Impaired regulation of nuclear calcium signals is a driver of chronic liver disease

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SUPPLEMENTAL METHODS

AAV8-mediated rescue experiment

An adeno-associated virus serotype 8 (AAV8) vector encoding the full-length mouse *Nup62* cDNA (NM_053074) under the control of the thyroxine-binding globulin (TBG) promoter (AAV8-TBG-m-Nup62-P2A-RFP) was used to restore NUP62 expression hepatocyte in CDAHFD-fed mice. An AAV8-TBG-RFP was used as the control. AAV8 vectors were produced by Vector Biolabs (Vector Biolabs Malvern, PA) at a titer of $1x10^{13}$ GC/ml. Chow- or CDAHFD-fed mice were randomly assigned to experimental groups. Mice received a single intravenous injection of AAV8 via the lateral tail vein at a dose of $2x10^{11}$ GC per mouse. AAV8-mediated expression of NUP62 was confirmed at 1, 2, and 3 weeks post-injection by immunoblotting and immunofluorescence. Following AAV8 administration, mice were maintained on either chow or CDAHFD for 3 weeks before undergoing partial hepatectomy (PHx). Mice were euthanized at 24 hours after PHx, and liver tissue were harvested for histology and biochemical analyses.

Partial hepatectomy

Eight-week-old *Itpr2*$^{-/-}$ and age-matched wild-type mice were fed with a CDAHFD for 4 weeks. In separate experiments, wild-type mice were fed the same CDAHFD and received either AAV8-Nup62 or AAV8-control prior to surgery. At the designated time point, mice underwent two-thirds partial hepatectomy (PHx) as previously described (1, 2). All animal procedures were approved by the Institutional Animal Care and Use Committees of Yale University and Veterans Affairs Connecticut Healthcare System. Mice were sacrificed at 24 hours after PHx. The regenerating liver was harvested for histological, immunofluorescence, or snap-frozen in liquid nitrogen for molecular and biochemical analyses.

Blood and liver tissue collection for biochemical and histological analyses

Mice were anesthetized in accordance with institutional ethical guidelines. Prior to liver harvesting, whole blood was drawn from the abdominal artery, allowed to clot at room temperature and then centrifuged at 3,000 rpm for 10 minutes at 4°C to obtain serum samples. Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by the Yale-New Haven Hospital (YNHH) Chemistry Laboratory. Following blood collection, the liver was carefully removed. Representative liver lobes were trimmed into 1 cm thick sections to ensure adequate fixation. The tissues were immediately placed in 10% neutral buffer formalin (NBF) (Sigma-Aldrich, St. Louis, MO, Cat# HT501128) and fixed at 4°C for 24 hours. After fixation, liver tissues were transferred to 70% ethanol prior to paraffin embedding, sectioning at 5 μm thickness, and staining with hematoxylin and eosin (H&E), Sirius Red, and Masson Trichrome at Yale Pathology

Tissue Services (YPTS). For hepatic triglyceride measurements, a portion of liver tissue (100 mg) was snap-frozen in liquid nitrogen and stored at -80°C. Triglyceride levels were measured by homogenizing liver samples in 5% NP-40 (Sigma-Aldrich), followed by heating and centrifugation. The supernatant was used to determine triglyceride concentrations using a commercial triglyceride quantification assay kit (Abcam, Waltham, MA, Cat# ab65336) according to the manufacturer's instructions. Triglyceride content was normalized to liver weight and expressed as mg triglyceride per gram of liver tissue.

BODIPY staining of lipid droplets

For lipid droplet staining, frozen liver samples were cryo-sectioned at 7-10 μm, mounted on glass slides, and fixed with 4% paraformaldehyde for 15 minutes. Mouse hepatocytes were plated on collagen-coated glass coverslip. After 5 hours of plating, cells were washed and fixed in 4% paraformaldehyde. After fixation, both slides and fixed cells were washed with PBS (Gibco, Grand Island, NY, REF-10010-023), and incubated with 1 μm BODIPY493/503 (Invitrogen, Carlsbad, CA, Cat# D3922) in PBS for 30 minutes, protected from light. After PBS washing, nuclei were stained with Hoechst 33342 (Invitrogen) for 10 minutes, washed with PBS, and mounted with Prolong Glass antifade mountant (Invitrogen, Cat# P36980). Images were acquired on a Zeiss LSM 880 confocal microscope with a C-Apochromat 40X/1.2 water objective for mouse hepatocytes and an EC Plan-Neofluar 10X/0.30 for liver section. Hoechst and BODIPY were imaged using 405 nm and 488 nm excitation, respectively.

