Supplementary Materials and Methods

Materials

The solution of iron-nitrilotriacetic acid complex was prepared as described previously.[1] MitoSOX was supplied from Molecular Probes (Carlsbad, CA). Anti-phospho-acetyl-CoA carboxylase (ACC), anti-phospho-AMPKα, anti-phospho-LKB1, and anti-LKB1 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Antibodies directed against AMPKα and farnesoid X receptor (FXR) were provided from Santa Cruz Biotechnology (Santa Cruz, CA) and R&D systems (Minneapolis, MN), respectively. Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse immunoglobulin (Ig) Gs were obtained from Zymed Laboratories (San Francisco, CA). Chenodeoxycholic acid (CDCA), GW4064, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), rhodamine123, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), anti-β-actin antibody, and other reagents were provided from Sigma (St. Louis, MO).

Patient Samples

Human liver tissues with fibrosis were obtained from 81 patients who had been diagnosed with liver fibrosis or cirrhosis by histologic examination and ultrasonography in 7 different hospitals in South Korea.[2] Certified pathologists evaluated the severity of fibrosis by Ishak fibrosis scoring system (range, 0–6; 0, no fibrosis; 1, fibrous expansion of some portal areas, with or without short fibrous septa; 2, fibrous expansion of most portal areas, with or without short fibrous septa; 3, fibrous expansion of most portal areas with occasional portal to portal [P-P] bridging; 4, fibrous expansion of portal areas with marked bridging [portal to portal (P-P) as well as portal to central (P-C)]; 5, marked bridging [P-P and/or P-C] with occasional nodules [incomplete cirrhosis]; 6, cirrhosis, probable or definite) in a blinded fashion.[3] Among the samples, those of mild (score of 3 or less, n = 10) and severe fibrosis (score of 5 or 6, n = 22) were used for analyses. This human investigation was performed after approval by the Institutional Review Board.

Immunohistochemistry

The paraffin-embedded tissue sections were deparaffinized with xylene and rehydrates with alcohols series. Antigen retrieval was performed in an incubator (37°C) for 10 minutes by proteinase K and autoclave (121°C) the sections for 10 minutes in citric acid buffer (10 mmol/L, pH 6.0). After cooling at room temperature for 20 minutes, the sections were washed in phosphate-buffered saline (PBS; 0.01 mol/L, pH 7.4). Endogenous peroxidase activity was quenched by immersion in 3% H_2O_2 for 15 minutes. After washing, the sections were pretreated with 10% normal donkey serum for 40 minutes to block nonspecific antibody binding and were incubated with anti-FXR (mouse, 1:50; Perseus Proteomics, Tokyo, Japan) or anti-LKB1 antibody (rabbit, 1:50; Thermo, Chicago, IL) overnight at 4°C. The sections were then treated with 2% normal donkey serum for 15 minutes and incubated with biotin-SP-conjugated affinity pure donkey anti-mouse IgG or anti-rabbit IgG (1:200; Jackson Immunolabs, West Grove, PA) for 2 hours. The labeling was done by using 3,3′-diaminobenzidine in PBS. The sections were mounted with Permount solution. The sections were examined using light microscope (DMRE, Leica Microsystems, Wetzlar, Germany), and images were acquired with Fluoview-II (Soft Imaging System GmbH, Muenster, Germany) attached on the microscope.[4]

For the double immunofluorescence for FXR and LKB1, the paraffin sections were deparaffinized, treated for antigen retrieval as mentioned above, and then incubated with 10% normal donkey serum for 40 minutes. Sections were incubated with a mixture of primary antibodies, mouse anti-FXR (1:50; Perseus Proteomisc) and rabbit anti-LKB1 antibody (1:50; Thermo), diluted in PBS overnight at 4°C. After several washings, they were incubated with Alexa488-conjugated donkey anti-mouse (1:200; Invitrogen, Carlsbad, CA) and Cy3-conjugated donkey anti-rabbit antibody (1:200; Jackson Immunolab) for 2 hours, washed with PBS, and cover-slipped using Vectashield containing DAPI (Vector labs, Burlingame, CA).[4] Multiple-labeled images were acquired using confocal microscope (Leica SP-5; Leica Microsystem).

