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Identification of the Niemann-Pick C1-like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor

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Abstract

Hepatitis C virus (HCV) is a leading cause of liver disease worldwide. With ~170 million individuals infected and current interferon-based treatment having toxic side-effects and marginal efficacy, more effective antivirals are critically needed[1]. Although HCV protease inhibitors were just FDA approved, analogous to HIV therapy, optimal HCV therapy likely will require a combination of antivirals targeting multiple aspects of the viral lifecycle. Viral entry represents a promising multi-faceted target for antiviral intervention; however, to date FDA-approved inhibitors of HCV cell entry are unavailable. Here we show that the cellular Niemann-Pick C1-Like 1 (NPC1L1) cholesterol uptake receptor is an HCV entry factor amendable to therapeutic intervention. Specifically, NPC1L1 expression is necessary for HCV infection as silencing or antibody-mediated blocking of NPC1L1 impairs cell-cultured-derived HCV (HCVcc) infection initiation. In addition, the clinically-available FDA-approved NPC1L1 antagonist ezetimibe[2,3] potently blocks HCV uptake *in vitro* via a virion cholesterol-dependent step prior to virion-cell membrane fusion. Importantly, ezetimibe inhibits infection of all major HCV genotypes *in vitro*,

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Author contributions

B.S.Jr. made the initial discovery. B.S.Jr. and S.L.U. designed the project, analyzed the results and wrote the manuscript. B.S.Jr., N.B., D.N.M, S.H., K.A.M. and X.Y. performed experimental work. B.S.Jr., S.L.U., M.I. and K.C. designed the hepatic xenorepopulation mouse experiments and N.H. performed the *in vivo* studies. W.A.A. was involved in the initial conception of the project and provided valuable expertise.

Conflict of interest

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/nature.

and *in vivo* delays the establishment of HCV genotype 1b infection in mice with human liver grafts. Thus, we have not only identified NPC1L1 as an HCV cell entry factor, but also discovered a new antiviral target and potential therapeutic agent.

HCV is thought to enter cells via receptor-mediated endocytosis beginning with interaction of the viral particle with a series of cell surface receptors, including tetraspanin CD81[4], scavenger receptor class B member I (SR-BI)[5] and tight-junction proteins claudin-1 (CLDN1)[6] and occludin (OCLN)[7,8], followed by clathrin-mediated endocytosis and fusion between the virion envelope and the endosomal membrane[9,10]. While the specifics of each interaction are not fully understood, we now recognize that multiple cellular factors as well as many components of the viral particle, not just the viral glycoproteins, participate in the entry process. For example, the HCVcc particle is associated with cellular lipoproteins (e.g. LDL and VLDL)[11,12] and enriched in cholesterol[13], the latter of which has been shown to be necessary for HCV cell entry[13,14]. Apart from cholesterol likely functioning in viral membrane stabilization and organization, the dependence of HCV infectivity on cholesterol led us to reason that cholesterol-uptake receptors might play a role in HCV cell entry.

NPC1L1, a 13 transmembrane cell surface cholesterol-sensing receptor (Fig. 1a) expressed on the apical surface of intestinal enterocytes and human hepatocytes, including Huh7 cells (Supplementary Fig. 1), is responsible for cellular cholesterol absorption and whole body cholesterol homeostasis[15,16]. Similar to what has been observed for other HCV entry factors[8], we observed down-regulation of NPC1L1 in HCVcc-infected Huh7 cultures. Specifically, as early as d 4 post-infection (p.i.) NPC1L1 protein levels were markedly reduced and remained down-regulated until the end of the experiment at d 12 p.i. (Fig. 1b). Having observed a correlation between NPC1L1 expression and HCV infection, we next determined if NPC1L1 expression levels affect HCV infection by transfecting Huh7 cells with short interfering RNAs (siRNAs) targeting NPC1L1 or the known HCV entry factors CD81 or SR-BI. Compared to cells transfected with an irrelevant-control siRNA, susceptibility to HCVcc infection was significantly reduced in CD81-, SR-BI- and NPC1L1-silenced cells (Fig. 1c). Inhibition was HCV-specific as silencing of these proteins had no effect on vesicular stomatitis virus G-protein pseudotyped particle (VSVGpp) infection (Supplementary Fig. 2a). Inhibition of HCV also correlated with NPC1L1 mRNA and protein reduction and was confirmed to be NPC1L1-specific and not the result of off-target effects (Fig. 1d,e, Supplementary Figs. 3 and 4a,b). Interestingly, although protein levels were only marginally reduced by siRNA knockdown, the effect on HCV was significant, highlighting the sensitivity of HCV to small changes in NPC1L1 levels. Importantly, since SR-BI mRNA expression has been shown to be reduced by NPC1L1 knockdown in non-hepatic cells[17] and SR-BI is an HCV entry factor[5], we confirmed that SR-BI expression was not adversely affected by NPC1L1 silencing in Huh7 cells (Supplementary Fig. 4c,d). Finally, NPC1L1 silencing had no effect on HCV subgenomic RNA replication, full length infectious HCVcc RNA replication, or secretion of *de novo* HCVcc (Supplementary Fig. 5).