Isolation of primary mouse hepatocytes

Mouse hepatocytes were isolated by collagenase perfusion in the Cell Isolation Core of the Yale Liver Center as described previously (3). Briefly, mouse livers were perfused with Hanks' A (5 mL/minutes) for 10 minutes and then Hanks' B medium containing collagenase type 2 (Worthington Biochemical Corporation, Lakewood, NJ, Cat# LS004177) for 10 minutes. Livers were then excised, minced, and passed through serial 40-μm nylon mesh filters (Component Supply, Sparta, TN, Cat# U-CMN-40-C) to yield primary hepatocytes in Leibovitz's L-15 (Gibco) medium suspension. The resultant cells were washed with cold L-15 medium by low-centrifugation at 60 x g for 2 minutes at 4°C and purified using a Percoll gradient. Crude hepatocytes pellets were resuspended in isotonic 45% Percoll (Sigma-Aldrich, Cat# P4937) and centrifuged at 60 x g for 2 minutes at 4°C. Cells were then resuspended in Williams' E Medium (Gibco, REF-12551-032) containing 5% heat-inactivated fetal bovine serum (Gibco, REF-A5670801), 1% Antibiotic-Antimycotic (Gibco, REF-15240-062), 1% GlutaMAX (Gibco, REF-

35050-061), 1% HEPES (Gibco, REF-15630-080), 4 mg/mL insulin (Gibco, REF-12585-014), 1 mM dexamethasone (Sigma-Aldrich, Cat# D4902), and assessed for cell viability before plating by 0.4% trypan blue stain (Gibco, REF-15250-061) exclusion. Cells with viability exceeding 90-95% are processed to plate on collagen-coated coverslips (for imaging) or MatTek collagen-coated glass bottom dishes (for Ca^{2+} signaling). Hepatocytes were cultured at 37°C in 5% CO_2 and used 4-6 hours after plating.

Human cell lines and CRISPR/Cas9-mediated generation of NUP62 knockout HepG2 cells

A new batch of human HepG2 cell line was obtained from American Type Culture Collection (ATCC; Manassas, VA). CRISPR/Cas9-generated ITPR2-KO HepG2 and CRISPR/Cas9-generated ITPR3-KO HepG2 were developed previously in our laboratory (4, 5). Wild-type, ITPR2-KO, and ITPR3-KO HepG2 cells were cultured and maintained under identical conditions in Eagle's Minimum Essential Medium (ATCC, REF-30-2003) supplemented with 10% heat-inactivated fetal bovine serum and 100 U/mL penicillin G, and 100 μg/mL streptomycin (Gibco, REF-15070063) at 37°C in a humidified incubator with 5% CO_2. Cells were passaged at 70-80% confluency and used for experiment between passages 5-20 to ensure consistency. For the generation of NUP62 knockout HepG2 cells, NUP62-KO HepG2 cells were established by using the CRISPR/Cas9 system. A commercially available CRISPR/Cas9 system was used to knockout NUP62 in HepG2 cells. NUP62 CRISPR/Cas9 KO plasmid (sc-416852) and NUP62 HDR plasmid (sc-416852-HDR) were obtained from Santa Cruz Biotechnology (Dallas, TX). Briefly, HepG2 cells were seeded and transfected with 1 μg of NUP62 HDR plasmid plus 1 μg of NUP62 CRISPR/Cas9 KO plasmid, which contains the guide RNA targeting NUP62 gene and the sequence encoding the Cas9 nuclease. In addition, a donor plasmid was co-transfected to facilitate homology-directed repair (HDR), enabling the insertion of red fluorescent protein (RFP) and puromycin resistance cassette at the targeted deletion site within NUP62 locus. During 24-48 hours post-transfection, enriched transfected cells incorporating RFP fluorescence cells were treated with 2 μg/mL puromycin (Gibco, REF-A11138-03). Surviving cells were diluted and seeded into 96-well plates to allow for single-cell clone expansion. On expansion of RFP-positive clones, genomic DNA was extracted and amplified by PCR using primers flanking the CRISPR/Cas9 target site. The PCR product were analyzed by Sanger sequencing at Keck DNA Sequencing Core, Yale University. In addition, RFP-positive clones were subjected to Western blot analysis to confirm successful knockout of NUP62. NUP62-KO HepG2 cells were subsequently cultured maintained in the presence of 1 μg/mL puromycin.

