Medicine

Hammerhead-type FXR agonists induce an eRNA FincoR that ameliorates nonalcoholic steatohepatitis in mice

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Abstract

The nuclear receptor, Farnesoid X Receptor (FXR/NR1H4), is increasingly recognized as a promising drug target for metabolic diseases, including nonalcoholic steatohepatitis (NASH). Protein coding genes regulated by FXR are well known, but whether FXR also acts through regulation of long non-coding RNAs (lncRNAs), which vastly outnumber protein-coding genes, remains unknown. Utilizing RNA-seq and GRO-seq analyses in mouse liver, we found that FXR activation affects the expression of many RNA transcripts from chromatin regions bearing enhancer features. Among these we discovered a previously unannotated liver-enriched enhancer-derived lncRNA (eRNA), termed *FincoR*. We show that *FincoR* is specifically induced by the hammerhead-type FXR agonists, including GW4064 and tropifexor. CRISPR/Cas9-mediated liver-specific knockdown of *FincoR* in dietary NASH mice reduced the beneficial effects of tropifexor, an FXR agonist currently in clinical trials for NASH and primary biliary cholangitis (PBC), indicating that that amelioration of liver fibrosis and inflammation in NASH treatment by tropifexor is mediated in part by *FincoR*. Overall, our findings highlight that pharmacological activation of FXR by hammerhead-type agonists induces a novel eRNA, *FincoR*, contributing to the amelioration of NASH in mice. *FincoR* may represent a new drug target for addressing metabolic disorders, including NASH.

eLife assessment

Using unbiased transcriptional profiling, the study reports a **fundamental** discovery of a novel hepatic lncRNA, FincoR, which regulates FXR. The **convincing** findings have therapeutic implications in the treatment of MASH. The authors use state-of-the-art methodology and use unbiased transcriptomic profiling and epigenetic profiling, including validation in mouse models and human samples.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease and a leading cause of liver transplants and liver-related death *(Friedman et al., 2018)*. NAFLD begins with simple steatosis but may further progress to a severe form, non-alcoholic steatohepatitis (NASH), and later, fatal cirrhosis and liver cancer *(Friedman et al., 2018)*. Despite its striking global increase and clinical importance, there is no approved drug for NASH. The urgent need for development of therapeutic agents for NASH has greatly increased research interest in the nuclear receptor, Farnesoid X Receptor (FXR, NR1H4) *(Evans & Mangelsdorf, 2014)*.

FXR is activated by its physiological ligands, bile acids (BAs), and regulates expression of genes involved in BA, lipid, and glucose metabolism and hepatic autophagy, which maintain metabolite levels and metabolic homeostasis *(Calkin & Tontonoz, 2012; Kliewer & Mangelsdorf, 2015; Lee et al., 2006; Lee et al., 2014; Seok et al., 2014)*. Ligand-activated FXR also protects against hepatic inflammation and liver injury *(Jung et al., 2020; Wang et al., 2008)*. The action of FXR, similar to other nuclear receptors, is achieved primarily by its binding to chromatin loci to regulate the transcription of target genes *(Calkin & Tontonoz, 2012; Lee et al., 2006; Lee et al., 2012; Thomas et al., 2010)*. Consistent with its crucial physiological functions, FXR is increasingly recognized as a promising drug target, particularly for liver diseases, such as NASH and primary biliary cholangitis (PBC) *(Abenavoli et al., 2018; Ali et al., 2015; Downes et al., 2003; Kremoser, 2021)*. For example, semi-synthetic or non-steroidal synthetic agonists of FXR, including obeticholic acid (OCA) and hammerhead-type agonists, such as tropifexor and cilofexor, are currently in clinical trials for NASH and PBC patients *(Abenavoli et al., 2018; Kremoser, 2021; Sanyal et al., 2023; Tully et al., 2017)*. However, how pharmacological activation of FXR mediates such beneficial therapeutic effects is poorly understood.

Non-protein-coding RNAs (ncRNAs) are one of the fascinating discoveries of modern biology *(Cech & Steitz, 2014)*. While a significant portion of the genome was initially thought to be “junk DNA”, it has been established that many non-coding regions give rise to functional non-coding RNAs *(Cech & Steitz, 2014)*. Of these ncRNAs, long non-coding RNAs (lncRNAs) are a group of transcripts longer than 200 nucleotides and play important roles in diverse biological processes *(Lam et al., 2014; Li et al., 2016; Mattick et al., 2023; Sartorelli & Lauberth, 2020; Statello et al., 2021)*. A group of lncRNAs are produced from genomic regions bearing epigenetic features of enhancers *(Hon et al., 2017; Mattick et al., 2023)*. This is consistent with the idea that many transcriptional enhancers actively transcribe noncoding RNAs that are referred to as eRNAs (>85k in humans and >57k in mice) *(Hirabayashi et al., 2019)*, some of which are functionally important for enhancer functions *(Lai & Shiekhattar, 2014; Li et al., 2016; Sartorelli & Lauberth, 2020)*. A majority of eRNAs have not yet been annotated in current lncRNA databases, such as GENCODE. Exploring eRNA landscapes and functions in diverse biology and disease states will facilitate our understanding of both lncRNAs and enhancers *(Li et al., 2016; Mattick et al., 2023)*. Indeed, the landscape of eRNAs in mouse liver has been minimally explored *(Fang et al., 2014)*, and has not been well-studied in response to specific nuclear receptor activation, such as by FXR. Moreover, functional roles of eRNAs *in vivo* in intact organisms is understudied.

FXR was shown to regulate expression of small non-coding microRNA (miR) genes, for example *miR-34a and miR-802 (Lee et al., 2010; Seok et al., 2021)*, but it has not been reported whether FXR achieves its function through regulating lncRNAs, which far outnumber miRs. Because FXR directly regulates expression of its target genes *(Lee et al., 2012; Thomas et al., 2010)*, we examined if FXR regulates enhancer-derived lncRNAs (eRNAs), and if such transcripts participate in its physiological or pharmacological functions. By utilizing RNA-seq and GRO-seq analyses of livers from mice treated with FXR ligands, we identified a set of FXR-regulated eRNAs. Among these, we focused on a highly induced and abundantly expressed eRNA that we referred to as FXR-induced non-coding RNA (*FincoR*) for functional studies. *FincoR* is highly enriched in mouse liver and is induced specifically by hammerhead-type FXR agonists, including GW4064 and tropifexor.

In vivo studies utilizing CRISPR/Cas9-mediated liver-specific knockdown of *FincoR* in dietary NASH mice indicated that *FincoR* is critically involved in mediating the beneficial pharmacological effects of tropifexor in reducing liver fibrosis and inflammation.

Results

Activation of FXR by GW4064 induces a novel eRNA, *FincoR*, in mouse liver

To identify enhancer-derived lncRNAs (eRNAs) potentially regulated by FXR, we first obtained a list of putative enhancers in mouse liver based on ENCODE H3K27ac ChIP-Seq data (ENCFF001KMI, see **methods**). Then, we performed ribo-depleted total RNA-seq in the livers from mice treated with a specific FXR agonist, GW4064, to identify transcripts produced from these enhancer regions (see **Supplementary Table S1**). To avoid confounding issues of RNA signals from genes, we specifically focused on intergenic enhancer regions (+/- 3kb from H3K27ac peak center) that harbor discernible RNA-Seq signals (RPKM > 1).

This genomic analysis resulted in identification of 190 high-confidence eRNAs in mouse liver. Among these, 14 eRNAs were upregulated and 5 were downregulated by GW4064 treatment (FDR < 0.05, Log2 FC > 1, **Figure 1A**) (see **Supplementary Table S2**). FXR-regulated eRNAs were produced adjacent to many genes with important roles in liver metabolism and disease, for example, *Hes1* (**Figure 1-figure supplement 1A, B**). One of the most robustly induced eRNAs was *FXR-induced non-coding RNA* (*FincoR)*, an unannotated novel transcript located on chromosome 19 (**Figure 1A, B, C**). Reverse transcription qPCR (RT-qPCR) confirmed that treatment with GW4064 substantially induced expression of *FincoR*, more than 10-fold in mouse liver, which is similar to induction of *Shp*, a well-known FXR target gene (**Figure 1D**) *(Claudel et al., 2005; Evans & Mangelsdorf, 2014; Goodwin et al., 2000)*. Induction of *FincoR* by GW4064 was transient, peaked within 1 h and then declined gradually, a pattern similar to that of *Shp* (**Figure 1-figure supplement 1C**). Expression of genes adjacent to *FincoR*, including *Gcnt1*, *Rfk, Pcsk5 and Prune2*, did not change after acute 1 h FXR activation as shown by RNA-seq (**Figure 1C**), and confirmed for *Gcnt1* by time course qPCR (**Figure 1-figure supplement 1C**).

We also found that the short-time GW4064 treatment resulted in 590 up-regulated genes and 500 down-regulated genes (FDR < 0.05) (**Figure 1-figure supplement 2A**). GO enrichment analysis of these differentially expressed genes (DEGs) revealed their roles in the regulation of triglyceride, fatty acid, and cholesterol metabolism (**Figure 1-figure supplement 2B**), which is consistent with known roles of FXR in these physiological processes *(Claudel et al., 2005)*.

Ligand-activated FXR directly activates transcription of eRNAs, including *FincoR*

We sought to examine, 1) if these eRNAs were directly activated by FXR, and 2) if the activation takes place transcriptionally using FXR liver-specific knockout (FXR-LKO) mice that were treated with GW4064 to determine if FXR was required for induction of eRNAs by GW4064. FXR-LKO mice were generated from FXR floxed mice (FXR-flox) **(Figure 2-figure supplement 1A**) and transcription of eRNAs was detected by global run-on sequencing (GRO-seq), a widely used method to detect nascent RNA transcription, including eRNAs *(Lam et al., 2014; Li et al., 2016; Sartorelli & Lauberth, 2020)*. GRO-seq showed that FXR-induced eRNAs were activated transcriptionally by GW4064 in FXR- flox mice, but such induction was abolished in FXR-LKO mice (**Figure 2A**). In particular, *FincoR* is robustly induced in the GRO-Seq analysis and its induction is dependent on hepatic FXR (**Figure 2B**). RT-qPCR confirmed the FXR-dependent expression of *FincoR*, similar to that of *Shp* (**Figure 2-figure supplement 1B**).

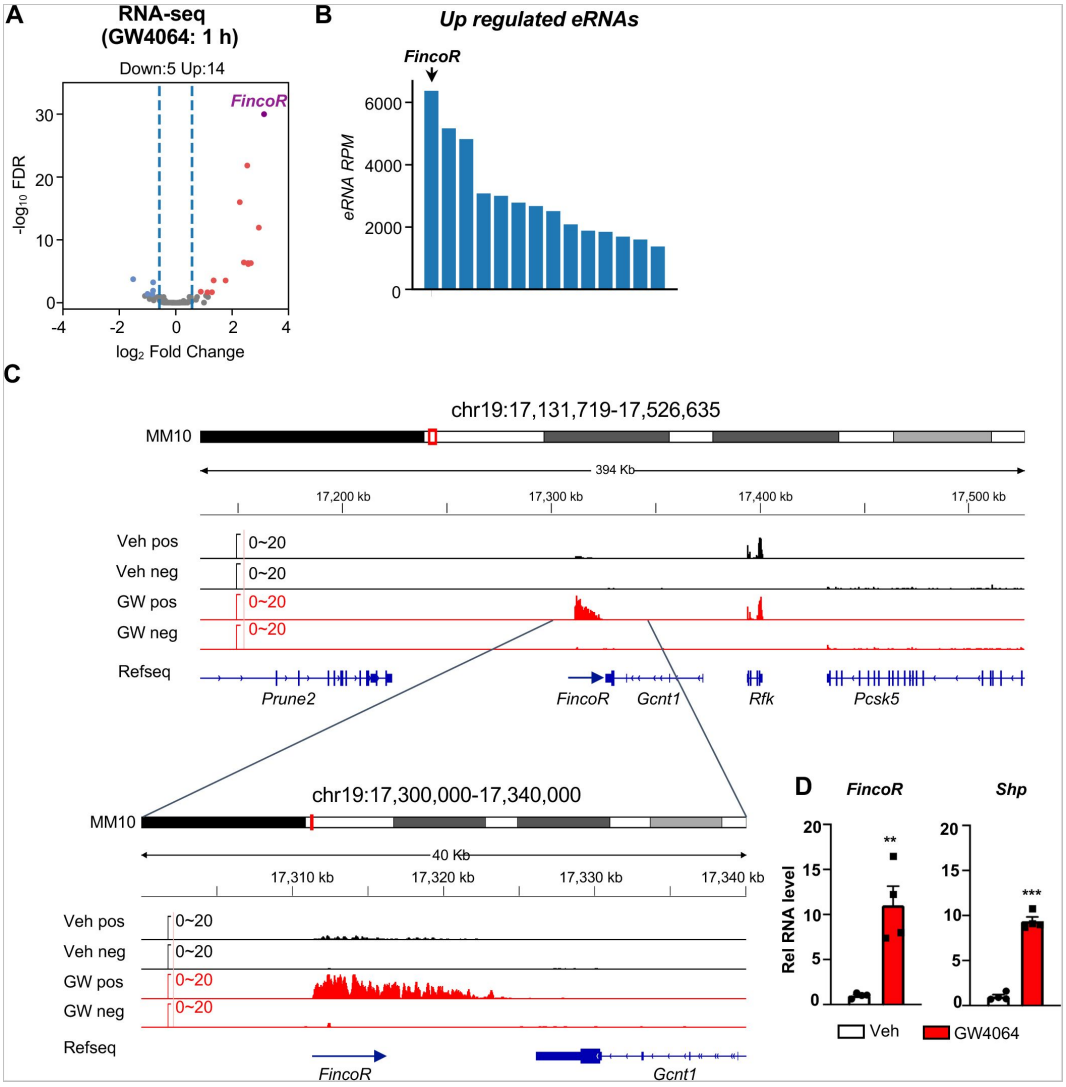

Figure 1.

Activation of FXR by GW4064 induces *FincoR*, a novel eRNA, in mouse liver.

(A) Volcano plot from RNA-Seq showing significantly induced eRNAs (*FincoR* is highlighed) in the livers of C57BL/6 male mice treated with GW4064 (*i.p*. injection, 30 mg/kg, 1 h) or vehicle. The *x* axis denotes $\log_2$ fold change (GW4064/Veh) of eRNAs and the *y* axis denotes -$\log_{10}$ FDR of eRNAs. (B) A Bar plot showing the reads per million (RPMs) of up regulated eRNAs by GW4064 treatment. (C) IGV genome browser track showing RNA-seq signals from vehicle or GW4064 treated samples around the *FincoR* locus and its neighboring regions. A zoom-in view of *FincoR* is shown below. Veh, vehicle; GW, GW4064; pos, positive strand; neg, negative strand; Refseq, reference sequence. (D) RT-qPCR data showing GW4064 induction of *FincoR* eRNA and *Shp* mRNA in the liver (n=4/group). Data are presented as mean ± SEM. Statistical significance was determined by the two-way ANOVA Sidak's multiple comparisons test with $^{**}p < 0.01$ and $^{***}p < 0.001$.