Because siRNA-mediated knockdown of NPC1L1 suggested inhibition of HCV infection at a step before RNA replication or secretion, we next assessed the susceptibility of HCV infection to antibody-mediated blocking of cell surface NPC1L1. Compared to cells treated

with irrelevant IgG control antibodies, HCVcc infection was significantly reduced in cells treated with an antibody specific for the known HCV cell entry factor CD81 (Fig. 1f). When we incubated cells with an NPC1L1-specific antibody, HCVcc infection was similarly reduced (Fig. 1f) and inhibition was HCV-specific as antibody-mediated blocking had no effect on VSVGpp entry (Supplementary Fig. 2b,c). To map NPC1L1-specific entry domains, we treated cells with antibodies targeting each of the three large extracellular loops (LELs) of NPC1L1 and observed that HCV infection was reduced only when NPC1L1 LEL1, but not LEL2 or LEL3, was blocked (Fig. 1g). Thus, NPC1L1 silencing and antibody-mediated blocking of NPC1L1 LEL1 reduced HCV infection as effectively as targeting other known HCV cell entry factors.

Ezetimibe is a 2-azetidinone class of drug that has been FDA-approved as a cholesterol-lowering medication[18]. Since ezetimibe has been shown to be a direct inhibitor of NPC1L1 internalization[19,20], we next used this high affinity, specific pharmacological agent as an alternate means of targeting NPC1L1 pre-, co- or post-infection while additionally evaluating its anti-HCV potential. Specifically, we performed HCVcc foci-reduction assays and quantified foci (i.e. clusters of ≥5 HCV E2-positive cells) following ezetimibe treatment. Ezetimibe reduced HCVcc foci formation in a dose-dependent manner when present prior to infection and then removed (Fig. 2a) or only during virus inoculation (Fig. 2b). However, when we added ezetimibe to cells post-infection (Fig. 2c), the initiation of HCV-positive foci was unaffected as would be expected for a viral entry inhibitor. Notably, the highest dose of ezetimibe (25 μM) reduced the foci size observed (i.e. 1 – 3 HCV E2-positive cells per foci) which resulted in a reduced number of ≥5 HCV E2-positive foci being counted suggesting NPC1L1 may also affect HCV cell-to-cell spread (data not shown). Dose-responsive time-of-addition-dependent inhibition of HCV infection was also evident when HCV RNA levels were measured (Supplementary Fig. 6). Importantly, ezetimibe sensitivity was also observed across a panel of HCVcc inter-genotypic clones containing the structural region of diverse HCV genotypes (1 – 7)[21] (Fig. 2d). Finally, because NPC1L1 and SR-BI are both involved in cellular cholesterol uptake and SR-BI has been reported to be a rate-limiting HCV cell entry factor[22], we over-expressed SR-BI prior to ezetimibe treatment to confirm that the dependence on NPC1L1 could not be overcome (Supplementary Fig. 7). Likewise, we confirmed that the potent anti-viral effect of ezetimibe was not due to drug-mediated cytotoxicity (Supplementary Figs. 2d,e and 8a), changes in cell proliferation (Supplementary Fig. 8b), reduction of the other known HCV cell surface receptors (Supplementary Fig. 8c–g), inhibition of HCV RNA replication (Supplementary Fig. 9a–c), or inhibition of virus secretion (Supplementary Fig. 9d). Hence, the data support the conclusion that direct pharmacological inhibition of NPC1L1 reduces HCV infection by directly inhibiting viral cell entry.

We next assessed if ezetimibe inhibits HCVcc binding or a post-binding step by examining cell-associated HCV RNA and protein expression from internalized RNA in vehicle- and ezetimibe-treated HCVcc-infected cultures. At 10 h p.i., a time before detectable HCV RNA replication occurs (Supplementary Fig. 10), ezetimibe did not affect bound/cell-associated HCV RNA levels (Fig 2e). In contrast, at later time points, HCV RNA expansion (Fig. 2e) and *de novo* NS5A protein expression (Fig. 2f,g) were reduced in ezetimibe-treated cultures,

suggesting HCV can efficiently bind to ezetimibe-treated cells, but that a post-binding step is prevented. To further test this hypothesis and determine when during the entry process NPC1L1 functions, we assessed the ability of ezetimibe to block HCVcc infection when added at various times post 4 °C virus binding. Ezetimibe retained inhibitory activity after temperature shift to 37 °C for up to 5 h (half-maximal inhibition at 4 h), confirming that NPC1L1 functions post-binding likely late in viral entry (Fig. 2h).

To determine if ezetimibe acts prior to fusion, we developed a fluorescence-based HCVcc fusion assay. Specifically, we labeled HCVcc with the hydrophobic fluorophore DiD[23], which incorporates into biological membranes and at high concentrations is self-quenching. Upon fusion of viral and target membranes, the DiD fluorophores diffuse away from each other causing dequenching, and the progression or inhibition of fusion can be measured in real time (Supplementary Fig. 11). Compared to NH_4Cl, an inhibitor of endosomal acidification[9], ezetimibe more potently inhibited $HCVcc^{DiD}$ fusion, such that by 12 h post-binding only ~10% $HCVcc^{DiD}$ dequenching was measured in ezetimibe-treated cultures as compared to vehicle-treated controls (Fig. 2i). Analogously, antibody-mediated inhibition of both CD81 and NPC1L1 also reduced $HCVcc^{DiD}$ fusion (Fig. 2j), indicating that the inhibition observed in ezetimibe-treated wells (Fig. 2i) was not drug-specific. Similar results were also observed using $HCVcc^{DiD}$ alternatively purified by iodixanol density gradient centrifugation (Supplementary Fig. 12a,b).