RNA Preparation From Formalin-Fixed, Paraffin Embedded Samples

Total RNA was extracted from the macro-dissected formalin-fixed, paraffin embedded (FFPE) samples with the RNeasy FFPE kit (Qiagen, Tokyo, Japan) following the manufacturer's instructions. Briefly, the sample sections were deparaffinized with xylene, washed with ethanol, and dried. Lysis buffer and proteinase K were added to the dried sections. Binding buffer was added to the lysate and transferred to a gDNA Eliminator spin column (Qiagen) to remove genomic DNA. After eliminating DNA, 100% ethanol was added to the flow-through. The samples were transferred to an RNeasy MinElute column (Qiagen) that binds total RNA. The purified RNA was eluted with 50 μL of RNase-free water.

RNA Isolation From Liver Tissues and Cells

Total RNA was isolated from cells using Trizol reagent (Invitrogen, Carlsbad, CA). The RNA (2 μg each) was reverse-transcribed using oligo-d$(T)_{16}$ primers to obtain complementary DNA.[4]

Real-time Polymerase Chain Reaction Assays

Real-time polymerase chain reaction (PCR) was carried out according to the manufacturer's instructions by using a Light CyclerDNA master SYBR green-I kit (Light-Cycler 2.0; Roche, Mannheim, Germany).[4] The relative levels of human FXR (sense: 5′-GGAACCAT-

ACTCGCAATACA-3′, antisense: 5′-TCGCATGTACATATCCATCA-3′); human FXRα1 (sense: 5′-AGACCACCATAAAGAAAGTG-3′, antisense: 5′-CAGTTAACAAGCCTGTATAC-3′); human FXRα2 (sense: 5′-AGACCACCATAAAGAAAGTG-3′, antisense: 5′-ACAAGCATTCAGCCAACATT-3′); human FXRα3 (sense: 5′-TCAAATTAGTCCTCACTGCA-3′, antisense: 5′-CAGTTAACAAGCCTGTATAC-3′); human FXRα4 (sense: 5′-TCAAATTAGTCCTCACTGCA-3′, antisense: 5′-ACAAGCATTCAGCCAACATT-3′); and human LKB1 (sense: 5′-GTCCTTGGTGTCTGG-3′, antisense: 5′-ACATCTGGTCGGCTG-3′) were normalized based on the level of glyceraldehyde-3-phosphate dehydrogenase using Lightcycler software 4.0 (Roche). After PCR amplification, a melting curve of each amplicon was determined to verify its accuracy.

The relative levels of human pri-miR-199a (sense: 5′-ATCGTCTCGGGAAGAGTGGT-3′, antisense: 5′-AAAATGACACTCACCTGCCG-3′); pre-miR-199a (sense: 5′- GCCAACCCAGTGTTCAGACT-3′, antisense: 5′-GCCTAACCAATGTGCAGACTACT-3′), miR-199a-3p (5′-ACAGTAGTCTGCATTGGTTA-3′); miR-34a (5′-TGGCAGTGTCTTAGCTGGTTGT-3′); miR-451 (5′-AAACCGTTACCATTACTGAGTT-3′); miR-802 (5′-CAGTAACAAAGATTCATCCTTGT-3′); miR-765 (5′-TGGAGGAGAAGGAAGGTGAT-3′); miR-29b (5′-TAGCACCATTTGAAATCAGTGTT-3′), and miR-132 (5′-TAACAGTCTACAGCCATGGTCG-3′) were normalized based on the level of RNU6B using Lightcycler software 4.0 (Roche). Mature miRNAs were amplified using each specific miR primer and miScript universal primer (Qiagen). After PCR amplifications, a melting curve of each amplicon was determined to verify its accuracy.

Extraction of HBV DNA in the Liver

The FFPE samples were sliced with disposable sterile blades in each paraffin block and deparaffinized with xylene, and 100% ethanol DNA was extracted using QIAamp FFPE DNA Mini Kit (Qiagen) according to the manufacturer's protocol. HBV DNA in the liver was amplified using the method described elsewhere with minor modification. Briefly, a reaction solution (25 μL) contained plasmid-safe ATP-dependent DNase-treated DNA template, 0.3 mmol/L dNTP, 3.5 μmol/L $MgCl_2$, 11.3 μL water, and 0.2 μmol/L selective primers. The amounts of HBV DNA were quantified using real-time PCR assays: the primers of HBV DNA were 5′-GTGTCTGCGGCGTTTTATCA-3′ (sense) and 5′-GACAAACGGGCAACATACCTT-3′ (antisense).

Cell Culture

HepG2 cells, a human hepatocyte-derived cell line, were supplied from American Type Culture Collection (Manassas, VA). Primary hepatocytes were isolated from rat liver according to the previously published method.[1] The cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37°C in humidified atmosphere with 5% CO_2. For all experiments, cells (1×10^6) were plated in a 6-well plastic dish for 2 to 3 days (ie, 80% confluence) and serum starved for 24 hours.

