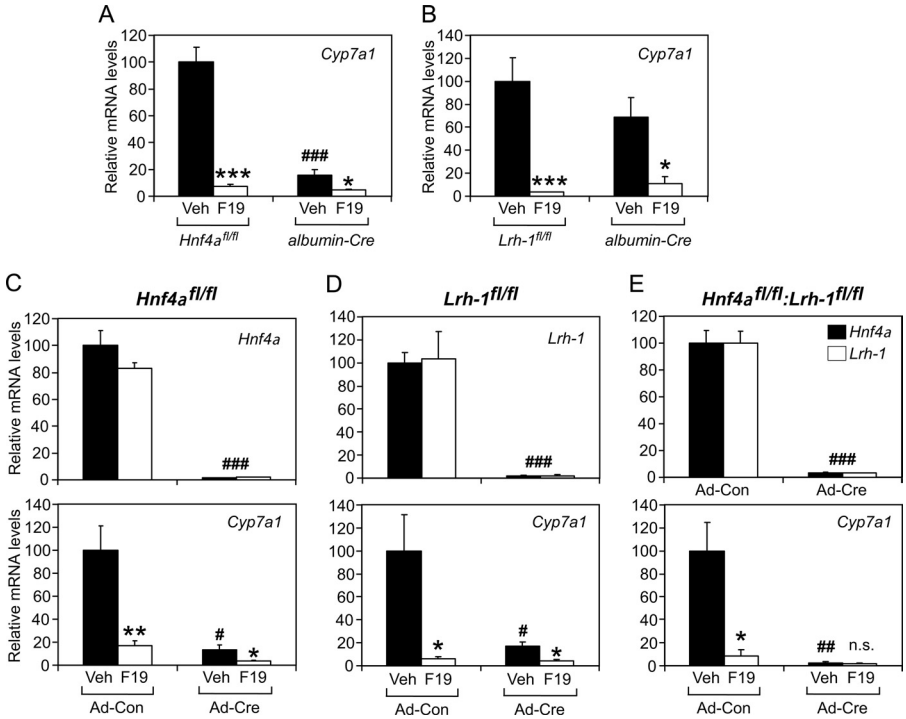

FIGURE 4. **HNF4α and LRH-1 maintain *Cyp7a1* expression and regulate FGF19-dependent repression.** Overnight-fasted *Hnf4α*$^{fl/fl}$ mice or their *albumin-Cre* littermates (*A*) and *Lrh-1*$^{fl/fl}$ mice or their *albumin-Cre* littermates (*B*) were treated with vehicle or FGF19 (1 mg/kg; intraperitoneal) for 6 h ($n = 6$). *Hnf4α*$^{fl/fl}$ (*C*), *Lrh-1*$^{fl/fl}$ (*D*), *Hnf4α*$^{fl/fl}$:*Lrh-1*$^{fl/fl}$ (*E*) mice were infected with control or Cre adenoviruses. Overnight-fasted mice ($n = 4–6$) were treated with vehicle or FGF19 (1 mg/kg; intraperitoneal) for 6 h. Hepatic *Hnf4α*, *Lrh-1* and *Cyp7a1* mRNA levels were tested by RT-qPCR. Values are means ± S.E. Statistical significance was determined by two-tailed *t* tests. (*) refers to differences between Veh and F19 groups. (#) refers to differences between two Veh groups. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$; #, $p < 0.05$; ##, $p < 0.005$; ###, $p < 0.0005$. *n.s.*, not significant.

knock-out models for *Hnf4α* and *Lrh-1*. Cre and/or FLAG-SHP were overexpressed in liver via adenoviral expression and FLAG-SHP binding was tested by ChIP. While knock-out of hepatic *Hnf4α* in *Hnf4α*$^{fl/fl}$ mice or knock-out of hepatic *Lrh-1* in *Lrh-1*$^{fl/fl}$ mice did not change FLAG-SHP binding to the *Cyp7a1* promoter in liver, knock-out of both genes in *Hnf4α*$^{fl/fl}$:*Lrh-1*$^{fl/fl}$ mice abolished FLAG-SHP binding (Fig. 3). These results show that SHP relies on both HNF4α and LRH-1 for binding to the *Cyp7a1* promoter. Although HNF4α and LRH-1 did not bind cooperatively to the *Cyp7a1* promoter in EMSA assays (Fig. 2*E*), we observed