Since not all viral membrane-incorporated DiD is self-quenched, DiD can also serve as a fluorescent tag to monitor virions during cell entry[24]. Taking advantage of this, we performed fluorescence microscopy analysis of $HCVcc^{DiD}$–infected cultures and noted that while little DiD was observed on the surface of vehicle-treated cells, indicative of successful viral entry and fusion, markedly more DiD was observed on the surface of ezetimibe-treated cells (Supplementary Fig. 12c,d). Together with the DiD-fusion data, this indicates that inhibition of NPC1L1 prevents HCVcc cell entry at or prior to virion:host cell fusion.

Since antibody-mediated blocking of only NPC1L1 LEL1 (Fig. 1g) reduced HCVcc infection, LEL1 has been shown to bind cholesterol[20,25] and infectious HCV particles are enriched in cholesterol[13,26], we next investigated whether the dependence of HCV cell entry on NPC1L1 might be related to the cholesterol contained within the HCV virions[13]. To address this hypothesis, we utilized JFH-1-based viruses that differ in their virion-associated cholesterol content and assessed their relative dependence on NPC1L1. Specifically, we show that HCV JFH-1 pseudotype particles (JFHpp) contain 94% less cholesterol than HCVcc JFH-1 while the cell culture-adapted virus JFH-1^{G451R}, which has a distinct density profile[27], contains ~50% more cholesterol than HCVcc JFH-1 (Fig. 3a). These cholesterol profiles are consistent with the fact that HCVpp are produced from 293T embryonal kidney cells, which do not produce cholesterol-associated lipoproteins[28], and are therefore compositionally distinct from HCVcc JFH-1, while HCVcc JFH-1^{G451R} contains a glycine-to-arginine mutation (G451R) in the viral E2 glycoprotein resulting in production of HCVcc having a narrower density range with a higher average mean density[27]. As expected, when CD81 was silenced, both HCVpp (Fig. 3b) and HCVcc JFH-1^{G451R} (Fig. 3c) cell entry was reduced; however, when NPC1L1 was silenced or inhibited by ezetimibe, the cholesterol-scarce JFHpp exhibited NPC1L1-independent cell entry and insensitivity to ezetimibe

inhibition (Fig. 3b and d, respectively). In contrast, the cholesterol-abundant HCVcc JFH-1^{G451R} exhibited enhanced NPC1L1-dependent cell entry and hypersensitivity to ezetimibe-mediated inhibition (Fig. 3c and e, respectively). Together, these data reveal a correlation between the amount of virion-associated cholesterol and dependence on NPC1L1 for HCV cell entry.

Finally, to assess the involvement of NPC1L1 in HCV cell entry *in vivo* we evaluated the ability of ezetimibe to inhibit infection of a genotype 1 clinical isolate in a hepatic xenorepopulation model of acute HCV infection[29]. Specifically, uPA-SCID mice repopulated with human hepatocytes were pre-treated via oral gavage with ezetimibe (10 mg kg^{-1} day^{-1}) or diluent alone for a total of 3 weeks, with treatment beginning 2 weeks, 1 week or 2 d prior to challenge with HCV genotype 1b-positive serum (Fig. 4a). Ezetimibe treatment delayed the establishment of HCV infection in mice pre-treated for 2 weeks prior to infection (Fig. 4b, $P = 0.0192$), confirming the ability of this FDA-approved drug to inhibit HCV infection *in vivo*. However, when mice were pre-treated for only 1 week prior to infection, ezetimibe was less effective at delaying infection ($P = 0.062$) and completely ineffective when treatment was initiated 2 d prior to challenge or after infection had been established (data not shown). Specifically, 100% of the nine control diluent-treated mice were HCV serum-positive 1 week following challenge, while 71% (five out of seven) and 43% (three out of seven) of mice treated with ezetimibe for 2 weeks and 1 week prior to infection were HCV-negative, respectively (Fig. 4b,c). Although the majority of ezetimibe-treated mice eventually became HCV-positive, of the five mice in the 2-week ezetimibe pre-treatment group that were HCV-negative at week 1, two were completely protected remaining HCV-negative at weeks 2 and 3 p.i. (and one mouse died during gavage) (Supplementary Fig. 13). Thus, similar to what was recently reported for another potential HCV entry inhibitor, erlotinib[30], ezetimibe was able to delay initial infection *in vivo*. Notably, since NPC1L1 is highly expressed on the apical surface of intestinal enterocytes[15,16], a significant amount of orally administered ezetimibe initially binds to these cells following oral administration[31]. Thus it is plausible that development of alternate non-oral delivery or drug-targeting methods might improve transport of ezetimibe to hepatocytes and increase its anti-HCV efficacy. Nevertheless, demonstration that ezetimibe can delay the establishment of HCV genotype 1 infection in mice confirms the involvement of NPC1L1 in HCV infection *in vivo* and highlights the therapeutic potential of further pursuing the refinement or development of anti-NPC1L1-based therapies[32] for the treatment of HCV.

