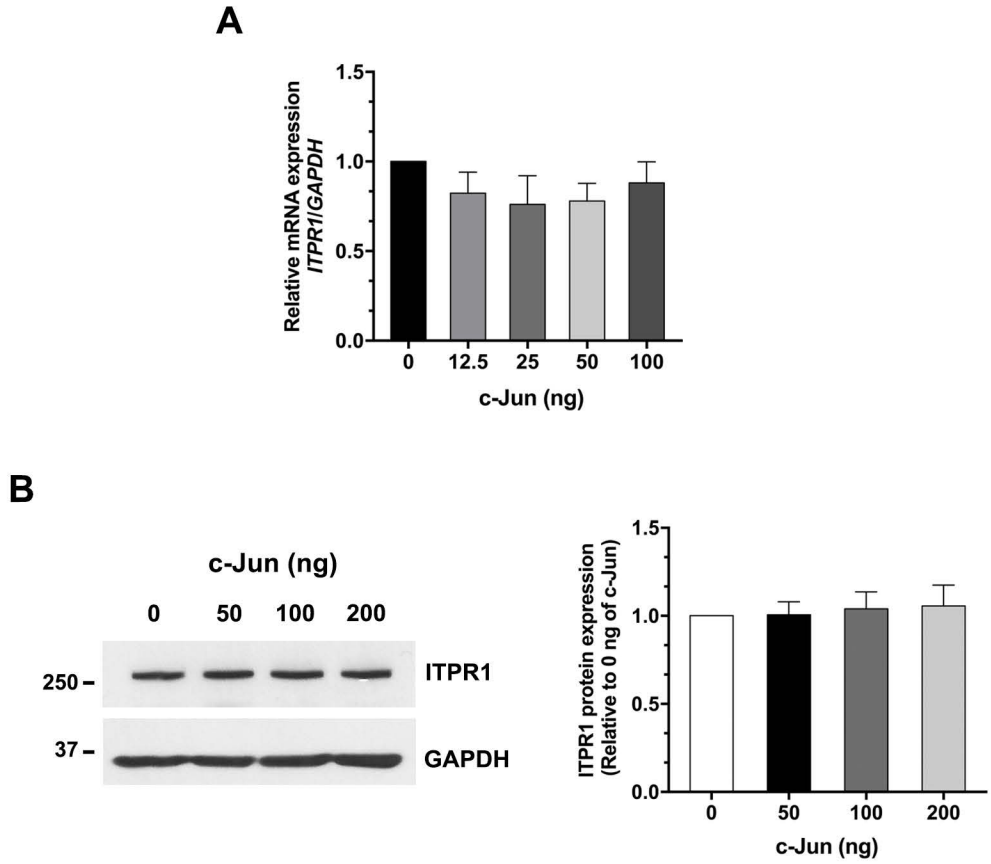


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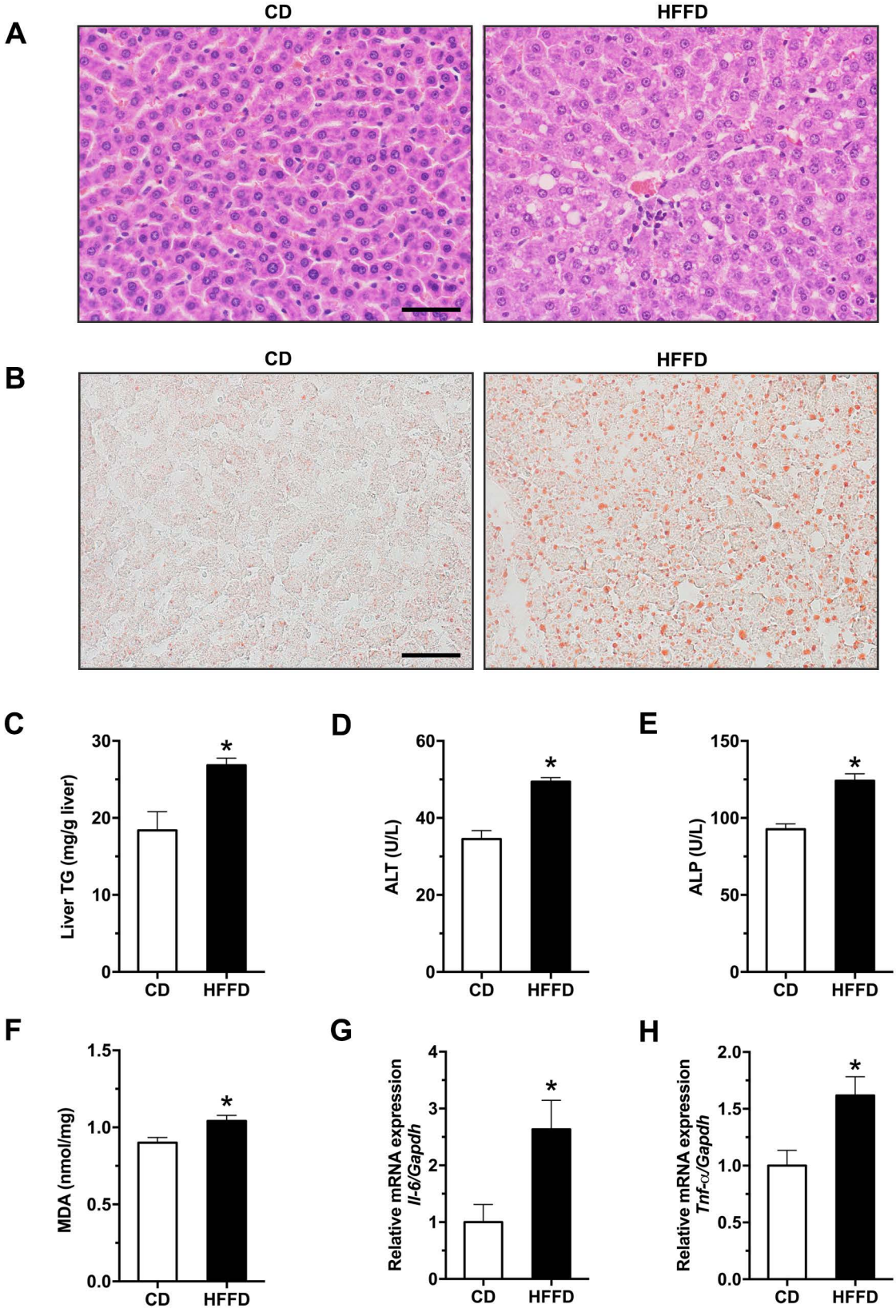


Khamphaya et al., Supporting. Fig. S2

**HEP-17-0946-R1 Supporting Information**

**Supporting Figure Legends**

**Supporting Figure S1. *ITPR*1 mRNA expression in Huh7 cells transfected with c-Jun expression plasmids. (A)** There is no change in *ITPR1* mRNA expression in Huh7 cells transfected with c-Jun expression plasmid for 24 hours (n = 4). **(B)** Representative immunoblot *(left panel)* and relative protein expression of ITPR1*(right panel)* in Huh7 cells transfected with c-Jun expression plasmids, and measured after 48 hours (n = 4 experiments).

**Supporting Figure S2. Male Sprague-Dawley rats fed a high-fat high-fructose diet for 12 weeks develop hepatic steatosis and liver damage. (A)** Representative photomicrographs of livers stained with H&E from rats fed a control diet (CD) or high-fat high-fructose diet (HFFD). **(B)** Oil red O staining shows lipid accumulation in livers of rats fed a HFFD (n = 6 animals). **(C)** Liver triglyceride (TG), **(D)** serum alanine aminotransferase (ALT), **(E)** serum alkaline phosphatase (ALP), and **(F)** malondialdehyde (MDA) as a lipid peroxidation marker are significantly higher in HFFD-fed rats compared with control group. *$p < 0.05$ (n = 5 animals). Relative mRNA expressions of **(G)** *Il-6* and **(H)** *Tnf-*$\alpha$ are significantly higher in livers of rats fed a HFFD. *$p < 0.05$ compared with CD (n = 4 animals).

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**EXPERIMENTAL PROCEDURES**

## Animal models

Eight-week-old male Sprague-Dawley rats weighting between 180-200 grams were obtained from the National Laboratory Animal Center, Mahidol University (Salaya, Thailand). After acclimatization period, rats were randomly assigned to receive either control diet (10% kcal as fat, Envigo, Cambridgeshire, UK) with reverse osmosis water or high-fat diet (60% kcal as fat, Envigo, Cambridgeshire, UK) with 30% w/v fructose in water for 12 weeks to develop NAFLD model.

The whole-body homozygous knockout *Itpr2*$^{-/-}$ mice in a C57BL/6 genetic background were used in this study. Nine- to ten-week-old male *Itpr2*$^{-/-}$ and their wild-type (WT) littermates underwent sham laparotomy or two-thirds hepatectomy (PH) as described previously (1).

All animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and all study procedures were approved by the Institutional Animal Care and Use Committee. The study protocol for rats challenged with high-fat, high-fructose diet was approved by the Institutional Animal Care and Use Committee (IACUC) of Faculty of Science, Mahidol University. The study protocol for *Itpr2*$^{-/-}$ mice was approved by Yale's IACUC.