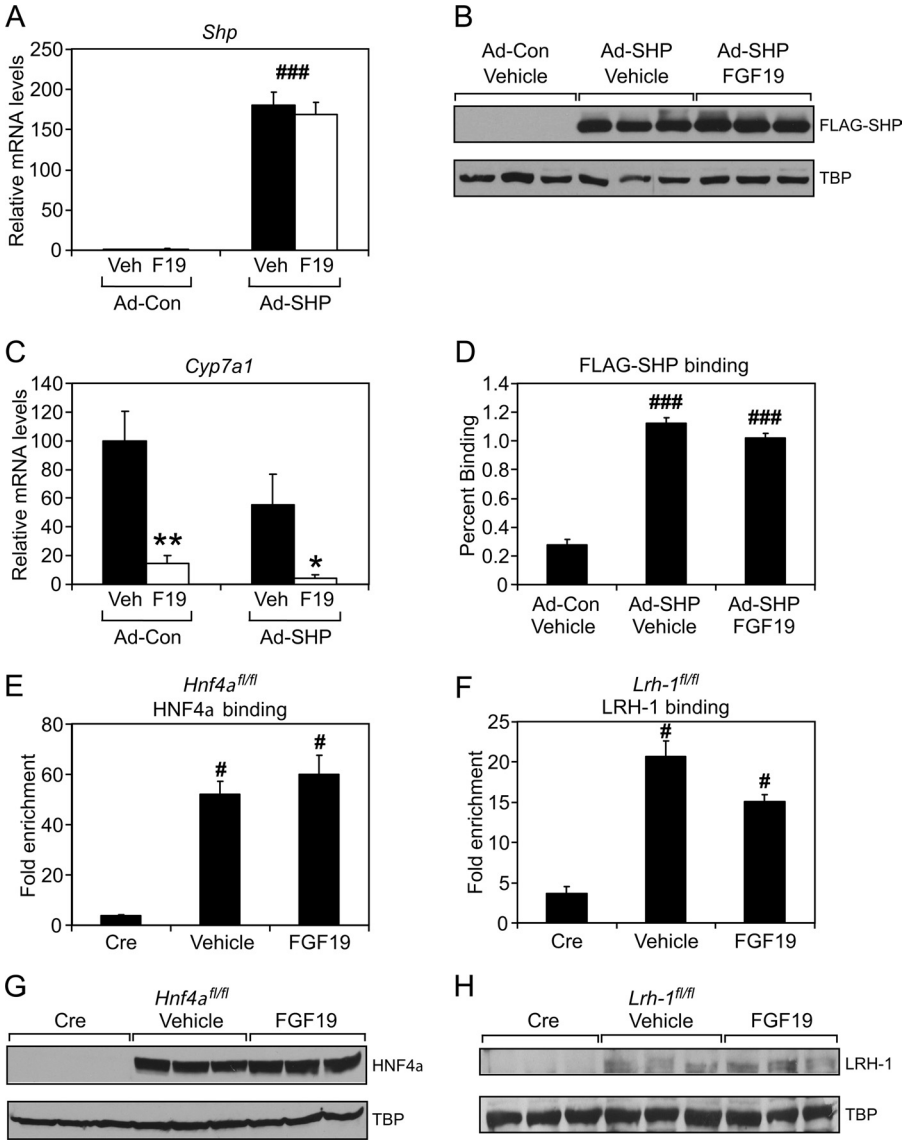

FIGURE 5. **FGF19 does not change SHP, HNF4α or LRH-1 binding to the *Cyp7a1* promoter.** FLAG-SHP was overexpressed in mouse liver via adenoviral expression. Mice ($n = 5–8$) were treated with vehicle or FGF19 (1 mg/kg; intraperitoneal) for 6 h. *Shp* mRNA levels (*A*), SHP protein levels (*B*), and *Cyp7a1* mRNA levels (*C*) are shown. FLAG-SHP binding to the *Cyp7a1* promoter was tested by ChIP ($n = 3$) (*D*). *E* and *F*, HNF4α and LRH-1 binding to the *Cyp7a1* promoter was tested by ChIP on liver samples ($n = 3$) from the experiments shown in Fig. 4, *A* and *B*. Albumin-Cre samples were included to show the specificity of the antibodies. *G* and *H*, nuclear HNF4α and LRH-1 protein levels are shown in triplicate. Values are means ± S.E. Statistical significance was determined by two-tailed *t* tests. (*) refers to differences between Veh and F19 groups. (#) refers to differences relative to Ad-Con or Cre groups. *, $p < 0.05$; **, $p < 0.005$; #, $p < 0.05$; ###, $p < 0.0005$.

reduced HNF4α binding in *Lrh-1*-deficient mice but no reciprocal change in LRH-1 binding in *Hnf4α*-deficient mice (supplemental Fig. S3). It is unclear how LRH-1 facilitates HNF4α binding to the *Cyp7a1* promoter.

