The Niemann-Pick C1-Like 1 cholesterol absorption receptor: a novel hepatitis C virus entry factor and potential therapeutic target

Bruno Sainz, Jr., Naina Barretto, Danyelle N. Martin, Nobuhiko Hiraga, Michio Imamura, Snawar Hussain, Katherine A. Marsh, Xuemei Yu, Kazuaki Chayama, Waddah A. Alrefai, and Susan L. Uprichard

Supplementary Methods

Cells

Huh7 cells[1] [also known as Huh7/scr cells[2,3]] were cultured as described by Yu and Uprichard[4]. 293T cells were purchased from the American Type Culture Collection (Manassas, VA). The Clone B HCV genotype 1b sub-genomic replicon (sg1b) Huh7 cells were obtained from C.M. Rice (Rockefeller University, NY) through the NIH AIDS Research and Reference Reagent Program and have been previously described[5]. HCV sg2a replicon cells were established as previously described[6,7] using the pSGR-JFH1[8] vector, kindly provided by T. Wakita, National Institute of Infectious Diseases, Tokyo, Japan.. All cells were cultured in complete Dulbecco's modified Eagle's medium (cDMEM) (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 100 units ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, and 2 mM L-glutamine (Gibco Invitrogen, Carlsbad, CA) and 500 μg ml^{-1} geneticin [(Invitrogen) for HCV replicon cells only].

HCVcc generation

The plasmids containing the full-length JFH-1 genome (pJFH1) and JFH-1-based inter-genotypic HCV clones have been previously described[8-11]. The JFH-1^{G451R} expression plasmid (pJFH-1^{G451R}), which contains a glycine-to-arginine mutation at amino acid residue 451 in the E2 glycoprotein[3], was generated by site-directed mutagenesis of pJFH1 using a complementary pair of oligomers of sequence 5'-CAA CTC TTC AAG GTG TCC TGA GAG GTT GGC C-3' and the QuikChange® II XL site-directed mutagenesis kit (Stratagene, Agilent Technologies, Santa Clara, CA), as per the manufacturer's instructions. Protocols for JFH-1 *in vitro* transcription and HCV RNA electroporation have been described elsewhere[4]. The HCVcc viral stocks were generated by infection of naïve Huh7 cells at a multiplicity of infection (MOI) of 0.01 focus forming units (FFU) $cell^{-1}$ using medium collected from Huh7 cells electroporated with *in vitro* transcribed HCV RNA as previously described[4].

For the generation of DiD-labeled JFH-1 HCVcc ($HCVcc^{DiD}$), 30 μl of a 1 mM stock of Vybrant® DiD cell-labeling solution (Invitrogen) was added to 6 ml of cDMEM (media control) or cDMEM containing 2.0×10^5 FFU ml^{-1} of HCVcc JFH-1 and incubated at 37

°C for 30 min in the dark. Prior to purification of the labeled media or virus, the total volume was reduced to 500 µl using Amicon® Ultra-4 Centrifugal Filter Units (Millipore, Billerica, MA) as per the manufacturer's instructions. The concentrated labeled media or virus was then purified to remove unincorporated DiD and non-specific DiD-lipid complexes by either iodixanol (OptiPrep™, Accurate Chemical, Westbury, NY) gradient purification as described by Sabahi A *et al.*[12] or by HiTrap™ Heparin HP affinity column purification (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) as described by Morikawa K *et al.*[13]. Heparin-bound fractions, which contain E2-heparin bound HCVcc[13], were eluted with 1X PBS containing 0.6 M NaCl (w/v) and subsequently concentrated using Amicon® Ultra-4 Centrifugal Filter Units (Millipore). HCV RNA copies and infectivity titers per microliter of concentrated elute or gradient fraction were quantified as previously described[4,12].

Reagents and antibodies

The mouse monoclonal antibody to human CD81 (mca1847; clone identity ID6) was purchased from AbD Serotec (Raleigh, NC) and the mouse monoclonal antibody to human SR-BI (Clone 25) from BD Biosciences (Franklin Lakes, NJ). The rabbit polyclonal antibody to human CLDN1 Loop 1 used for flow cytometric analysis was purchased from AbCam (Cambridge, MA). The mouse monoclonal antibody to human OCLN (Clone OC-3F10) and mouse monoclonal antibody to human CLDN1 (Clone 1C5-D9) used for immunofluorescence staining were purchased from Invitrogen and Abnova Corp. (Taipei, Taiwan), respectively. The rabbit monoclonal and polyclonal antibodies to human NPC1L1 targeting extracellular loops 1, 2 and 3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), Cayman Chemicals (Ann Arbor, MI) and Y. Ioannou (Bsn4052[14], Mount Sinai School of Medicine, NY), respectively. The rabbit polyclonal antibody to human NPC1L1 used for western blot analysis was purchased from Cell Signaling Technology (Danvers, MA). The human monoclonal antibody to the HCV E2 glycoprotein (C1) and the mouse monoclonal antibody to the HCV NS5A protein (E910) have both been previously described[1,15,16]. The monoclonal mouse antibody to the HCV NS3 protein (Clone 9-G2) was obtained from ViroGen (Watertown, MA). The HRP-conjugated anti-human, anti-mouse and anti-rabbit secondary antibodies were purchased from Pierce (Rockford, IL). The anti-mouse Alexa-555- and anti-rabbit Alexa-488-conjugated antibodies were from Invitrogen. Negative control mouse and rabbit IgG antibodies were from Santa Cruz Biotechnology. The cell-tracking fluorescent probe 5-chloromethylfluorescein diacetate (CMFDA) was purchased from Invitrogen and used to label live Huh7 cells (5µM) as described by Krieger *et al.*[17]. Ezetimibe (Zetia® 10 mg tablets, Schering Corporation, Kenilworth, NJ) was used at indicated concentrations and are consistent with previous published reports[18-20] and are additionally in line with patient daily intake concentrations of 10 mg day^{-1} (i.e. 2.0 – 3.3 µg ml^{-1} serum). Lovastatin (Sigma, St. Louis, MO) and the nucleoside polymerase inhibitor 2'-*C*-methylcytidine[21,22] (a gift from Michael J. Otto, Pharmasset, Inc., Princeton, NJ) were resuspended to concentrations of 100 mM or 10 mM, respectively in DMSO (Sigma) and stored at –20 °C.

DNA constructs

The human SR-BI expression plasmid (pZeo_huSR-BI) was cloned by PCR amplifying the human SR-BI coding sequence (cds) with primers 5'AGG CAA GCT TGC CGC CAT GGG CTG CTC CGC CAA AGC GCG CTG GG 3' (sense) and 5' CCA GTC TAG ACT ACA GTT TTG CTT CCT GCA GCA CAG AGC CC 3' (anti-sense). This product was cloned as a HindIII/XbaI fragment into HindIII/XbaI-digested pZeoSV2(–) (Invitrogen). The JFH-1 E1/E2 glycoprotein expression plasmid (pCDNA3.1_JFHcE1/E2) was cloned by PCR amplifying the polyprotein residues 167 – 751 of pJFH-1 with primers 5'-GAA TTC ATG GGG AAC CTA CCC GG-3' (sense) and 5'-CTC TAG ACT ATG CTT CGG CCT GG-3' (anti-sense). This product was cloned as an EcoRI/XbaI fragment into EcoRI/XbaI-digested pCDNA3.1 (Invitrogen). The vesicular stomatitis virus (VSV) G glycoprotein expression plasmid (pCDNA3.1_VSVG) was cloned by PCR amplifying the VSVG coding sequence from pET-3c (Novagen, Gibbstown, NJ) with primers 5'- GAA TTC ATG AAG TGC CTT TTG TAC TTA GCC -3' (sense) and 5'-CTC TAG ATT ACT TTC CAA GTC GGT T-3' (anti-sense). This product was cloned as an EcoRI/XbaI fragment into EcoRI/XbaI-digested pCDNA3.1 (Invitrogen).

The human NPC1L1 expression plasmid (pCDNA3.1_huNPC1L1) was cloned by PCR amplifying the NPC1L1 cds with primers 5'-GGA TCC ATG GCG GAG GCC GGC CTG AGG-3' (sense) and 5'-GAA TTC ACT TGA CGG CGG GTA AC-3' (anti-sense) from RNA purified from primary human hepatocytes. This PCR product was cloned as a BamHI/EcoRI fragment into BamHI/EcoRI-digested pCDNA3.1 (Invitrogen). pCDNA3.1_huNPC1L1$^{\Delta s26623}$ contains six silent point mutations in pCDNA3.1_huNPC1L1 that ablate the binding of the NPC1L1-specific small interfering RNA oligonucleotide s26623 (see below) but maintain the wild-type amino acid sequence of NPC1L1. It was generated by sequential mutagenesis using the QuikChange® site-directed mutagenesis kit (Stratagene, Agilent Technologies) with complementary pair of oligomers of sequences 5'-CCC TGA CCT CTG GGT TGG CGG TGA TCC TTG ACT TCC TCC-3'and 5'-CCC TGA CCT CTG GGT TGG CGG TCA TTT TGG ACT TCC TCC TGC-3' as per the manufacturer's recommendations.

Pseudotyped retrovirus production and infections

Pseudotyped viruses were produced as previously described[23]. Briefly, pseudotyped viruses were generated by co-transfection of DNA vectors encoding HCV JFH-1 or VSV envelope glycoproteins, with an Env-deficient HIV vector carrying a luciferase reporter gene (pNL4-3-Luc-R$^-$-E$^-$) into 293T producer cells. The plasmids used for pseudotyped virus generation have been described previously[23]. Supernatants were collected 48 h post-transfection, filtered through a 0.45 μm-pore-size filter (BD Biosciences), aliquoted, frozen and subsequently titered using the QuickTiter Lentivirus Titer Kit (Cell Biolabs, Inc., San Diego, CA) according to the manufacturer's instructions. Infectivity titers were determined 72 h p.i. by lysing infected cultures in 20 μl of lysis reagent to measure luciferase activity (Promega, Madison, WI) using a FLUOstar Optima microplate reader (BMG Labtechnologies Inc, Durham, NC).