Transient Transfection

The constructs encoding for FXR and a dominant negative mutant of AMPKα (DN-AMPK) were kindly provided by Drs Bart Staels (Institut Pasteur de Lille, Lille, France)[5] and J. Ha (Kyunghee University, Seoul, Korea),[1] respectively. Cells were transfected with the plasmid using FuGENE HD (Roche, Indianapolis, IN). pCDNA3.1 was used for mock transfection.

LKB1 3′ Untranslated Region Luciferase Assay

The plasmid containing Luc-LKB1-3′ untranslated region (UTR) (Product ID: HmiT017794-MT01) was specifically synthesized (GeneCopoeia) and was used in luciferase reporter assay. The plasmid contains firefly luciferase that is fused to the 3′UTR of human LKB1 and Renilla luciferase that functions as a tracking gene. Luciferase activity assays were performed following manufacturer's protocols. Briefly, HepG2 cells were seeded in 6-well plates, cotransfected with pre-miR-199a-3p and LKB1-3′UTR. Following transfection, media were exchanged after 12 hours, and the cells were harvested after 36 hours. Firefly and Renilla luciferase activities were measured sequentially using the dual luciferase assay kit (GeneCopoeia) using POLAR Star Omega plate reader (BMG LABTECH, Ortenberg, Germany). The activities were normalized with those of Renilla luciferase and expressed as relative luciferase activity units. Data represent 3 independent experiments, each performed in triplicate.

Immunoblot Analysis

Cell lysates were prepared according to previously published methods.[4] Protein bands of interest were developed using the ECL chemiluminescence system (Amersham, Buckinghamshire, UK). Equal loading of protein was verified by immunoblotting for actin. Relative protein levels were determined by scanning densitometry. At least 3 replicates were used for each experiment.[4]

Animal Treatment

Animal experiments were conducted under the guidelines of the Institutional Animal Use and Care Committee at Seoul National University. Male ICR mice (6 weeks old) and C57BL/6 mice (8 weeks old) were purchased from Samtako Company (Osan, Korea) and housed at 20°C ± 2°C with 12-hour light/dark cycles and a relative humidity of 50% ± 5% (Tecniplast, Varese, Italy) under filtered, pathogen-free air, with food (Purina, Seongnam, Korea) and water available ad libitum. The ICR mice were treated with either CDCA or GW4064

(intraperitoneally, 30 mg/kg, 6 hours), and the livers were excised for assays. In another set of experiment, C57BL/6 mice were given an intraperitoneal dose of CCl_4 in corn oil (0.6 mL/kg) twice a week for 5 weeks. Another group of mice received the same doses of CCl_4 in combination with GW4064 (30 mg/kg in corn oil, intraperitoneally twice a week for 5 weeks). Some mice were injected only with 30 mg/kg GW4064 as control, whereas others received the respective amount of corn oil (vehicle) as vehicle control. Livers were excised 24 hours after last administration of CCl_4; partial livers were fixed, embedded in paraffin, and processed for histology. Serial liver sections were stained with H&E.

LKB1 Immunohistochemistry in FXR Knockout Mice

Immunohistochemistry was performed with the paraffin-embedded liver samples that had been obtained from wild-type and FXR knockout mice injected with a single dose of CCl_4 or vehicle.[6] The same treatment samples that had been used for a study previously published were provided by Drs Zhipeng Meng and Wendong Huang.[6]

MTT Assay

HepG2 cells were plated at a density of 1×10^5 cells per well in a 48-well plate to measure the degree of cell survival.[4] After treatment, viable cells were stained with MTT (0.25 μg/mL, 2 hours). The media were then removed, and formazan crystals produced in the wells were dissolved by the addition of 300 μL dimethylsulfoxide. Absorbance was measured at 540 nm using an ELISA microplate reader (Tecan, Research Triangle Park, NC). Cell viability was defined relative to untreated control [ie, viability (% control) = 100 $\times$ (absorbance of treated sample)/(absorbance of control)].[1] The cells were treated with 10 μmol/L arachidonic acid (AA) for 12 hours, washed with minimum essential medium, and then incubated with 5 μmol/L iron for indicated time period. The cells were treated with indicated concentrations of CDCA or GW4064 for 1 hour and were continuously incubated with AA + iron.