HNF4α and LRH-1 Are Essential Regulators of the Cyp7a1 Promoter in Vivo—We next used the conditional knock-out mice to examine the contribution of *HNF4α* and *LRH-1* to *FGF19*-mediated repression of *Cyp7a1*. In albumin-Cre liver-specific *Hnf4α*-knock-out mice, basal *Cyp7a1* mRNA levels were reduced (Fig. 4*A*). However, FGF19 treatment further reduced *Cyp7a1* expression (Fig. 4*A*). As described (15, 16), *Lrh-1* deficiency in livers of albumin-Cre mice did not significantly alter *Cyp7a1* mRNA levels. FGF19 treatment repressed *Cyp7a1* transcription in *Lrh-1* liver knock-out mice (Fig. 4*B*).

To avoid compensation that might occur due to disruption of the *Hnf4α* and *Lrh-1* genes during liver development, we acutely disrupted the *Hnf4α* and/or *Lrh-1* genes via adenoviral Cre expression in liver. Acute knock-out of hepatic *Hnf4α* in *Hnf4α*$^{fl/fl}$ mice gave results similar to albumin-Cre knock-out: *Cyp7a1* basal mRNA levels were reduced and FGF19-mediated *Cyp7a1* repression was intact (Fig. 4*C*). Surprisingly, acute knock-out of hepatic *Lrh-1* in *Lrh-1*$^{fl/fl}$ mice differed from the albumin-Cre knock-out. When *Lrh-1* was knocked out acutely, basal *Cyp7a1* mRNA levels were significantly decreased. However, FGF19 treatment further repressed *Cyp7a1* expression (Fig. 4*D*). In double *Hnf4α*:*Lrh-1* liver knock-out mice, basal *Cyp7a1* mRNA levels were severely reduced and not further repressed by FGF19 treatment (Fig. 4*E*). Taken together, the