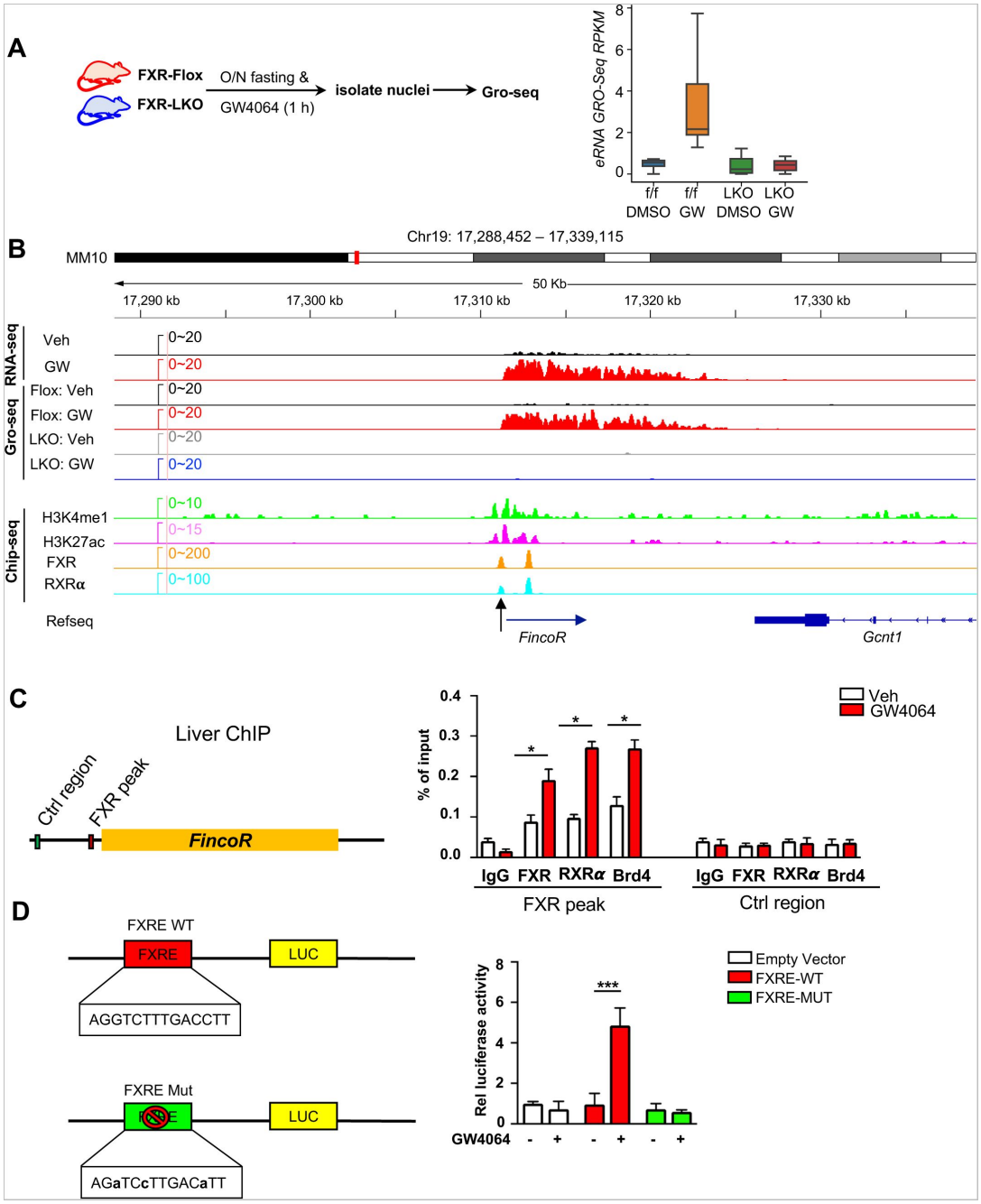

Figure 2.

Ligand-activated FXR directly activates transcription of eRNAs including *FincoR* in the liver.

(A) Left: experimental outline. FXR-Flox and LKO male mice were fasted overnight and treated with vehicle or GW4064, and livers were collected 1 h later with nuclei isolated for Gro-seq (n=2/group). Right: a boxplot shows the Gro-Seq signals for GW4064 up-regulated eRNAs in different conditions. RPKM: reads per kbp per million. (B) IGV genome browser track showing RNA-seq, Gro-seq and ChIP-seq signals in the *FincoR* locus. An arrow at the bottom points to the main enhancer FXR ChIP-seq peak that overlaps the start site of *FincoR*. (C) ChIP assays were performed in the same liver samples described in **Fig. 1A** to detect FXR, RXR**α**, and BRD4 occupancy at the FXR binding peak region close to the transcription start site of *FincoR* (black arrow in **Fig.2B**). (D) HepG2 cells were transfected with luciferase reporter expressing wild type FXRE or mutant FXRE (see materials and methods) for 24 h before treatment with GW4064 for an additional 6 h. Relative luciferase activities are shown. (C-D) Data are presented as mean ± SEM. Statistical significance was determined by the Student's t test with $*p < 0.05$ and $***p < 0.001$.

We next examined if FXR binds to the enhancers that produce the identified eRNAs by analyzing published mouse liver ChIP-Seq data for FXR (**Supplementary Table S3**) *(Lee et al., 2012; Thomas et al., 2010)*. FXR binding was strongly enriched at the enhancers associated with FXR-induced eRNAs as were the enhancer marks H3K27ac and H3K4me1 (**Figure 2-figure supplement 1C**). The binding of FXR and the presence of histone marks at the *FincoR* enhancer region determined by ChIP-seq as compared with nascent transcripts detected by Gro-seq is shown in **Figure 2B**. These analyses support the conclusion that activation of FXR transcriptionally induces this series of eRNAs via chromatin binding at these enhancers, including *FincoR*.

We validated FXR binding at the enhancer region that produces *FincoR* using mouse liver ChIP (**Figure 2C**). We also examined binding of a well-known DNA binding partner of FXR, Retinoid x Receptor alpha (RXRα/NR2B1) *(Evans & Mangelsdorf, 2014; Zheng et al., 2018)*, and bromodomain-containing protein 4 (BRD4), an acetylated histone reader protein that often binds at active enhancers *(Chen et al., 2016; Li et al., 2016; Rahnamoun et al., 2018; Sartorelli & Lauberth, 2020)* and is a transcriptional coactivator of FXR *(Jung et al., 2020)*. GW4064 treatment resulted in substantial increases in recruitment of both FXR and RXRα to the enhancer region close to the transcription start site of *FincoR* (arrow shown below in **Figure 2B**), whereas binding was not detected at a control region (**Figure 2C**). We also found that BRD4 occupancy was increased at this enhancer region after GW4064 treatment (**Figure 2C**). These results indicate that GW4064 activation of FXR leads to increased occupancy of the FXR/RXRα heterodimer and BRD4 to the enhancer region to upregulate *FincoR* eRNA in the liver.

We identified an inverted repeat1 (IR1) motif that is known to bind FXR *(Calkin & Tontonoz, 2012; Lee et al., 2006)* within the major FXR binding peak near the start site of *FincoR* (arrow shown below in **Figure 2B**), which we also refer to as FXRE (**Figure 2B, C**). We examined the functionality of this IR1 motif for mediating transcriptional activation by GW4064 using reporter assays (**Figure 2D**). We cloned the region containing the IR1 motif into the pGL4.23 luciferase reporter and generated a mutated IR1 motif construct as a comparison (**Figure 2D**). After transfection into human hepatic HepG2 cells, GW4064 treatment significantly elevated the luciferase activity of the reporter with the wild type IR1 motif, but not with the mutated IR1 motif (**Figure 2D**). Together, these results suggest that GW4064-activated FXR directly upregulates *FincoR* expression.

FincoR is a liver-specific nucleus-enriched eRNA

Because enhancers and eRNAs generally act in a tissue-specific manner *(Li et al., 2016; Sartorelli & Lauberth, 2020)*, we examined the tissue-specific expression of *FincoR* in mice. Strikingly, *FincoR* is highly expressed in the liver and it is expressed at extremely low levels in most other tissues, except for a detectable, but still fairly low, level in the lung (**Figure 3A**). GW4064 treatment resulted in induction of *FincoR* specifically in the liver (**Figure 3A**). The level of *FincoR* detected in primary mouse hepatocytes (PMHs) isolated from GW4064-treated mouse liver was similar to that in the liver tissue from the same mouse, suggesting that the majority of *FincoR* is present in hepatocytes (**Figure 3B**).

By using the 5' and 3' rapid amplification of cDNA ends (RACE), we identified one transcript of *FincoR* that is of approximately 3.7 kb in length (**Figure 3C**). However, based on RNA-seq, the length of *FincoR* is over 10 kb (**Figure 1C**), suggesting there are likely additional multiple RNA isoforms that we were not able to identify by RACE. We next analyzed the coding potential of *FincoR* utilizing a comparative genomic program, PhyloCSF. While adjacent genes *Gcnt1* and *Prune2* (**Figure 3D, E**) were correctly predicted to encode proteins, *FincoR* did not contain a potential protein-coding open reading frame (**Figure 3E**). Consistent with this bioinformatic prediction, a vector expressing *FincoR* failed to produce any proteins in an in vitro transcription/translation assay (**Figure 3F**) confirming that the *FincoR* transcript is a noncoding RNA. *FincoR* transcripts were enriched by binding to oligo-dT beads, suggesting that *FincoR* is 3' polyadenylated (**Figure 3G**). Further, *FincoR* was detected in the nuclear compartment and

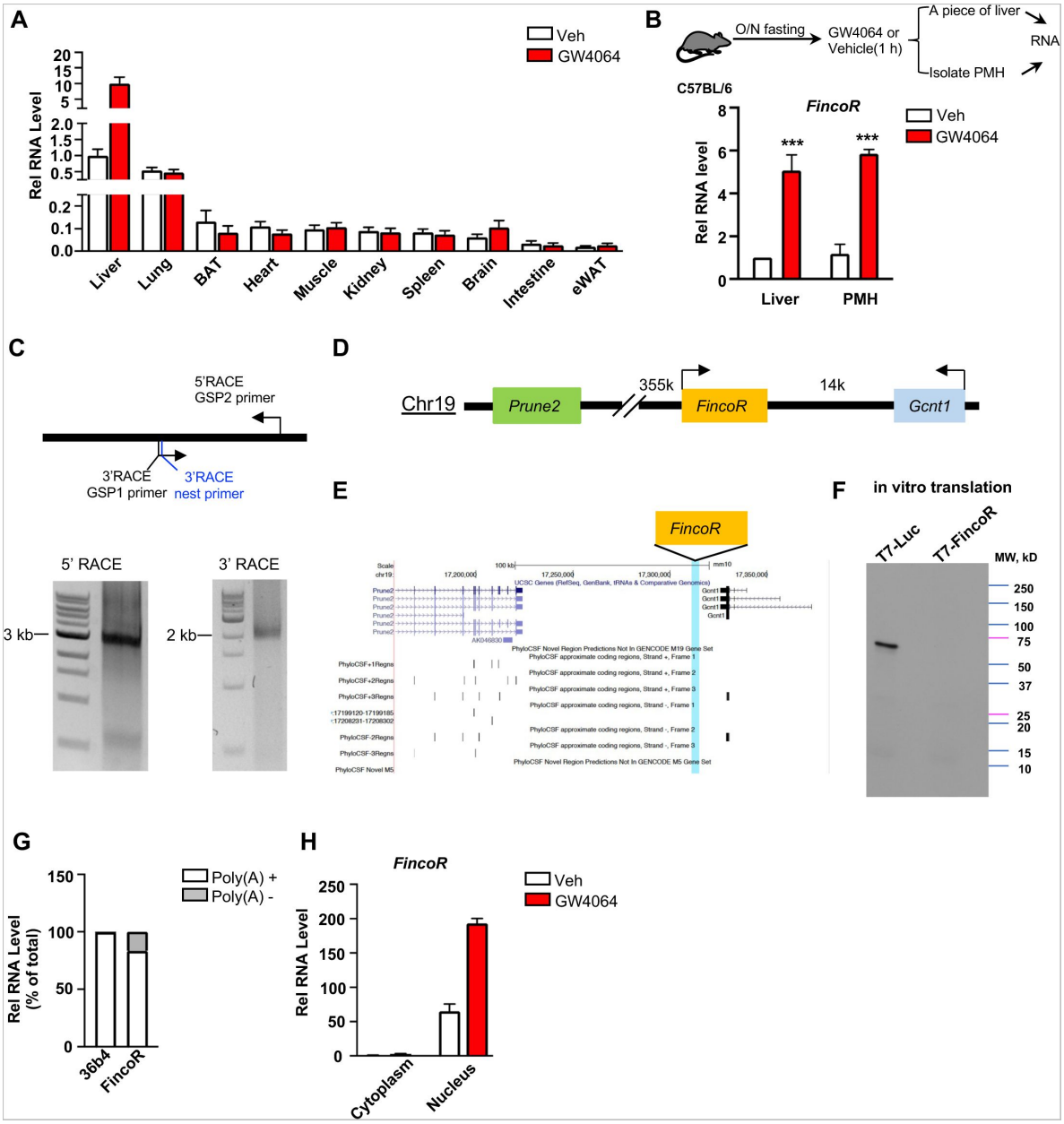

Figure 3.

FincoR is a liver-specific nucleus-enriched eRNA.

(A) Expression levels of *FincoR* in various tissues after GW4064 treatment. Data from C57BL/6 male mice fasted overnight and *i.p.* injected with vehicle or GW4064 (30 mg/kg) for 1 h (n=2/group). (B) C57BL/6 male mice were fasted overnight and injected *i.p.* with vehicle or GW4064 (30 mg/kg) for 1 h. One small piece of liver was snap-frozen for later RNA isolation and the remaining part was used for immediate primary hepatocyte isolation. Then, RNAs were extracted from liver or primary hepatocytes and *FincoR* expression was measured (n=3/group). (C) Agarose gel electrophoresis of PCR products generated in 5' (left) and 3' (right) RACE of *FincoR* in liver samples. Primer locations are shown. RACE, rapid amplification of cDNA ends. GSP: gene specific primer. (D) A schematic diagram showing location of *FincoR* relative to nearby genes in the mice genome. (E) PhyloCSF analysis of the coding potential of *FincoR*. (F) In vitro translation of *FincoR* using the Promega Transcend Non-Radioactive Translation Detection Systems. Luciferase is used as a control for coding RNA. (G) qPCR analysis of *FincoR*, *36b4* in Poly(A)+ and Poly(A)-RNA fractions from GW4064 treated mouse liver. (H) *FincoR* identified in the subcellular fractions using cellular fractionation assays. Primary hepatocytes were isolated from GW4064 or DMSO treated mice and the cytoplasm and nucleus fractions of these hepatocytes were separated and both fractions were subjected to RNA extraction and qPCR.

GW4064 treatment increased the nuclear abundance of *FincoR* (**Figure 3H**), consistent with its potential transcriptional regulatory function. Together, these results from molecular biochemical characterization studies reveal that *FincoR* is a liver-enriched nuclear polyadenylated eRNA.

FincoR is induced specifically by the hammerhead-type synthetic FXR agonists

To determine whether induction of the FXR-induced *FincoR* is ligand-specific, we examined the effects of several hammerhead-type synthetic FXR agonists, including GW4064, cilofexor, and tropifexor; a semi-synthetic agonist, OCA; and a non-hammerhead-type gut-specific agonist, fexaramine (**Figure 4A**) *(Downes et al., 2003; Fang et al., 2015)*. Remarkably, treatment with each of the hammerhead type agonists for 1 h resulted in a robust induction of *FincoR* (**Figure 4B**), whereas *FincoR* levels were unchanged after treatment with OCA or fexaramine for 1 h (**Figure 4B**). Treatment with OCA for 4 h or even one-week treatment with OCA failed to induce hepatic *FincoR* in mice, while expression of *Shp* was significantly induced (**Figure 4C, D**). Acute feeding with a diet supplemented with 0.5% cholic acid (CA), a primary BA, for 6 h also failed to induce *FincoR* expression (**Figure 4E**). Collectively, these results demonstrate that *FincoR* is induced specifically by hammerhead-type FXR agonists.

Generation of CRISPR/Cas9-mediated *FincoR* liver-specific knockdown mice

To explore the functional role of hepatic FincoR, we utilized the CRISPR/Cas9 technique to generate *FincoR* liver-specific knockdown (FincoR-LKD) mice (**Figure 5-figure supplement 1A, B**). Adenoviral-mediated sgRNA expression in Cas9 mice resulted in downregulation of *FincoR* specifically in the liver by about 60%, but not in other tissues (**Figure 5B**). As FXR is a key regulator of bile acid, cholesterol, lipid, and glucose metabolism and *FincoR* is specifically regulated by FXR, *FincoR* may have a role in the metabolic process in physiology and disease. We examined liver triglyceride, cholesterol, bile acid, glycogen and serum non-esterified fatty acids (NEFA) but *FincoR* downregulation did not result in any significant changes under physiological conditions (**Figure 5C**).