Meanwhile, herein we demonstrate that NPC1L1 is an HCV cell entry factor which functions post-binding, at or prior to fusion. These findings, together with the facts that NPC1L1 is a cellular cholesterol receptor, the HCV particle is enriched in cholesterol, and relative dependence on NPC1L1 is correlated with HCV particle cholesterol levels support and expand upon previous reports suggesting that virion cholesterol plays a role during HCV cell entry[13,14,26]. Whether NPC1L1 directly interacts with HCV or indirectly participates in HCV entry by removing virion-associated cholesterol to perhaps reveal protected viral glycoprotein binding sites or confer a required conformational change remains to be determined. Lastly, since NPC1L1 is only expressed on the surface of human and primate

hepatocytes[33,34], this discovery additionally highlights NPC1L1 as a potential HCV tropism determinant, which may facilitate the future development of a small animal model of HCV infection.

Methods

Cell-culture-propagated HCV (HCVcc)

Plasmids containing the full-length JFH-1 genome (pJFH1)[36], full-length JFH-1 genome with a glycine-to-arginine mutation at amino acid residue 451 in the E2 glycoprotein (pJFH-1^{G451R})[37] and the eight intergenotypic clones (described in[21]) were XbaI linearized, transcribed using MEGAscript T7 (Ambion) and 10 μg *in vitro* transcribed RNA was electroporated (BioRad) into Huh7 cells[38]. We generated HCVcc viral stocks by infecting naïve Huh7 cells at a multiplicity of infection (MOI) of 0.01 focus forming units (FFU) cell^{-1} with medium from Huh7 cells electroporated with *in vitro* transcribed RNA from pJFH-1-based vectors , as described[38].

Treatments and analysis

Huh7 cultures were established as previously described[38]. We performed RNA silencing experiments by reverse transfection (Lipofectamine™ RNAiMAX, Invitrogen) of Huh7 cells with indicated siRNAs. Transfected cells were infected with HCVcc at indicated times post-transfection. For antibody experiments, we treated cells with 36 μg ml^{-1} of indicated antibodies prior to and during infection. For ezetimibe inhibition experiments, cells were vehicle-treated or treated with increasing concentrations of ezetimibe prior to infection (PRE), during the time of virus inoculation (CO), and/or following virus inoculation (POST), as indicated. The ezetimibe concentrations of 3.125 – 30 μM (i.e. 1.5 – 12.28 μg ml^{-1} culture medium) used in this study are consistent with previous published reports[19,20,39] and are additionally in line with patient daily intake concentrations of 10 mg day^{-1} (i.e. 2.0 – 3.3 μg ml^{-1} of serum). For RTqPCR analysis, we isolated total cellular RNA from triplicate wells at indicated times post-infection or transfection. For HCV E2-positive foci analysis, we fixed infected cells with 4% paraformaldehyde (w/v) 72 h p.i., and immunocytochemical staining for HCV E2 was performed. See Supplementary Methods for further details.

HCV infection in chimeric mice

All mouse studies were conducted with protocols approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan. Human hepatocyte-transplanted mice generated in severe combined immunodeficient (SCID)/urokinase plasminogen activator (uPA) mice were purchased from PhenixBio[40]. For acute HCV infection experiments, we stably transplanted male uPA/SCID mice with human hepatocytes (purchased from BD Biosciences)[35] and treated them daily with 10 mg kg^{-1} ezetimibe via oral gavage of a 0.02 mg ml^{-1} solution of ezetimibe resuspended in corn oil (100 μl 20g^{-1}) for a total of 3 weeks, with treatment initiation beginning at indicated times prior to infection[16]. Control mice were treated via oral gavage with corn oil alone (100 μl 20g^{-1}). A total of 4 – 7 mice were included in each group. On day 0 we intravenously inoculated mice with HCV human serum containing

1.0×10^5 copies of HCV genotype 1b. We obtained mouse serum samples on indicated days for HCV RNA or human albumin determination by RTqPCR and Alb-II Kit (Eiken Chemical), respectively.

Statistics

Data are presented as the means ± standard deviation (SD). We determined significant differences by one-way analysis of variance (ANOVA) followed by Tukey's post hoc *t* test (GraphPad Prism© Software). To compare categorical variables we used a 2-tailed Fisher's exact test (SPSS v18, Chicago, IL). In all cases a P value < 0.05 was considered statistically significant.