Treatment of cells and infections

Naïve Huh7 and Huh7 cells harboring HCV subgenomic replicons were seeded and cultured as described above and by Yu *et al.*[24]. For antibody blocking assays, naïve cells were treated with 36 μg ml^{-1} of antibodies for one h prior to infection and subsequently throughout the course of infection. For ezetimibe-mediated acute HCVcc inhibition assays, cells were vehicle-treated or treated with increasing concentrations of ezetimibe 6 h prior to infection (PRE), during the time of infection for 12 h (CO), and/or immediately following infection for 12 to 72 h (POST). For HCVcc infections of transiently-transfected cells, Huh7 cells were first electroporated (Biorad, Hercules, CA), as described[7], with either a control vector pZeoSV2(–) (Invitrogen) or a vector expressing human SR-BI (pZeo_huSR-BI) 48 h prior to infection. HCVcc infections were performed at indicated MOIs (i.e. 0.1, 0.5, or 1.0 FFU $cell^{-1}$). HCVcc chronically-infected Huh7 cells were established by infecting monolayers of naïve Huh7 cells with HCVcc JFH-1 at an MOI of 0.01 FFU $cell^{-1}$. Infected cultures were maintained for an additional 10 days to allow HCV RNA to reach steady-state levels, as confirmed by RTqPCR analysis. Chronically-infected cultures were then treated as indicated. For RTqPCR analysis, total cellular RNA was extracted in 1X Nucleic Acid Purification Lysis Solution (Applied Biosystems, Foster City, CA) from triplicate wells at indicated times p.i. For HCV E2-positive foci assay analysis, medium was removed and cells were fixed with 4% paraformaldehyde (w/v) (Sigma) 72 h p.i. and immunocytochemical staining for HCV E2 was performed as described below.

Immunocytochemical staining of HCV foci

Immunocytochemical staining of HCV E2-positive foci has been previously described[4]. Briefly, fixed cells were first incubated with 1X PBS containing 0.3% (v/v) hydrogen peroxide (Fisher, Fairlawn, NJ) to block endogenous peroxidase. Following three rinses with 1X PBS, cells were blocked for 1 h with 1X PBS containing 0.5% (v/v) Triton X-100 (Fisher), 3% (w/v) bovine serum albumin (BSA) (Sigma) and 10% (v/v) FBS. The HCV E2 glycoprotein was detected by incubation at room temperature with 1X PBS containing 0.5% (v/v) Triton X-100 and 3% (w/v) BSA and a 1:500 dilution of the human monoclonal anti-HCV E2 antibody C1. Bound C1 was subsequently detected by a 1 h incubation with a 1:1000 dilution of an HRP-conjugated anti-human antibody (Pierce) followed by a 30 min incubation with an AEC detection substrate (BD Biosciences). Cells were washed with dH_2O and foci were quantified and photographed using a Zeiss Axiovert microscope (Carl Zeiss, Germany).

RNA interference and infections

Pools of small interfering RNA oligonucleotides (siRNAs) targeting human NPC1L1 (Silencer® Select siRNAs s26623, s26633, and s26634) were purchased from Ambion (Austin, TX). Individual siRNAs directed against human NPC1L1, human CD81, human SR-BI and EGFP (Control siRNA) were purchased from Ambion or Qiagen (Valencia, CA). Huh7 cells, seeded in 75-cm^2 BioCoat™ flasks (BD Biosciences) and differentiated in the presence of cDMEM supplemented with 1% DMSO (Sigma) for 20 days were reverse transfected using Lipofectamine™ RNAiMAX Transfection Reagent as per the manufacturer's instructions. Briefly, for each well a transfection mix consisting of 1 μl

RNAiMAX and 70 nM siRNA in OptiMem (Invitrogen) was incubated for 20 min at room temperature. For siRNA resistant experiments, a transfection mix consisting of 1 µl Lipofectamine™ 2000 (Invitrogen), 70 nM of siRNA s26623 and 1 µg of pCDNA3.1, pCDNA3.1_huNPC1L1, or pCDNA3.1_huNPC1L1$^{\Delta s26623}$ in OptiMem (Invitrogen) was incubated for 20 min at room temperature. Sixty thousand Huh7 cells were then seeded with the transfection mix in 96-well BioCoat™ plates. Twenty-four h post-seeding 200 µl of fresh cDMEM supplemented with 1% DMSO (Sigma) was added to each well. At indicated times post-transfection, cultures were either mock-inoculated or inoculated with JFH-1 HCVcc at an MOI of 0.05 FFU cell^{-1}, and total cellular RNA was harvested for extraction in 1X Nucleic Acid Purification Lysis Solution (Applied Biosystems) from triplicate wells 48 h p.i. for RTqPCR analysis. To assess protein knockdown, at indicated times post-transfection cultures were either trypsinized for flow-cytometric analysis, fixed in 4% PFA (v/v) for indirect immunofluorescence analysis or lysed in 1.25% Triton X-100 lysis buffer for western blot analysis.

Flow cytometric analysis of cell surface receptors

Untreated, vehicle-treated, ezetimibe-treated, siRNA-transfected or transiently-transfected Huh7 cells were resuspended in 150 µl of FACS buffer (1X PBS containing 2% (v/v) FBS, 0.3% (w/v) NaN_3 and 1 mM EDTA) and incubated for 60 min at 4 °C with a 1:100 dilution of antibodies specific for CD81 (AbD Serotec), SR-BI (BD BioSciences), CLDN1 (AbCam), or NPC1L1 (Santa Cruz Biotechnology). Following three rinses with FACS buffer, bound antibodies were detected by incubation for 1 h at 4 °C with phycoerythrin (PE)-conjugated anti-mouse (BD Pharmingen) (for CD81 and SR-BI) or anti-rabbit (Santa Cruz Biotechnology) (for NPC1L1 and CLDN1) antibodies at a dilution of 1:200. Cells stained with irrelevant immunoglobulin G (IgG) antibodies and respective PE-conjugated secondary antibody served as negative controls. Cells were washed three times, fixed in FACS buffer containing 4% (w/v) PFA, and analyzed by flow cytometry using the DakoCytomation CyAn system (Dako, Carpinteria, CA) and Summit Software v4.3 (Dako).

Indirect immunofluorescence analysis

Huh7 cells were fixed with 4% PFA (Sigma) at indicated times and immunofluorescence analysis was performed as previously described[25]. Briefly, fixed cultures were rinsed three times with 1X PBS, permeabilized with 50% Methanol/50% acetone (v/v) (Fisher) and subsequently blocked for 1 h with 1X PBS containing 3% (w/v) bovine serum albumin (BSA) (Sigma) and 10% (v/v) FBS. Cells were stained with a 1:500 dilution of indicated primary antibody overnight at 4 °C, followed by incubation with a 1:750 dilution of an anti-mouse Alexa-555 (for HCV NS5A, CLDN1 or OCLN) or anti-rabbit Alexa-488 (for NPC1L1) conjugated secondary antibody (Molecular Probes) for 1 h at room temperature. Cell nuclei were stained by Hoechst dye. Bound antibodies were visualized via confocal microscopy (630X, Zeiss LSM 510, Germany) and compared to negative control samples stained with irrelevant mouse or rabbit IgG control antibody (Santa Cruz Biotechnology) and appropriate Alexa-555- or Alexa-488-conjugated secondary antibody. Images were analyzed using Zeiss LSM Alpha Imager Browser v4.0

software (Zeiss), and brightness and contrast were adjusted using Adobe®Photoshop® (San Jose, CA).

RNA isolation and RTqPCR analysis

Total intracellular RNA was purified using an ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems), as per the manufacturer's instructions. One μg of purified RNA was used for cDNA synthesis using the TaqMan reverse transcription reagents (Applied Biosystems), followed by SYBR green RTqPCR using an Applied Biosystems 7300 real-time thermocycler (Applied Biosystems). Thermal cycling consisted of an initial 10 minute denaturation step at 95 °C followed by 40 cycles of denaturation (15 sec at 95 °C) and annealing/extension (1 min at 60 °C). HCV, HIV, human GAPDH, murine GAPDH, human NPC1L1 or human SR-BI RNA levels were determined relative to standard curves comprised of serial dilutions of plasmids containing the JFH-1 HCV cDNA, the Env-deficient HIV backbone containing a luciferase reporter gene (pNL4-3-Luc-R^--E^-) or the human GAPDH, murine GAPDH, human NPC1L1 or human SR-BI cds, respectively. The PCR primers used to amplify each respective amplicon were: Universal HCV primers[26] 5'-GCC TAG CCA TGG CGT TAG TA -3' (sense) and 5'- CTC CCG GGG CACTCG CAA GC-3' (anti-sense), HIV[27](sense) 5'-AGT TGG AGG ACA TCA AGC AGC CAT GCA AAT-3' and (anti-sense) 5'-TGC TAT GTC AGT TCC CCT TGG TTC TCT-3', human GAPDH[1] 5'-GAA GGT GAA GGT CGG AGT C-3' (sense) and 5'-GAA GAT GGT GAT GGG ATT TC-3' (anti-sense), murine GAPDH[12] 5'-TCTGGAAAGCTGTGGCGTG-3' (sense) and 5'-CCAGTGAGCTTCCCGTTCAG-3' (antisense), human NPC1L1[28] 5'-TAT GGT CGC CCG AAG CA-3' (sense) and 5'-TGC GGT TGT TCT GGA AAT ACT G-3' (anti-sense) and human SR-BI[29] 5'-TCG CAG GCA TTG GAC AAA CT-3' (sense) and 5'-CTC CTT ATC CTT TGA GCC CTT TT-3' (anti-sense). Additional primers are referenced in[23].

Western blot analysis

Cells were harvested in 1.25% Triton X-100 lysis buffer (Triton X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA) supplemented with a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Fifty micrograms of protein was resolved by SDS-PAGE and transferred to Hybond nitrocellulose membranes (Amersham Pharmacia, Piscataway, NJ). Membranes were sequentially blocked with 5% nonfat milk, incubated with a 1:1000 dilution of a polyclonal rabbit anti-human NPC1L1 antibody (Cell Signaling Technology) or a 1:1000 dilution of monoclonal mouse anti-HCV NS3 antibody (Clone 9-G2, ViroGen), washed 3 times with 1X PBS containing 0.05% Tween20 (v/v), incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (Pierce), and washed again to remove unbound antibody. Bound antibody complexes were detected with SuperSignal chemiluminescent substrate (Pierce).

Cell proliferation and cytotoxicity bioluminescence assays

The ViaLight® Plus Cell Proliferation assay kit (Lonza, Walkersville, MD), which incorporates bioluminescent detection of cellular ATP as a measure of cell viability and proliferation, was used according to the manufacturer's instructions. Briefly, vehicle-

treated and ezetimibe-treated cultures were lysed in Cell Lysis reagent for 10 min. One hundred µl of culture medium was transferred to white 96-well plates (BD Biosciences) containing ATP detection reagent, and luminescence, expressed as relative light units (RLU), was measured (FLUOstar OPTIMA, BMG Labtech). To assess drug-induced cellular toxicity, a bioluminescence-based assay (The Toxilight BioAssay Kit, Lonza) that measures adenylate kinase (AK) released from damaged cells was used as per the manufacturer's instructions. Briefly, 20 µl of supernatant was collected on indicated days and transferred to white 96-well plates (BD Biosciences). One hundred µl of AK detection reagent was then added to each well, and luminescence (RLU) was measured (Fluostar OPTIMA).