To explore the molecular signatures and pathways affected by FincoR, we examined global gene expression by RNA-seq analysis in mouse liver after *FincoR* knock-down (**Figure 5D**). While *FincoR* was markedly downregulated, the neighboring genes, such as *Gcnt1, Rfk, Pcsk5 and Prune2*, were largely unchanged (**Figure 5D; Figure 5-figure supplement 1C**). The RNA-seq analysis revealed 18 up-regulated genes and 53 downregulated genes in FincoR-LKD liver (**Supplementary Table S4; Figure 5E**). GO analysis of those downregulated genes indicated that these genes were enriched in pathways involved in fatty acid oxidation, organelle organization and metabolic process (**Figure 5F**). Among the down-regulated genes, *Ppp1r3g*, which has a role in controlling glycogen synthesis, and *Igfbp2*, which functions in insulin resistance, were markedly reduced (**Supplementary Table S4; Figure 5-figure supplement 1D, E**). Among the up-regulated genes, expression was substantially increased for *Eda2r*, which is a member of the tumor necrosis factor receptor superfamily and is involved in inflammation, the immune response, and development (**Figure 5-figure supplement 1F**). *Fndc1*, which is involved in fibronectin matrix remodeling, was also suppressed by *FincoR* (and thus upregulated upon its KO, **Figure 5-figure supplement 1G**). These studies suggest that *FincoR* has a role in modulating metabolic homeostasis by regulating genes involved in metabolism and inflammation.

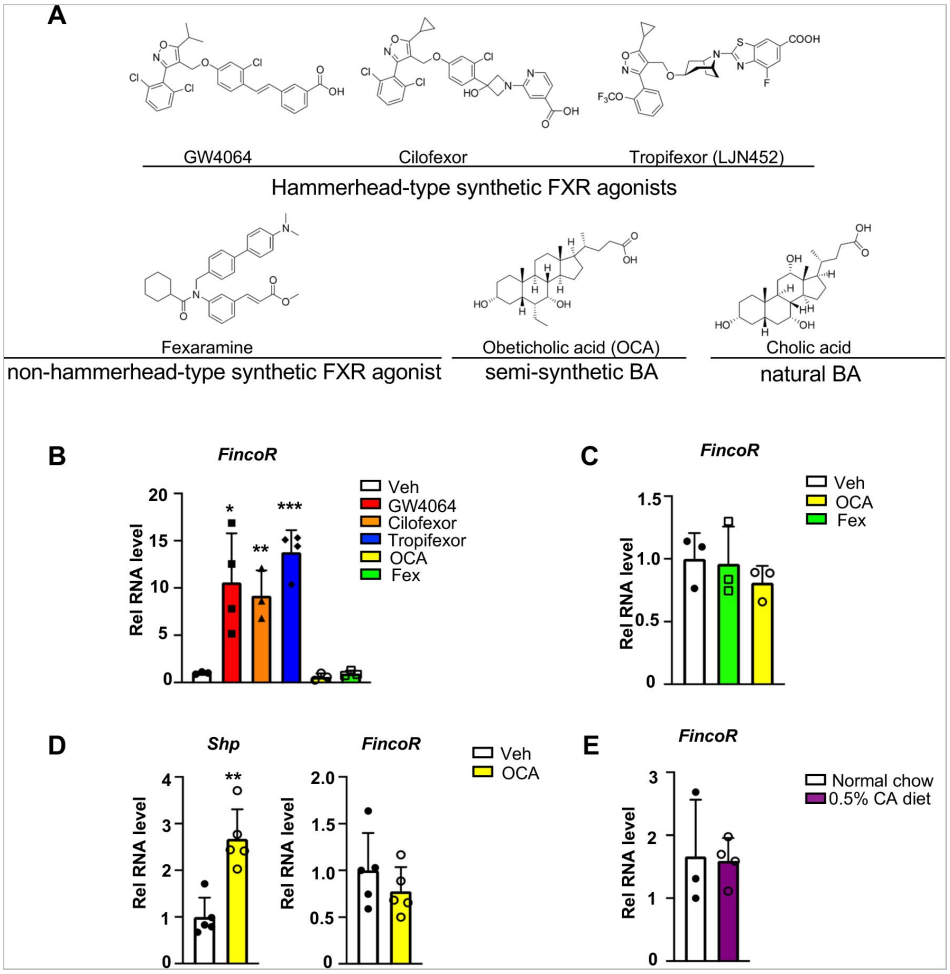

Figure 4.

FincoR is induced by the hammerhead class of non-steroidal FXR agonists, including GW4064 and tropifexor.

(A) The chemical structures of the FXR agonists including the hammerhead class of synthetic FXR agonists, non-hammerhead-type synthetic agonists, semi-synthetic BA and natural BA. BA: bile acid. (B) qPCR data showing *FincoR* expression levels in C57BL/6 mice liver respectively treated with GW4064 (30 mg/kg), Cilofexor (30 mg/kg), Tropifexor (0.5 mg/kg), Fexaramine (100 mg/kg), or OCA (20 mg/kg) for 1 h (n=3~4/group). (C) *FincoR* expression levels in C57BL/6 mice liver treated with OCA (20 mg/kg) or Fexaramine (100 mg/kg) for 4 hrs (n=3/group). (D) Expression of *FincoR* in C57BL/6 mice liver after daily treatment with OCA (20 mg/kg) for 7 days (n=5/group). *Shp* gene mRNA was measured as a positive control. (E) Expression of *FincoR* in C57BL/6 mice fed with 0.5% cholic acid (CA) diet for 6 hrs (n=3~4/group). In panels B-E, all mice underwent over-night fasting. (B-E) Data are presented as mean ± SEM. Statistical significance was determined by the Student's t test with *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

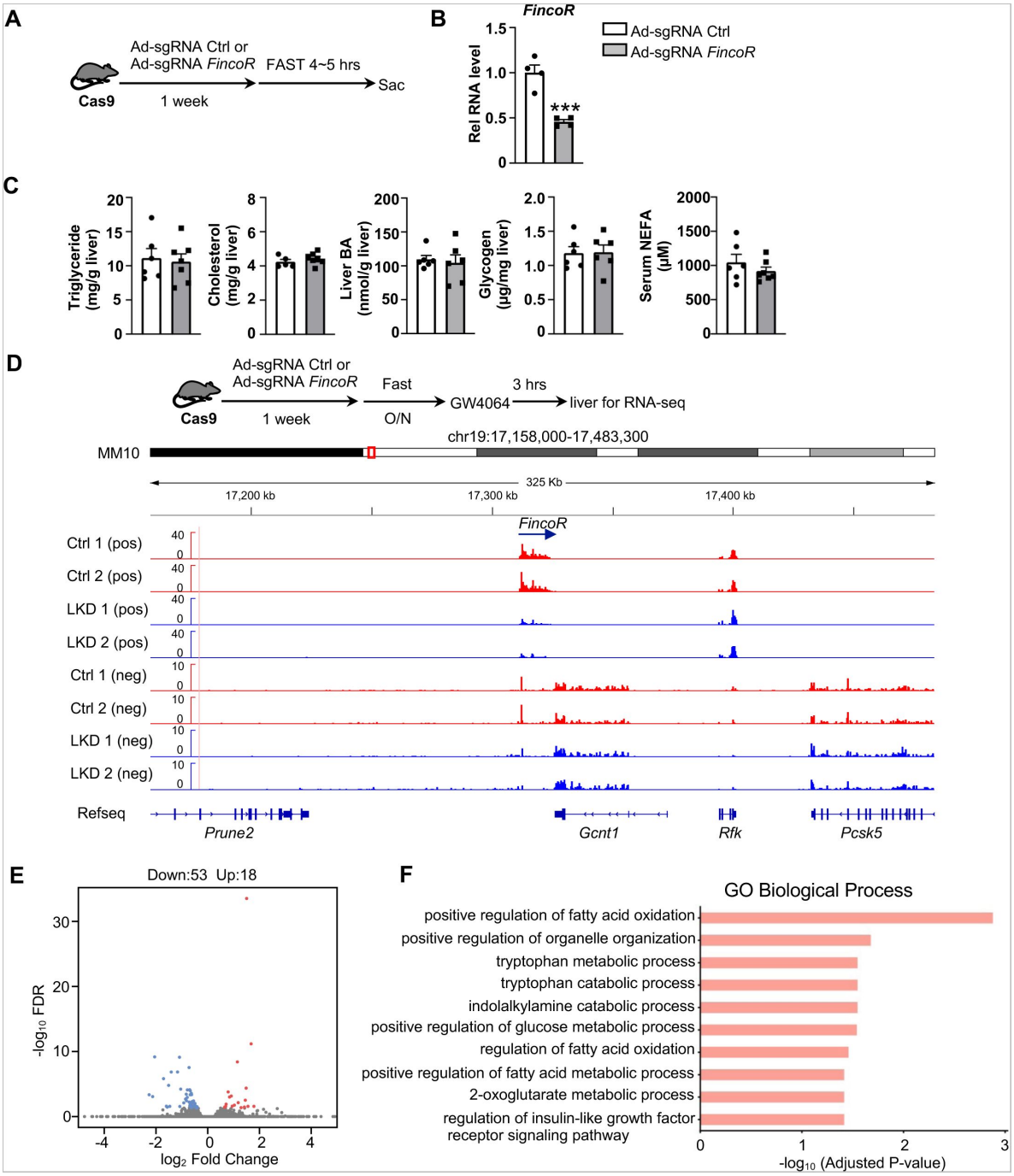

Figure 5.

Generation of CRISPR/Cas9-mediated *FincoR* liver-specific knockdown mice.

(A) Experimental scheme: male Cas9 mice were infected with adenovirus expressing sgRNA for *FincoR* or a control for 1 week. Then the liver and serum were collected from these mice after 4~5 hrs of fasting. (B) The expression of *FincoR* in the liver was measured by qPCR (n=4/group). (C) Hepatic triglyceride, cholesterol, bile acid, glycogen and serum NEFA were measured (n=5~7/group) (D) Male Cas9 mice were infected with adenovirus expressing sgRNA for *FincoR* or control for 1 week. Then these mice were fasted overnight and treated with GW4064 for 3 h before tissue collection. RNA-seq profiles of expression of hepatic *FincoR* and the adjacent genes were shown (n=2/group). (E) Genome-wide changes in mRNA expression shown in a volcano plot. The numbers refer to the number of genes up- or down-regulated by 2-fold or more with an adjusted *p*-value < 0.01. (F) Gene ontology analysis of biological pathways using DAVID Tools for genes downregulated after *FincoR* knockdown. (B) Data are presented as mean ± SEM. Statistical significance was determined by the Student's t test with ***$p < 0.001$.

Amelioration of hepatic steatosis mediated by tropifexor is independent of *FincoR* in diet-induced NASH mice

Tropifexor, also known as LJN452, is a highly potent hammerhead type FXR agonist that is currently under clinical trials for NASH and PBC patients *(Kremoser, 2021; Sanyal et al., 2023; Tully et al., 2017)*. Because *FincoR* is induced specifically by the hammerhead class of FXR agonist (**Figure 4**) and has a potential role in the regulation of metabolism and inflammation (**Figure 5**), we hypothesized that *FincoR* may play a role in tropifexor-mediated beneficial effects on reducing NASH pathologies in mice.

We utilized a mouse model that had been fed the Amylin Liver NASH-promoting (AMLN) diet *(Hernandez et al., 2019; Sun et al., 2022; Zhao et al., 2018)*, and examined the potential impact of liver-specific downregulation of *FincoR* on tropifexor's effects on NASH pathology. Cas9 mice fed the AMLN diet for 12 weeks were injected via tail veins with adenovirus expressing control sgRNA or *FincoR* sgRNA, respectively, and then treated daily with tropifexor (0.3 mg/kg) for 12 days (**Figure 6A**). In these mice, as a technical validation of RNA induction and knockdown, *FincoR* levels were significantly increased by FXR agonist tropifexor, and the increase was blocked by adenovirus expressing sgRNA for *FincoR* (**Figure 6B**).

We then examined the effect of tropifexor treatment and *FincoR* downregulation on hepatic steatosis in these mice. Tropifexor treatment markedly reduced neutral lipids determined by Oil Red O staining of liver sections (**Figure 6C**) and liver TG levels (**Figure 6D**), and these beneficial effects on reducing fatty liver were not altered by *FincoR* downregulation. Also, *FincoR* downregulation had little effect on liver cholesterol and gallbladder BA levels, although gallbladder BA levels were reduced by tropifexor (**Figure 6D**).

Consistent with the phenotypes, hepatic expression of key genes involved in BA synthesis was dramatically reduced by tropifexor treatment (i.e., *Cyp7a1 and Cyp8b1*), which is consistent with decreased gallbladder BA levels mediated by this agonist (**Figure 6E**). Similarly, tropifexor also lowered lipid synthesis genes (*Srebp1c*, *Lpin1*, *Scd1*), consistent with decreased liver TG levels. However, downregulation of *FincoR* did not result in changes in mRNA levels of these genes (**Figure 6E**). These results indicate that tropifexor-mediated beneficial effects on reducing hepatic steatosis are independent of *FincoR*.

FincoR facilitates alleviation of liver inflammation by tropifexor in diet-induced NASH

Tropifexor ameliorated fibrotic NASH pathologies in preclinical studies *(Hernandez et al., 2019; Tully et al., 2017)* and has recently concluded phase 2 clinical trials for NASH patients *(Sanyal et al., 2023)*. We, therefore, further examined the effects of *FincoR* downregulation on altering other NASH pathologies, including hepatocellular apoptosis, liver fibrosis and inflammation.

In the same AMLN diet-fed mice as described above (**Figure 6**), analyses of liver sections revealed that tropifexor treatment reduced hepatocyte swelling/ballooning (H&E staining), decreased numbers of apoptotic cells (TUNEL staining), alleviated fibrosis (Sirius red staining), and lowered infiltration of macrophages (F4/80 staining) (**Figure 7A**). Remarkably, these tropifexor-mediated beneficial effects on NASH pathologies were all markedly diminished by *FincoR* downregulation (**Figure 7A**). This was consistently found in various liver lobes and two representative pictures from two different lobes are shown (**Figure 7A**). In control experiments, *FincoR* downregulation in vehicle-treated mice did not result in marked changes in NASH pathologies (**Figure 7-figure supplement 1**). These results demonstrate that *FincoR* is required for tropifexor-mediated beneficial effects on reducing NASH pathologies, specifically reducing hepatic inflammation, fibrosis and hepatocyte apoptosis. Consistent with these results, serum ALT and AST

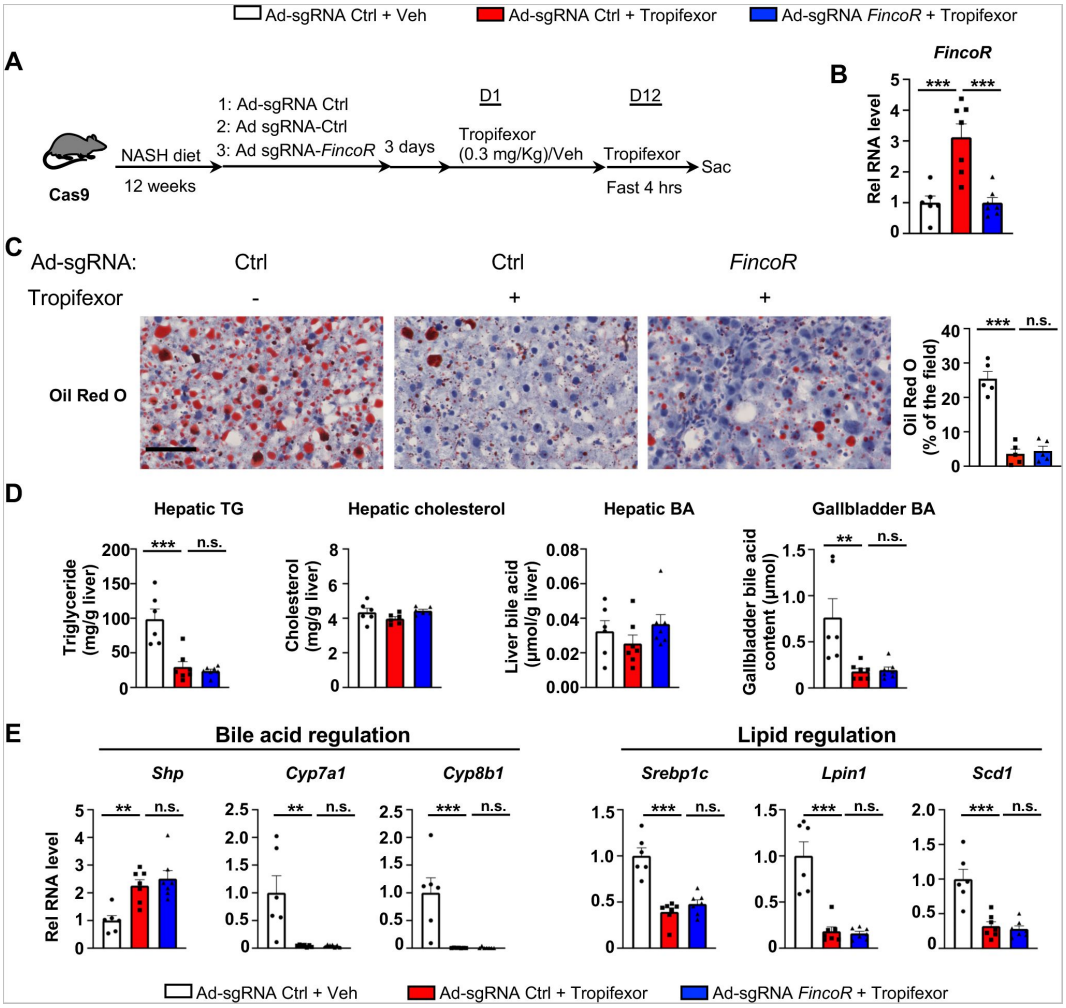

Figure 6.