Additional methods

Detailed methodology is described in the Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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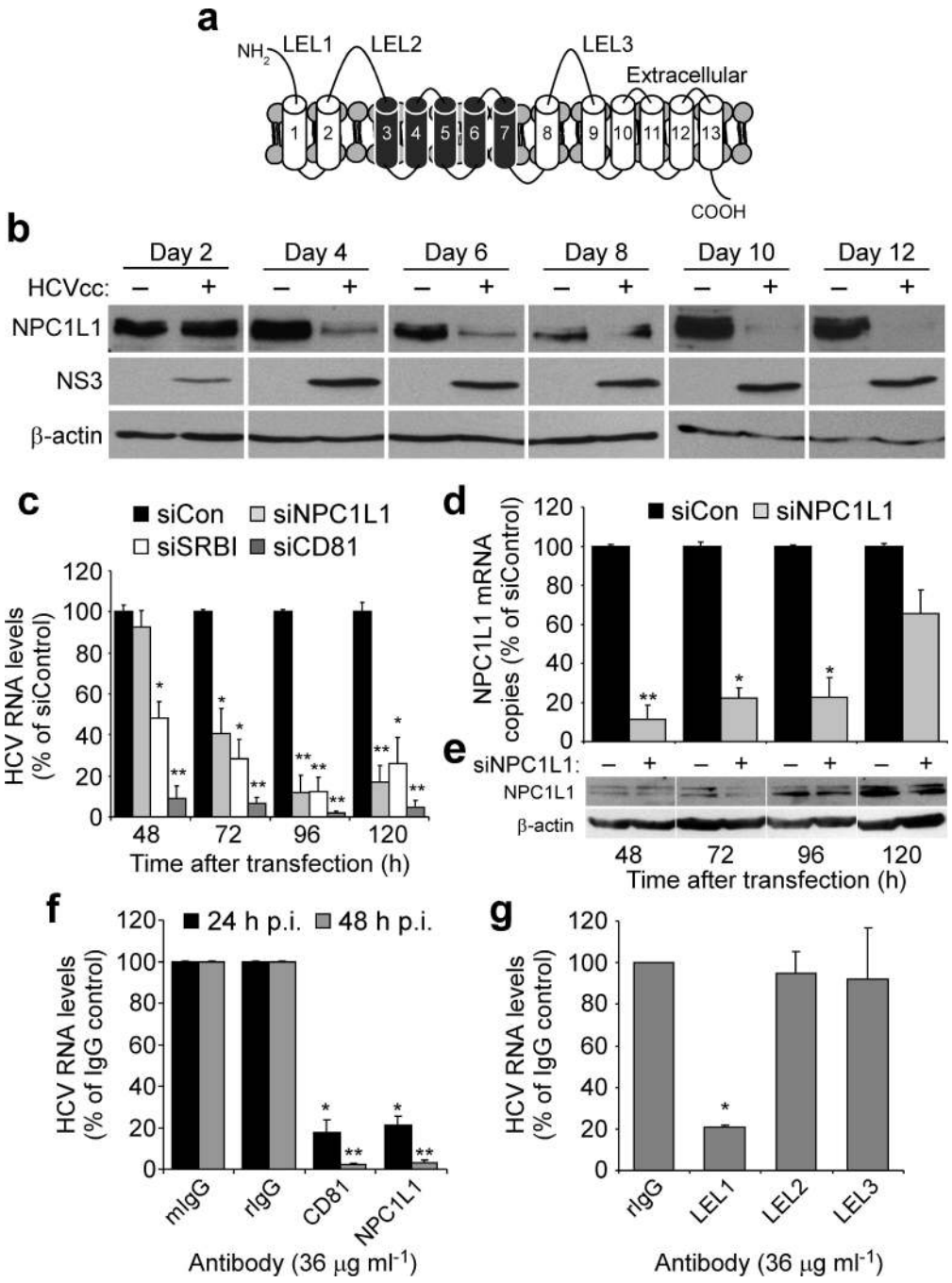

Figure 1.

NPC1L1 plays a role in HCVcc infection. (**a**) NPC1L1 topology. (**b**) Immunoblot of NPC1L1, HCV NS3, and β-actin in Huh7 cells mock-infected or infected with HCVcc at an MOI of 3.0 FFU cell^{-1} over the course of 12 d. (**c–e**) Huh7 cells were mock-transfected or transfected with irrelevant control (siCon), SR-BI-specific, CD81-specfic, or NPC1L1-specific siRNAs and subsequently infected with HCVcc at an MOI of 0.05 FFU cell^{-1} at indicated times post-transfection. (**c**) Forty-eight h p.i. HCV RNA was quantified by RTqPCR and data normalized to GAPDH. Results are graphed as a percentage of infection

achieved in siCon-transfected cultures. (**d**) NPC1L1 transcript levels were quantified by RTqPCR, normalized to GAPDH and are graphed as a percentage of the maximum number of copies determined in siCon-transfected cultures at each time point examined. (**e**) Immunoblot of NPC1L1 and β-actin protein expression in siCon-transfected (–) and siNPC1L1-transfected cultures (+). (**f,g**) Huh7 cells were treated with 36 μg ml^{-1} of indicated antibodies for 1 h prior to and during HCVcc infection at an MOI of 0.05 FFU $cell^{-1}$. HCV RNA levels were determined by RTqPCR analysis 24 (**f**) or 48 (**f** and **g**) h p.i. Data were normalized to GAPDH levels and results are graphed as a percentage of infection achieved in respective IgG control-treated cultures. In all cases, significant differences relative to controls (one-way ANOVA and Tukey's post hoc *t* test) are denoted as * $P < 0.05$ or ** $P < 0.01$. All results are graphed as means ± SD for triplicate samples. The data presented are representative of three independent experiments.