HCVcc^{DiD} fusion assay and imaging

Huh7 cells were seeded and cultured as described[4]. Unless otherwise indicated, mock-infected and infected cultures were vehicle-treated or treated with NH_4Cl (10 mM), ezetimibe (30 µM), IgG control antibody (36 µg ml^{-1}), anti-CD81 antibody (36 µg ml^{-1}), or anti-NPC1L1 LEL1 antibody (36 µg ml^{-1}) for one h prior to inoculation, during HCVcc synchronization at 4 °C and during the course of infection. For DiD dequenching analysis, vehicle-treated or treated cultures were mock-infected with cDMEM^{DiD} or infected with HCVcc^{DiD} JFH-1 at an MOI of 5.0 FFU cell^{-1} for 1 h at 4 °C. Plates were then brought to 37 °C and immediately placed in a Fluostar OPTIMA microplate reader (BMG Labtech) and DiD dequenching was measured at 640 nm (excitation) and 670 nm (emission) for 250 cycles in kinetic mode. DiD relative fluorescence units (RFU) values measured in wells devoid of cells were subtracted from reported RFU values. In addition, RFU values measured in cDMEM^{DiD} mock-infected (see **Supplementary Fig. 11b**) and untreated and uninfected cultures (background blank control) were additionally subtracted from RFU values. For imaging, fluorescent images were recorded (630X, Zeiss LSM 510) by exciting CMFDA and DiD with FITC or RITC filters, respectively. Z stack images were analyzed using Zeiss LSM Alpha Imager Browser v4.0 software (Zeiss), and brightness and contrast were adjusted using Adobe®Photoshop® (San Jose, CA).

Quantification of HCV cholesterol content

Ten milliliters of cDMEM or 10 ml of cDMEM containing JFHpp, HCVcc JFH-1 and HCVcc JFH-1^{G451R} with titers of 3.9×10^9 Lentiviral particles (LP) ml^{-1}, 2×10^5 FFU ml^{-1} and 5×10^5 FFU ml^{-1}, respectively, were reduced to 500 µl using Amicon® Ultra-4 Centrifugal Filter Units (Millipore) as per the manufacturer's instructions. Concentrated media or media containing virus was then purified using a HiTrap™ Heparin HP affinity columns (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) as described by Morikawa K *et al*[13]. Heparin-bound fractions, which contain E2-heparin bound HCV[13], were eluted with 1X PBS containing 0.6 M NaCl (w/v) and subsequently concentrated using Amicon® Ultra-4 Centrifugal Filter Units (Millipore). RNA was purified following standard protocols[30] by the guanidine thiocyanate (GTC) method using 1.6X GTC containing 2 µg of murine liver RNA, and HCV or HIV genome copies µl^{-1} of eluted fraction were determined by RTqPCR. Equal genome copies µl^{-1} of elute for each virus and an equal volume of heparin column-purified cDMEM (media background control)

were used in a fluorometric cholesterol detection assay as per the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI).

Establishment, infection and treatment of hepatic xenorepopulated mice

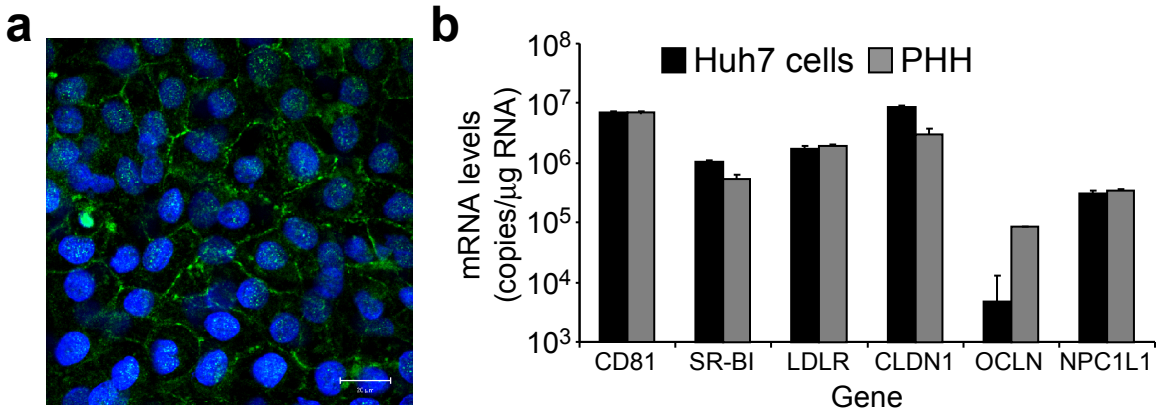
All mouse studies were conducted at Hiroshima University. Human hepatocyte-transplanted mice were generated in severe combined immunodeficient (SCID)/urokinase plasminogen activator (uPA) mice, purchased from PhenixBio (Hiroshima, Japan)[31]. Primary human hepatocytes were purchased from BD Bioscience. All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan. For acute HCV infection experiments, male uPA/SCID mice stably transplanted with human hepatocytes were treated via oral gavage with ezetimibe (10 mg kg^{-1}) resuspended in corn oil (100 µl 20 g^{-1}) starting 2 weeks, 1 week, or 2 d prior to infection or 2 d post-infection. Control mice were treated via oral gavage with corn oil (100 µl 20 g^{-1}). A total of 4 – 7 male mice were included in each group. The mice were intravenously inoculated on d 0 with HCV human serum containing 1.0×10^5 copies of HCV genotype 1b. Serum samples were obtained on indicated days for HCV RNA and/or human albumin determination by RTqPCR and Alb-II Kit (Eiken Chemical, Tokyo, Japan), respectively.

Supplementary References

1. Zhong, J. et al. Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci U S A* **102**, 9294-99 (2005).
2. Gastaminza, P., Kapadia, S.B. & Chisari, F.V. Differential biophysical properties of infectious intracellular and secreted hepatitis C virus particles. *J Virol* **80**, 11074-81 (2006).
3. Zhong, J. et al. Persistent hepatitis C virus infection in vitro: coevolution of virus and host. *J Virol* **80**, 11082-93 (2006).
4. Yu, X. & Uprichard, S.L. Cell-based hepatitis C virus infection fluorescence resonance energy transfer (FRET) assay for antiviral compound screening. *Curr Protoc Microbiol* **Chapter 17**, Unit 17 5 (2010).
5. Blight, K.J., Kolykhalov, A.A. & Rice, C.M. Efficient initiation of HCV RNA replication in cell culture. *Science* **290**, 1972-1974 (2000).
6. Uprichard, S.L., Chung, J., Chisari, F.V. & Wakita, T. Replication of a hepatitis C virus replicon clone in mouse cells. *Virol J* **3**, 89 (2006).
7. Krieger, N., Lohmann, V. & Bartenschlager, R. Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. *J Virol* **75**, 4614-4624 (2001).
8. Kato, T. et al. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology* **125**, 1808-17 (2003).
9. Kato, T. et al. Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. *J Med Virol* **64**, 334-9 (2001).
10. Wakita, T. et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* **11**, 791-6 (2005).
11. Gottwein, J.M. et al. Development and characterization of hepatitis C virus genotype 1-7 cell culture systems: role of CD81 and scavenger receptor class B type I and effect of antiviral drugs. *Hepatology* **49**, 364-77 (2009).
12. Sabahi, A. et al. The rate of hepatitis C virus infection initiation in vitro is directly related to particle density. *Virology* **407**, 110-9 (2010).
13. Morikawa, K. et al. The roles of CD81 and glycosaminoglycans in the adsorption and uptake of infectious HCV particles. *J Med Virol* **79**, 714-23 (2007).
14. Davies, J.P., Scott, C., Oishi, K., Liapis, A. & Ioannou, Y.A. Inactivation of NPC1L1 causes multiple lipid transport defects and protects against diet-induced hypercholesterolemia. *J Biol Chem* **280**, 12710-20 (2005).
15. Law, M. et al. Broadly neutralizing antibodies protect against hepatitis C virus quasispecies challenge. *Nat Med* **14**, 25-7 (2008).
16. Lindenbach, B.D. et al. Complete replication of hepatitis C virus in cell culture. *Science* **309**, 623-6 (2005).
17. Krieger, S.E. et al. Inhibition of hepatitis C virus infection by anti-claudin-1 antibodies is mediated by neutralization of E2-CD81-claudin-1 associations. *Hepatology* **51**, 1144-57 (2009).
18. Ge, L. et al. The cholesterol absorption inhibitor ezetimibe acts by blocking the sterol-induced internalization of NPC1L1. *Cell Metab* **7**, 508-19 (2008).
19. Chang, T.Y. & Chang, C. Ezetimibe blocks internalization of the NPC1L1/cholesterol complex. *Cell Metab* **7**, 469-71 (2008).