Immunohistochemical analyses of ITPR2 and NUP62

Human liver biopsies from individual patients with MASH, AAH, HBV, HCV, cirrhosis, and histologically normal livers were obtained from the Department of Pathology at YNHH. Liver biopsy or resection specimens were previously stained with H&E and were independently reviewed, and the histological diagnosis was confirmed by an experienced liver pathologist (M.E.R). Formalin-fixed, paraffin-embedded (FFPE) liver tissue sections were used to analyze ITPR2 and NUP62 expression in hepatocytes. Liver biopsies from 3-6 patients under each condition were evaluated. FFPE liver sections were deparaffinized in xylene and rehydrated through a graded series of ethanol (100, 95, 90, 80, and 70%) in distilled water. Antigen retrieval was performed by incubating sections in 10 mM sodium citrate buffer (pH 6.0) (Sigma-Aldrich, Cat# C9999) in a streamer for 20 minutes. Subsequent steps employed the Dako Envision+System-HRP, a labeled polymer anti-mouse with 3'3'-diaminobenzidine (DAB) detection (Dako, Carpinteria, CA, REF-K4006), in a combination with the CSA II Rabbit Link (Dako, REF-K15101) as described (5). Tissue sections were treated with peroxidase block (Dako), then blocked with Protein Block (Dako) for 5 minutes at room temperature followed by the incubation with ITPR2 antibody (a gift from Richard Wojcikiewicz, Department of Pharmacology, SUNY Upstate Medical University, Syracuse, NY) (1:25 dilution) and NUP62 (Cell Signaling Technology, clone E4Y5P, Cat# 24588) (1:100 dilution) overnight at 4°C in a humidified chamber. After incubation with primary antibodies, the signal was developed using CSA II, Biotin-free catalyzed amplification system. Immunoreactivity was visualized using DAB and counterstained with hematoxylin QS (Vector Laboratories, Newark, CA, Cat# H-3404). Slides were dehydrated and mounted with coverslips using VectaMount Express Mounting Medium (Vector Laboratories, Cat# H-5700). Images were acquired using a digitalized bright-field Olympus BX51 microscope with Olympus CellSens digital imaging software with the same settings with a 20X objective for ITPR2 and a 40X objective for NUP62. Quantitative analysis for ITPR2 and NUP62 was quantitated in each individual human liver biopsy using ImageJ analysis software (National Institutes of Health (NIH), Bethesda, MD). For quantification of ITPR2, the percentage of DAB-positive staining area in liver sections was quantified and expressed as ITPR2-positive area (%). NUP62 expression was quantified as the percentage of NUP62-positive cells relative to the total number of nuclei. To minimize bias, a different investigator performed the quantification.

Cytoplasmic and nuclear protein extraction

Cytoplasmic and nuclear protein fractions of mouse hepatocytes or HepG2 cells were extracted using the Minute cytoplasmic and nuclear extraction kit for cells (Invent Biotechnologies,

Plymouth, MN, Cat# SC-003) according to the manufacturer's instructions. Briefly, adherent cells (90% confluence) were washed twice with ice-cold PBS and incubated on ice with ice-cold cytoplasmic extraction buffer containing Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL, REF-78442) on ice for 10 minutes. After harvesting cells, the suspension was vortexed vigorously for 15 seconds, then centrifuged 16000 g for 5 minutes at 4°C. The supernatant (cytosolic fraction) was collected and stored -80°C. The remaining pellet was resuspended in ice-cold nuclear extraction buffer supplemented with protease and phosphatase inhibitors, vortexed for 15 seconds every 1 minute, over a 5-minutes period while on ice. The suspension was then applied to a pre-chilled filter cartridge, centrifuged at 16000 g for 30 seconds at 4°C. The resulting eluate (the nuclear fraction) was collected and stored -80°C. Cytoplasmic and nuclear protein concentrations were determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific, REF-23227).