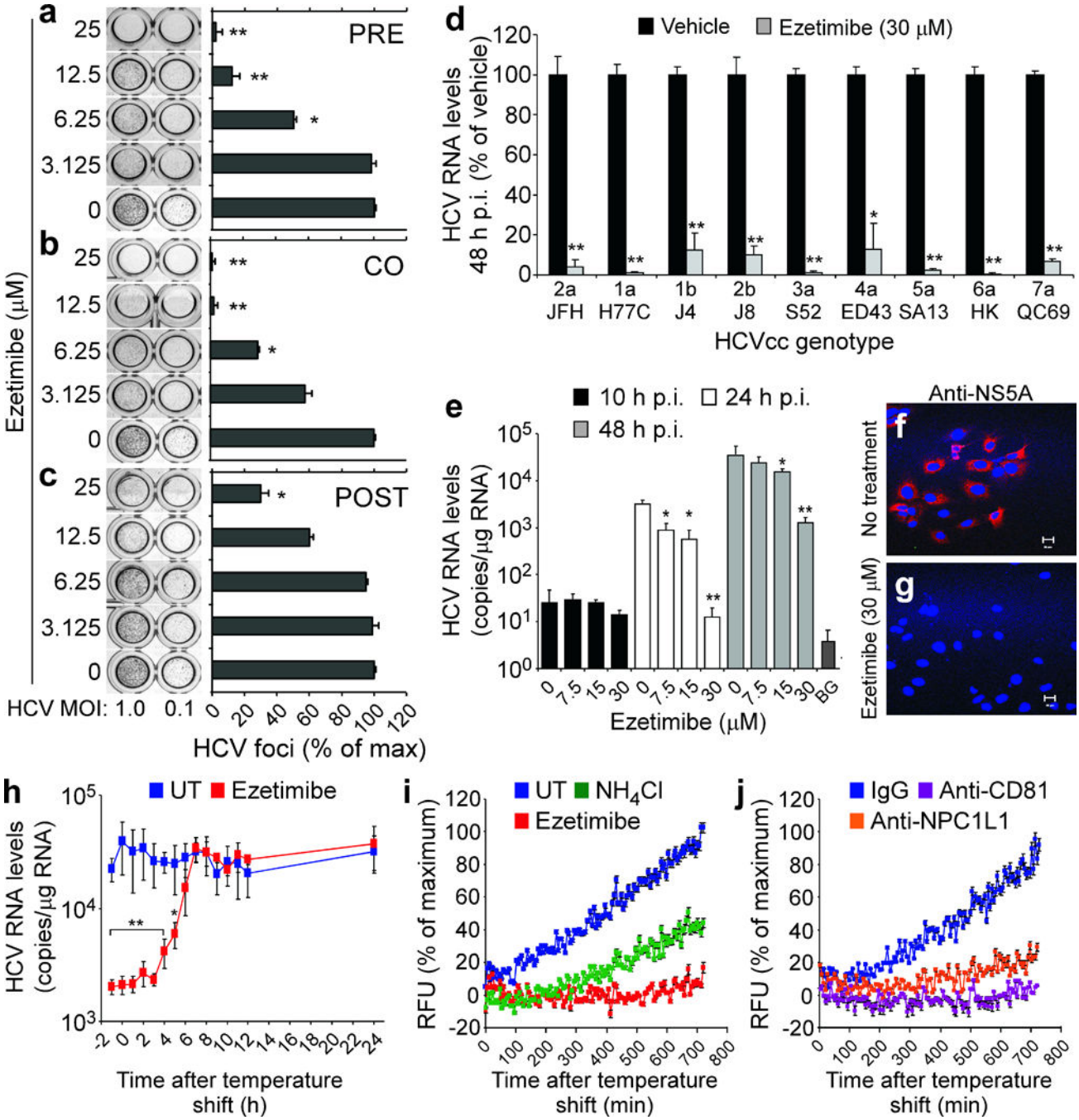

Figure 2.

Ezetimibe-mediated inhibition of NPC1L1 reduces HCV entry at a post-binding pre-fusion step. (**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 or 0.1 FFU cell^{-1}. HCV foci were quantified 72 h p.i. and are expressed as a percentage of the foci obtained in vehicle-treated (0 μM) cultures ± SD (n = 3). (**d–g**) Huh7 cells were treated with vehicle or indicated concentrations of ezetimibe

beginning 1 h prior to and during infection with (**d**) HCVcc containing the structural region of the indicated genotypes or (**e–g**) HCVcc JFH-1 at an MOI of 0.1 FFU cell^{-1}. HCV RNA was quantified by RTqPCR at the indicated times p.i. and data normalized to GAPDH. Results are graphed as a percentage of infection in vehicle-treated cultures or as mean HCV RNA copies/μg total cellular RNA ± SD (n = 3). Assay background (BG) = HCV RNA level detected in uninfected samples. (**f,g**) Indirect immunofluorescence analysis of HCV NS5A in (**f**) vehicle-treated and (**g**) ezetimibe-treated cultures 24 h p.i. Scale bar = 20 μm. (**h**) Synchronized infections in Huh7 cells were treated with vehicle or ezetimibe (30 μM) at the indicated times. Thirty hours p.i. RNA was harvested. HCV RNA was quantified by RTqPCR, normalized to GAPDH and displayed as mean HCV RNA copies/μg total cellular RNA ± SD (n = 3). Significant reduction in HCV relative to vehicle-treated cultures (one-way ANOVA and Tukey's post hoc t test) is denoted as * P < 0.05 or ** P <0.01. (**i,j**) Huh7 cells were treated with vehicle (UT), 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}) beginning 1 h prior to inoculation with HCVcc^{DiD} (MOI of 5.0 FFU cell^{-1}). HCV fusion was measured by DiD dequenching every 6 min. Results are graphed as a percentage of maximum background-corrected relative fluorescence units (RFU) achieved in vehicle-treated or IgG control-treated cultures. All data presented are representative of three independent experiments.