In diet-induced NASH mice, tropifexor-mediated beneficial effects on reducing hepatic steatosis are largely independent of *FincoR*.

(A-E) Male Cas9 mice were fed a NASH diet for 12 weeks. The mice were randomly assigned to 3 groups and infected with adenovirus expressing sgRNA for *FincoR* or control. Three days later, the mice were treated with tropifexor (0.3 mg/kg) or vehicle for 12 days. The mice were administered tropifexor or vehicle and fasted for 4 h before tissues were collected. (A) Experimental scheme. (B) Hepatic *FincoR* expression was measured (n=6~7/group). (C) Oil Red O staining of liver sections. Scale bar (50 μm). Image analyses were done using Image J and the areas of stained field were quantified (n=5/group) (D) Hepatic TG, hepatic cholesterol, gallbladder BA and hepatic BA levels were measured (n=6~7/group). (E) mRNA levels in the liver of the indicated genes involved in bile acid regulation and lipid regulation (n=6~7/group). (B, D and E) Data are presented as mean ± SEM. Statistical significance was determined by the one-way ANOVA (Sidak's multiple comparisons test) with *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Ad, adenovirus; H & E, hematoxylin and eosin; TG, triglyceride; Veh, vehicle; ns, not significant.

levels, indicators of liver damage, were significantly elevated after *FincoR* downregulation (**Figure 7B**). Protein levels of key inflammatory markers, IL1β and CCL2, in liver extracts were also elevated after *FincoR* downregulation (**Figure 7C**).

Consistent with the phenotypes from histological analyses, hepatic expression of fibrosis and inflammation was altered by *FincoR* knockdown. For example, tropifexor treatment reduced mRNA levels of several genes that promote fibrosis (*Col1a1*, *Col1a2*, *Acta2*) and hepatic inflammation (*Eda2r*, *Ifng*, *Ccl3)*, whereas these reductions were largely reversed by *FincoR* downregulation (**Figure 7D**). We also detected increased expression of inflammatory genes (*Ccl2*, *Ccr2*, *Lcn2)* and an extracellular matrix remodeling gene *(Fndc1)* in liver with *FincoR* knockdown (**Figure 7D**). Tropifexor treatment suppresses hepatic apoptosis by reducing pro-apoptotic genes (*Ctsb*, *Ctss*) and upregulating anti-apoptotic genes such as *Bcl2 (Warren et al., 2019)*. Importantly, these effects were significantly reversed by *FincoR* downregulation (**Figure 7D**). Collectively, these results demonstrate that in diet-induced NASH mice, pharmacological activation of FXR by tropifexor reduced fibrosis, apoptosis, and inflammation, which was dependent, at least in part, on the induction of *FincoR*.

FincoR expression is increased in chronic liver disease with hepatic inflammation and liver injury

To determine whether expression of *FincoR* is altered in chronic liver disease, we utilized mouse models of NAFLD/NASH and cholestatic liver injury. Hepatic *FincoR* levels were significantly increased in mice fed with a high fat diet (HFD) for 12 weeks (**Supplementary Figure S1A**) and in mice fed a HFD with high fructose (HFHF) in drinking water for 12 weeks (**Supplementary Figure S1B**). Elevated hepatic *FincoR* levels were also observed in mice treated with α-naphthylisothiocyanate (ANIT), a chemical inducer of liver cholestasis *(Jung et al., 2020; Kim et al., 2016)* (**Supplementary Figure S1C**), and in mice with bile duct ligation (BDL), a surgical method to induce cholestatic liver injury (**Supplementary Figure S1D**).

The sequence of *FincoR* is moderately conserved between mice and humans as displayed in the UCSC genome browser (**Supplementary Figure S1E**). Annotation in the NCBI genome data viewer of the human sequence region with similarity to mouse *FincoR* revealed an functionally uncharacterized human lncRNA, XR_007061585.1, in this region **(Supplementary Figure S1F)**. However, whether this sequence (or an as yet to be identified lncRNA) is functional or not has not been determined. To explore the potential changes or role of lncRNA XR_007061585.1 in human liver pathological conditions, we measured hepatic levels of the transcripts in PBC and NAFLD patients. Compared to normal individuals, hepatic lncRNA XR_007061585.1 levels were elevated in patients with PBC or NAFLD, but not in severe NASH-fibrosis patients (**Supplementary Figure S1G, H**). These results demonstrate that hepatic levels of a potential human analog of *FincoR* are elevated in NAFLD and PBC patients, as *FincoR* is in mouse models of chronic liver disease with hepatic inflammation and liver injury. However, whether human lncRNA XR_007061585.1 is analogous to mouse *FincoR* in terms of functions and mechanisms, and whether elevated XR_007061585.1 levels may have a role in the disease progression or may be an adaptive response to liver injury remains to be determined.

Discussion

FXR maintains metabolic homeostasis by transcriptional regulation of genes. Direct regulation of protein-coding genes by FXR, including *Shp*, is well characterized. In this study we show that FXR also mediates its functions by induction of lncRNA genes, which vastly outnumber protein-coding genes. We further show that pharmacological activation of FXR by hammerhead-type agonists induces a liver-specific enhancer-derived lncRNA, which we named *FincoR*, that contributes to reduction of NASH pathologies in mice.

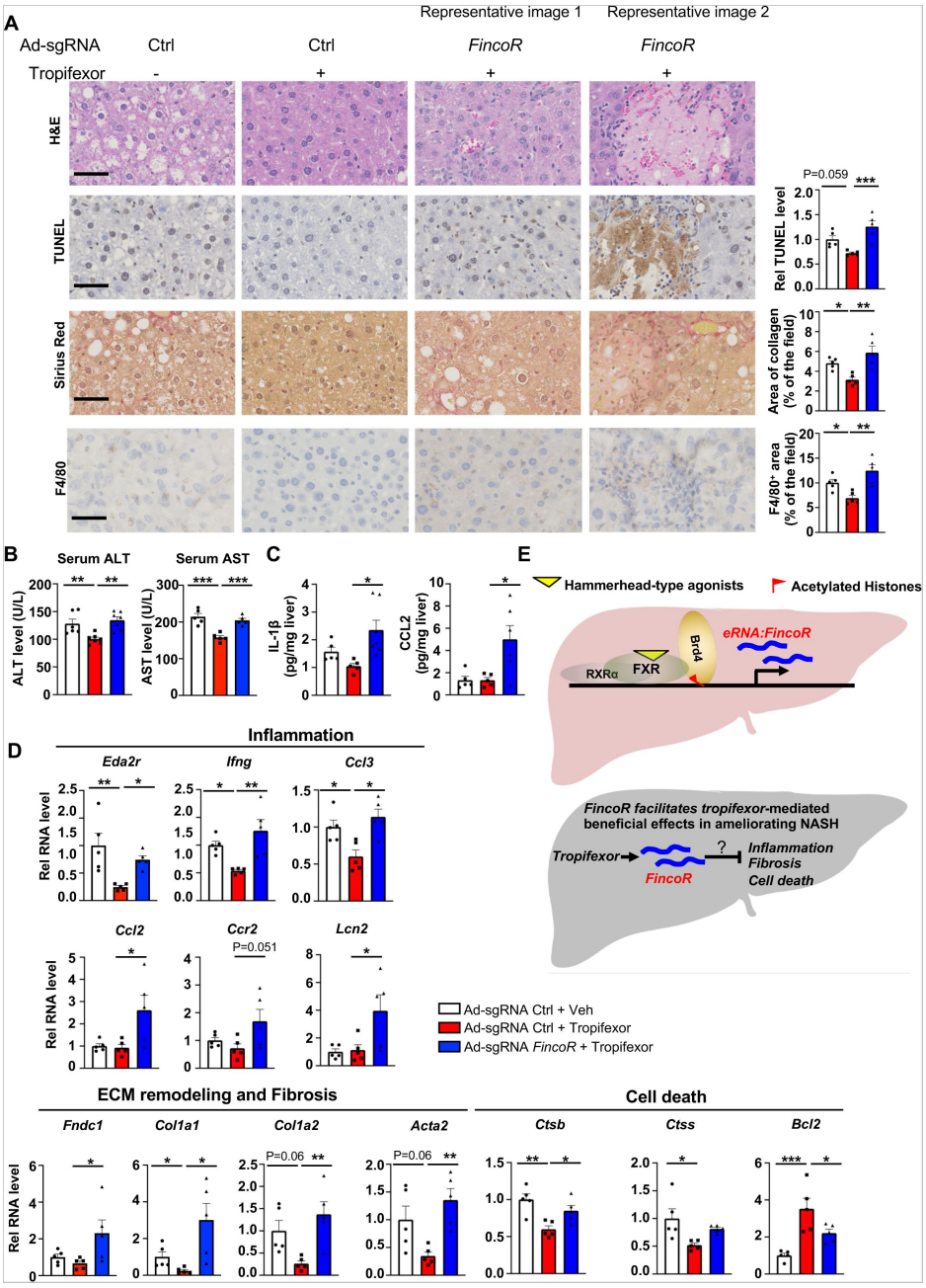

Figure 7.

In diet-induced NASH mice, tropifexor-mediated beneficial effects on reducing liver fibrosis and inflammation are diminished by *FincoR* downregulation.

(A) Representative images from H & E, F4/80, Sirius Red and TUNEL staining of liver sections from the same cohort of mice described in **Figure 6**. Scale bar (50 μm). Image analyses were done using Image J and the area of collagen staining, TUNEL and F4/80 levels were quantified (n=5/group). (B) Serum ALT and AST levels were measured (n=5/group). (C) IL-1β and CCL2 levels in the liver tissues were determined by ELISA (n=5/group). (D) mRNA levels in the liver of the indicated genes involved in inflammation, fibrosis and cell death (n=5/group). (E) Model: *FincoR* is a liver-enriched eRNA that is induced specifically by hammerhead-type FXR agonists (top). In diet-induced NASH mice, *FincoR* is required for tropifexor-mediated beneficial effects on reducing hepatic inflammation, fibrosis, and cell death with the mechanisms to be determined (bottom). (A-D) Data are presented as mean ± SEM. Statistical significance was determined by the one-way ANOVA (Sidak's multiple comparisons test) with *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

FincoR is specifically induced by the hammerhead class of FXR agonists, such as GW4064, cilofexor, and tropifexor. GW4064 is the mother compound of these isoxazole-type hammerhead ligands but is not an ideal therapeutic agent because of its poor water solubility and pharmacokinetics *(Abel et al., 2010)*. In contrast, tropifexor and cilofexor have better pharmacokinetics and are generally well-tolerated in clinical trials for NASH and PBC patients *(Abenavoli et al., 2018; Kremoser, 2021; Sanyal et al., 2023; Tully et al., 2017)*, but the underlying mechanisms for their beneficial effects are poorly understood. Intriguingly, a recent study showed that the gene signature regulated by tropifexor-activated FXR appears to be broader than that of OCA, partly because the tropifexor backbone allows a more favorable interaction of FXR with coactivators or epigenomic modulators *(Hernandez et al., 2019)*. Further, tropifexor was shown to regulate distinct sets of genes in experimental NASH as compared to other FXR agonists, particularly genes involved in fibrosis, inflammation, and oxidative stress *(Hernandez et al., 2019)*. Utilizing CRISPR/Cas9-mediated liver-specific knockdown of *FincoR* in diet-induced NASH mice, we demonstrate that beneficial effects on reducing liver fibrosis, inflammation, and apoptosis mediated by tropifexor were largely dependent on *FincoR* (Model, **Figure 7E**).

While this study focused on regulation of *FincoR* by pharmacological activation of FXR, physiological and pathological regulations of *FincoR* appear to be complex. Although *FincoR* can be induced by the hammerhead class of FXR agonists, it was not induced by the endogenous FXR ligand, cholic acid. This implies that *FincoR* may not contribute to the physiological functions of FXR. In an effort to investigate the potential role of FincoR in the pathological conditions, we observed that *FincoR* levels were elevated in mouse models of cholestasis and NASH as well as in human PBC and NAFLD patients, where BA metabolism is dysregulated. Since different BAs can activate or repress the gene-regulating function of FXR *(Wahlstrom et al., 2016)*, altered BA composition in these pathological conditions may contribute to induction of *FincoR*. Further, binding peaks for multiple nuclear receptors, FXR, LXR, PPARα, RXRα, and HNF-4α, were detected in the *FincoR* locus so that regulation of *FincoR* likely involves the combinatorial regulation by multiple nuclear receptors (**Supplementary Figure S2**). Interestingly, occupancy of the nuclear receptor PPARα at the enhancer region was increased in fasted mice (**Supplementary Figure S3**). It will be interesting to investigate whether and how *FincoR* is differently regulated by these nuclear receptors in response to physiological and pathological cues.

The roles of regulatory RNAs in liver function and diseases and their potentials as therapeutic targets are increasingly being appreciated *(Brocker et al., 2020; Li et al., 2021; Sallam et al., 2016; Zhao et al., 2018)*. For example, a critical role for an oxysterol nuclear receptor LXR-induced lncRNA, *LeXis*, in feedback modulation of cholesterol biosynthesis has been shown *(Sallam et al., 2016)*. Recently, the role of a lncRNA, *Pair*, in liver phenylalanine metabolism has been demonstrated *(Li et al., 2021)*. Enhancer RNAs are a less characterized class of lncRNAs and are highly associated with enhancer functions in gene regulation *(Li et al., 2016)*. Numerous studies have revealed transcriptional roles for eRNAs in various cellular processes *(Lai & Shiekhattar, 2014; Lam et al., 2014; Li et al., 2016; Sartorelli & Lauberth, 2020)* but most previous eRNA studies have used cultured cells *(Hsieh et al., 2014; Li et al., 2013)*, with only a few *in vivo* studies in mouse models *(Mirtschink et al., 2019; Tang et al., 2023)*. In our current study, through integrative analysis of transcriptome and histone mark ChIP-Seq, we identified a group of FXR- regulated eRNAs, including the highly induced *FincoR*. Our current work characterized the role of *FincoR* in gene regulation and in mediating beneficial pharmacological effects of tropifexor in NASH, representing important progress in understanding the roles of eRNAs *in vivo*. Future work is warranted to elucidate the exact mechanisms by which *FincoR* facilitates action of FXR agonists to alleviate inflammation, fibrosis and apoptosis. RNA inside the cells usually associates with different RNA-binding proteins (RBPs) *(Gerstberger et al., 2014)*. We identified potential binding proteins of *FincoR* using the ATtRACT database (*Giudice et al., 2016*). The top four candidates for *FincoR* binding are KHDRBS1, RBM38, YBX2 and YBX3 (**Supplemental Table S5**). KHDRBS1 and RBM38 have been reported to have important roles in RNA processing (*Bielli et al., 2011; Zou et al., 2021*). YBX2 and YBX3 belong to the Y-box (YBX) protein family, which have

been linked to diverse forms of RNA metabolism and many other processes, including cell proliferation, DNA repair, stress responses, development, and inflammation (*Kleene, 2018*). Whether these predicted RBPs interact with *FincoR* and how they contribute to phenotypes should be investigated in future experimentation to understand the mechanisms involved in *FincoR*-regulated hepatocyte function.