Coimmunoprecipitation of nuclear proteins

Coimmunoprecipitation was performed using the Capturem IP and Co-IP kit (Takara Bio, San Jose, CA, Cat# 635721) according to the manufacturer's instructions. Briefly, nuclear fraction from HepG2 cells or mouse hepatocytes were extracted using Minute cytoplasmic and nuclear extraction kit. IP Lysis/Equilibration buffer supplemented with protease inhibitor cocktails was added to nuclear protein (500-1000 μg) lysate, then incubated with 5-10 μg of ITPR2 antibody (Santa Cruz Biotechnology, clone A-5, Cat# sc-398434) or NUP62 (Invitrogen, Cat# PA5-21882) overnight 4°C gently with on an end-over-end rotator. Next, antibody-bound target complexes were loaded onto the Capturem column and incubated for 1 hour at 4°C on an end-over-end rotator, then centrifuged at 1000 g for 1 minute at room temperature. The Capturem column was washed with the wash buffer to remove unbound and nonspecific proteins. Immunoprecipitated protein complexes were eluted with the elution buffer under non-denaturing conditions. Eluted protein was subsequently analyzed by Western blotting or downstream analysis.

Coimmunoprecipitation and mass spectrometry analysis

Primary mouse hepatocytes were isolated from wild-type C57BL/6 and the nuclear and cytoplasmic fractions were isolated using the NE-PER nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific, REF-78833). Coimmunoprecipitation was performed using the co-immunoprecipitation kit (Thermo Fisher Scientific, REF-26149) using the anti-ITPR2 antibody (Santa Cruz Biotechnology, clone A-5, Cat# sc-398434). Nuclear fraction of HepG2 cells were extracted using Minute cytoplasmic and nuclear extraction kit (Invent Biotechnologies) and

coimmunoprecipitation was performed using the Capturem IP and Co-IP kit (Takara Bio). The eluted co-IP protein complexes from the nuclear fraction of mouse hepatocytes and HepG2 cells were subsequently submitted for mass spectrometry for protein identification at Keck Foundation Biotechnology (Mass Spectrometry and Proteomics) Resource Laboratory, Yale University. Mass spectrometry data analysis was performed using Scaffold software (Version 5.0) (Proteome Software, Portland, OR).

Proximity Ligation Assay

Proximity ligation assay (PLA) was performed using the Duolink *In Situ* Red Starter kit mouse/rabbit (Sigma-Aldrich, Cat# DUO92101) according to the manufacturer's instructions. Briefly, mouse hepatocytes ($1x10^5$ cells) or HepG2 cells ($2x10^5$ cells) were cultured on 18 mm glass coverslips coated with collagen for cell-imaging (Neuvitro Corporation, Camas, WA, Cat# GG-18-1.5H-collagen). After 4-5 hours of plating, mouse hepatocytes were fixed with 4% paraformaldehyde (PFA) (Electron Microscopy Science, Hatfield, PA) in TBS for 10 minutes at room temperature. HepG2 cells were fixed with 4% PFA after 48 hours of plating. Cells were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in TBS for 10 minutes. After washing, cells were blocked with Duolink blocking solution (DUO82007) in a pre-heated humidity chamber at 37°C for 1 hour. Cells were then incubated with a pair of primary antibodies: mouse anti-ITPR2 (Santa Cruz Biotechnology, clone A-5, Cat# sc-398434) (1:20 dilution) and rabbit anti-Nup62 (Invitrogen, Cat# PA5-21882) (1:50 dilution) diluted in Duolink antibody diluent (DUO82008) and incubated overnight at 4°C in a humidity chamber. After washing in Wash buffer-A (DUO82046), cells were incubated with Duolink *in situ* PLA probe anti-rabbit Plus (DUO92002) (1:5 dilution) and Duolink *in situ* PLA probe anti-mouse Minus (DUO92004) (1:5 dilution) in Duolink antibody diluent at 37°C for 1 hour in a humidity chamber. Following additional washes in Wash buffer-A, ligation buffer containing ligase (DUO82029) (1:40 dilution) and connector oligonucleotides was added, and cells were incubated at 37°C for 30 minutes. After ligation incubation, cells were washed and the amplification buffer containing polymerase enzyme (DUO82030) and fluorescent-label oligonucleotides (DUO82010) was applied at 37°C for 100 minutes protected from light. After amplification incubation, cells were washed with Wash buffer-B (DUO82048), counterstained with Duolink *in situ* mounting medium with DAPI (DUO82040). Negative controls included omitting one of primary antibodies or probes to confirm the specificity of PLA signals. The PLA signals representing protein-protein proximity events appeared as distinct red fluorescent dots and were visualized using a Zeiss LSM 880 (Axio Observer) confocal fluorescence microscope. Z-stack images were acquired with a Plan-Apochromat 63X/1.4 oil immersion objective and 405 nm, and

561 nm excitation lasers for DAPI and Texas Red, respectively. Images were post-processed using Imaris software (Version 10.2) (Oxford Instruments, Belfast, United Kingdom).