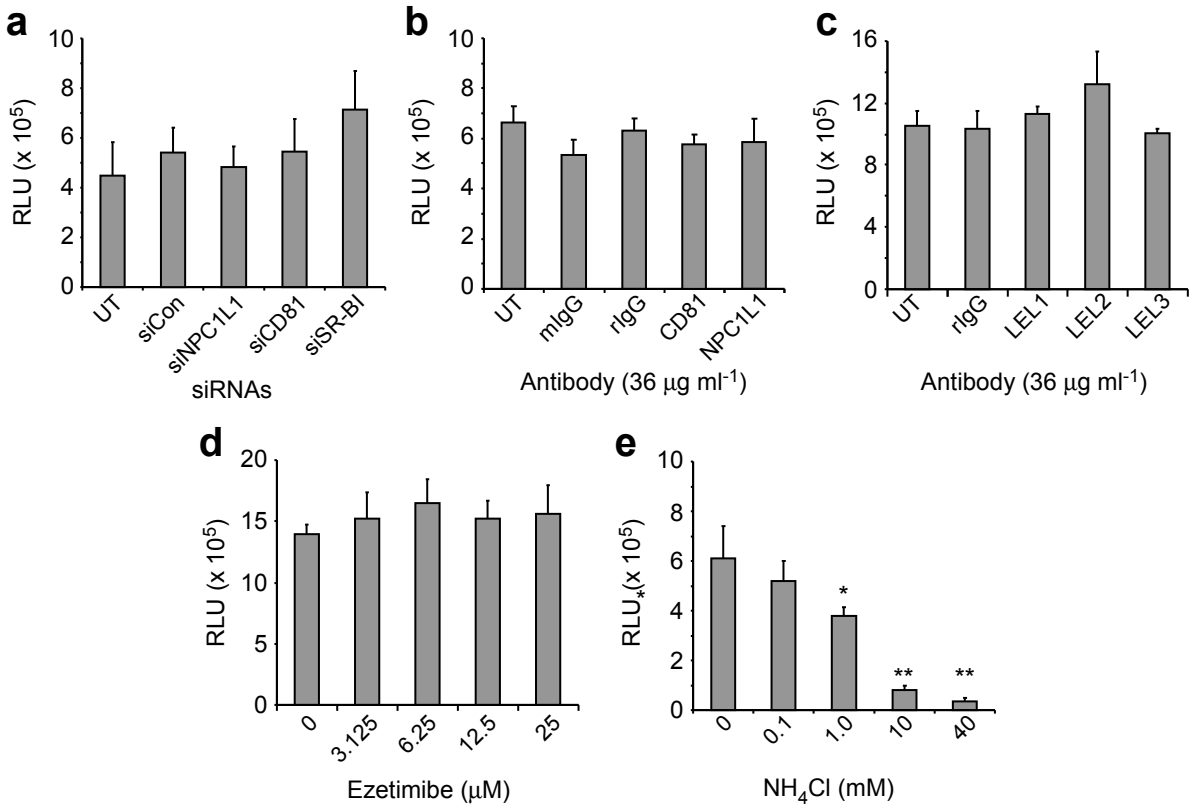
20. Weinglass, A.B. et al. Extracellular loop C of NPC1L1 is important for binding to ezetimibe. *Proc Natl Acad Sci U S A* **105**, 11140-5 (2008).
21. Mathy, J.E., Ma, S., Compton, T. & Lin, K. Combinations of cyclophilin inhibitor NIM811 with hepatitis C Virus NS3-4A Protease or NS5B polymerase inhibitors enhance antiviral activity and suppress the emergence of resistance. *Antimicrob Agents Chemother* **52**, 3267-75 (2008).
22. McCown, M.F. et al. The hepatitis C virus replicon presents a higher barrier to resistance to nucleoside analogs than to nonnucleoside polymerase or protease inhibitors. *Antimicrob Agents Chemother* **52**, 1604-12 (2008).
23. Sainz, B., Jr., Barretto, N. & Uprichard, S.L. Hepatitis C Virus infection in phenotypically distinct Huh7 cell lines. *PLoS ONE* **4**, e6561 (2009).
24. Yu, X., Sainz, B., Jr. & Uprichard, S.L. Development of a cell-based hepatitis C virus infection fluorescent resonance energy transfer assay for high-throughput antiviral compound screening. *Antimicrob Agents Chemother* **53**, 4311-9 (2009).
25. Sainz, B., Jr., TenCate, V. & Uprichard, S.L. Three-dimensional Huh7 cell culture system for the study of Hepatitis C virus infection. *Virol J* **6**, 103 (2009).
26. Komurian-Pradel, F. et al. Strand specific quantitative real-time PCR to study replication of hepatitis C virus genome. *J Virol Methods* **116**, 103-6 (2004).
27. Christopherson, C., Sninsky, J. & Kwok, S. The effects of internal primer-template mismatches on RT-PCR: HIV-1 model studies. *Nucleic Acids Res* **25**, 654-8 (1997).
28. Feng, D., Ohlsson, L. & Duan, R.D. Curcumin inhibits cholesterol uptake in Caco-2 cells by down-regulation of NPC1L1 expression. *Lipids Health Dis* **9**, 40 (2010).
29. Ploss, A. et al. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature* **457**, 882-6 (2009).
30. Chomczynski, P. & Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**, 156-9 (1987).
31. Tateno, C. et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* **165**, 901-12 (2004).
32. Matsumura, T. et al. Amphipathic DNA polymers inhibit hepatitis C virus infection by blocking viral entry. *Gastroenterology* **137**, 673-81 (2009).

***Sainz et al.*, Supplementary Figure 1**



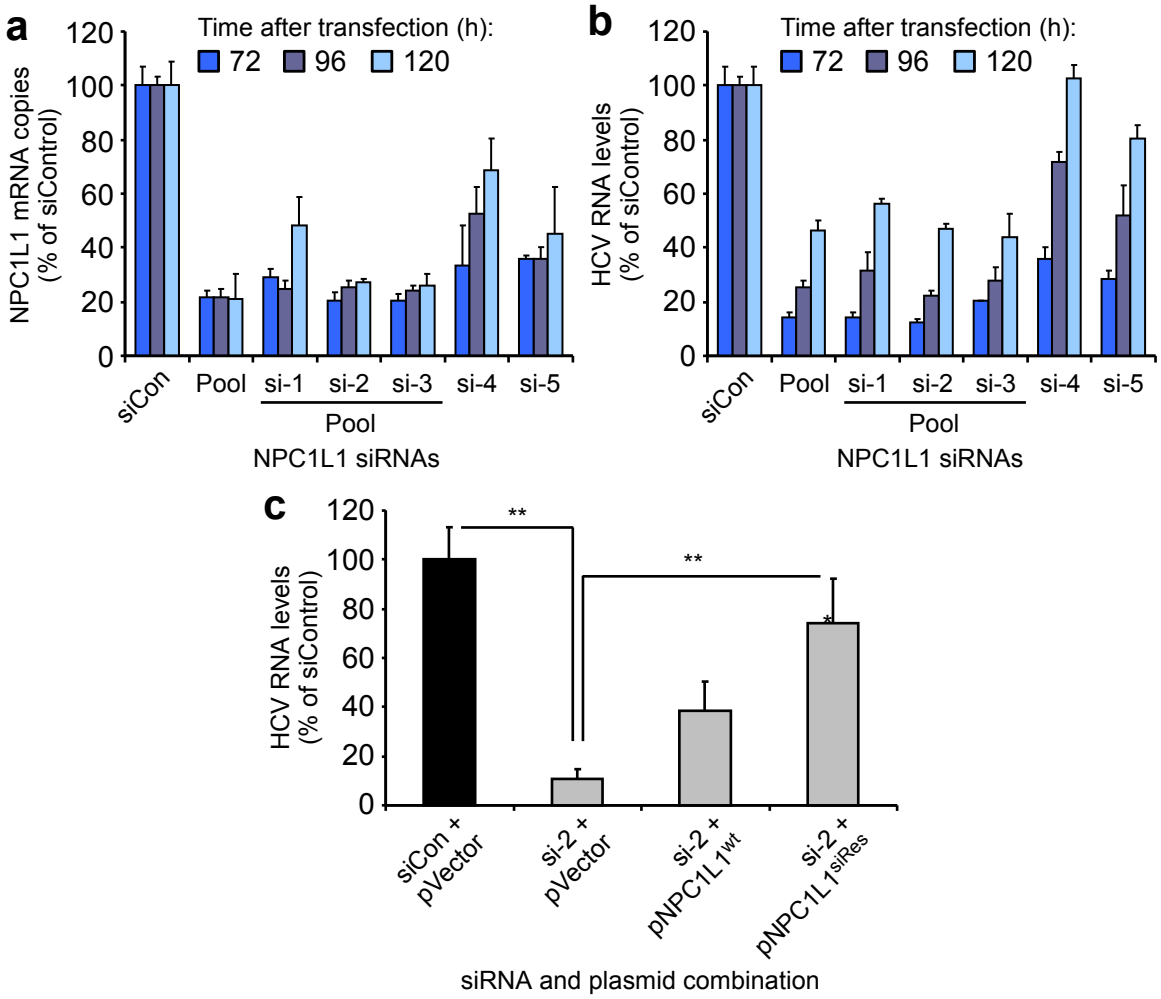
Supplementary Figure 1 Expression levels of NPC1L1 and other HCV receptors in Huh7 cells. (**a**) Confocal analysis of NPC1L1 expression levels on Huh7 cells. Fixed cells were bound with rabbit anti-human NPC1L1 LEL1 monoclonal antibody and stained with anti-rabbit Alexa 488-conjugated secondary antibody. The scale bar = 20 µm. (**b**) Total RNA was extracted from Huh7 cells and primary human hepatocytes (PHH), and mRNA levels of human CD81, SR-BI, low-density lipoprotein receptor (LDLR), CLDN1, OCLN and NPC1L1 were determined by RTqPCR analysis and compared to a plasmid standard curve encoding the open reading frame of each respective gene. Values were normalized to GAPDH and data are displayed as mRNA copies/µg total cellular RNA. Results are graphed as means ± SD for quadruplicate qPCR samples.

***Sainz et al.,* Supplementary Figure 2**



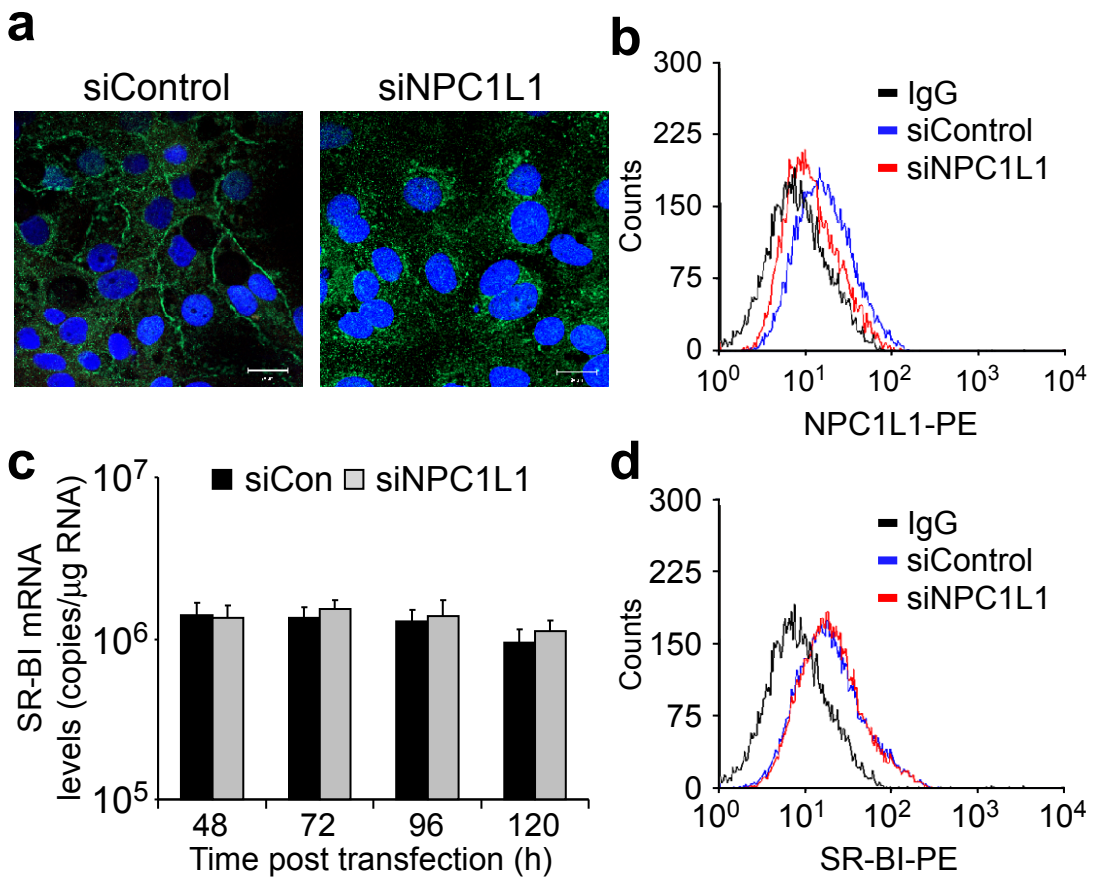
Supplementary Figure 2 siRNA transfection, antibody blocking and ezetimibe treatment do not affect entry of VSVGpp into Huh7 cells. Huh7 cells were either (**a**) reverse transfected with 70 nM of a negative control siRNA (siCon) or SR-BI-specific, CD81-specfic or NPC1L1-specific siRNAs, (**b,c**) treated with 36 μg ml^{-1} of indicated antibodies, (**d**) treated with increasing doses of ezetimibe or (**e**) NH_4Cl, an inhibitor of endosomal acidification, prior to infection with VSVGpp. VSVGpp infection was determined by measuring expression of the virally encoded luciferase reporter gene 72 h p.i and are expressed as relative light units (RLU) ± SD for triplicate samples. Significant reductions in VSVGpp RLU values relative to control-treated cultures were determined by one-way ANOVA and Tukey's post hoc *t* test. Significant reductions are denoted by a single asterisk ($P < 0.05$) or double asterisks ($P < 0.01$).