**Biochemical analysis**

Blood was drawn from the abdominal artery, allowed to clot and centrifuged at 3,000 rpm at 4°C for 10 minutes to obtain serum samples. Serum alanine aminotransferase and alkaline phosphatase were measured using the commercial kit, Human Gesellschaft für Biochemica und Diagnostica mbH (Wiesbaden, Germany).

**Oil Red O staining**

For determination of hepatic lipid accumulation, frozen liver sections were cut at 10-μm thickness, fixed in 10% neutral buffered formalin (Sigma-Aldrich, St Louis, MO, USA) at room temperature for 5 minutes and stained with Oil Red O for 10 minutes. The tissue sections were then rinsed with 60% isopropanol and mounted with 85% glycerol. For determination of lipid accumulation in Huh7 cells, cells plated on the coverslips were fixed with 10% neutral buffered formalin for 30 minutes at room temperature and stained with Oil Red O for 60 minutes. The coverslips were then rinsed with 60% isopropanol, washed with PBS for 3 times, and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). All images were captured using an Olympus BX53 digital microscope with the CellSens digital imaging software (Olympus, Tokyo, Japan) with the same settings.

**Liver triglyceride**

Frozen liver tissue was assayed for triglycerides according to the manufacturer's protocol using the triglyceride quantification kit (Abcam, Cambridge, MA, USA). Briefly, liver triglyceride was extracted from rat liver tissues (100 mg) by homogenizing. Samples were then heated and centrifuged at 12,000 rpm to remove any insoluble material. The supernatant was diluted 10-fold with sterile ultra-pure water, and mixed with triglyceride reaction mixture. The absorbance of samples was

measured using a Multiskan GO microplate spectrophotometer (Thermo Scientific, Rockford, IL, USA).

**Malondialdehyde assay**

Lipid peroxidation was determined from liver tissue homogenate according to the manufacturer's protocol using lipid peroxidation (MDA) assay kit (Abcam, Cambridge, MA, USA). Briefly, 10 mg liver tissue or Huh7 cells ($1x10^6$ cells) were homogenized in MDA lysis buffer, and centrifuged at 12,000 rpm. Thiobarbituric acid solution was then added into each tube containing the standard malondialdehyde bis-dimethyl acetal solution and the sample. After incubation at 95°C for 60 minutes, the absorbance of standards and samples was measured at 532 nm.

**Cell culture**

The human Huh7 cell line was originally acquired from Japanese Collection of Research Bioresources (Tokyo, Japan) (2). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin G, and 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA). The human HepG2 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HepG2 cells were cultured in Eagle's Minimum Essential Medium (ATCC) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 100 U/ml penicillin G, and 100 μg/ml streptomycin (Gibco), at 37°C, 5% $CO_2$.

**Generation of CRISPR/Cas9-mediated ITPR2-knockout HepG2 cell line.**

The ITPR2 knockout HepG2 cell line was established by using the CRISPR/Cas9 system. Control CRISPR/Cas9 plasmid, a non-targeting 20 nt scramble guide RNA (gRNA) designed as a negative control, IP3R-II CRISPR/Cas9 KO plasmid and IP3R-II HDR plasmid were obtained from Santa Cruz Technology. Briefly, HepG2 cells were cultured in 6-well dishes to 70-80% confluence. Cells were cotransfected with 1.5 μg of HDR plasmid plus 1.5 μg of Cas-9 and 9 μl of FuGENE HD Transfection Reagent (Promega, Madison, WI, USA) per well. Since IP3R-II HDR plasmid contains an RFP gene, RFP was used as a fluorescent marker to sort transfected cells. Seventy-two hours post-transfection, cells were pelleted in PBS plus 2% FBS and sorted in 6-well plates using fluorescence-activated cell sorting (FACS) with a FACSAria II cell sorter (BD BioSciences, San Jose, CA, USA). Single cells from RFP-expressing cells were expanded to obtain individual clones. Cells were then cultured in the presence of puromycin (1 μg/ml). A Western blot was performed to confirm a bi-allelic knockout of the ITPR2 gene.