Live-cell nuclear Ca^{2+} signaling

To measure nuclear Ca^{2+} signaling, mouse hepatocytes or HepG2 cells were incubated with a membrane-permeable, photoactivatable caged InsP3 analog (ci-IP3/PM or caged InsP3) (TOCRIS Biosciences, Minneapolis, MN, Cat# 6210) in a combination with the fluorescent Ca^{2+} indicator Fluo-4/AM (Invitrogen, REF-F14201). This approach enables spatially restricted release of InsP3 within the nucleus, allowing precise stimulation of InsP3-mediated Ca^{2+} release within the nucleus. Briefly, mouse hepatocytes ($3x10^5$ cells) were plated onto 35 mm collagen coated-glass MatTek dishes (No.1.5 coverslip) (MATTEK, Ashland, MA, Cat# P35G-Col-1.5-14-C) and imaged after 6 hours of plating. To monitor nuclear Ca^{2+} signaling in HepG2 cells, cells ($1.5x10^5$ cells) were plated on 35 mm glass MatTek dishes (No.1.5 coverslip) (MATTEK, Cat# P35G-1.5-20-C) and Ca^{2+} signaling were measured 48 hours after plating. On the day of nuclear Ca^{2+} signaling imaging, cells were co-incubated with 5 μm Fluo-4/AM and 1 μm ci-IP3/PM in the presence of 10% FBS/EMEM for 30 minutes in a 37°C incubator. After loading, cells were washed twice with HEPES buffer (pH 7.4). Live-cell Ca^{2+} imaging was performed using a Bruker Opterra II swept-field confocal microscope equipped with a 405 nm UV laser to photo-release of InsP3 as described (5, 6). Fluo-4 was excited at 488 nm, and emission was collected at 505-530 nm. For spatially restricted InsP3 uncaging within the nucleus, a region of interest (ROI, 12x14 pixels) was demarcated within the nucleus for InsP3 uncaging. For mouse hepatocytes, simultaneous time-lapse imaging was performed using a Plan-Apo 40X/1.15 water immersion objective and a 488 nm laser with a collection rate of 2 Hz to acquire images before uncaging InsP3 (10 seconds) and after uncaging InsP3 (200 seconds) with 405 nm laser (a total uncaging duration time of 360 milliseconds) confined to a region of interest. For HepG2 cells, time-lapse imaging was performed using a Plan-Apo 60X/1.2 water immersion objective and a 488 nm laser with an acquisition rate of 2 Hz using the same ROI size and image acquisition before and after uncaging InsP3. The total uncaging duration time was 200 milliseconds. To determine the origin of nuclear Ca^{2+} signaling, time-lapse imaging was conducted using a Plan-Apo 100X/1.45 oil immersion objective and a 488 nm laser at a collection rate of 5 Hz. A 20x20 pixel ROI was defined within the center of nucleus for photo-release of InsP3. Fourteen images were acquired before uncaging and 76 images after uncaging InsP3. The total uncaging duration time was 40 milliseconds. Negative controls included mock-loading cells (Fluo-4 only). Changes in Fluo-4 fluorescence were recorded and quantified

using Bruker's Prairie View software (Version 5.4). Changes in fluorescence (F) were normalized by the initial fluorescence (F0) and were expressed as (F/F0) × 100%.