***Sainz et al.*, Supplementary Figure 3**



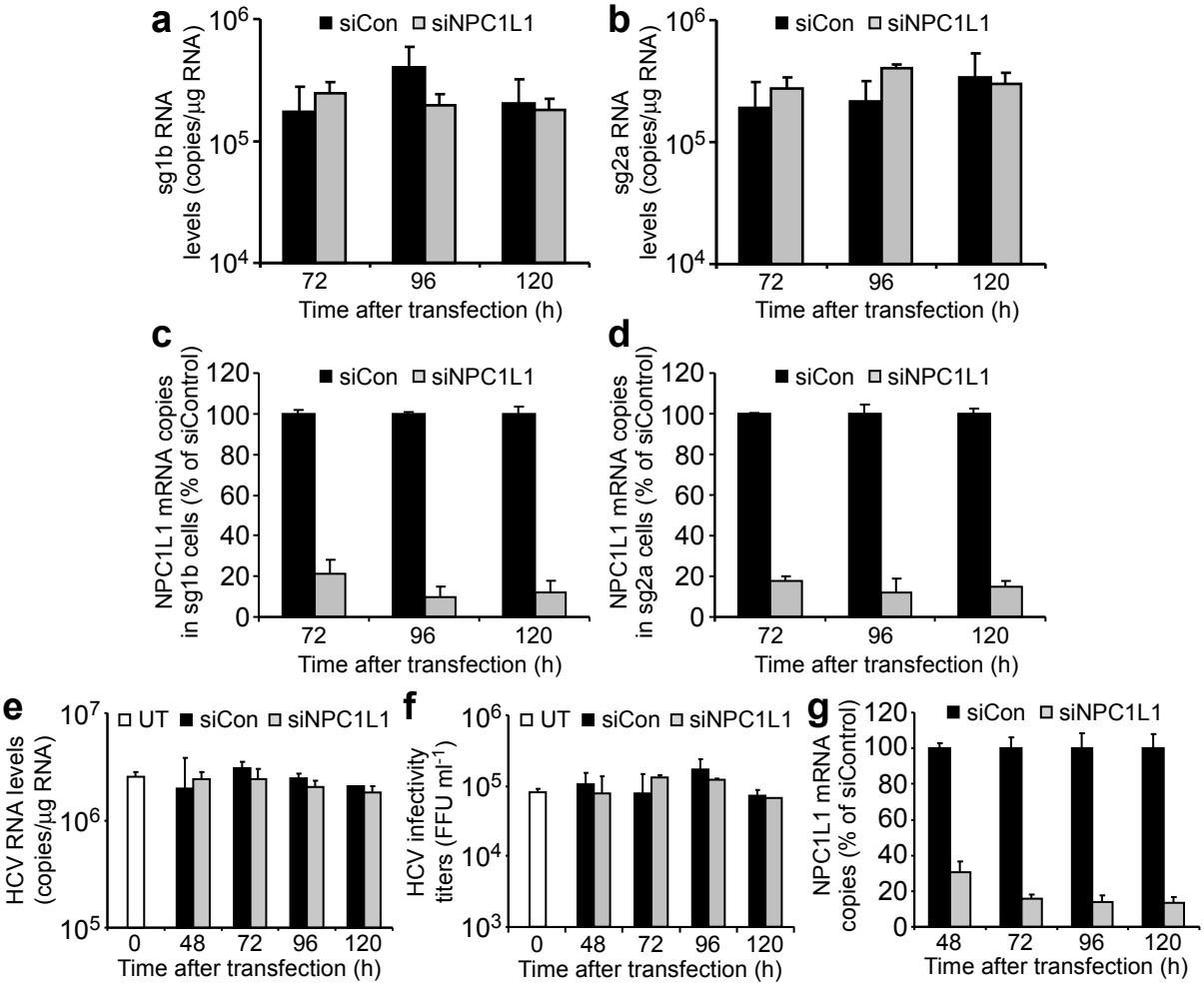
Supplementary Figure 3 Inhibition of HCVcc infection correlates with the degree of NPC1L1 silencing and can be restored by the expression of siRNA-resistant NPC1L1. (**a, b**) Huh7 cells were reverse transfected with 70 nM of negative control siRNA (siCon), a pool of three siRNAs (Pool), or individual siRNAs (si-1 through si-5) targeting NPC1L1 and subsequently HCVcc-infected at an MOI of 0.05 FFU cell^{-1} at indicated times post-transfection. Total cellular RNA was harvested 48 h p.i. and NPC1L1 mRNA and HCV RNA levels were quantified by RTqPCR and normalized to GAPDH. (**a**) NPC1L1 mRNA copies are displayed as a percentage of the copies detected in siCon-transfected cultures at each time point. (**b**) HCV RNA levels are graphed as a percentage of HCV RNA levels in siCon-transfected cultures. (**c**) Huh7 cells were reverse co-transfected with 70 nM siCon or an NPC1L1-specific siRNA (si-2) and a vector control (pVector), a plasmid encoding the wild-type NPC1L1 coding sequence (pNPC1L1^{wt}) or a plasmid encoding an siRNA-resistant NPC1L1 coding sequence (pNPC1L1^{siRes}). Seventy-two h post-tranfection cells were HCVcc-infected at an MOI of 0.05 FFU cell^{-1} and 48 h p.i., total cellular RNA was harvested. HCV RNA was quantified by RTqPCR, normalized to GAPDH and HCV RNA levels are graphed as a percentage of HCV RNA detected in siCon-transfected cultures. All results are graphed as means ± SD for triplicate samples. Significant differences were assessed by one-way ANOVA and Tukey's post hoc *t* test (** $P < 0.01$).

Sainz et al., **Supplementary Figure 4**



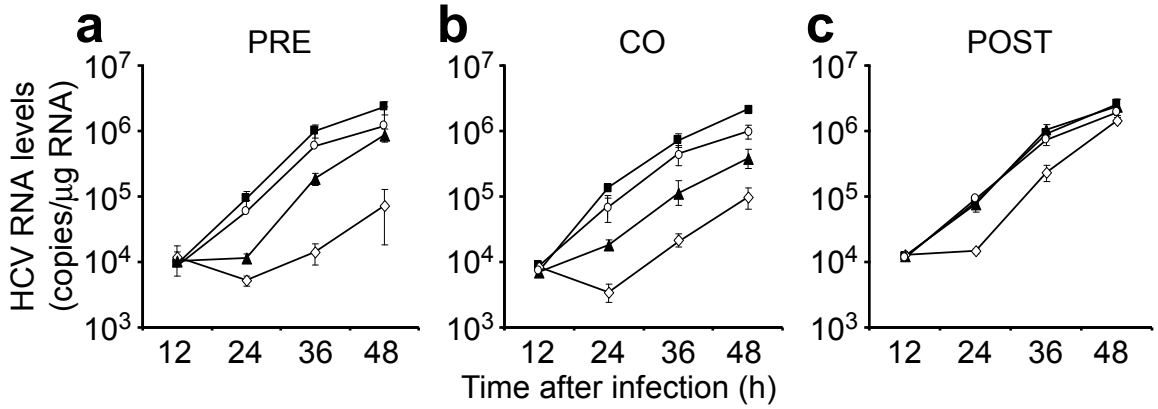
Supplementary Figure 4 NPC1L1 and SR-BI expression in siNPC1L1-silenced Huh7 cells. (**a–d**) Huh7 cells transfected with a negative control siRNA (siControl) or an siRNA targeting NPC1L1 (siNPC1L1) were fixed, trypsinized, or lysed 96 h post-transfection for (**a**) indirect immunofluorescence (IF) analysis, (**b** and **d**) flow cytometric analysis or (**c**) RNA analysis. For IF analysis, fixed cells were incubated with rabbit anti-human NPC1L1 LEL1 monoclonal antibody and stained with anti-rabbit Alexa 488-conjugated secondary antibody. The scale bar = 20 µm. For flow cytometric analysis, siControl- (blue line) and siNPC1L1-transfected (red line) cells were trypsinized and stained with (**b**) a rabbit monoclonal antibody against human NPC1L1 LEL1 and respective anti-rabbit secondary antibody conjugated with PE or (**d**) a mouse monoclonal antibody against human SR-BI and respective anti-mouse secondary antibody conjugated with PE. Black lines represent cells stained with a (**b**) monoclonal rabbit or (**d**) mouse IgG control primary antibody and respective PE-conjugated secondary antibody. (**c**) SR-BI mRNA copies in siCon-transfected (black bars) and siNPC1L1-transfected (grey bars) cultures were determined by RTqPCR, normalized to GAPDH and data are displayed as SR-BI mRNA copies/µg total cellular RNA. Results are graphed as means ± SD for triplicate samples. The data presented are representative of three independent experiments. Significant reductions in SR-BI mRNA levels relative to siCon-transfected cultures at each time point were assessed by one-way ANOVA and Tukey's post hoc *t* test. No significant differences ($P > 0.05$) in SR-BI mRNA levels were observed in siNPC1L1-silenced Huh7 cells.