There is limitation of some of our approaches that cannot fully dissect the underlying mechanisms of *FincoR*. Currently, the function of eRNA loci can be attributed to a functional eRNA transcript, or binding of transcription factors to this region, or the transcription process itself, or some combination of these effects (*Li et al., 2016*; *Sartorelli & Lauberth, 2020*). In our studies to decrease expression of *FincoR*, the region containing the FXR binding site was deleted (**Figure 5-figure supplement 1A, right panel**). While the expression of *FincoR* transcripts was significantly reduced, we cannot rule out whether decreased binding of FXR or decreased transcription contribute to the observed changes in phenotype. To directly investigate the function of *FincoR* transcripts, downregulation of the transcript with antisense oligonucleotides together with overexpression of *FincoR* in the liver will be required in future experiments.

FXR is increasingly recognized as an important therapeutic target for enterohepatic diseases, but the development of clinically applicable and more targeted FXR-based therapy is still challenging. In this study, we provided the first characterization of an eRNA, *FincoR*, induced by pharmacological activation of FXR and show that *FincoR* has a beneficial role in reducing liver fibrosis and inflammation in dietary NASH mice. Complete understanding of the function and mechanisms of *FincoR* may provide novel insights for the development of desirable therapy for NASH and other chronic liver diseases.

Materials and Methods

Animal experiments

All animal studies were performed according to procedures approved by the Institutional Animal Care & Use Committee at the University of Illinois at Urbana-Champaign and were in accordance with National Institutes of Health guidelines. Mice were maintained in 12/12 h light/dark cycles and fed standard rodent chow. FXR-LKO mice were generated by breeding FXR floxed mice with Albumin-Cre mice (The Jackson Lab). *FincoR*-LKD mice were generated as previously reported (*Zhao et al., 2018*). Briefly, Cas9 transgenic mice (JAX #024858) were injected via the tail vein with adenoviruses (approximately 5×10^8 PFU) expressing two sgRNAs targeting *FincoR* (sgRNA1: *GGGTTAAGAGCTGTAGGCTG* and sgRNA2: ACTTCTATGTCCAACAACCG). The sequences of sgRNAs were designed using a CRISPR design tool (*http://crispr.mit.edu/*).

Mice were given a single dose of vehicle or 30 mg/kg GW4064 (in corn oil, Tocris Bioscience, #2473) after overnight fasting. Mice were treated with 0.5 mg/kg tropifexor (in corn oil, MedChem Express, HY-107418), 30 mg/kg cilofexor (in corn oil, MedChemExpress, HY-109083), 100 mg/kg fexaramine (in 0.5% methylcellulose, MedChem Express, HY-10912), and 20 mg/kg OCA (in 0.5% methylcellulose, MedChem Express, HY-12222) as indicated. C57BL6 mice were fed with a chow diet containing 0.5% CA for 6 h (*Jung et al., 2020*).

To induce cholestasis, mice were treated by gavage with 75 mg/kg α-naphthyl isothiocyanate (ANIT) for 48 hours as previously reported (*Jung et al., 2020*; *Kim et al., 2016*; *Kim et al., 2020*). Cholestasis was also induced by bile duct ligation or sham operation in mice for 24 hours or 72 hours (*Li et al., 2018*).

To induce dietary obesity, mice were fed a high-fat diet (TD88137; Harlan Teklad) or a high-fat diet with 25% fructose in water (high fat/high fructose) for 12 weeks (*Seok et al., 2021*).

To investigate the effect of liver-specific downregulation of *FincoR* on NASH, male Cas9 mice were fed the AMLN diet (Research Diets, D09100310, 40 kcal% fat, 2% cholesterol, 20 kcal% fructose) for 12 weeks and then, were injected with adenovirus expressing control sgRNA or sgRNA targeting FincoR. Administration of tropifexor was started 3 days later and given at 0.3 mg/kg dissolved in corn oil.

RNA-Seq

C57BL/6 mice were fasted overnight and *i.p.* injected with vehicle or GW4064 (30 mg/kg) for 1 h, and livers were collected (n = 4 mice for either vehicle or treated group). Total RNA from each liver was extracted by RNeasy kit (Qiagen), and two randomly selected mice liver RNAs were pooled for RNA-seq (two RNA-seq reactions from 4 mice livers for either vehicle or treated group). RNA-seq was performed as previously described *(Byun et al., 2018; Byun et al., 2020; Seok et al., 2018)*. Ribosomal RNA was removed with the Ribozero HMR Gold kit (Illumina). The sequencing library was generated following methods described below.

Construction of strand-specific RNAseq libraries

Construction of the RNAseq libraries and sequencing on the Illumina NovaSeq 6000 were performed at the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign. After DNase digestion, purified total RNAs were analyzed on a Fragment Analyzer (Agilent) to evaluate RNA integrity. The total RNAs were converted into individually barcoded polyadenylated mRNAseq libraries with the Kapa HyperPrep mRNA kit (Roche). Libraries were barcoded with Unique Dual Indexes (UDI's) which have been developed to prevent index switching. The adaptor-ligated double-stranded cDNAs were amplified by PCR for 8 cycles with the Kapa HiFi polymerase (Roche). The final libraries were quantitated with Qubit (Thermo Fisher) and the average cDNA fragment sizes were determined on a Fragment Analyzer. The libraries were diluted to 10 nM and further quantitated by qPCR on a CFX Connect Real-Time qPCR system (Bio-Rad) for accurate pooling of barcoded libraries and maximization of number of clusters in the flowcell.

Sequencing of libraries in the NovaSeq

The barcoded RNAseq libraries were loaded on one SP lane on a NovaSeq 6000 for cluster formation and sequencing. The libraries were sequenced from one end of the fragments for a total of 100 bp. The fastq read files were generated and demultiplexed with the bcl2fastq v2.20 Conversion Software (Illumina, San Diego, CA). The quality of the demultiplexed fastq files was evaluated with the FastQC software, which generates reports with the quality scores, base composition, k-mer, GC and N contents, sequence duplication levels and overrepresented sequences.

GRO-seq

To harvest the nuclei from mouse liver cells, the liver was harvested at indicated time and washed with a cold swelling buffer (10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 2 U/ml Superase-In). The nuclei were prepared by Dounce homogenization in cold swelling buffer and filtered using a cell strainer (100 µm, BD Biosciences). Nuclei were collected by centrifugation at 400 x g for 10 min, then resuspended in the lysis buffer (swelling buffer with 10% glycerol and 1% IGEPAL) and incubated on ice for 5 min. Nuclei were washed twice with the lysis buffer and resuspended at a concentration of 10^8 nuclei/ml in the freezing buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA). We then followed our previous method *(Li et al., 2013; Oh et al., 2021)* to conduct nuclear run-on and GRO-Seq library preparation. Briefly, the nuclei in freezing buffer were subjected to the nuclear run-on reaction by mixing with an equal volume of run-on buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 300 mM KCl, 20 units of Superase-In, 1% sarkosyl, 500 µM ATP, GTP, Br-UTP and 2 µM CTP) for 5 min at 30 °C. The BrU-labeled run-on RNAs were extracted by TRIzol and purified by anti-BrdU agarose beads (Santa Cruz Biotech, sc-

32323 AC). The run-on RNAs were then subjected to end repair by T4 PNK, poly-adenylation, and then to cDNA first strand synthesis by a custom primer (oNTI223) that allows circularization of the cDNA. This cDNA then was re-linearized by Ape1 (NEB), size selected by TBE gel electrophoresis and the products of the desired size were excised (~320–350 bp) for final library prep and sequencing. GRO-Seq samples were run on a NextSeq 500 sequencer from Illumina with a single-end 80 nt model.

Histological analyses

For histology, tissues were dissected and immediately fixed in 10% formalin overnight and processed for paraffin embedding and H&E staining. Paraffin-embedded liver sections were incubated with F4/80 antibody, and antibody was detected using a peroxidase-based method (Abcam, ab64238). Liver collagen was detected by Sirius Red staining (Abcam, ab246832) and apoptosis was detected by TUNEL staining (Millipore, S7100). For Oil Red O staining, liver tissue was frozen in OCT compound (Sakura Finetek, 4583), sectioned, and stained. Liver sections were imaged with a NanoZoomer Scanner (Hamamatsu) and quantification was done using NIH ImageJ.

Metabolic analyses

Hepatic levels of TG (Sigma, MAK266), cholesterol (Sigma, MAK043), glycogen (Biovision, K646-100), total BA levels (Diazyme, DZ042A), serum NEFA (Sigma, MAK044), serum ALT (Sigma, MAK052) and serum AST (Sigma, MAK055) were determined according to the manufacturer's instructions. Mouse liver IL-1β (R&D systems, MLB00C) and CCL2 (R&D systems, DY479-05) were detected by commercially available ELISA kit.

Liver samples of PBC and NAFLD patients

Liver specimens from normal organ donors, and patients with PBC or NAFLD were obtained from the Liver Tissue Procurement and Distribution System. The samples were unidentifiable, and thus, ethical approval was not required. Hepatic XR_007061585.1 levels were measured by qPCR.

Mouse liver ChIP

Liver ChIP assay was performed as described previously (*Jung et al., 2020*; *Seok et al., 2018*). Briefly, chromatin extracts were prepared from FXR floxed and FXR-LKO mouse livers after treatment of the mice with GW4064 which was followed by preclearing and immunoprecipitation using control IgG or FXR antibody (Novus Biologicals, NBP2-16550; Santa Cruz, sc-25309), RXRα antibody (Proteintech, catalog no. 21218-1-AP), or BRD4 antibody (Bethyl Laboratories, catalog #A301-985A50). Enrichment in chromatin precipitates of gene sequences was measured by qRCR using primers listed (**Supplementary Table S6**).

Primary mouse hepatocytes and HepG2 cells

Primary hepatocytes were isolated from C57BL/6 mice by collagenase (Worthington Biochemical Corporation, LS004188) perfusion and maintained in William's E Medium with primary hepatocyte maintenance supplements (Gibco #CM4000) in six-well plates as described previously (*Jung et al., 2020*; *Kim et al., 2015*). HepG2 cells were maintained in DMEM supplemented with 10% fetal bovine serum at 37°C and 5% CO_2.

Luciferase reporter assay

The enhancer region containing the FXR binding element was amplified by PCR and cloned into the pGL4.23 vector (Promega). HepG2 cells were transiently transfected with FXR (100 ng/well) and RXRα (5 ng/well) in combination with reporters containing the wild type FXRE or mutated FXRE (200 ng/well) (**Supplementary Table S6**). β-galactosidase plasmid (200 ng/well) was also

transfected as an internal control. Cells were treated with GW4064 or DMSO after transient transfection. Six hours later, the cells were harvested. All reporter assays were repeated at least three times in triplicates.

Race

5' and 3' RACE assays were performed using a SMARTer RACE kit (Clontech) according to the manufacturer's instructions. The resulting PCR products were separated by electrophoresis in agarose gels and cloned into the pRACE vector provided by the kit. The transcription start sites and end sites of *FincoR* were determined by sequencing. The gene-specific primers used for 5'- and 3'-RACE are listed in **Supplementary Table S6**.

In vitro transcription and translation (TNT)

Expression plasmids for luciferase and *FincoR* were mixed with a Coupled Reticulocyte Lysate System (Promega). After incubating at 30°C for 60 min, translated products were separated on 4-20% gradient SDS polyacrylamide gels and transferred to PVDF membranes. Chemiluminescent detection of in vitro translated protein was performed following the manufacturer's protocol (Promega).

RT-qPCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, 74104) and 2 μg of RNA was reverse transcribed and mRNA expression was normalized relative to that of 36B4. The qPCR primers are shown in **Supplementary Table S6**.

Subcellular fractionation

Using a Cytoplasmic and Nuclear RNA Purification Kit (Norgen, Thorold, ON, Canada), the cytoplasm and nucleus fractions from primary hepatocytes isolated from livers of mice treated with GW4064 or DMSO were separated, and both fractions were subjected to RNA extraction and qRT-PCR.

Immunoblotting analysis

Total liver lysates were prepared as described before *(Sun et al., 2022)*. Antibodies for immunoblotting for β-ACTIN (#4970) were purchased from Cell Signaling Technology. Antibodies for immunoblotting for FXR (sc-25309) were purchased from Santa Cruz.

FincoR polyadenylation study

Total RNA was prepared using the RNeasy mini prep kit (Qiagen). Poly(A)+ and poly(A)-RNA was separated using a Dynabeads™ mRNA Purification Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Briefly, total RNA was incubated with the Dynabeads/binding buffer suspension at room temperature for 5 min and the reaction tubes were placed on a magnet until solution was clear. The supernatant containing poly(A)-RNA was saved. The beads with poly(A)+ RNA were washed three times with washing buffer provided by the kit. RNA was extracted from the supernatant and beads respectively using Trizol. qPCR was performed to analyze levels of *FincoR* and 36b4 in the poly(A)+ and poly(A)-RNA fractions.

Genomics Analysis

RNA-Seq and GRO-Seq reads were mapped to the mouse reference genome mm10 with STAR aligner *(Dobin et al., 2013)*. Transcript quantifications were done with the HOMER tool set *(Heinz et al., 2010)* and enhancer RNAs were identified based on H3K27ac ChIP-Seq in mouse liver. Briefly, intergenic H3K27ac ChIP-Seq peaks were selected as putative enhancer regions in mouse liver. Then +/- 3kb regions around putative enhancers with RNA-Seq signal (>1 RPKM) were

considered as putative eRNAs in mouse livers. Overlapped eRNAs regions were merged, and redundant ones were removed. In addition, any +/- 3kb extended eRNAs regions that overlapped with protein-coding genes were further removed to avoid transcriptional readthrough from genes. We used DESeq2 *(Love et al., 2014)* to identify significantly regulated eRNAs with a cutoff of (FDR < 0.05, Log2FC > 1). Public ChIP-Seq datasets were obtained from ENCODE or GEO (**Supplementary Table S3**).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9. Statistical differences were evaluated using the two-tailed unpaired Student's t-test for comparisons between two groups, or ANOVA and appropriate post hoc analyses for comparisons of more than two groups. Statistical methods and corresponding *p* values for data shown in each panel are indicated in figure legends.

Data availability

RNA-seq and Gro-seq data were deposited in GEO under the following accession numbers GSE 221986.