Terminal Transferase-Mediated dUTP Nick-End Labeling Assay

Terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed using the DeadEnd Colorimetric TUNEL System, according to the manufacturer's instruction.[1] HepG2 cells were fixed with 10% buffered formalin in PBS at room temperature for 30 minutes and permeabilized with 0.2% Triton X-100 for 5 minutes. After washing, each sample was incubated with biotinylated nucleotide and terminal deoxynucleotidyltransferase in equilibration buffer at 37°C for 1 hour. The reaction was stopped by immersing the samples in 2X saline sodium citrate buffer for 15 minutes. Endogenous peroxidases were blocked by immersing the samples in 0.3% H_2O_2 for 5 minutes. The samples were treated with horseradish peroxidase-labeled streptavidin solution (1:500) and incubated for 30 minutes. Finally, the samples were developed using the chromogen, H_2O_2, and diaminobenzidine for 10 minutes. The samples were washed and examined under light microscope ($\times$200). The counting of TUNEL-positive cells was repeated 3 times, and the percentage from each counting was calculated.

Measurement of Hydrogen Peroxide Production

The fluorescence intensity in the cells was measured using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).[1] HepG2 cells were stained with 10 μmol/L DCFH-DA for 30 minutes at 37°C. Hydrogen peroxide generation was assessed by measuring the intensity of dichlorofluorescein fluorescence. In each analysis, 10,000 events were recorded.[1]

Determination of Reduced Glutathione Content

Reduced glutathione (GSH) content in the cell was quantified using a commercially available GSH determination kit (Oxis International, Portland, OR).[1]

Flow Cytometric Analysis of Mitochondrial Membrane Potential

Mitochondrial membrane potential (MMP) was measured with rhodamine 123, a membrane-permeable cationic fluorescent dye. The fraction with low rhodamine 123 fluorescence intensity (M1 fraction) represents mitochondrial damage and dysfunction. Cells were treated as specified, stained with 0.05 μg/mL rhodamine 123 for 1 hour, and harvested by trypsinization. After washing with PBS containing 1% fetal bovine serum, the change in MMP was monitored using a BD FACSCalibur flow cytometer (BD Biosciences). In each analysis, 10,000 events were recorded.[1]

Measurement of Mitochondrial Superoxide Production

MitoSOX is a live-cell-permeable and mitochondrial-localizing superoxide indicator. After treatment with AA + iron, HepG2 cells were stained with 5-μmol/L MitoSOX for 10 minutes at 37°C. The fluorescence intensity in the cells was measured using a BD FACSCalibur flow cytometer. In each analysis, 10,000 events were recorded.[1]

Statistical Analysis

One-way analysis of variance tests were used to assess the significance of differences among treatment groups. For each statistically significant effect of treatment, the Newman–Keuls test was used for comparisons between multiple group means. The data were expressed

as means ± standard error of mean. The criterion for statistical significance was set at $P < .05$ or $P < .01$.