Confocal and gated stimulated emission depletion (gSTED) imaging

Mouse hepatocytes or HepG2 cells were plated on 25 mm collagen coated-glass coverslips for cell-imaging (Neuvitro Corporation). Both cell types were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Cat# 15710) in tris-buffered saline (TBS) and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in TBS. After washing in TBS, non-specific binding was blocked with TBS containing 1% bovine serum albumin (BSA) (Sigma-Aldrich), 0.05% Tween-20, and 5% normal goat serum (Vector Laboratories, Cat# S1000) for 1 hour. For co-immunolabelling, cells were incubated with primary antibodies: ITPR2 (Santa Cruz Biotechnology, clone A-5, Cat# sc-398434) (1:25 dilution), NUP62 (Cell Signaling Technology, clone E4Y5P, Cat# 24588 or NUP62 (Invitrogen, Cat# PA5-21882) (1:50 dilution), or Lamin B1 (Abcam, Cat# ab16048) (1:100 dilution) were incubated overnight at 4°C. Following washes, secondary antibodies (Invitrogen): goat anti-rabbit IgG Alexa Fluor Plus 555 (1:500 dilution) and goat anti-mouse IgG Alexa Fluor Plus 488 (1:500) were applied for a 1-hour incubation at room temperature. Hoechst 33342 (Invitrogen, Cat# H3570) (1:1000 dilution) was used for nuclear staining. Coverslips were mounted with Prolong Glass antifade mountant (Invitrogen, Cat# P36980). Images were acquired on a Zeiss LSM 880 Airyscan Fast confocal microscope with 63X/1.4 NA oil-immersion objective using the Airyscan detector and processing algorithm. For gSTED, abberior STAR 580, goat anti-mouse IgG (Cat# ST580-1001, 1:300 dilution) and abberior STAR 635P, goat anti-rabbit IgG (Cat# ST635P-1002, 1:300 dilution) (abberior GmbH, Göttingen, Germany) were used as secondary antibodies. Images were acquired on a Leica SP8 Gated STED 3X super resolution microscope (Leica Microsystems, Mannheim, Germany) and collected at 100X magnification and with 775 nm depletion laser. Images were post-processed with the deconvolution express function of the Huygens software (Scientific Volume Imaging (SVI), Hilversum, The Netherlands).

Immunofluorescence staining of liver section

Mouse frozen liver tissue were embedded in optimal cutting temperature (OCT) compound (Electron Microscopy Sciences), sectioned into serial section (7 µm) using a cryostat and mounted onto glass slides. Sections were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 10 minutes, permeabilized with 0.1% Triton X-100, and blocked before incubation with the following primary antibodies: proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology, clone PC-10, Cat# SC-56, 1:25 dilution), HNF4α (Cell Signaling Technology, clone C11F12, Cat#

3131, 1:100 dilution), or β-catenin (GeneTex, Cat# GTX101435, 1:100 dilution) overnight at 4°C, followed by incubation with goat anti-mouse Alexa Fluor 488, goat anti-rabbit Alexa Fluor 555, or goat anti-rabbit Alexa Fluor 647 for 1 hour at room temperature. Nuclei were counterstained with Hoechst 33342 (1:1000) and section were mounted with Prolong Glass antifade mountant (Invitrogen). Images were acquired on a Zeiss LSM 880 Airyscan confocal microscope using identical laser power, gain, and offset setting across all experiment groups. Image analysis was performed using Imaris software (version 11.0.2, Oxford Instrument). For quantitative analysis of β-catenin nuclear accumulation, nuclei were segmented based on Hoechst fluorescence, and β-catenin signal within nuclear region was quantified. For PCNA hepatocytes proliferation. HNF4α-positive hepatocytes nuclei were identified using spot creation, and PCNA-positive cells were quantified based on fluorescence intensity.

Photoactivation of photoactivatable green fluorescence protein (PA-GFP)

HepG2 cells were seeded on 6-well glass bottom black plate (Cellvis, Mountain View, CA, Cat# P06-1.5H-N). Forty-eight hours after seeding, cells were co-transfected with pPAGFP-N1 and pcDNA3-mRFP to monitor transfection efficiency using Mirus TransIT-LT1 transfection reagent (Mirus Bio LLC, Madison, WI, Cat# MIR-2300). The pPAGFP-N1 (Addgene plasmid# 11909, a gift from Jennifer Lippincott-Schwartz, Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MA) and pcDNA3-mRFP (Addgene plasmid# 13032, a gift from Doug Golenbock, University of Massachusetts, Worcester, MA) were purchased from Addgene (Watertown, MA). Cells were then cultured under 37°C, 5% CO_2 for 24 hours prior to imaging. For live-cell imaging photoactivation experiments, cells expressing PA-GFP and mRFP were maintained in phenol-red free live cell fluorescence imaging medium (FluoroBrite DMEM, Gibco, REF-A18967-01) supplemented with 10% FBS and GlutaMAX, pre-warmed to 37°C. Live-cell photoactivation and imaging were performed using a Bruker Opterra II swept-field confocal microscope equipped with a Plan-Apochromat 100X/1.45 NA oil immersion objective with perfect focus system (PFS), a 405 nm laser for photoactivation, and a 488 nm and 561 nm lasers for GFP and RFP imaging, respectively. Cells were imaged in a temperature- and CO_2 -controlled chamber throughout the experiment. Time-lapse imaging was performed using a 488 nm laser at 1 Hz to monitor GFP fluorescence before (10 seconds) and after photoactivation (180 seconds) with 405 nm laser (total photoactivation duration: 1 second) confined to a region of interest (ROI, 22x22 pixels) within the nucleus. Microscope control and image acquisition were performed using Bruker's Prairie View software (Version 5.4). Changes in GFP fluorescence for nuclear or cytosolic regions were analyzed using Bruker's Prairie View

software (Prairie Technologies, Middleton, WI) and ImageJ software (National Institutes of Health).