Acknowledgements

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Supplementary Figure Legends

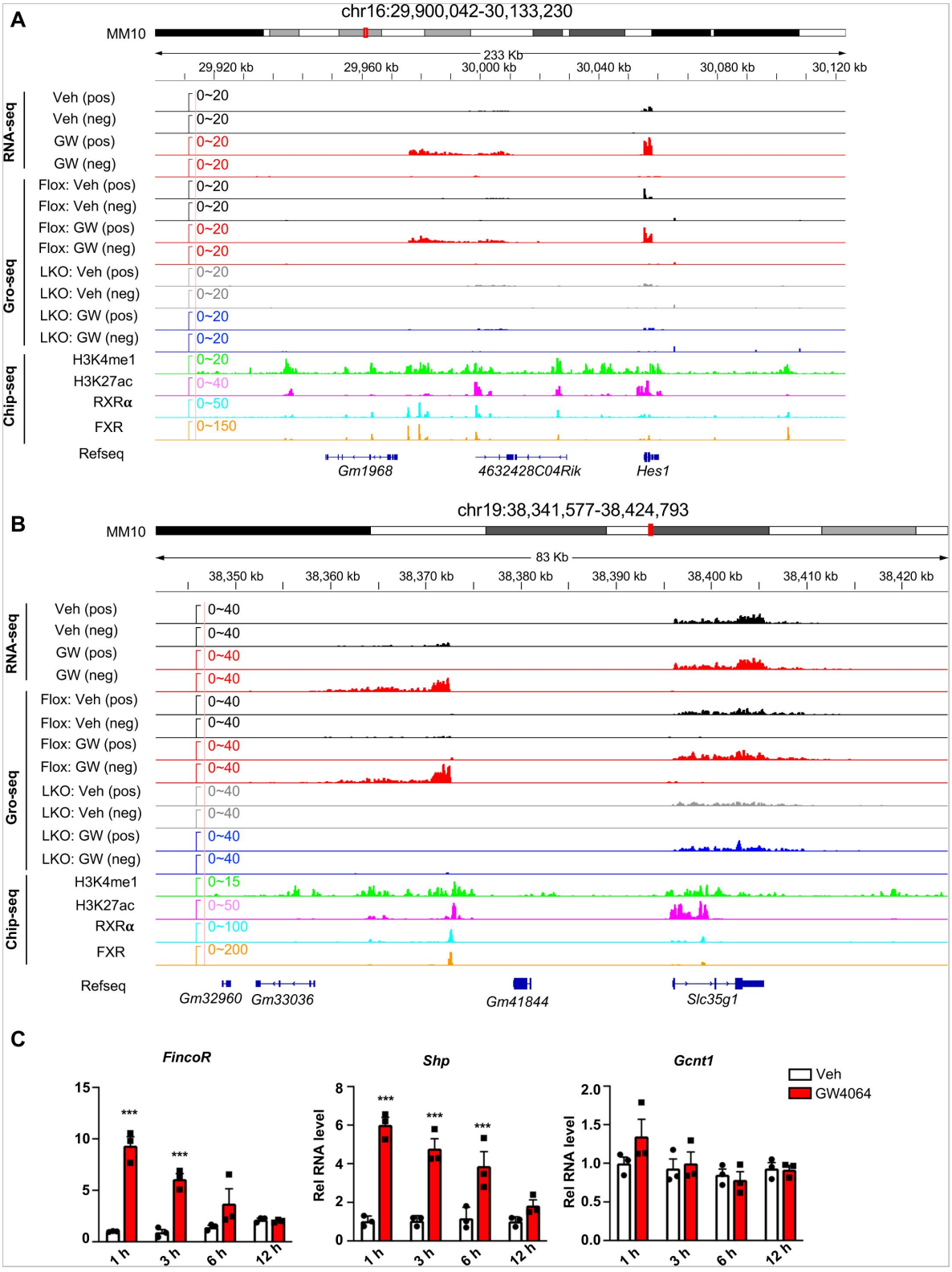


Figure 1-figure supplement 1.

(A, B) Examples of FXR-regulated eRNAs produced near the genes *Hes1* (A) and *Slc35g1* (B) were shown. (C) Time course expression of *FincoR*. C57BL/6 male mice were fasted overnight and injected *i.p.* with vehicle or GW4064 (30 mg/kg) for 1, 3, 6,12 h. Livers were collected at the indicated time points (n=3/group) and *FincoR* and mRNA levels of *Shp* and *Gcnt1* were measured. Data are presented as mean ± SEM. Statistical significance was determined by the two-way ANOVA Sidak's multiple comparisons test with *$p < 0.05$ and ***$p < 0.001$.

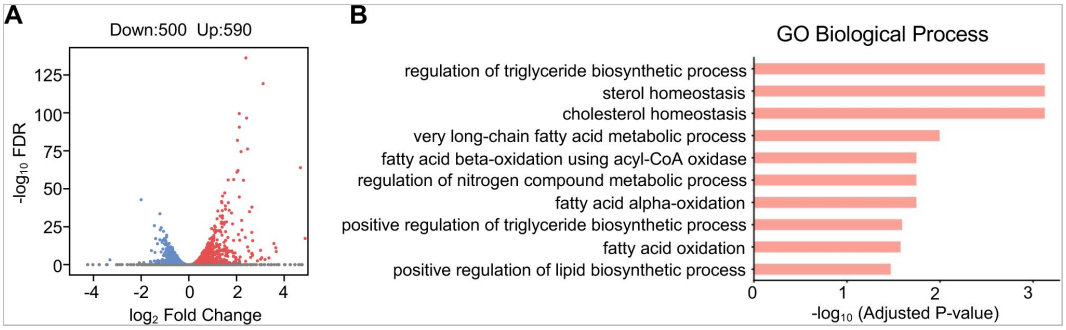


Figure 1-figure supplement 2.

(A) Volcano plot showing the differential expressed genes (DEGs) after GW4064 treatment (DESeq2 FDR<0.05). The numbers refer to the number of genes up- or down-regulated. (B) Bar plot showing the enriched Gene Ontology in terms of biological processes for up-regulated genes.

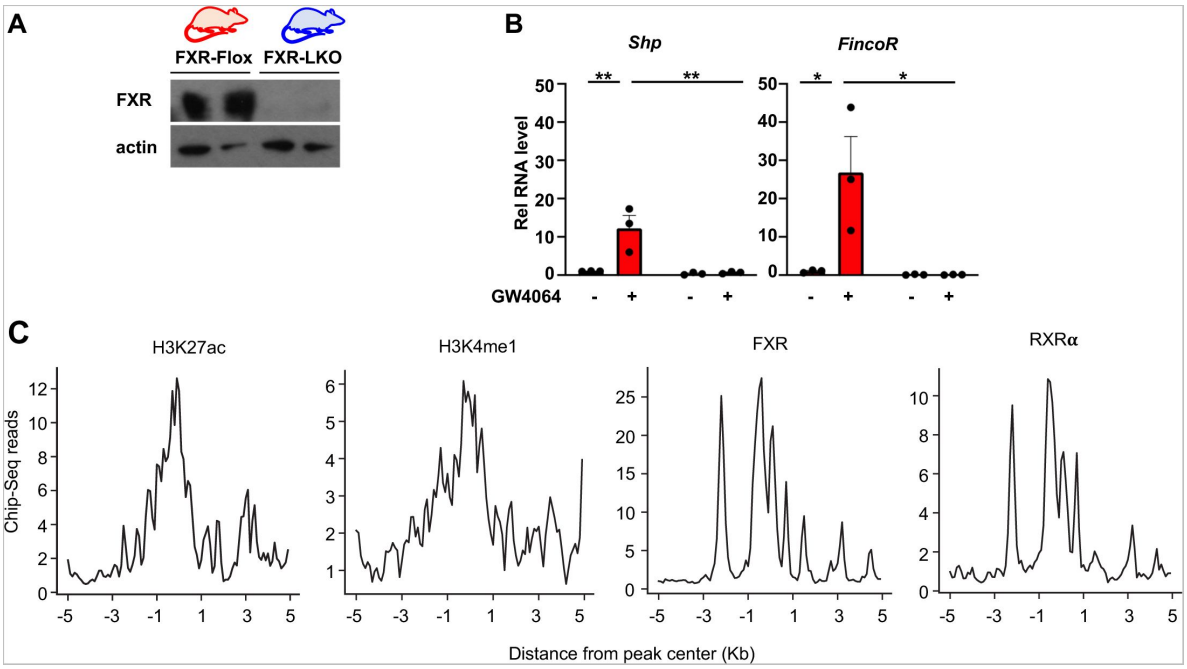


Figure 2-figure supplement 1.

(A) FXR protein levels in the livers isolated from FXR-Flox and FXR-LKO mice are shown. (B) Validation of hepatic FXR-dependent induction of *FincoR* by qPCR (n = 3 mice). The *Shp* gene was used as a control. Data are presented as mean ± SEM. Statistical significance was determined by the two-way ANOVA Tukey's multiple comparisons test with *$p < 0.05$ and **$p < 0.01$. (C) Metagene plots showing H3K27ac, H3K4me1, FXR, and RXRα ChIP-Seq profiles centered on up-regulated eRNAs.

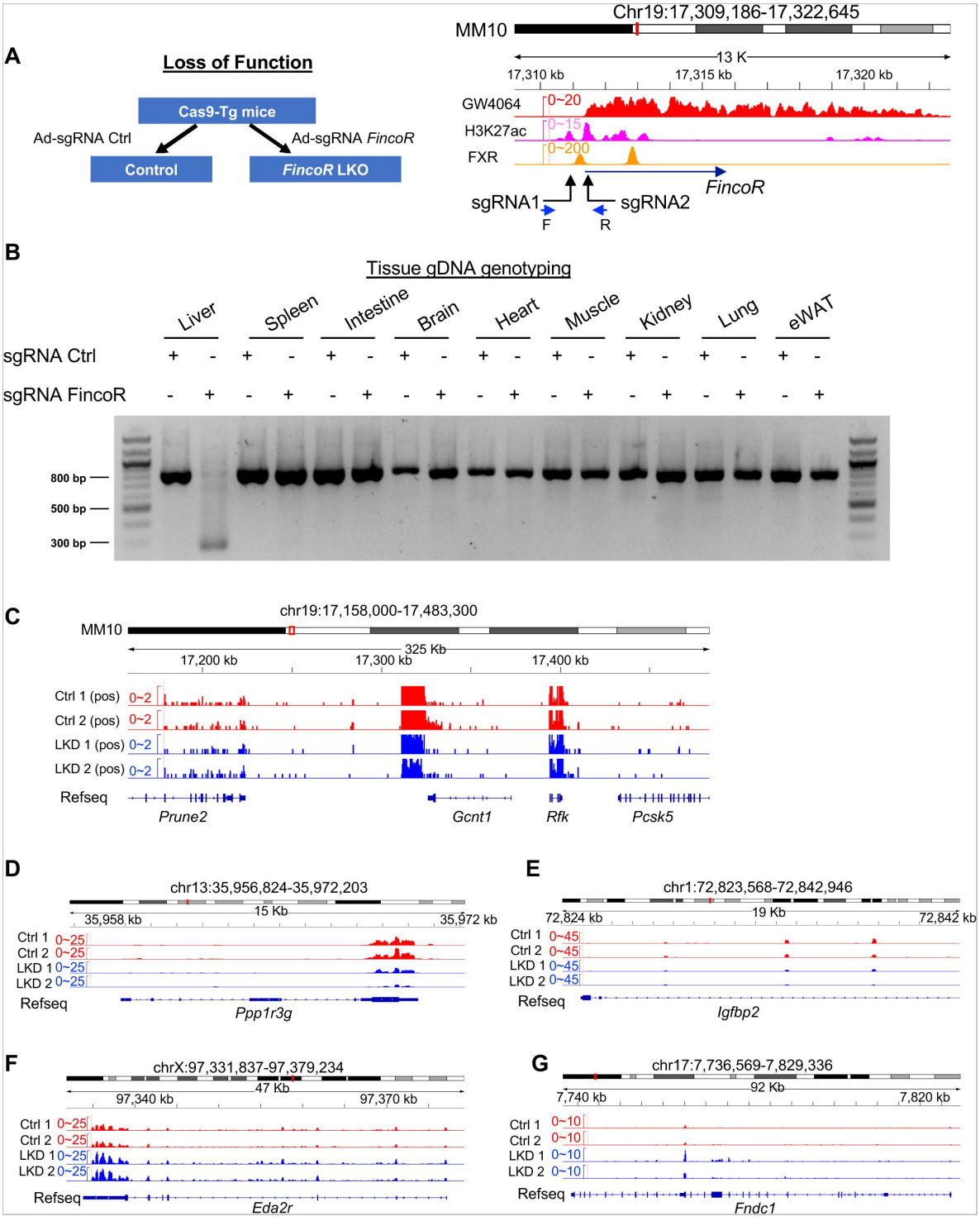


Figure 5-figure supplement 1.

(A) Left: experimental scheme for the *FincoR* loss of function experiments. Right: illustration demonstrating the sequence targeted by the sgRNA in relation to the transcriptional and epigenetic profile. (B) The genomic DNAs from liver, spleen, intestine, brain, heart, muscle, kidney, lung and adipose tissue were isolated and PCR was performed using the primers (**Supplementary Table S6**) to verify tissue-specific knock-out. (C-G) RNA-seq profiles of expression of hepatic *Prune2* (C), *PPP1r3g* (D), *Igfbp2* (E), *Eda2r* (F) *and Fndc1* (G) are shown (n=2/group).

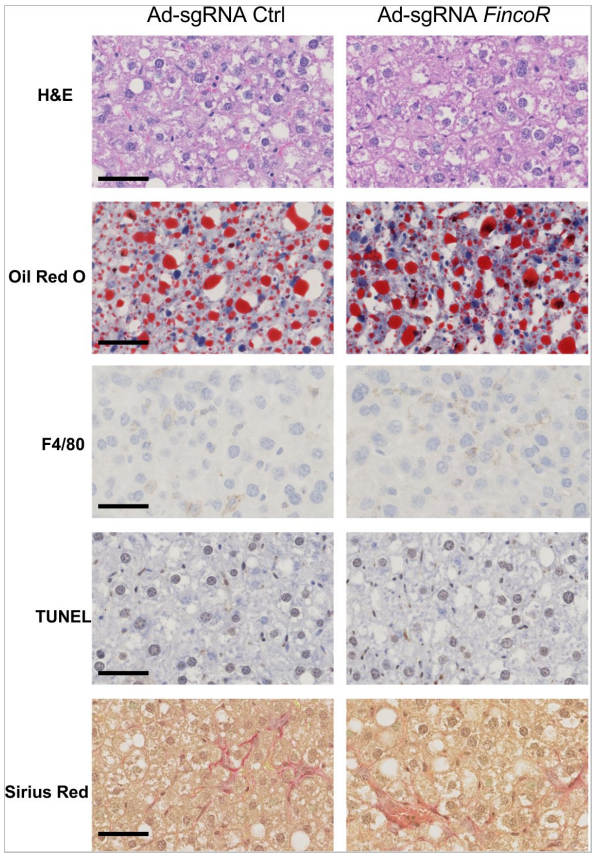
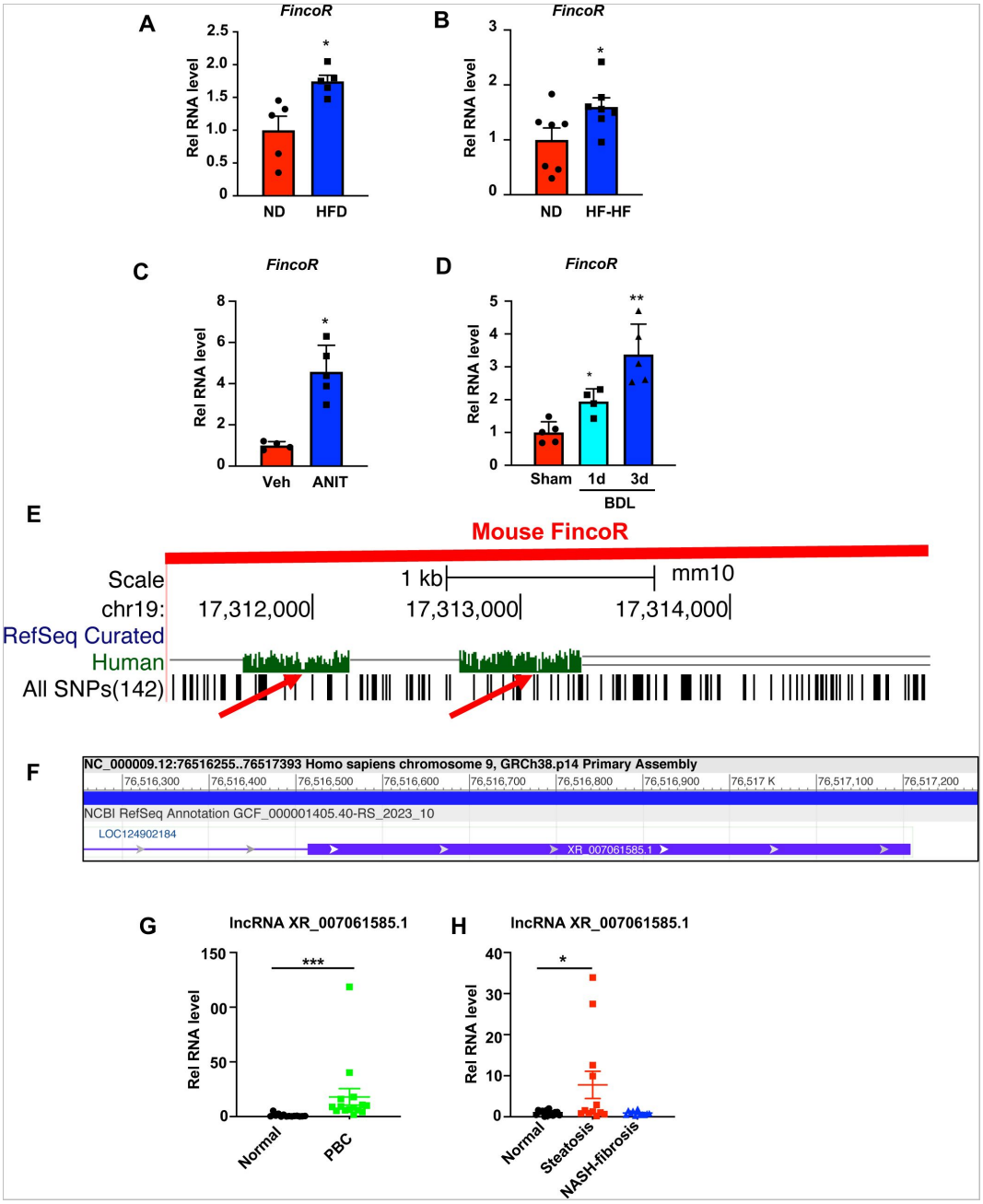


Figure 7-figure supplement 1.