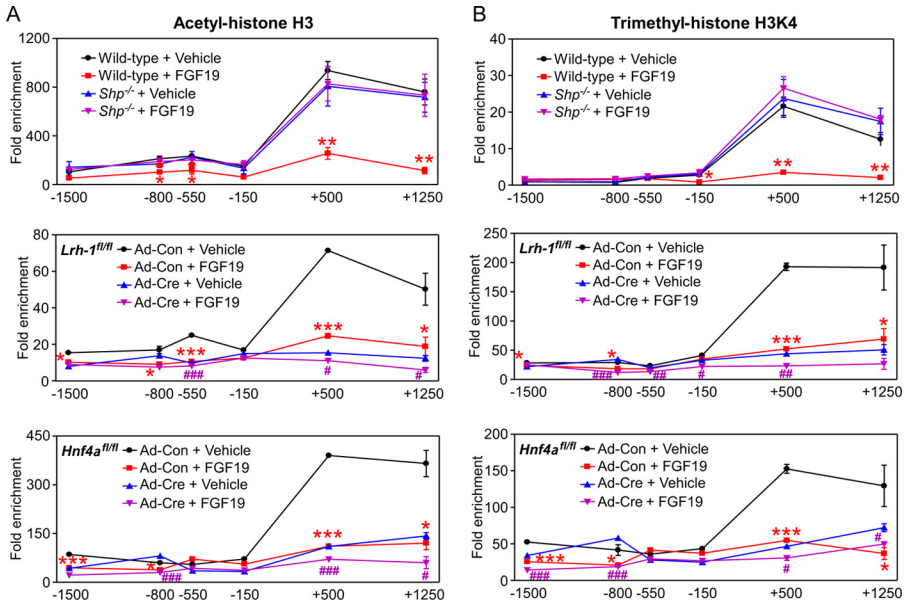

FIGURE 6. **FGF19 reduces histone H3 acetylation and H3K4 trimethylation on the *Cyp7a1* promoter.** Histone H3 acetylation (*A*) and histone H3K4 trimethylation (*B*) on the *Cyp7a1* promoter were tested by ChIP on liver samples ($n = 3$) from the mouse experiments shown in Figs. 1 and 4. Values are means ± S.E. Statistical significance was determined by two-tailed *t* tests. (*) refers to differences between wild-type or Ad-Con vehicle and FGF19 groups. (#) refers to differences between Ad-Cre vehicle and FGF19 groups. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$; #, $p < 0.05$; ##, $p < 0.005$; ###, $p < 0.0005$.

acute knock-out studies show that HNF4α and LRH-1 cooperate in regulating the *Cyp7a1* promoter *in vivo*. Moreover, they show that the presence of either HNF4α or LRH-1 is sufficient for repression of the *Cyp7a1* promoter by the FGF19/SHP pathway.

FGF19 Does Not Regulate Nuclear Receptor Binding to the Cyp7a1 Promoter—To test whether SHP binding to the *Cyp7a1* promoter is regulated by FGF19, FLAG-SHP protein was overexpressed in liver via adenoviral expression (Fig. 5, *A* and *B*). FGF19 treatment did not change nuclear FLAG-SHP protein levels (Fig. 5*B*). SHP overexpression caused only a trend toward decreased *Cyp7a1* expression while FGF19 treatment markedly repressed *Cyp7a1* levels (Fig. 5*C*), demonstrating that the FGF19-dependent repression mechanism is functional in this SHP-overexpression system. Surprisingly, FGF19 treatment did not change FLAG-SHP binding to the *Cyp7a1* promoter (Fig. 5*D*). Similarly, FGF19 treatment failed to alter the binding of either HNF4α or LRH-1 to the *Cyp7a1* promoter (Fig. 5, *E* and *F*) or the nuclear levels of these proteins (Fig. 5, *G* and *H*). These results show that FGF19 does not regulate SHP, HNF4α, and LRH-1 binding to the *Cyp7a1* promoter.

FGF19 Causes Histone Deacetylation and Demethylation on the Cyp7a1 Promoter—To gain insight into how FGF19 represses the *Cyp7a1* promoter, histone modifications on the promoter were examined. Histone H3 acetylation, a mark of active transcription, was repressed by FGF19 in wild-type but not $Shp^{-/-}$ mice (Fig. 6*A*, *top panel*). Knock-out of *Hnf4α* or *Lrh-1* led to depletion of histone H3 acetylation (Fig. 6*A*, *middle* and *lower panels*), which agrees with the decreased *Cyp7a1* mRNA levels. FGF19 treatment further reduced acetylation in all these knock-out strains (Fig. 6*A*). Interestingly, FGF19 caused histone H3 deacetylation on either side of the HNF4α/LRH-1 binding site at −150 bp but not at the binding site itself. Additional ChIP assays showed that histone H3 levels are low at the −150 bp position (supplemental Fig. S4). We speculate that the HNF4α/LRH-1/SHP complex recruits deacetylases that then act on both upstream and downstream histones.