Immunoblotting analysis

Liver tissues or cells were lysed in RIPA buffer (Thermo Fisher Scientific, REF-89900) supplemented with Halt protease and phosphatase inhibitors (Thermo Fisher Scientific), homogenized 30 seconds, incubated on ice for 30 minutes, and centrifuged at 14,000 g for 15 minutes at 4°C. Protein concentrations were determined using BCA protein assay kit (Thermo Fisher Scientific). Equal amount of protein (50 μg) were mixed with NuPAGE LDS sample buffer (Invitrogen, REF-NP0007) with NuPAGE sample reducing agent (Invitrogen, REF-NP0004), heated at 70°C for 10 minutes, and resolved by NuPAGE 4%-12% Bis-Tris gradient gel (Invitrogen, REF-NP0335) with MOPS SDS running buffer (Invitrogen, REF-NP0001) plus NuPAGE antioxidant (Invitrogen, REF-NP0005). Proteins were transferred to 0.45 μm nitrocellulose membrane (Bio-Rad, REF-2304793) using wet transfer method. Membrane were blocked with Intercept blocking (TBS-based) buffer (LICORbio, Part# 927-60001) for 2 hours at room temperature, then incubated overnight at 4°C with primary antibodies diluted in Intercept antibody diluent buffer (LICORbio, Part#927-65001). The antibodies used included: ITPR1 (Invitrogen, Cat# PA1-901, 1:1000 dilution), ITPR2 (Santa Cruz Biotechnology, clone A5, Cat# SC-398434, 1:500 dilution), ITPR3 (BD Transduction Laboratories, Cat# 610313, 1:500 dilution), GAPDH (Santa Cruz Biotechnology, clone 6C5, Cat# SC-32233, 1:3000 dilution), NUP62 (Santa Cruz Biotechnology, clone C-9, Cat# SC-48373, 1:250 dilution), NUP62 (Invitrogen, Cat# PA5-21882, 1:1000 dilution), Lamin A/C (Invitrogen, clone-4L8Q0, Cat# MA5-35284, 1:1000 dilution), Lamin B1 (Abcam, Cat# ab16048, 1:1000 dilution), Calreticulin (Cell Signaling Technology, clone D3E6, Cat# 12238, 1:1000 dilution), α-SMA (Abcam, Cat# ab124964, 1:5000 dilution), β-catenin (GeneTex, Cat# GTX101435, 1:1000 dilution). After washing in TBS-Tween-20, membranes were incubated with fluorophore-conjugated secondary antibodies: IRDye680RD Donkey anti-rabbit (LICORbio, Lincoln, NE, Part# 926-68073, 1:10000 dilution) or IRDye800CW (LICORbio, Part# 925-32213, 1:10000 dilution), IRDye800CW Donkey anti-mouse (LICORbio, Part# 925-32212, 1:10000 dilution) or goat anti-rabbit IgG (H+L) Alexa Fluor Plus 555 (Invitrogen, Cat# A32732, 1:5000 dilution) or goat anti-mouse IgG (H+L) Alexa Fluor Plus 647 (Invitrogen, Cat# A32728, 1:5000 dilution) for 1 hour at room temperature, protected from light. After final washes, western blot images were developed using fluorescent detection using Odyssey Imaging System (LICORbio) or iBright FL1500 Imaging System (Thermo Fisher Scientific) in fluorescence mode. Band intensities were quantified using Image Studio software (Version 5.5) (LICORbio).

Fluorescent images were converted to grayscale for clarity and consistent visualization. Protein loading was normalized to GAPDH.

