LETTER

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A genetically humanized mouse model for hepatitis C virus infection

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Hepatitis C virus (HCV) remains a major medical problem. Antiviral treatment is only partially effective and a vaccine does not exist. Development of more effective therapies has been hampered by the lack of a suitable small animal model. Although xenotransplantation of immunodeficient mice with human hepatocytes has shown promise, these models are subject to important challenges. Building on the previous observation that CD81 and occludin comprise the minimal human factors required to render mouse cells permissive to HCV entry *in vitro*[4], we attempted murine humanization via a genetic approach. Here we show that expression of two human genes is sufficient to allow HCV infection of fully immunocompetent inbred mice. We establish a precedent for applying mouse genetics to dissect viral entry and validate the role of scavenger receptor type B class I for HCV uptake. We demonstrate that HCV can be blocked by passive immunization, as well as showing that a recombinant vaccinia virus vector induces humoral immunity and confers partial protection against heterologous challenge. This system recapitulates a portion of the HCV life cycle in an immunocompetent rodent for the first time, opening opportunities for studying viral pathogenesis and immunity and comprising an effective platform for testing HCV entry inhibitors *in vivo*.

Humans and chimpanzees are the only species permissive to HCV infection. The basis for this highly restricted tropism is not completely understood, but may result from viral dependence on host factors present in only a few cell types. Murine cells are resistant to HCV entry, show inefficient viral replication, and may be blocked at later life cycle steps. HCV enters hepatocytes through the combined action of at least four host molecules: CD81 (ref. 1), scavenger receptor type B class I (SCARB1)[2], claudin 1 (CLDN1)[3] and occludin (OCLN)[4]. We have previously shown that CD81 and OCLN comprise the minimal human factors required for HCV uptake by rodent cells[4]. This led to the hypothesis that expression of these human orthologues could render mice susceptible to HCV infection *in vivo*. We therefore constructed recombinant adenoviruses encoding human CD81, SCARB1, CLDN1 and/or OCLN. Intravenous delivery of these vectors resulted in 100 to 1,000-fold overexpression of the corresponding messenger RNA in the murine liver and strong expression of all four proteins with the expected subcellular distribution (Supplementary Fig. 1). We determined that 18–25% of murine hepatocytes expressed human CD81 and OCLN together, and approximately 5% of cells expressed all four heterologous genes (Supplementary Fig. 2b–d). These results encouraged us to investigate infection of these animals. Unfortunately, HCV replication in mouse cells is inefficient *in vitro* and *in vivo*[5–9]. Consistent with this, challenge of mice expressing all four human factors with a firefly luciferase (Fluc)-encoding HCV genome (Jc1FLAG2(p7Fluc2A)) did not yield bioluminescent signal above background (Supplementary Fig. 3a). Direct measurement of Jc1FLAG2(p7Fluc2A) genome levels by polymerase chain reaction with quantitative reverse transcription (qRT–PCR) demonstrated a slight increase in HCV RNA in the serum (at 3 h) and liver (at 3 and 24 h); at 72 h, however, the signal was reduced to background (Supplementary Fig. 3b–d). These data highlight the difficulty of detecting HCV infection in cell types that do not support robust replication. In mouse cells, this defect may result from incompatibility between the viral replication machinery and murine factors and/or from exacerbated murine innate antiviral responses. Furthermore, adenoviral gene delivery strongly induces interferon-stimulated mouse genes, including viperin (also known as *Rsad2*), *Ifi44*, *Mx1*, *Oas1*, *Cxcl10* and *Eif2ak2* (also known as *PKR*), creating an environment that mimics recombinant IFN-α treatment (Supplementary Fig. 4) and may antagonize HCV replication[10].

As an alternative approach, we constructed a bicistronic HCV genome expressing CRE recombinase (Bi-nlsCre-Jc1FLAG2, abbreviated HCV-CRE), which activates a loxP-flanked luciferase reporter in the genome of the Gt(ROSA)26Sor$^{tm1(Luc)Kaelin}$ (Rosa26-Fluc) mouse[11]. Hydrodynamic delivery of HCV-CRE RNA into Rosa26-Fluc mice led to reporter signal in the liver, indicating that CRE recombinase is active in the context of the HCV genome (Supplementary Fig. 5). Delivery of a polymerase-defective HCV-CRE RNA produced similar results, indicating that significant CRE production was derived from initial translation without the need for replication (Supplementary Fig. 5). To test whether mice could be infected by authentic HCV particles, we generated Rosa26-Fluc animals expressing human CD81 and OCLN, or all four human entry factors, and inoculated these mice with cell-culture-derived HCV-CRE. In mice expressing all four transgenes, luciferase signals increased longitudinally, peaked at approximately 72 h after infection, and decreased thereafter; mice lacking the transgenes did not show significant reporter activity (Fig. 1b and Supplementary Fig. 6). All mice expressing at least human CD81 and OCLN could be successfully infected. Loss of signal after 72 h was probably due to strong antivector immunity, as evidenced by the increased frequencies of natural killer (NK) cells (Supplementary Fig. 7a); depletion of NK cells before adenovirus injection prolonged luminescence activity (Supplementary Fig. 7c). Bioluminescent signals were dependent on the doses of both adenovirus and HCV-CRE (Supplementary Fig. 6). HCV core, NS3 or NS5A could not be detected (data not shown), probably owing to inefficient viral replication in murine hepatocytes. To estimate the number of HCV-infected liver cells, we therefore used an indicator mouse strain in which CRE leads to activation of a nuclear-localized green fluorescent protein/β-galactosidase (GNZ) reporter (B6.129-Gt(ROSA)26Sor^{tm1Joe}/J, abbreviated Rosa26-GNZ)[12]. We observed HCV-CRE infection in approximately 4–5% of murine hepatocytes expressing human CD81 and OCLN (Fig. 1c and Supplementary Fig. 2h) and up to 20% expressing all four factors (Fig. 1c). We did not observe HCV uptake in non-parenchymal liver cells. These frequencies are consistent with previous estimates from primary human hepatocyte cultures[13,14] and HCV-positive patient tissues[15], although our system represents a single cycle infection and does not fully reflect the natural equilibrium. Taken together, these data provide— to our knowledge for the first time—evidence that mice can be rendered susceptible to HCV infection by a defined set of human genes.