References

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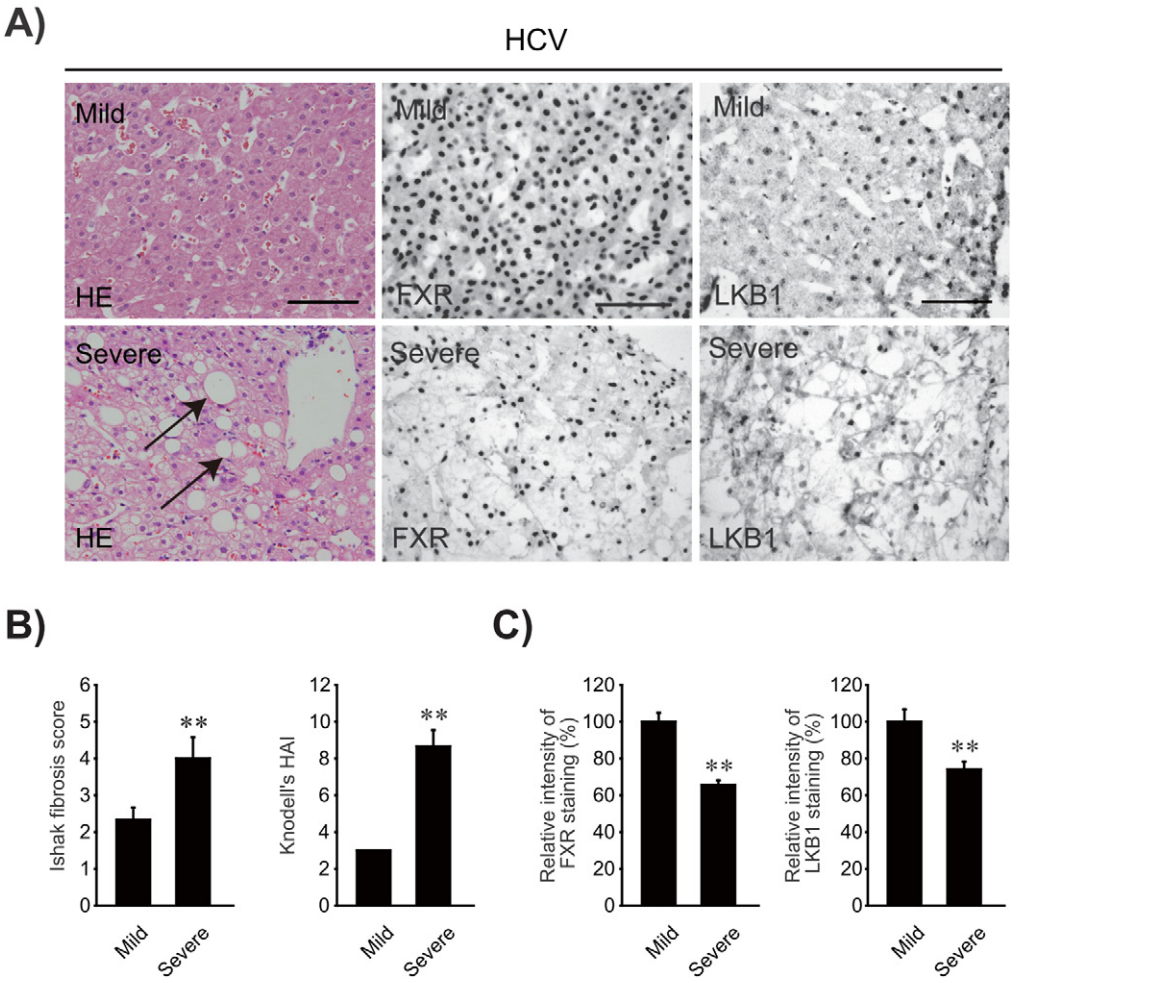
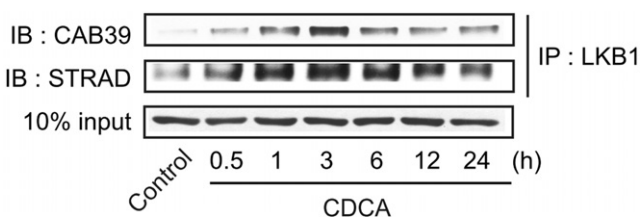


Supplementary Figure 1. FXR and LKB1 expression in the liver of HCV patients with mild or severe fibrosis. (*A*) H&E and immunohistochemical stainings for FXR and LKB1. *Arrows* indicate eosinophilic necrosis of hepatocytes (*scale bar*, 100 μm). (*B*) The degrees of hepatocyte death, inflammation, and fibrosis in the human liver samples. Knodell histologic activity index and Ishak fibrosis scores were measured in the patient samples (n = 3). (*C*) The staining intensities of FXR and LKB1 were quantified in the liver sections. For *panels B* and *C*, values represent the mean ± standard error of mean (significantly different as compared with mild fibrosis, $^{**}P < .01$).

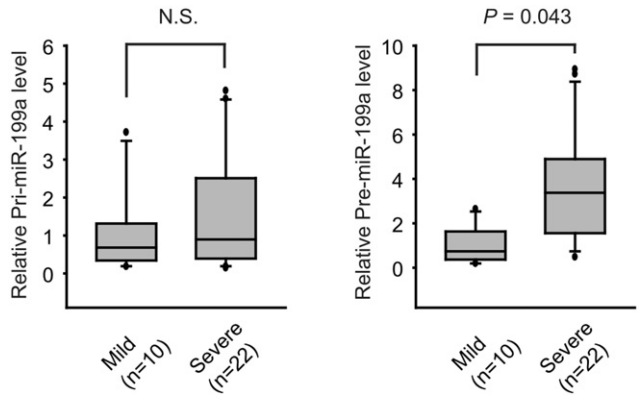
Supplementary Table 1. The Candidate Target Genes of miR-199a-3p That Are Associated With Cell Survival