RNA isolation and TaqMan real-time PCR

Total RNA was extracted by TRIzol reagent (Invitrogen) and was purified with RNeasy MinElute Cleanup kit (QIAGEN). One microgram of total RNA was reverse-transcribed into cDNA using the iScript cDNA Synthesis kit (Bio-Rad, Cat# 1708891). Quantitative real-time PCR was performed using TaqMan Gene Expression Assay (Applied Biosystems; *Itpr2* (Mm00444937_m1), *Nup62* (Mm01700766_m1), and *Actb* (Mm00607939_s1)) on a QuantStudio 3 Real-Time PCR System. Gene expression levels were normalized to *Actb*. Relative mRNA expression was calculated and is presented as fold change relative to the control group.

Luciferase reporter gene assay of human ITPR2/Luc reporter

HepG2 cells ($2x10^5$ cells/well) were plated onto 24-well plates (Corning Costar, Tewksbury, MA). Twenty-four hours after plating, cells were transfected with a 2-kb human ITPR2 luciferase promoter (5) or pGL4 luciferase reporter vector (Promega) served as an empty vector, and co-transfected with the NFAT3/NFATc4 expression plasmid along with a Renilla luciferase (pRL-TK vector) (Promega, Madison, WI) as a control for transfection efficiency using FuGENE HD transfection reagent (Promega, Cat# E2311). Human pEGFP-C1 NFAT3 was purchased from Addgene (Addgene plasmid# 10961, a gift from Toren Finkel (Laboratory of Molecular Biology, NHLBI, National Institutes of Health, Bethesda, MA). Forty-eight hours post-transfection, cells were lysed in passive lysis buffer. Cell lysates were harvested to measure firefly and Renilla luciferase activity using the dual-luciferase reporter assay system (Promega). Luciferase activity was measured using the Synergy 2 multi-mode microplate reader (BioTek, Winooski, VT). The reporter gene assay was carried out in triplicate. Luciferase promoter activity was calculated by normalizing the firefly luminescence to the Renilla luciferase activity, and the ratio of promoter construct over control is presented.

Cell proliferation assay

For image-based detection of proliferating cells, NUP62-KO HepG2 or wild-type HepG2 cells ($2x10^5$ 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, REF-C10337) according to the manufacturer's instructions. Images were acquired on a Zeiss

LSM 880 confocal microscopewith a C-Apochromat 40X/1.2 water objective. Hoechst and 5-ethynyl-2'-deoxyuridine (EdU) were imaged using 405 nm and 488 nm excitation, respectively.

Bulk RNA sequencing and enrichment analysis

Total RNA was isolated from HepG2 cells transduced with adenovirus encoding mRFP-InsP3R-LBD-3NLS (ViraQuest, Davis, CA) for 48 hours to buffer nuclear InsP3 and ITPR2-KO HepG2 cells (three biological replicates/each) using the RNeasy Plus Mini kit (QIAGEN, Germantown, MD). For mouse hepatocytes, total RNA was isolated from wild-type C57BL/6 or *Itpr2*$^{-/-}$ mice aged 10-12 weeks overlaid with collagen and treated with recombinant mouse EGF (R&D systems, Minneapolis, MN, Cat# 2028-EG) (100 ng/mL) or recombinant mouse HGF (R&D systems, Cat# 2207-HG) (100 ng/mL) for 6 hours (three biological replicates/each) using TRIzol reagent (Invitrogen, REF-15596018), followed by RNeasy-MinElute-Cleanup kit (QIAGEN, REF-74204). RNA concentration and purity were assessed using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific), and RNA integrity was evaluated by Agilent Bioanalyzer. All RNA samples have RNA Integrity Numbers (RIN) ranging from 9-10 and were processed for cDNA and library preparation and sequencing by Yale Center for Genome Analysis. Raw sequencing reads were evaluated for quality. Low-quality reads were trimmed and adaptor contamination were removed using Trim Galore (v0.5.0). Trimmed reads were mapped to the mouse reference genome (GRCm38) using HISAT2 (v2.1.0) (7). Gene expression levels were quantified using StringTie (v1.3.3b) (8) with gene models (M15) from the GENCODE project. Differentially expressed genes were identified using DESeq2 (v 1.22.1) (9). Genes with an adjusted *p*-value (FDR) < 0.05 were considered significantly differentially expressed. Significantly differentially expressed genes were uploaded to Ingenuity Pathway Analysis (IPA, QIAGEN) for canonical pathway analysis. IPA was used to identify enriched pathways and biological functions. Pathways with a -log10(*p*-value) > 1.3 and/or z-score > 2 were considered significantly enriched.

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