**Human *ITPR2* promoter and reporter plasmid constructs**

A 2-kb DNA fragment containing the human *ITPR2* proximal promoter region was amplified by PCR using a BAC Clone (CH230-105F11), accession no. AC024093 (RP11-791I2) obtained from Children's Hospital of Oakland Research Institute (BacPac resources, Oakland, CA, USA) as a template. A promoter construct containing -1579 to +421 fragment (positions relative to transcriptional starting site) from GenBank Accession NM_002223 was achieved with the following primers; forward: 5′-TCCCAGCTTCAAGCAATTCTC-3′; reverse: 5′-GGACTACAGCGGCCA AGAG-3′. PCR was performed using Phusion DNA Polymerase and purified using

QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA). The 2000-bp DNA fragment containing the human *ITPR2* promoter region was digested with KpnI and XhoI and cloned into a pGL4.17 luciferase vector (Promega). The human *ITPR2*/Luc reporter was verified by DNA sequencing at the W. M. Keck Biotechnology Resource Laboratory at Yale University. MatInspector program (www.genomatix.de) was used to analyze the human *ITPR2* promoter sequence, and a putative AP1 was identified.

**Transient transfection and luciferase reporter gene assay**

HepG2 cells ($2x10^5$ cells/well) or Huh7 cells ($1x10^5$ cells/well) were plated onto 24-well plates 24 hours prior to transfection. Luciferase reporters for the human ITPR2 and the expression plasmid for c-Jun were transiently transfected into HepG2 cells or Huh7 cells, along with a renilla luciferase (Promega) as a control for transfection efficiency using FuGENE HD Transfection Reagent (Promega). After transfection for 48 hours, cells were lysed in passive lysis buffer (Promega). Cell lysates were harvested to measure firefly and renilla luciferase activity using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Luciferase activity was measured using the Synergy 2 Multi-Mode Microplate Reader with injectors (BioTek, Winooski, VT, USA). The reporter gene assay was carried out in triplicate. Results are presented as firefly luciferase activity normalized to renilla luciferase activity.

**c-Jun overexpression**

Huh7 cells ($4x10^5$ cells/well) were plated onto 6-well plates 24 hours prior to transfection. Cells were transfected with the expression plasmid for c-Jun (a gift from Dr. Meenakshisundaram Ananthanarayanan, Yale University) using FuGENE HD Transfection Reagent (Promega). At 24 and 48 hours after transfection, cells were

harvested for RNA and protein extraction, respectively.

**c-Jun silencing**

c-Jun was suppressed by the human c-Jun-specific siRNA (Ref. # SO-2600663G, Dharmacon, Lafayette, CO, USA). The c-Jun target sequences are *i)* 5′-GAGCGGACCUUAUGGCUAC-3′, *ii)* 5′-GAACAGGUGGCACAGCUUA-3′, *iii)* 5′-GAAACGACCUUCUAUGACG-3′, *iv)* 5′-UGAAAGCUCAGAACUCGGA-3′. A non-targeting siRNA (scrambled-siRNA, Ref. #4390846) was purchased from Ambion (Waltham, MA, USA) and used as a negative control. Huh7 cells ($4x10^5$ cells/well) were plated onto 6-well plates for 24 hours and transfected with 30 pmol of c-Jun-siRNA or scrambled-siRNA with Lipofectamine RNAiMax Transfection Reagent (Invitrogen). Knockdown efficiency of c-Jun was confirmed in total cell lysates by Western blotting.

**Palmitic acid treatment**

Huh7 cells ($4x10^5$ cells/well) were plated onto 6-well plates and cultured for 24 hours. Cells were starved for 24 hours before treatment with bovine serum albumin (BSA) alone or with 500 μM of palmitic acid (XF Palmitate-BSA FAO substrate, Agilent Seahorse XF Technologies, Cedar Creek, TX, USA) for another 24 hours. For the c-Jun knockdown experiment with palmitic acid treatment, after Huh7 cells were transfected with scrambled-siRNA or c-Jun-siRNA for 24 hours, cells were starved for 2 hours and then treated with 500 μM of palmitic acid for another 24 hours. Palmitic acid-induced lipid accumulation was subsequently verified by Oil Red O staining as described above.

**RNA isolation and TaqMan real-time PCR**

Total RNA was extracted by TRIzol reagent (Invitrogen) and was purified using RNeasy MinElute Cleanup kit (QIAGEN) according to the manufacturer's protocol. One microgram of total RNA was reverse-transcribed into cDNA using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). All primers and probes were TaqMan Gene Expression Assays purchased from Applied Biosystems (Applied Biosystems, Foster city, CA, USA): *ITPR2* (Hs00181916_m1), *c-JUN* (Hs01103582_s1), *GAPDH* (Hs02758991_g1), *Il-6* (Rn01410330_m1), *Tnf-*$\alpha$ (Rn01525859_g1), *Gapdh* (Rn01775763_g1), *Gapdh* (Mm99999915_g1), *Itpr2* (Mm00444937_m1), *c-Jun* (Mm00495062_s1), and *c-Fos* (Mm00487425_m1). Real-time PCR reactions were performed in triplicate in an ABI 7500 Sequence Detection System (Applied Biosystems). The expression of target genes was normalized to *GAPDH/Gapdh* and quantification of relative expression was determined by the Pfaffl's method (3).