***Sainz et al.*, Supplementary Figure 5**



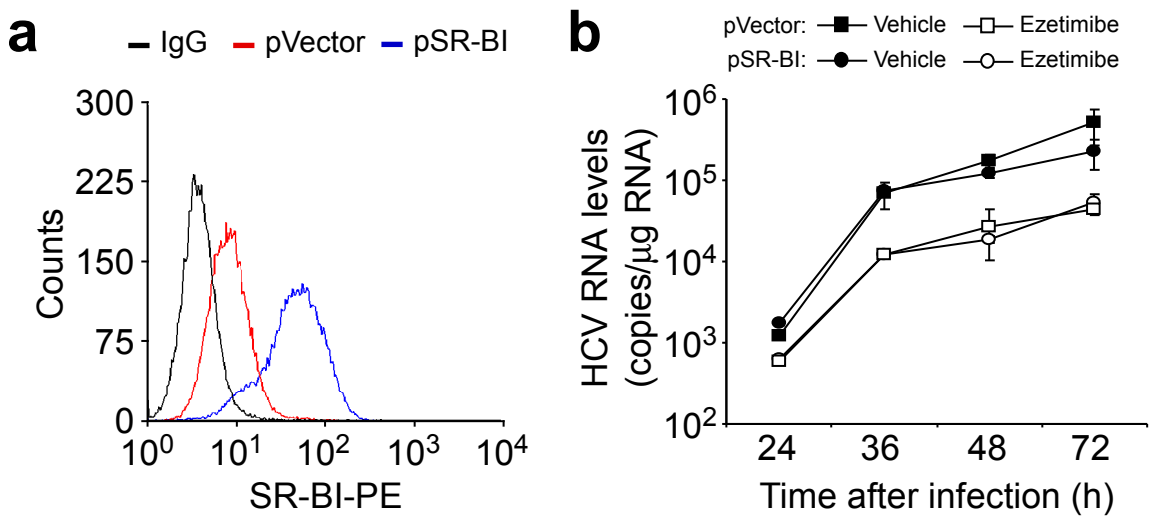
Supplementary Figure 5 NPC1L1 knockdown does not inhibit subgenomic replicon HCV RNA replication, full-length HCVcc RNA replication or secretion of infectious HCVcc. (**a** and **c**) HCV sg1b replicon cells, (**b** and **d**) HCV sg2a replicon cells or (**e–g**) Huh7 cells chronically infected with HCVcc were reverse transfected with 70 nM of a negative control siRNA (siCon) or an siRNA targeting human NPC1L1 (siNPC1L1). At indicated times post-transfection total intracellular RNA was extracted. HCV RNA and NPC1L1 mRNA levels were quantified by RTqPCR and normalized to GAPDH. (**a,b** and **e**) HCV RNA levels are displayed as HCV RNA copies/µg total cellular RNA. Significant reductions in HCV RNA levels relative to siCon-transfected cultures at each time point were assessed by one-way ANOVA and Tukey's post hoc *t* test. A reduction was noted in sg1b replicon cells 96 h post-transfection ($P = 0.08$) in this particular experiment but not in two other independent experiments. (**c,d** and **g**) Intracellular NPC1L1 mRNA copies are displayed as a percentage of the maximum number of copies determined in cells transfected with siCon at each time point examined. (**f**) HCVcc infectivity titers, expressed as mean FFU ml^{-1} ± SD ($n = 3$), were determined as described in Supplementary Methods. Significant differences in HCV titers relative to siCon-transfected cultures at each time point were assessed by one-way ANOVA and Tukey's post hoc *t* test. No significant differences ($P > 0.05$) were observed. All results are graphed as means ± SD for triplicate samples. The data presented are representative of three independent experiments.

***Sainz et al.,* Supplementary Figure 6**



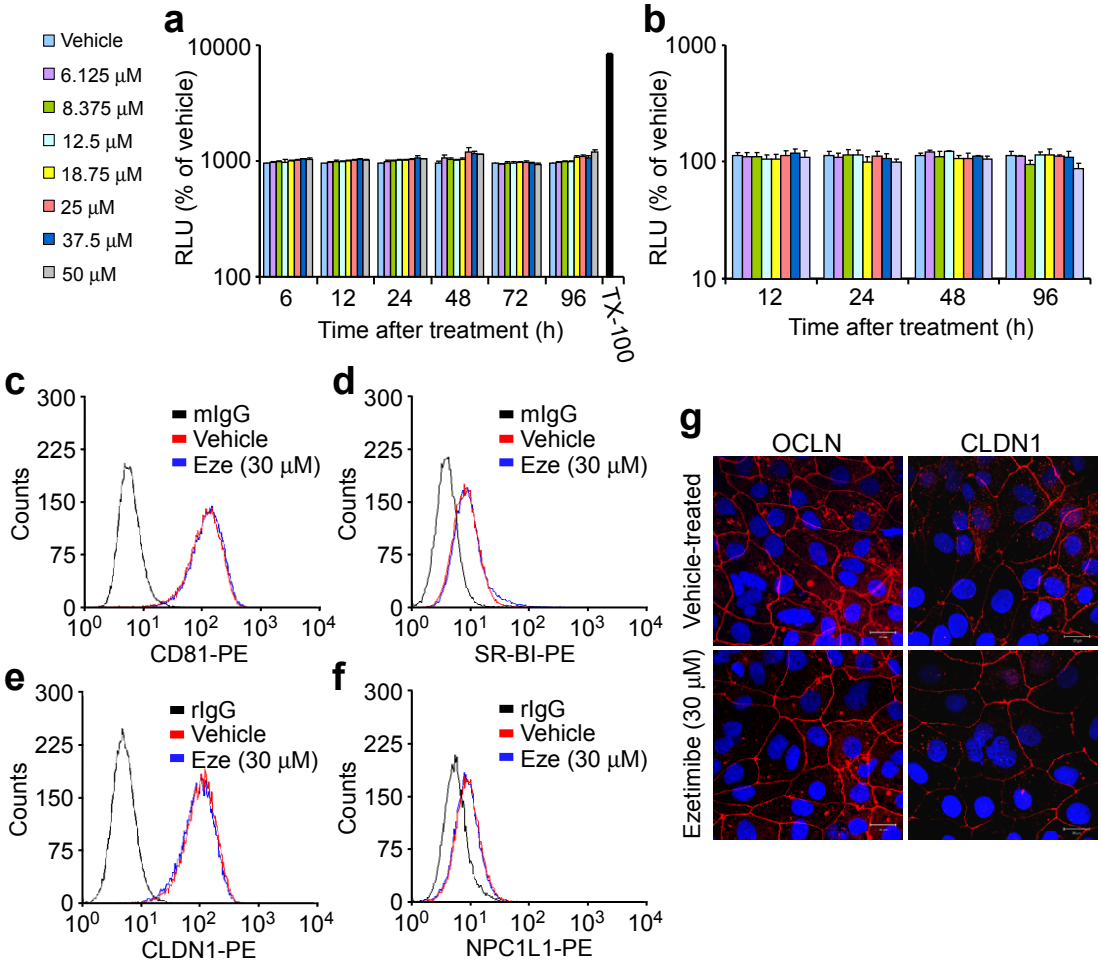
Supplementary Figure 6 Ezetimibe-mediated inhibition of NPC1L1 reduces HCV infection. (**a–c**) Huh7 cells were vehicle-treated or treated with increasing concentrations of ezetimibe (**a**) for 6 h prior to infection and then removed (PRE), (**b**) for 12 h coincident with viral inoculation and then removed (CO), or (**c**) following viral inoculation (POST) with HCVcc at an MOI of 1.0 FFU cell^{-1}. Intracellular RNA was collected at the indicated times p.i. and HCV RNA was quantified by RTqPCR, normalized to GAPDH and are displayed as HCV RNA copies/μg total cellular RNA (0 μM, closed squares, 7.5 μM, open circles; 15 μM, closed triangles; 30 μM, open diamonds). Results are graphed as means ± SD for triplicate samples.

***Sainz et al.*, Supplementary Figure 7**



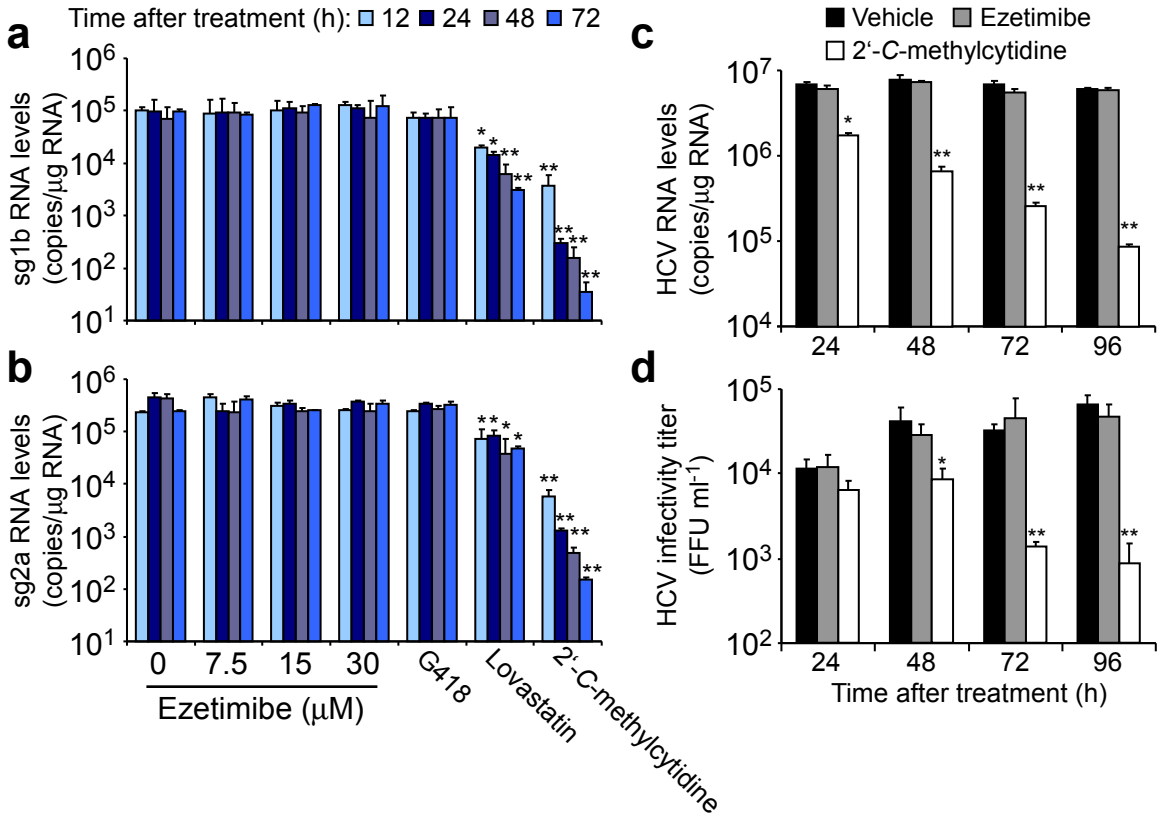
Supplementary Figure 7 Over-expression of SR-BI does not reverse HCVcc NPC1L1-dependence. (**a,b**) Huh7 cells were transfected with a vector control expression plasmid (pVector) or an SR-BI expression plasmid (pSR-BI). Forty-eight h post-transfection, cells were either trypsinized for (**a**) flow cytometric analysis or (**b**) infected with HCVcc at an MOI of 0.01 FFU cell^{-1}. For flow cytometric analysis, trypsinized cells were stained with a mouse antibody against human SR-BI or a mouse IgG control primary antibody and an anti-mouse secondary antibody conjugated with PE. Intracellular RNA was collected at the indicated times p.i. and HCV RNA was quantified by RTqPCR, normalized to GAPDH and is displayed as HCV RNA copies/µg total cellular RNA (vehicle-treated, closed symbols; ezetimibe-treated (30 µM), open symbols). Results are graphed as means ± SD for triplicate samples.