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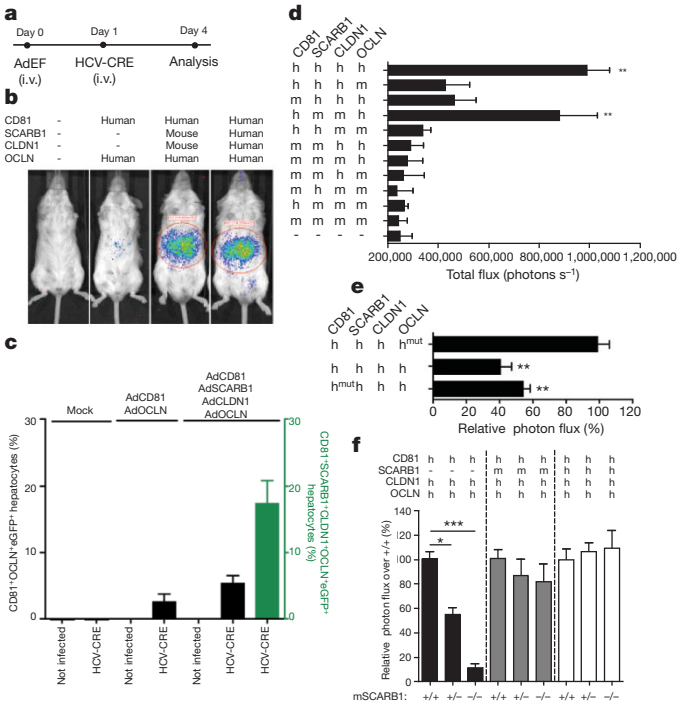

Figure 1 | Genetic requirements for HCV entry *in vivo*. **a**, Timeline for administration of adenovirus and HCV-CRE. **b**, HCV entry into Rosa26-Fluc mice 96 h after injection of medium ($n = 20$), 10^{11} adenovirus particles encoding CD81 and OCLN ($n = 10$) or 10^{11} particles encoding CD81, SCARB1, CLDN1 and OCLN of the indicated species ($n = 20$ each). Bioluminescence was measured at 72 h after injection of 2×10^7 50% tissue culture infectious doses ($TCID_{50}$) of HCV-CRE. **c**, Frequency of HCV-infected hepatocytes. Rosa26-GNZ mice were injected with medium or adenovirus encoding human CD81, mKate-SCARB1, Cerulean-CLDN1 and Venus/YFP-OCLN. Seventy-two hours after injection of 2×10^7 $TCID_{50}$ HCV-CRE, the frequency of infected hepatocytes was determined by flow cytometry. Data are per cent infected ($eGFP^+$) cells relative to either CD81/OCLN transduced (left axis) or CD81/SCARB1/CLDN1/OCLN transduced hepatocytes (right axis) **d**, Rosa26-Fluc mice were injected with human (h) and murine (m) entry factors 24 h before injection of HCV-CRE ($n \geq 4$). **e**, Entry factor mutants reduce infection *in vivo*. Combined delivery of human (h) and mutant human (h^{mut}), hCD81/F186L/E188K or hOCLN/mEL2 ($n = 3$). **f**, mSCARB1 is an essential HCV entry factor *in vivo*. Rosa26-Fluc mice were crossed with mSCARB1$^{-/-}$ mice and offspring was injected with adenoviruses encoding human CD81, SCARB1, CLDN1 and OCLN as indicated, and 24 h later with 2×10^7 $TCID_{50}$ HCV-CRE ($n = 5$–7). Data represent mean ± standard deviation (s.d.). Statistical significance was calculated by Kruskal–Wallis one-way analysis of variance (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