**Immunoblotting**

Liver tissue and cells were lysed with RIPA buffer (Thermo Scientific) containing protease and phosphatase inhibitor cocktail (Thermo Scientific). The protein concentration was determined using Pierce BCA protein assay kit (Thermo Scientific). In brief, protein samples were loaded onto NUPAGE Novex 4% - 12% Bis-Tris gradient gel and were electrophoresed with MOPS running buffer (Invitrogen). Proteins were transferred onto nitrocellulose membrane (Bio-Rad) and incubated with primary antibodies against ITPR2 (a gift from Dr. Richard Wojcikiewicz, SUNY, Syracuse, NY, USA), c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA, USA), JNK and p-JNK (Thr183) (Cell Signaling Technology, Danvers, MA, USA), GAPDH (Ambion), and $\beta$-actin (Sigma). Band intensities were quantified and

analyzed using ImageJ analysis software developed at National Institutes of Health (NIH, Bethesda, MD, USA). The amount of GAPDH or β-actin was used as the internal control to normalize for quantitative protein expression.

**Cell proliferation assay**

For image-based detection of proliferating cells, ITPR2 CRISPR-knockout or CRISPR control HepG2 cells (1.7 x $10^5$ cells/well) were plated onto coverslip in 6-well plates. The detection of fluorescent labeling of proliferating cells was carried out using the Click-iT EdU Alexa Fluor 488 Imaging kit (Invitrogen) according to the manufacturer's instructions. For colorimetric immunoassay, the quantification of cell proliferation was measured by BrdU incorporation using the BrdU Cell Proliferation ELISA kit (Roche) according to the manufacturer's instructions.

**Nuclear calcium imaging**

ITPR2 CRISPR-knockout or CRISPR control HepG2 cells (1.7 x $10^5$ cells/well) were plated onto coverslip in 6-well plates and transfected with a nuclear targeted calcium-sensing protein, GCaMP3 plasmid (a gift from Dr. Anant Parekh, University of Oxford, England) using FuGENE HD Transfection Reagent (Promega). Transfected nuclear-GCaMP3. Coverslip with transfected GCaMP3 were transferred to a custom-built perfusion chamber on the stage of an LSM710 confocal microscope (Carl Zeiss, Inc., Thornwood, NY, USA), and cells were then perfused with a HEPES buffer while stimulated with 20 μm adenosine triphosphate (ATP). This ATP concentration was used because nuclear $Ca^{2+}$ signals were elicited in this cell type (4). Nuclear $Ca^{2+}$ signaling was monitored with a Zeiss 710 confocal microscope.

**Liver histology and immunohistochemistry**

The liver specimens fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin (H&E). Immunohistochemical analyses of c-Jun and ITPR2 expression were performed in liver biopsies from histologically normal livers and from patients with steatosis and NASH. Liver biopsies from 3-6 patients under each condition were evaluated. Sodium citrate buffer (pH 6.0) (Sigma) was used as an antigen retrieval solution. Tissue sections were treated with peroxidase block (Dako, Agilent Technologies, Santa Clara, CA, USA), blocked with Protein Block (Dako) for 5 minutes at room temperature followed by the incubation with ITPR2 (1:25) and c-Jun (1:200) overnight at 4°C. After incubation with primary antibodies, the signal was developed using CSAII, Biotin-Free Catalyzed Amplification System (Dako). Liver sections were mounted, and images were captured using a digitalized Olympus BX51 microscope with the same settings. Quantitative analysis for c-Jun and ITPR2 was quantitated in each individual human liver biopsy using ImageJ analysis software (NIH).

**Human ethics statement**

Human liver tissue biopsies were obtained under the auspices of protocols approved by the Institutional Review Board on the Protection of the Rights of Human Subjects (Yale University), which was conducted in accordance with the Declaration of Helsinki. The Human Investigation Committee protocol number is HIC-1304011763.

**Statistical analysis**

All data are expressed as means ± standard error of mean (S.E.M). GraphPad Prism 7 Software (GraphPad, La Jolla, CA) was used for data analysis. Statistical analyses

were performed using the Student's t-test when appropriate or the One-way analysis of variance (ANOVA) when three or more groups were compared and followed by Dunnett's multiple comparison test. Differences with $p < 0.05$ was considered statistically significant.

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