The effects of *FincoR* downregulation on NASH pathologies.

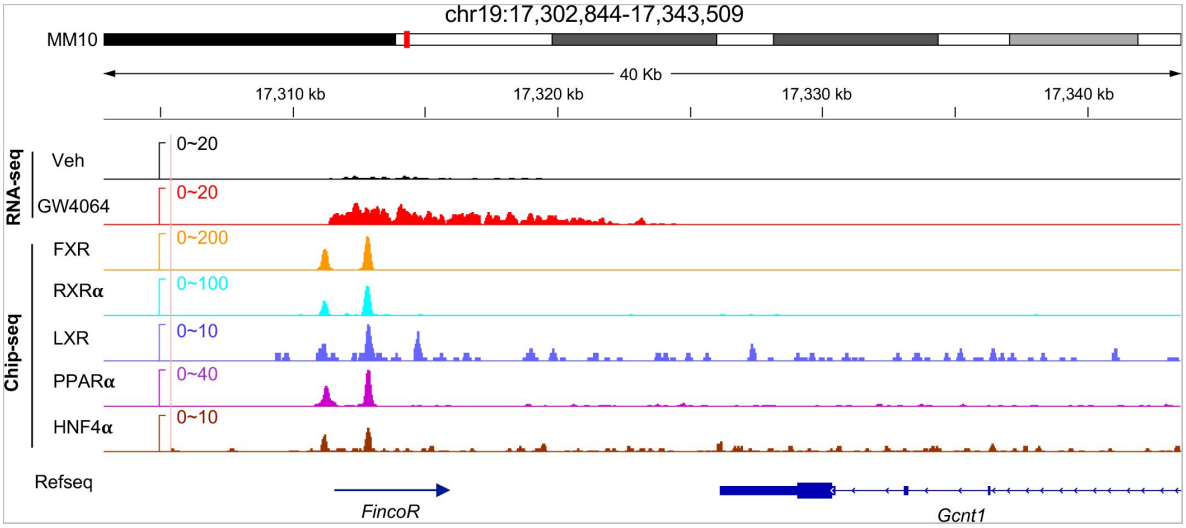
Male Cas9 mice were fed with a NASH diet for 12 weeks. Then these mice were randomly assigned to 2 groups and infected with adenovirus expressing sgRNA for *FincoR* or control. The tissues were collected 2 weeks later. Liver histology analysis was performed and representative images were shown. Scale bar (50 μm).



Supplementary Figure S1.

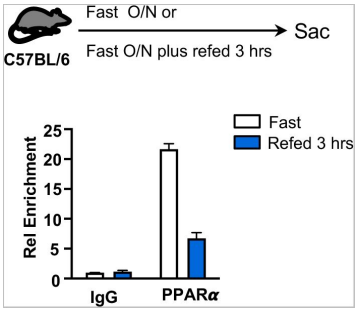
Hepatic expression of *FincoR* is elevated in liver disease associated with inflammation and fibrosis.

(A) C57BL/6 mice were fed with a high fat diet for 12 weeks. The liver RNAs were extracted and *FincoR* expression was measured (n=5/group). (B) C57BL/6 mice were fed with a high fat diet with high fructose water for 12 weeks. The liver RNAs were extracted and *FincoR* expression was measured (n=7/group). (C) C57BL/6 mice were treated with ANIT (75 mg/kg) for 48 h and then sacrificed after 5 h of fasting. The liver RNAs were extracted, and *FincoR* expression was measured (n=4~5/group). (D) C57BL/6 mice were bile duct ligated for 1 day or 3 days. They were then sacrificed after 5 h of fasting. The liver RNAs were extracted and *FincoR* expression was measured (n=4~5/group). (E) *FincoR* conservation between mice and human as displayed in the UCSC Genome Browser. Red arrows indicate the conserved region. (F) Human lncRNA XR_007061585.1 with sequence similarity to mouse *FincoR* annotated in the NCBI genome data viewer. (G) Expression of lncRNA XR_007061585.1 in liver samples from normal individuals or patients with primary biliary cholangitis (PBC) (n=14~15/group). (H) Expression of lncRNA XR_007061585.1 in liver samples from normal individuals or patients with NAFLD-associated steatosis (n=12~15/group). (A-D, G-H) Data are presented as mean ± SEM. Statistical significance was determined by the Student's t test with $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.



Supplementary Figure S2.

Hepatic genome browser tracks of FXR, RXRα, LXR, PPARα, and HNF4α binding peaks at the *FincoR* locus.



Supplementary Figure S3.

PPARα occupancy in the *FincoR* enhancer region.

C57BL/6 male mice were fasted overnight with or without refeeding for 3 h and then sacrificed. ChIP assays were performed in liver samples to detect PPARα occupancy at the FXR binding peak region close to the transcription start site of *FincoR*.

Supplemental Tables

Supplemental Table S2 is attached as an Excel file.

Supplemental Table S4 is attached as an Excel file.

Name	Total reads	Uniquely mapped reads
WT Liver Veh RNA-Seq rep1	50887391	38072659
WT Liver GW RNA-Seq rep1	54020150	40595856
WT Liver Veh RNA-Seq rep2	51268164	38898913
WT Liver GW RNA-Seq rep2	51899892	39487241
Flox Liver Veh GRO-Seq	62960996	30305910
Flox Liver GW GRO-Seq	59210073	26116795
FXR KO Liver Veh GRO-Seq	34153537	14379417
FXR KO Liver GW GRO-Seq	36430101	15008281
WT Liver RNA-Seq rep1	96262381	81801494
WT Liver RNA-Seq rep2	104979915	88365481
FincoR KD Liver RNA-Seq rep1	91654076	75346167
FincoR KD Liver RNA-Seq rep2	90211420	74745807

Supplemental Table S1.

Sequencing data generated in this study

target	accession ID
FXR	https://genome.ucsc.edu/cgi-bin/hgCustom?hgsid=1577671033_RPWVA7jbx3rDswKOeo2dazTsXxe0&hgct_table=ct_FxrLiver_2971
H3K27ac	ENCFF001KMI
H3K4me1	ENCFF001KNF
LXR	GSM864669
RXRα	GSM864674
PPARα	GSM864671
HNF4α	GSM2055887

Supplemental Table S3.

Public ChIP-Seq dataset

RBP	Length	Motif	Domain	number of binding sites
KHDRBS1	7	UAAAAAG	KH	1
RBM38	7	UUGUGUG, GUGUGUG	RRM	3
YBX2	7	CACACCA	CSD	11
YBX3	7	CACACCA	CSD	11
PCBP1	6	CUUUCC	KH	1
KHSRP	6	UGCAUG	KH	2
PTBP1	6	CUCUCU	RRM	1
PTBP2	6	CUCUCU	RRM	1
4KZD	5	GAAAC	N/A	1
4KZE	5	GAAAC	N/A	1
4Q9Q	5	GAAAC	N/A	1
DAZL	5	GUUCU	RRM	6
MSI1	5	GUAGU	RRM	1
TLR3	5	AAAGG	LRR;TIR	12

Supplemental Table S5

Predicted RNA binding proteins (RBPs) binding to *FincoR*

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
FincoR	GCAAAGCACCTTCTAGCACA	GTCAGGGAGCTAACGAATGC
Gcnt1	GCATCGCATCCTGCTTTGATA	GGTCTGCCTTAACCCGACTC
Shp	TCTGCAGGTCGTCCGACTAT	CAGGCAGTGGCTGTGAGAT
Cyp7a1	GGGATTGCTGTGGTAGTGAGC	GGTATGGAATCAACCCGTTGTC
Cyp8b1	CCTCTGGACAAGGGTTTTGTG	GCACCGTGAAGACATCCCC
Srebp1c	GCAGCCACCATCTAGCCTG	CAGCAGTGAGTCTGCCTTGAT
Lpin1	CATGCTTCGGAAAGTCCTTCA	GGTTATTCTTTGGCGTCAACCT
Scd1	TTCTTGCGATACACTCTGGTGC	CGGGATTGAATGTTCTTGTCGT
Ifng	GCG TCA TTG AAT CAC ACC TG	GAC CTG TGG GTT GTT GAC CT
Ccl3	TTCTCTGTACCATGACACTCTGC	CGTGGAATCTTCCGGCTGTAG
Ccl2	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
Ccr2	AAGAGGGCATTGGATTCACCACA	GCCGTGGATGAACTGAGGTAACA
Lcn2	GCAGGTGGTACGTTGTGGG	CTCTTGTAGCTCATAGATGGTGC
Col1a1	ATCGGTCATGCTCTCTCCAAACCA	ACTGCAACATGGAGACAGGTCAGA
Col1a2	CCTTTGTCAGAATACTGAGCAGC	GTAACTTCGTGCCTAGCAACA
Acta2	TCGGATACTTCAGCGTCAGGA	GTCCCAGACATCAGGGAGTAA
Eda2r	CACACTGCATAGTCTGCCCTC	GCCTTCTGGACCCGATTGA
Fndc1	GGGAGACATGGCAAACCTGT	TGGTAGGAGAGTATGTGGTGG
Ctsb	TCCTTGATCCTTCTTTCTTGCC	ACAGTGCCACACAGCTTCTTC
Ctss	GAAGTACGGCGTCTCATCTGG	CATGCCCACTTGGTAGGTATG
Bcl2	GTCGCTACCGTCGTGACTTC	CAGACATGCACCTACCCAGC
36b4	CCCTGAAGTGCTCGACATCA	TGCGGACACCCTCCAGAA
XR_007061585.1	TTGTCATCAAGCCCTGTTCA	TCTGCTTTGTCTGAGGACCA

B. List of primers used for mutagenesis

	Forward Primer (5'-3')	Reverse Primer (5'-3')
Mutation	GTGAATTCTCTCCTAGaTCcTTGACaTTCCAAGTC ATGTAC	GTACATGACTTGGAAtGTCAAgGAtCTAGGAGAGAATTC AC

Supplemental Table S6.

Primer sequences used in this study

C. List of primer sequences for mouse ChIP-qPCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
FincoR	TCCTAGGTCTTTGACCTTCCAA	TGTGCCCACTGGTGAAATAG
Non-specific region	GTGTACACGCCCAAACTTGA	GTTCACCTCTCTAGCTCACCT

D. List of primer used for RACE

	Gene specific primer (5'-3')
5'RACE	GATTACGCCAAGCTT GGGAACTCACGTCTGGAAGCAGAAGCTG
3'RACE	GATTACGCCAAGCTT GGACACTCGGGAATTGACAGTGGGTCAGG
3'RACE nest primer	GATTACGCCAAGCTT GTGGGGGATAGCACAAGCGCCAGGATGC

E. List of primer used for genotyping

	Forward Primer (5'-3')	Reverse Primer (5'-3')
	GTGATTTCCTTTCCCACAGC	GTCAGGGAGCTAACGAATGC

Supplemental Table S6. (continued)

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Reviewer #1 (Public Review):

Summary:

In their article, the authors delve into the therapeutic potential of a newly identified liver-specific lncRNA, FincoR, regulated by the Farnesoid X Receptor (FXR) and induced by the agonist tropifexor, in treating nonalcoholic steatohepatitis (NASH). They demonstrate that FincoR significantly enhances tropifexor's effectiveness in reducing liver fibrosis and inflammation in NASH, presenting it as a promising therapeutic target. The manuscript revisions broaden the study to include both mouse and human data, showing elevated FincoR levels in various mouse models of liver disease and identifying a similar lncRNA in humans, potentially indicating a conserved therapeutic mechanism. This research offers valuable insights into FincoR's role in NASH and suggests further exploration into its functions and mechanisms in liver disease treatment.

Strengths:

This study enhances our understanding of FincoR, a liver-specific lncRNA, and its therapeutic potential in treating NASH through a multifaceted research approach. The revised manuscript further strengthens this contribution by incorporating additional experiments and human relevance, summarized as follows: 1) The use of GRO-seq and RNA-seq technologies has provided an in-depth and unbiased view of the transcriptional alterations driven by the FXR agonist tropifexor, especially emphasizing FincoR's pivotal role. 2) The research expands on the original findings by including diverse mouse models of NAFLD/NASH and cholestatic liver injury. These models demonstrate significant increases in

hepatic FincoR levels across various conditions, such as diets high in fat and fructose, chemical induction of liver cholestasis with ANIT, and surgical induction via bile duct ligation. This broadened scope underscores FincoR's involvement in liver disease mechanisms beyond the initial models of FXR knockout (KO) and FincoR liver-specific knockdown (FincoR-LKD). 3) Incorporation of tropifexor, an FDA-approved FXR agonist, alongside these experimental models bridges experimental findings to potential therapeutic applications for NASH patients. 2) The manuscript revision includes promising data on the sequence similarity between mouse FincoR and a human locus, identifying a partially conserved human lncRNA (XR_007061585.1) with elevated levels in NAFLD and PBC patients. This addition enhances the study's relevance to human health. 3) The study's design, with the inclusion of both negative and positive controls and now enriched with a wider array of mouse models and human data, ensures that the observed therapeutic effects can be confidently attributed to FincoR's modulation by tropifexor.

Weaknesses:

The authors acknowledge that certain questions remain unanswered within the scope of this study on FincoR, due to feasibility and technical challenges. While it's important to note that such limitations are rooted in the practical and technical complexities, these unresolved issues might limit the study's immediate impact. The decision to focus on the discovery and initial characterization of FincoR, is strategically but not scientifically justified.

https://doi.org/10.7554/eLife.91438.2.sa1

Reviewer #2 (Public Review):

Summary:

Nonalcoholic fatty liver disease (NASH), recently renamed as metabolic dysfunction-associated steatohepatitis (MASH) is a leading cause of liver-related death. Farnesoid X receptor (FXR) is a promising drug target for treating NASH and several drugs targeting FXR is under clinical investigation for its efficacy in treating NASH. The authors intended to address whether FXR mediates its hepatic protective effects through regulation of lncRNAs, which would provide novel insights into the pharmacological targeting of FXR for NASH treatment. The authors went from an unbiased transcriptomics profiling to identify a novel enhancer-derived lncRNA FincoR enriched in the liver and showed that the knockdown of FincoR in a murine NASH model attenuated part of the effect of tropifexor, an FXR agonist, namely inflammation and fibrosis, but not steatosis. This study provides a framework how one can investigate the role of noncoding genes in pharmacological intervention targeting a known protein coding genes. Given that many disease-associated genetic variants are located in the non-coding regions, this study, together with others, may provide useful information for improved and individualized treatment for metabolic disorders.

Strengths:

The study leverages both transcriptional profile and epigenetic signatures to identify the top candidate eRNA for further study. The subsequent biochemical characterization of FincoR using FXR-KO mice combined with Gro-seq and Luciferase reporter assays convincingly demonstrates this eRNA as a FXR transcriptional targets sensitive to FXR agonists. The use of in vitro culture cells and the in vivo mouse model of NASH provide multi-level evaluation of the context-dependent importance of the FincoR downstream of FXR in regulation of functions related to liver dysfunction.