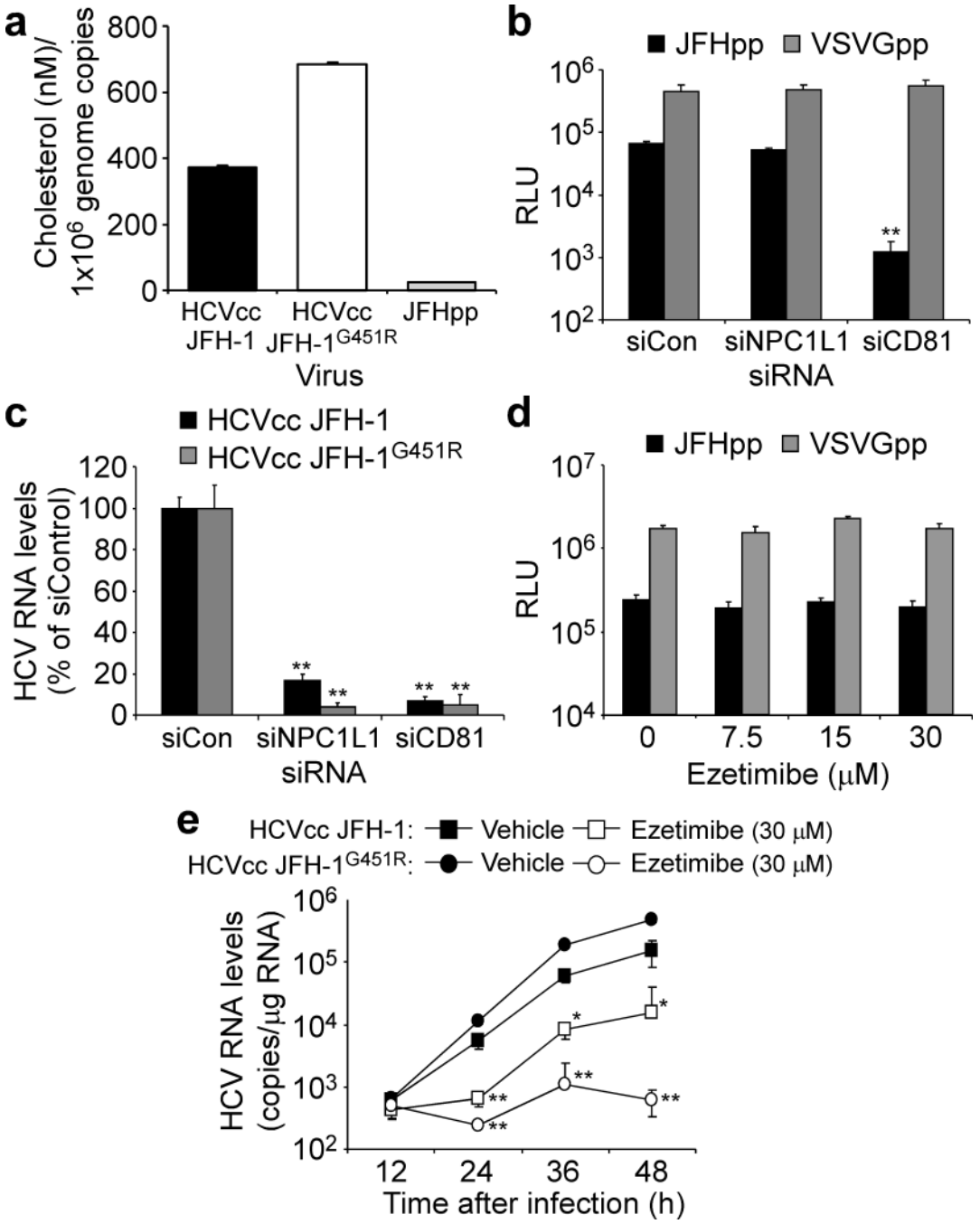

Figure 3.

NPC1L1-mediated HCV cell entry is cholesterol-dependent. (**a**) Cholesterol content of HCVcc JFH-1, HCVcc JFH-1^{G451R} and JFHpp determined by a fluorometric cholesterol quantification assay, after HiTrap™ Heparin HP affinity column purification. Cholesterol content is graphed as cholesterol (nM) per 1×10^6 genome copies determined by RTqPCR. (**b,c**) Huh7 cells were mock-transfected or transfected with indicated siRNA, knockdown was confirmed by RTqPCR (data not shown) and cultures were infected with (**b**) equal titers of JFHpp or VSVGpp or (**c**) HCVcc JFH-1 or HCVcc JFH-1^{G451R} at an MOI of 0.05 FFU