***Sainz et al.*, Supplementary Figure 8**



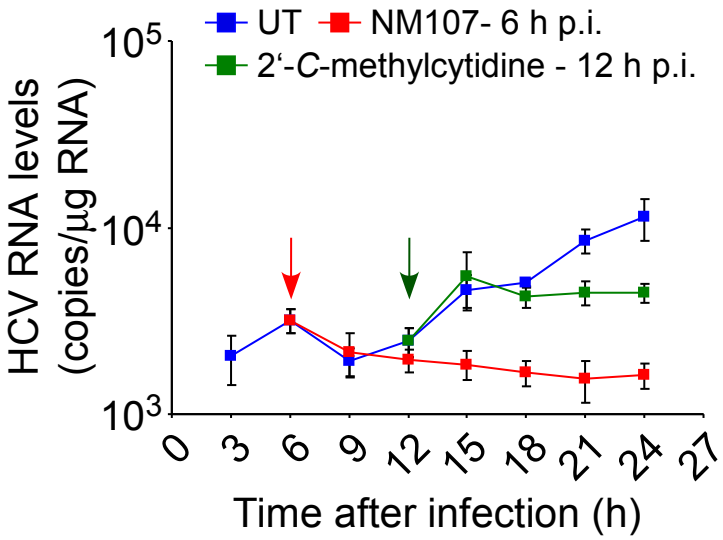
Supplementary Figure 8 Ezetimibe is non-cytotoxic, does not affect cell proliferation and does not reduce cell surface expression of HCV receptors on Huh7 cells. (**a,b**) At indicated time post-treatment, (**a**) culture supernatant was harvested and ezetimibe-induced toxicity was determined by measuring cellular release of adenlyate kinase (AK) using the ToxiLight® Non-destructive Cytotoxicity luminescence assay kit, or (**b**) cultures were lysed and bioluminescence detection of cellular ATP was performed using the ViaLight® Plus Cell Proliferation luminescence-based assay kit as a quantitative measure of cell viability. Triton X-100-lysed Huh7 cells served as positive control for maximum AK release from Huh7 cells. Results are presented as a percentage of relative luminescence (RLU) compared to vehicle-treated cultures. (**c–g**) Flow cytometric analysis of HCV receptors on Huh7 cells vehicle-treated (red line) or treated with ezetimibe (30 µM, blue line) for 24 h. Cells were stained with (**c**) a mouse antibody against CD81, (**d**) a mouse antibody against SR-BI, (**e**) a rabbit antibody against CLDN1 or (**f**) a rabbit antibody against NPC1L1 LEL1 and respective anti-mouse or anti-rabbit secondary antibody conjugated with PE. Black lines represent cells stained with species-specific control primary antibody and respective PE-conjugated secondary antibody. (**g**) Confocal immunofluorescence analysis of OCLN or CLDN1 protein expression on Huh7 cells vehicle-treated or treated with ezetimibe (30 µM) for 24 h. Fixed cells were stained with respective antibodies and counterstained with Hoechst stain (blue) and an Alexa 555-conjugated secondary antibody (red). Scale bar = 20 µm.

***Sainz et al.*, Supplementary Figure 9**



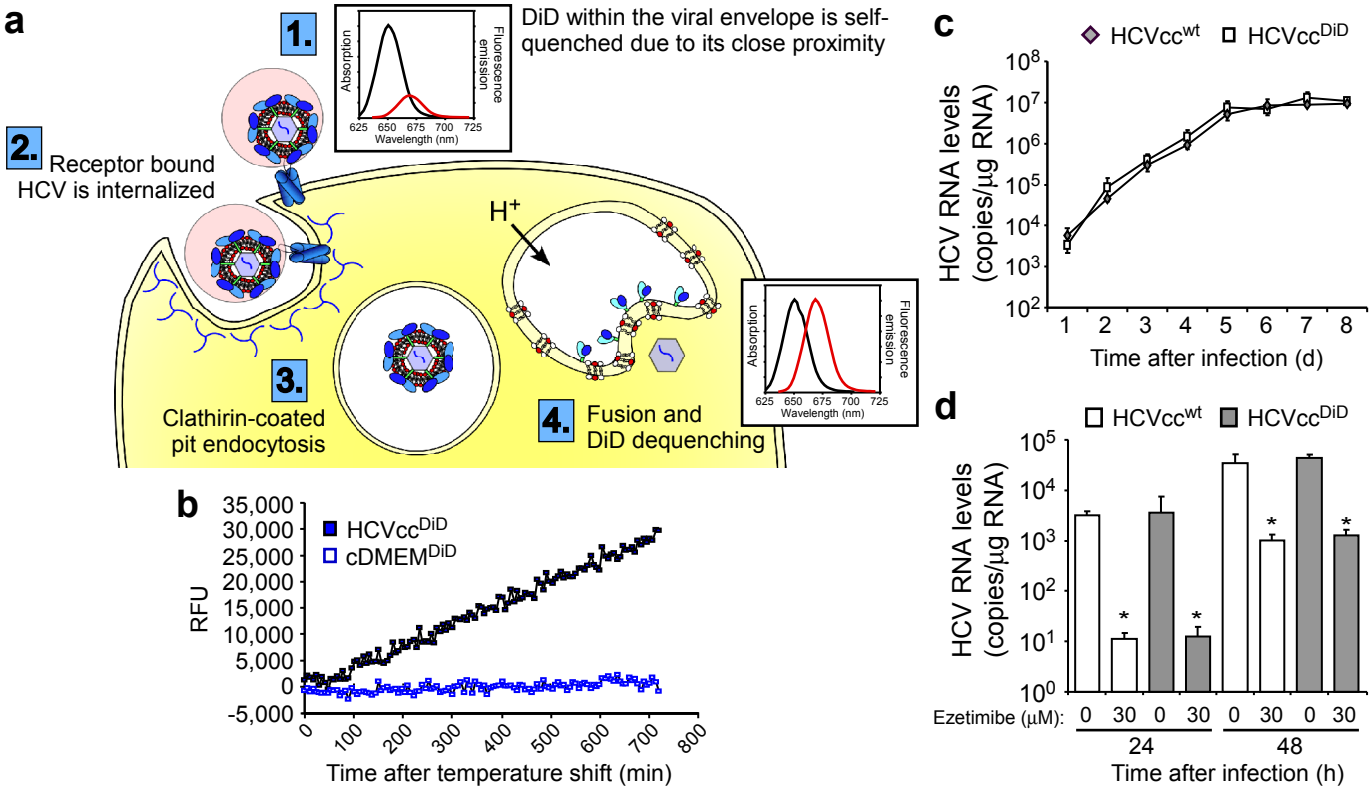
Supplementary Figure 9 Ezetimibe does not inhibit HCV subgenomic RNA replication, steady state HCVcc RNA levels or secretion of infectious HCVcc. (**a**) HCV sg1b replicon cells (**b**) or HCV sg2a replicon cells were vehicle-treated or treated with the indicated concentrations of ezetimibe, G418 (500 µg ml^{-1}), Lovastatin (15 µM), or 2'-*C*-methylcytidine (12.5 µM) for 72 h. (**c,d**) Huh7 cultures were inoculated with HCVcc at an MOI of 0.01 FFU cell^{-1}. Infected cultures were maintained for an additional 10 d to allow HCV RNA to reach steady-state levels. Cultures were then vehicle-treated, treated with ezetimibe (30 µM) or 2'-*C*-methylcytidine (12.5 µM). Fresh medium containing specified treatments was replenished every day throughout the course of the experiment. (**a–c**) Intracellular RNA was collected from triplicate wells at indicated times post-treatment and HCV RNA was quantified by RTqPCR, normalized to GAPDH and is displayed as HCV RNA copies/µg total cellular RNA. Results are graphed as means ± SD for triplicate samples. (d) HCVcc-containing supernatant was collected from triplicate wells at indicated times post-treatment and HCV infectivity titers were determined as described in Supplementary Methods. Infectivity titers are expressed as mean FFU ml^{-1} ± SD for triplicate samples. Significant reductions in HCV RNA copies or supernatant infectivity titers compared to vehicle-treated cultures at each time point (one-way ANOVA and Tukey's post hoc *t* test) are denoted by a single asterisk ($P < 0.05$) or double asterisks ($P < 0.01$). The data presented are representative of three independent experiments.