Weaknesses:

Future work to dissect the detailed mechanisms by which FincoR facilitates action of FXR and its agonists is warranted. A more direct approach to alter eRNA levels, e.g., overexpression of FincoR in the liver would provide important data to interpret its functional regulation.

https://doi.org/10.7554/eLife.91438.2.sa0

Author Response

The following is the authors' response to the original reviews.

> ***eLife assessment:***
>
> *The authors report a novel hepatic lncRNA FincoR regulating FXR with therapeutic implications in the treatment of MASH. The findings are important and use an appropriate methodology in line with the current state-of-the-art, with convincing support for the claims.*
>
> ***Public Reviews:***
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> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *In the article titled "Hammerhead-type FXR agonists induce an eRNA FincoR that ameliorates nonalcoholic steatohepatitis in mice," the authors explore the role of the Farnesoid X Receptor (FXR) in treating metabolic disorders like NASH. They identify a new liver-specific long non-coding RNA (lncRNA), FincoR, regulated by FXR, notably induced by agonists such as tropifexor. The study shows that FincoR plays a significant role in enhancing the efficacy of tropifexor in mitigating liver fibrosis and inflammation associated with NASH, suggesting its potential as a novel therapeutic target. The study makes a promising contribution to understanding the role of FincoR in alleviating liver fibrosis in NASH, providing initial insights into the mechanisms involved. While it offers a valuable starting point, there is potential for further exploration into the functional roles of FincoR and their specific actions in human NASH cases. Building upon the current findings to elucidate more detailed mechanistic pathways through which FincoR exerts its therapeutic effects in liver disease would elevate the research's significance and potential impact in the field.*
>
> *Strengths:*
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> *This study stands out for its comprehensive and unbiased approach to investigating the role of FincoR, a liver-specific lncRNA, in the treatment of NASH. Key strengths include: 1) The application of advanced sequencing methods like GRO-seq and RNA-seq offered a comprehensive and unbiased view of the transcriptional changes induced by tropifexor, particularly highlighting the role of FincoR. 2) Utilizing a genetic mouse model of FXR KO and a FincoR liver-specific knockdown (FincoR-LKD) mouse model provided a controlled and relevant environment for studying NASH, allowing for precise assessment of tropifexor's therapeutic effects. 3) The inclusion of tropifexor, an FDAapproved FXR agonist, adds significant clinical relevance to the study. It bridges the gap between experimental research and potential therapeutic application, providing a direct pathway for translating these findings into real-world clinical benefits for NASH patients. 4) The study's rigorous experimental design, incorporating both negative and positive controls, ensured that the results were specifically attributable to the action of FincoR and tropifexor.*

Weaknesses:

The study presents several notable weaknesses that could be addressed to strengthen its findings and conclusions: 1) The authors focus on FincoR, but do not extensively test other lncRNAs identified in Figure 1A. A more comprehensive approach, such as rescue experiments with these lncRNAs, would provide a better understanding of whether similar roles are played by other lncRNAs in mitigating NASH. 2) FincoR was chosen for further study primarily because it is the most upregulated lncRNA induced by GW4064. Including another GW4064-induced lncRNA as a control in functional studies would strengthen the argument for FincoR's unique role in NASH. 3) The study does not conclusively demonstrate whether FincoR is specifically expressed in hepatocytes or other liver cell types. Conducting FincoR RNA-FISH with immunofluorescent experiments or RT-PCR, using markers for different liver cell types, would clarify its expression profile. 4) Understanding the absolute copy number of FincoR is crucial. Determining whether there are sufficient copies of FincoR to function as proposed would lend more credibility to its suggested role. 5) The manuscript, although technically proficient, does not thoroughly address the relevance of these findings to human NASH. Questions like the conservation of FincoR in humans and its potential role in human NASH should be discussed.

Reviewer #2 (Public Review):

Summary:

Nonalcoholic fatty liver disease (NASH), recently renamed as metabolic dysfunctionassociated steatohepatitis (MASH) is a leading cause of liver-related death. Farnesoid X receptor (FXR) is a promising drug target for treating NASH and several drugs targeting FXR are under clinical investigation for their efficacy in treating NASH. The authors intended to address whether FXR mediates its hepatic protective effects through the regulation of lncRNAs, which would provide novel insights into the pharmacological targeting of FXR for NASH treatment. The authors went from an unbiased transcriptomics profiling to identify a novel enhancer-derived lncRNA FincoR enriched in the liver and showed that the knockdown of FincoR in a murine NASH model attenuated part of the effect of tropifexor, an FXR agonist, namely inflammation and fibrosis, but not steatosis. This study provides a framework for how one can investigate the role of noncoding genes in pharmacological intervention targeting known protein-coding genes. Given that many disease-associated genetic variants are located in the non-coding regions, this study, together with others, may provide useful information for improved and individualized treatment for metabolic disorders.

Strengths:

The study leverages both transcriptional profile and epigenetic signatures to identify the top candidate eRNA for further study. The subsequent biochemical characterization of FincoR using FXR-KO mice combined with Gro-seq and Luciferase reporter assays convincingly demonstrates this eRNA as a FXR transcriptional target sensitive to FXR agonists. The use of in vitro culture cells and the in vivo mouse model of NASH provide multi-level evaluation of the context-dependent importance of the FincoR downstream of FXR in the regulation of functions related to liver dysfunction.

Weaknesses:

As discussed, future work to dissect the mechanisms by which FincoR facilitates the action of FXR and its agonists is warranted. It would be helpful if the authors could base this on the current understanding of eRNA modes of action and the observed biochemical features of FincoR to speculate potential molecular mechanisms explaining the observed functional phenotype. It is unclear if this eRNA is conserved in humans in

> *any way, which will provide relevance to human disease. Additionally, the eRNA knockdown was achieved by deletion of an upstream region of the eRNA transcription. A more direct approach to alter eRNA levels, e.g., overexpression of FincoR in the liver would provide important data to interpret its functional regulation.*

We thank the Editor and Reviewers for their constructive comments. We believe we have addressed all of the issues (detailed below) and the revisions have greatly strengthened the manuscript.

> ***Reviewer 1:***
>
> *The study presents several notable weaknesses that could be addressed to strengthen its findings and conclusions:*
>
> *(1) The authors focus on FincoR, but do not extensively test other lncRNAs identified in Figure 1A. A more comprehensive approach, such as rescue experiments with these lncRNAs, would provide a better understanding of whether similar roles are played by other lncRNAs in mitigating NASH.*
>
> *(2) FincoR was chosen for further study primarily because it is the most upregulated lncRNA induced by GW4064. Including another GW4064-induced lncRNA as a control in functional studies would strengthen the argument for FincoR's unique role in NASH.*
>
> *(3) The study does not conclusively demonstrate whether FincoR is specifically expressed in hepatocytes or other liver cell types. Conducting FincoR RNA-FISH with immunofluorescent experiments or RT-PCR, using markers for different liver cell types, would clarify its expression profile.*
>
> *(4) Understanding the absolute copy number of FincoR is crucial. Determining whether there are sufficient copies of FincoR to function as proposed would lend more credibility to its suggested role.*

Response to 1 - 4): We thank Reviewer 1 for the positive comments on the strength of our work, including the open-ended approach, the novel eRNA FincoR and its strong relevance to liver disease. We also value the constructive feedback provided by the reviewer and agree that additional studies are important to fully understand the mechanisms of FincoR and the functional significance of other FXR-induced lncRNAs. In this manuscript we report the discovery and initial characterization of FincoR, as well as its potential function in FXR action in response to hammerhead agonists, but a number of interesting questions are raised. Future experiments, as suggested by reviewer, will be needed to examine the role of other FXR-induced lncRNAs, the potential role of FincoR induction by other nuclear receptors with binding sites at FincoR, whether FincoR is expressed in liver cell types in addition to hepatocytes, and the expression abundance of FincoR. These are all excellent suggestions for future experimentation which we feel are beyond the scope of the present report. For example, generating a genetic CRISPR/Cas9 of another lncRNA is not trial as it takes a significant amount of work with murine models. Also, we did not mean to exclude if other lncRNAs induced by FXR also bear functions. Technically, rescue experiment is not possible as FincoR RNA can be potentially very long (~10 kb if estimated by RNA-seq pattern in Fig.1C), and it is not feasible now to properly express it by exogenous vectors to ensure the expression levels are similar to endogenous ones. We therefore consider that these important questions are more suitable for future work to fully address. Our belief is that a comprehensive exploration of FXR-regulated lncRNAs holds the potential to unveil novel insights crucial for the development of therapies targeting NASH and other metabolic diseases. The study of FincoR is the beginning of this area of research.

> *(5) The manuscript, although technically proficient, does not thoroughly address the relevance of these findings to human NASH. Questions like the conservation of FincoR in humans and its potential role in human NASH should be discussed.*

Response: These are important questions. To respond to the reviewer's comment, new experiments are presented in our final revised manuscript in which we utilized mouse models of NAFLD/NASH and cholestatic liver injury to determine FincoR's role in these diseases. Hepatic FincoR levels were significantly increased in mice fed with high fat diet (HFD) for 12 weeks (Supplementary Figure S1A) and in mice fed a HFD with high fructose (HFHF) in drinking water for 12 weeks (Supplementary Figure S1B). Elevated hepatic FincoR levels were also observed in mice treated with α-naphthylisothiocyanate (ANIT), a chemical inducer of liver cholestasis (Supplementary Figure S1C), and in mice with bile duct ligation (BDL), a surgical method to induce cholestatic liver injury (Supplementary Figure S1D).

In terms of the human relevance, we have provided additional information and figures showing that there is sequence similarity between mouse FincoR and a human loci. FincoR sequence is moderately conserved between mice and humans as displayed in the UCSC genome browser (Supplementary Figure S1E). Annotation of these conserved human sequences revealed that they overlap with a functionally uncharacterized human lncRNA XR_007061585.1 (Supplementary Figure S1F). Further, we conducted qRT-PCR experiment from human patient's RNA samples, which demonstrated that hepatic lncRNA XR_007061585.1 levels are elevated in patients with NAFLD and PBC, but not in severe NASH-fibrosis patients (Supplementary Figure S1G, H). These results demonstrate that hepatic levels of a potential human analog of FincoR are elevated in NAFLD and PBC patients, which is consistent with FincoR's upregulation in mouse models of chronic liver disease with hepatic inflammation and liver injury. Whether human lncRNA XR_007061585.1 is entirely analogous to mouse FincoR in terms of functions and mechanisms, and whether the elevation of this human lncRNA has a role in liver disease progression or is an adaptive response to liver injury remains to be determined.

> ***Reviewer #2 (Recommendations For The Authors):***
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> *(1) In the introduction Line 96, "..., while the vast majority are transcribed into ncRNAs" may not be accurate. Please refer to Pointing and Haerty Annu Rev 2022 for a related discussion.*

Response: We would like to thank the reviewer for pointing out this inaccurate information in the introduction. We have changed the content in the text, "While a significant portion of the genome was initially thought to be "junk DNA", it has been established that many non-coding regions give rise to functional non-coding RNAs."

> *(2) Figure 5: the authors should provide a clear illustration demonstrating the sequence targeted by the sgRNA in relation to the transcriptional and epigenetic profile (i.e., RNAseq and H3K27ac ChIP-seq data).*

Response: The illustration (Figure 5-figure supplement 1A, right panel) demonstrating the sequence targeted by the sgRNA has been updated as suggested by the reviewer.

> *In this model, the upstream of FincoR is deleted, leading to the inhibition of FincoR transcription. Does the deleted region include FXR binding sites? If so, would the phenotype be due to the deletion of these binding sequences, rather than the decreased FincoR transcripts? Accordingly, the limitation or alternative interpretation should be discussed.*

Response: The reviewer made a good point. The deleted region includes FXR binding sites so that we cannot rule out decreased binding of FXR or decreased transcription of the region per se, in addition to the decreased levels of FincoR, to bear a role in the phenotypic changes we observed. In the final revision, we have added discussion of this alternative (6th paragraph in the revised discussion section).

> *(3) Figure 6C, the images should be accompanied by quantification. It appears the FincoR-KD shows a visible difference as compared to Tropifexor-treated control mice, which does not match entirely what is written in the results.*

Response: The quantitation of Oil Red O staining has been done as suggested by the reviewer (Figure 6C). The result is consistent with the triglyceride result showing that tropifexor treatment markedly reduced neutral lipids determined by Oil Red O staining of liver sections (Figure 6C) and liver TG levels (Figure 6D) and these beneficial effects on reducing fatty liver were not altered by FincoR.

> *(4) Figure 7, does AST show the same pattern as ALT? As indicated from Line 335, "tropifexor treatment reduced mRNA levels of several genes that promote fibrosis (Col1a1, Col1a2, ...)". Fig. 7D does not seem to match the description of Col1a1. Authors may need to modify the results.*

Response: AST has been measured and has the same pattern as ALT. The new data have been added to Figure 7B. Col1a1 expression has been re-measured and the results have been updated in Figure 7D.

> *(5) Is FincoR level reduced in NASH conditions?*

Response: We thank the Reviewer for this question. We now added new data to examine the levels of FincoR in mouse liver disease models and also examined levels of a potential human analog of FincoR in human liver specimens from PBC, NAFLD, and NASH patients. Please see our new data and description above in the response to comment 5 by Reviewer 1 (most data now included in the new Supplementary Figure S1).

> *(6) Please provide information on the conservation of FincoR (DNA and RNA) in humans. This would be important to provide the human disease relevance.*

Response: As described above in the response to comment 5 of reviewer 1, a human loci shows sequence similarity to mouse FincoR and this conserved region has an annotated uncharacterized human lncRNA. We also examined the levels of this human homolog in human diseased liver samples. Our new results demonstrate that hepatic levels of a potential human analog of FincoR are elevated in NAFLD and PBC patients, which is consistent with FincoR's upregulation in mouse models of chronic liver disease with hepatic inflammation and liver injury. Whether human lncRNA XR_007061585.1 is entirely analogous to mouse FincoR in terms of functions and mechanisms, and whether the elevation of this human lncRNA has a role in liver disease progression or is an adaptive response to liver injury remains to be determined.

> *(7) Several discussion points for the authors' consideration:*
>
> *(7.1) human-mouse conservation as alluded to in #6;*

Response: Potential human-mouse conservation is discussed with new data in the last paragraph of the Results section.

> *(7.2) potential molecular mechanism involved in FincoR-regulated hepatocyte function;*

Response: We thank Reviewer for this comment. We have added more discussion as shown below: “RNA inside the cells usually associates with different RNA-binding proteins (RBPs). To predict those potential binding proteins of FincoR. Additional bioinformatic analysis identified proteins that potentially binding FincoR, including KHDRBS1, RBM38, YBX2 and YBX3 (Supplemental Table S5). These findings and potential functions of the binding proteins are discussed in the 5th paragraph of the discussion section in the final revised manuscript. Whether these predicted RBPs interact with FincoR and the underlying mechanisms will need to be investigated in future experimentation to understand the mechanisms involved in FincoR-regulated hepatocyte function.”

> *(7.3) any disease-associated SNPs in the FincoR locus.*

Response: No SNPs were noted in the annotation of the human loci with sequence similarity to mouse FincoR in the NCBI genome data viewer.

> *(7.4) the in vitro induction of FincoR is transient but in vivo this occurs after 12 days of drug treatment. How do the authors reconcile the differential induction patterns?*

Response: To clarify, the induction of FincoR after a single dose of GW4064 in vivo was transient, peaked within 1 h and then declined gradually (Figure 1-figure Supplement 1C). In the tropifexor treatment protocol (also in vivo), the mice were treated daily with tropifexor for 12 days so that the multiple doses maintained FincoR induction. The beneficial effect of tropifexor by inducing FincoR, therefore, accumulated over the 12 days.

It is worthy to note that we failed to see induction of FincoR in isolated primary mouse hepatocytes treated with GW4064 in vitro. We can only detect FincoR in primary hepatocytes isolated from GW4064-treated mice liver. This may be due to the loss of key factors mediating FincoR induction in the cultured primary hepatocytes.