Similar results were obtained for two other active transcription marks - histone H3 lysine 4 trimethylation (Fig. 6*B*) and histone H4 acetylation - as well as for RNA polymerase II recruitment (supplemental Fig. S5). These changes correlate with FGF19-mediated repression of the *Cyp7a1* promoter. However, treatment of primary mouse hepatocytes with trichostatin A (histone demethylase inhibitor), nicotinamide (sirtuin inhibitor), 5-azacytidine (5- DNA methylation inhibitor), BIX-01294 (histone H3K9 methyltransferase G9a inhibitor), tranylcypromine (histone H3K4 demethylase LSD1 inhibitor) or dimethyl-oxoglutarate (deoxygenase/jumonji demethylase inhibitor) failed to block FGF19-mediated repression of *Cyp7a1* (supplemental Fig. S5), suggesting either redundancy or the involvement of other pathways. We conclude that HNF4α and LRH-1 maintain the *Cyp7a1* promoter in an active transcriptional state whereas SHP is essential for its repression by FGF19 through pathways that remain to be defined.

DISCUSSION

In this report, we use acute, liver-specific knock-out mice to show that both HNF4α and LRH-1 serve as crucial regulators of *Cyp7a1*. The effects of HNF4α and LRH-1 on *Cyp7a1* are 2-fold. First, they cooperate in inducing basal *Cyp7a1* transcription. Second, they both contribute to SHP recruitment and the repression of *Cyp7a1* by FGF19. Thus, there is surprising overlap in the actions of these two transcription factors on this promoter. Our finding that HNF4α regulates basal *Cyp7a1* expression is consistent with a previous study in which the *Hnf4α* gene was disrupted in liver using an albumin-Cre driver (17). However, our results with acute LRH-1 knock-out differ from those using albumin-Cre, in which *Cyp7a1* mRNA levels

were either unchanged or increased (15, 16). It seems likely that albumin-Cre-mediated disruption of *Lrh-1* during liver development induces compensatory mechanisms that maintain *Cyp7a1* transcription.

We previously showed that SHP is required for FGF15-mediated repression of *Cyp7a1*. Here we show similar results with FGF19. Notably, FGF19 did not alter the binding of HNF4α, LRH-1 or SHP to the *Cyp7a1* promoter. In contrast to a previous report showing that FGF19 increases SHP stability by preventing its ubiquitination (25), we did not observe FGF19-mediated changes in SHP concentrations. We conclude that the repression of *Cyp7a1* promoter by FGF19 is not mediated by changes in the occupancy of HNF4α, LRH-1, or SHP on the *Cyp7a1* promoter.

Elimination of HNF4α or LRH-1 markedly reduced active transcription histone marks on the *Cyp7a1* promoter. FGF19 also down-regulated these histone modifications in a SHP-dependent manner. The most dramatic changes occurred in histone H3 and H4 acetylation and histone H3K4 trimethylation. Minor effects on repressive histone marks histone H3 K9 and K27 methylation, were also observed (supplemental Fig. S5). Inhibition of histone deacetylases or demethylases failed to block *Cyp7a1* repression by FGF19 (supplemental Fig. S5). It seems likely that the FGF19/SHP pathway regulates multiple downstream pathways that impact *Cyp7a1* repression.

It remains to be determined precisely how FGF19 cooperates with SHP to repress *Cyp7a1*. As discussed above, our data show that SHP is constitutively present at the *Cyp7a1* promoter, so FGF19 does not appear to promote SHP recruitment. A likely possibility is that FGF19 causes recruitment of co-repressor complexes that require the presence of SHP. In this regard, SHP has been shown to interact with factors that can modify chromatin (26–28). It is interesting that the two promoters that are repressed by FGF19 (*Cyp7a1* and *cholesterol 12α-hydroxylase*) contain overlapping HNF4α and LRH-1 binding sites (29). This suggests that these two factors provide the context necessary for SHP-mediated repression.

In summary, we show that HNF4α and LRH-1 cooperate in regulating basal expression and FGF19/SHP-mediated repression of *Cyp7a1* in liver. Understanding how FGF19 represses *Cyp7a1* has important therapeutic implications for the treatment of primary bile acid malabsorption disease characterized by the excess production of bile acids.

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