***Sainz et al.*, Supplementary Figure 10**



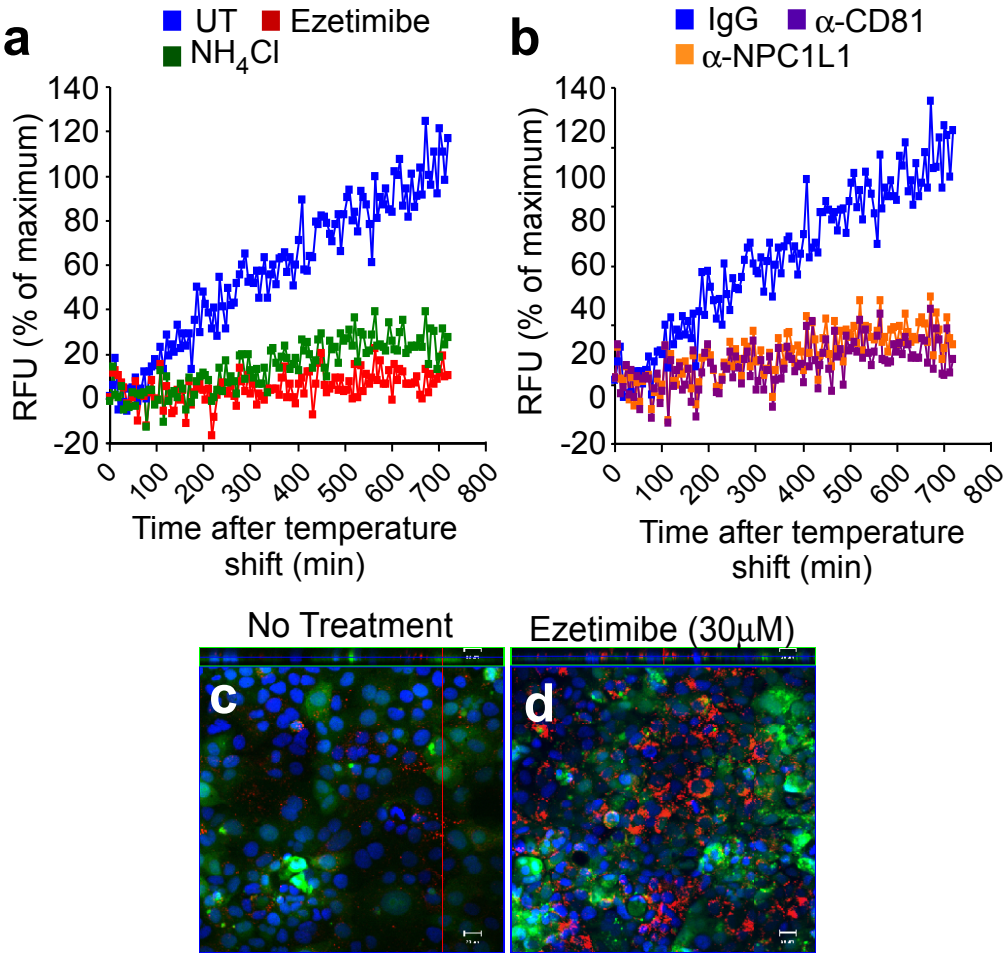
Supplementary Figure 10 Kinetics of HCV RNA replication post-infection. Huh7 cells were infected with HCVcc at an MOI of 3.0 FFU cell^{-1} and at indicated times post-infection (arrows), cultures were treated with the polymerase inhibitor 2'-*C*-methylcytidine (25 µM) to block HCV RNA replication. Intracellular RNA was extracted at indicated times p.i., HCV RNA was quantified by RTqPCR, normalized to GAPDH and is displayed as HCV RNA copies/µg total cellular RNA. Results are graphed as means ± SD for quadruplicate RTqPCR samples. The data indicate that HCV RNA expansion begins ~12 h p.i. UT = untreated.

Sainz et al., **Supplementary Figure 11**



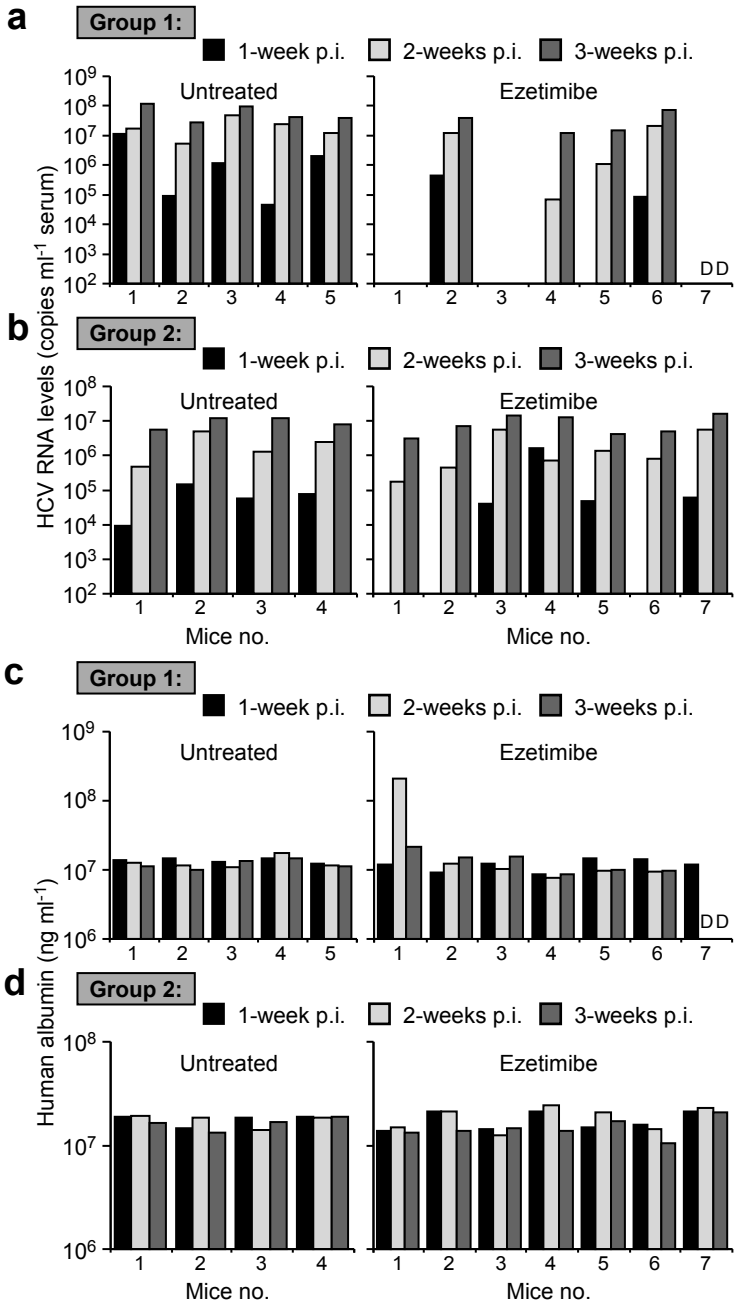
Supplementary Figure 11 HCVcc^{DiD} RNA replication and susceptibility to ezetimibe is equivalent to that of HCVcc^{wt}. (**a**) Diagram of HCVcc^{DiD} cell entry and fusion. Red circles represent DiD fluorophores. (**b**) Confirmation of signal specificity for HCVcc fusion was determined by inoculating Huh7 cells with DiD-labeled, heparin column-purified HCVcc (HCVcc^{DiD}; MOI of 5.0 FFU cell^{-1}) or an equal volume of DiD-labeled and heparin column-purified cDMEM (cDMEM^{DiD}). DiD dequenching was determined every 6 min over the course of 12 h in a fluorescence plate reader. (**c**) To confirm infectivity of HCVcc^{DiD}, Huh7 cells were infected with wild-type (wt) HCVcc^{wt} or HCVcc^{DiD} at an MOI of 0.1 FFU cell^{-1}. At indicated times post-infection total intracellular RNA was extracted and HCV RNA was quantified by RTqPCR, normalized to GAPDH and is displayed as HCV RNA copies/µg total cellular RNA. Results are graphed as means ± SD for triplicate samples. (d) To confirm addition of DiD did not alter HCVcc sensitivity to ezetimibe inhibition, Huh7 cells were treated with vehicle or ezetimibe (30 µM) beginning 1 h prior to and during 6 h inoculation with HCVcc^{wt} or HCVcc^{DiD} at an MOI of 0.1 FFU cell^{-1}. Treatment was continued until intracellular RNA was extracted at indicated times post-infection. HCV RNA was quantified by RTqPCR, normalized to GAPDH and is displayed as HCV RNA copies/µg total cellular RNA. Results are graphed as means ± SD for triplicate samples. Significant reduction in HCV infectivity relative to control-treated cultures (one-way ANOVA and Tukey's post hoc *t* test) is denoted by a single asterisk ($P < 0.01$).

***Sainz et al.*, Supplementary Figure 12**



Supplementary Figure 12 Iodixanol-purified HCVcc^{DiD} fusion kinetics in Huh7 cells. To confirm that the DiD fluorescence observed with HiTrap Heparin HP affinity column-purified HCVcc^{DiD} was specific, we labeled HCVcc with DiD and alternatively purified the labeled virus by iodixanol gradient purification. (**a,b**) Monolayers of Huh7 cells were then treated with vehicle (UT), NH_4Cl (10 mM), ezetimibe (30 μM), IgG control antibody (36 μg ml^{-1}), a mouse antibody against CD81 (36 μg ml^{-1}) or a rabbit antibody against NPC1L1 LEL1 (36 μg ml^{-1}) for 1 h prior to inoculation with iodixanol gradient purified HCVcc^{DiD} (MOI of 5.0 FFU cell^{-1}), during 1 h inoculation at 4 °C and continued during the 12 h assay post-temperature shift to 37 °C. HCV fusion, as measured by DiD dequenching, was determined every 6 min over the course of 12 h at 37 °C. Results are graphed as a percentage of maximum background- and control media-corrected RFU values achieved in vehicle-treated or IgG control-treated cultures. (**c,d**) Confocal images of the apical plane of CMFDA-stained Huh7 cells 24 h after inoculation with HCVcc^{DiD} in the (**c**) absence or (**d**) presence of ezetimibe (30 μM). Small horizontal panels represent x-z sections (apical = top; basal = bottom) of larger x-y sections, which were compiled by taking 0.5 μm steps through corresponding x-y sections. Green lines indicate the plane from which the z section was taken. Scale bar = 20 μm. Blue = Hoechst-stained nuclei, red = DiD-labeled HCVcc, green = CMFDA-stained Huh7 cells.

Sainz et al., **Supplementary Figure 13**



Supplementary Figure 13 Ezetimibe delays the establishment of HCV infection in hepatic xenorepopulated mice. (**a–d**) Human hepatocyte-transplanted uPA-SCID mice[32] were pre-treated with diluent alone ($n = 4 – 5$) or ezetimibe ($n = 7$, 10 mg kg^{-1} day^{-1}), via oral gavage, starting (**a,c**) 2 weeks or (**b,d**) 1 week prior to infection. The mice were intravenously inoculated on day 0 with human serum containing 1.0×10^5 genome copies of HCV genotype 1b. Serum samples were obtained weekly for 3 weeks post-infection for (**a,b**) HCV RNA and (**c,d**) human albumin determination. Graphed are human albumin (ng ml^{-1} of serum) and HCV RNA levels (genome copies ml^{-1} of serum) from diluent- or ezetimibe-treated mice at indicated times p.i. The lower limit of HCV RNA detection is equal to 100 genomic copies ml^{-1} of serum. D = deceased.