cell^{-1}. JFHpp and VSVGpp infection was determined 72 h p.i and is expressed as relative light units (RLU) ± SD (n = 3). HCV RNA levels were determined by RTqPCR 48 h p.i., normalized to GAPDH and are graphed as a percentage of maximum determined in siCon-transfected cultures. (**d,e**) Huh7 cells were treated with vehicle or increasing concentrations of ezetimibe beginning 1 h prior to inoculation with (**d**) equal titers of JFHpp or VSVGpp or (**e**) HCVcc JFH-1 or HCVcc JFH-1^{G451R} at an MOI of 0.05 FFU cell^{-1}. JFHpp and VSVGpp infection was determined 72 h p.i and is expressed as RLU ± SD (n = 3). HCV RNA was quantified by RTqPCR, normalized to GAPDH and is displayed as mean HCV RNA copies/μg total cellular RNA ± SD. Significant reductions in RNA or RLU values relative to siCon-transfected or vehicle-treated cultures (one-way ANOVA and Tukey's post hoc t test) are denoted as * $P < 0.05$ or ** $P < 0.01$.

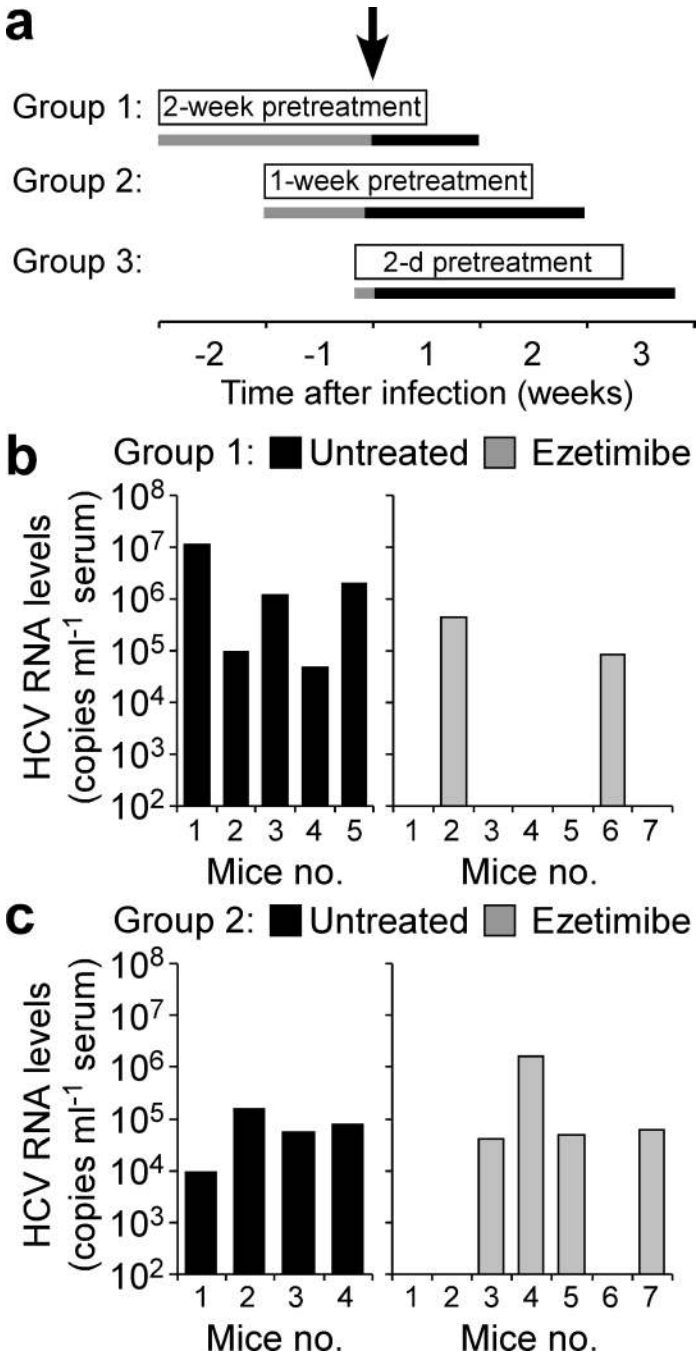

Figure 4.

Ezetimibe delays the establishment of HCV infection in hepatic xenorepopulated mice. (**a**) Schematic diagram of experiment in which uPA-SCID mice transplanted with human hepatocytes[35] were pre-treated with diluent alone ($n = 4 – 5$) or ezetimibe ($n = 7$, 10 mg kg^{-1} day^{-1}), via oral gavage, starting 2 weeks, 1 week or 2 d prior to infection (indicated by grey bars). The mice were intravenously inoculated on d 0 with HCV human serum containing $1.0×10^5$ genome copies of HCV genotype 1b (indicated by arrow) and treatments were continued as indicated (black bars). (**b,c**) Serum samples were obtained weekly for

three weeks post-infection for HCV RNA determination. Graphed are HCV RNA levels (genome copies ml^{-1} of serum) one week post-infection from mice pre-treated for (**b**) two weeks or (**c**) one week. The lower limit of HCV RNA detection is equal to 10^2 genomic copies ml^{-1} of serum. A 2-tailed Fisher's exact test was performed to compare categorical variables. In all cases $P < 0.05$ was used to reject the null hypothesis that the distribution of HCV-positive/HCV-negative mice between ezetimibe-treated and nine diluent-treated mice at specific weeks post-infection were the same.