Target	Molecular properties	Functions	References
STK11[a]	Protein kinase	Essential role in response to metabolic stress. Plays a role in the protection of hepatocytes from free radical-induced injury by activating AMPK, an intracellular sensor of energy status.	1, 7
FIGF	Growth factor	Growth factor active in angiogenesis, lymphangiogenesis, and endothelial cell growth, stimulating their proliferation and migration.	8
PAWR[a]	Transcriptional repressor	Proapoptopic protein capable of apoptosis in various cell types. In cancer, sensitizing the cells to diverse apoptotic stimuli and causing regression of tumors in animal models.	9
PRDX1	Enzyme	Involved in redox regulation of the cell. Reduces peroxides with reducing equivalents provided through the thioredoxin system.	10
MAP3K4[a]	Protein kinase	Component of MAPK cascades; central regulator of cell fate.	11
MAP3K5[a]	Protein kinase	Component of MAPK cascades; central regulator of cell fate.	11
CYB5R4	Oxidoreductase	NADH-cytochrome b5 reductase involved in endolasmic reticulum stress response pathway. Plays a critical role in protecting cells against oxidant stress, possibly by protecting the cell from excess buildup of ROS.	12
PAK4	Protein kinase	Known target of miR-199a-3p. Activates the MAPK pathway.	13
mTOR	Protein kinase	Known target of miR-199a-3p. Regulator of AKT1 activation for cell survival.	14

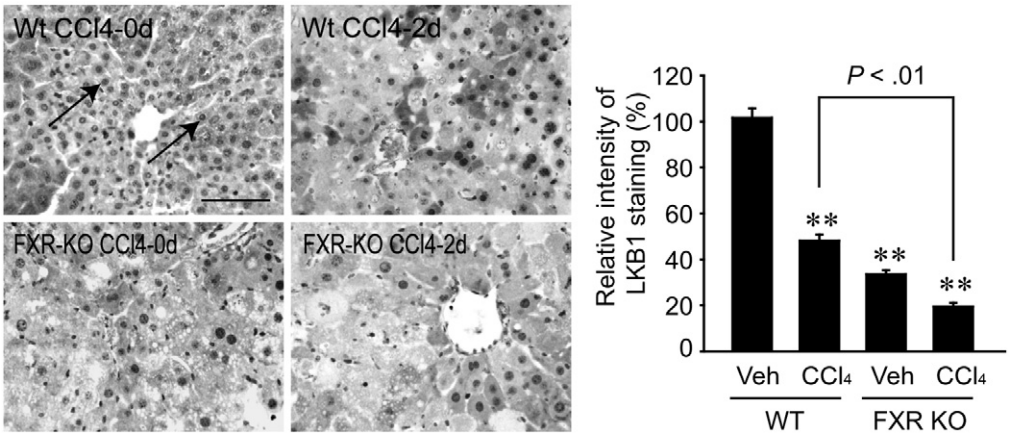
NOTE. Seven candidate targets were selected from the Microcosm or Targetscan 5.1 prediction results based on both their potential functions and the presence of conserved miRNA binding sites. PAK4 and mTOR are known targets of miR-199a-3p.
CYB5R4, cytochrome b5 reductase 4; FIGF, c-fos induced growth factor; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; PAK4, p21 protein (Cdc42/Rac)-activated kinase 4; PAWR, PRKC, apoptosis, WT1, regulator; PRDX1, peroxiredoxin1; STK11, serine/threonine kinase 11.
[a]Four of the targets present in both the Microcosm and Targetscan results.



Supplementary Figure 2. LKB1 binding with CAB39 and STRAD by FXR ligand treatment. Either CAB39 or STRAD was immunoblotted on the LKB1 immunoprecipitates from HepG2 cells treated with 100 μmol/L CDCA for the indicated times.



Supplementary Figure 3. The levels of pri-miR-199a or pre-miR-199a in HBV patients with fibrosis. The levels of pri-miR-199a or pre-miR-199a were measured in the livers of HBV patients with mild or severe fibrosis. Values represent the mean ± standard error of mean. NS, not significant.



Supplementary Figure 4. LKB1 expression in the liver of wild-type or FXR knockout (KO) mice treated with CCl_4. Immunohistochemistry for LKB1 was done on the liver sections of wild-type or FXR KO mice treated with a single dose of CCl_4 (0.75 mL/kg, intraperitoneally, for 48 hours). The same paraffin blocks had been used for a different study.[6] The relative LKB1 intensities were quantified (n = 3 animals/each group). Data represent the mean ± standard error of mean of 3 replicates (treatment mean significantly different from vehicle-treated control, $^{**}P < .01$).