Previous studies in Chinese hamster ovary (CHO) and mouse fibroblast (NIH3T3) cells have shown that CD81 and OCLN are the primary determinants of HCV tropism at the level of entry[4]. To investigate the *in vivo* determinants of species restriction, we delivered combinations of human or mouse CD81, SCARB1, CLDN1 and OCLN to Rosa26-Fluc mice. In accordance with the *in vitro* data, HCV permissivity increased 6–10-fold in mice expressing human CD81 and OCLN, compared to those expressing only murine factors. Notably, addition of exogenous human or mouse SCARB1/CLDN1 (Fig. 1d) boosted permissivity compared to human CD81/OCLN alone (Fig. 1b), indicating that low endogenous entry factor levels may limit infection. Although overexpression of mouse CD81 or OCLN in combination with the other human entry factors permits low-level uptake, human CD81 and OCLN are required for optimal infection. To verify authentic co-receptor function, we delivered entry factors bearing mutations previously shown to disrupt their activities. CD81 mutations F186L and E188K are known to abrogate soluble E2 binding and to reduce HCV uptake by human hepatocytes[16]. Indeed, expression of mutant human CD81 reduced *in vivo* entry efficiency by about 50% (Fig. 1e). A human OCLN chimaera in which the second extracellular loop is replaced with the murine sequence has been shown to impair HCV entry *in vitro* by approximately fourfold[4]. Concordantly, HCV susceptibility of mice expressing the chimaeric molecule was reduced by approximately 50% (Fig. 1e). These data indicate that infection of murine hepatocytes *in vivo* is dependent on the native functions of human CD81 and OCLN.

Unlike the human-restricted entry factors, mouse SCARB1 seems to support HCV uptake *in vitro*[4] and *in vivo* (Fig. 1b). To validate the importance of this endogenous factor, we crossed mice with a disruption in *Scarb1* (SCARB1$^{-/-}$)[17] to the Rosa26-Fluc background. Expression of human CD81, CLDN1 and OCLN in mouse SCARB1$^{-/-}$ Rosa26-Fluc mice reduced HCV-CRE infection efficiency by ~90%, as compared to SCARB1$^{+/+}$, and ~50% compared to SCARB1$^{+/-}$ mice (Fig. 1f). This reduction seems to be a direct consequence of murine SCARB1 deficiency, and not transduction efficiency, as expression levels of CD81, CLDN1 and OCLN were similar in wild-type (Supplementary Fig. 1g) and SCARB1 knockout mice (Supplementary Fig. 8). Complementing mouse SCARB1$^{-/-}$ Rosa26-Fluc mice with human CD81, CLDN1 and OCLN and SCARB1 of either human or mouse origin fully restored infectivity (Fig. 1f). These data provide the first direct evidence that SCARB1 is a bona fide HCV entry factor *in vivo*. Taken together, these results indicate that infection of humanized mice occurs via authentic entry pathways, and highlight the value of this system for studying HCV co-receptor biology *in vivo*.

Despite considerable efforts, the lack of preventative or therapeutic HCV vaccines remains a major clinical challenge. Delivery of antibodies via passive immunization might be particularly suitable to block HCV entry, possibly limiting graft re-infection during liver transplantation and potentially boosting treatment success. Antibodies against E2 and CD81 have been shown to block HCV infection in cell culture[13,18] and human liver chimaeric mice[19,20]. We investigated passive immunization in the genetically humanized model. Delivery of anti-CD81 antibodies resulted in a dose-dependent inhibition of HCV-CRE infection, whereas isotype control immunoglobulins had no effect (Fig. 2a, d). Similarly, pre-incubation of HCV-CRE with antibodies directed against E2 (clone 3/11), but not an isotype control, significantly inhibited infection (Fig. 2b, e). These data further affirm that HCV is taken up in a glycoprotein-specific fashion *in vivo* and suggest the utility of this model for evaluation of passive immunization strategies.

Induction of broad-spectrum adaptive immunity is a key goal of HCV vaccine research. A promising candidate is a recombinant vaccinia virus (rVV) vector expressing HCV proteins, which has been shown to induce adaptive responses in rodents and chimpanzees[21]. We confirmed that HCV infection in our mouse model was observed across a panel of chimaeras expressing the structural proteins of diverse HCV genotypes (Fig. 3a). To evaluate this candidate in genetically humanized animals, Rosa26-Fluc mice were immunized intraperitoneally with 1×10^7 plaque forming units (p.f.u.) of rVV expressing the HCV proteins C-E1-E2-p7-NS2 (strain HCV-1, genotype 1a)[22]. Robust titres of anti-E2 antibodies (Fig. 3b) and decreased susceptibility to heterologous challenge with HCV-CRE expressing the structural proteins of genotypes 1b, 2a, or 4a (Fig. 3c) were observed. Protection directly correlated with the levels of anti-E2 antibodies in the serum. Challenge with genotype 1b (strain Con1) HCV-CRE virus, the envelope proteins of which are closely related to those of HCV-1, resulted in complete protection in two out of ten animals (Fig. 3d–f). Pooled sera of immunized—but not non-immune—mice neutralized diverse HCV genotypes *in vitro* (Supplementary Fig. 9) and reduced infection by 50% when transferred passively (Fig. 2c, f). These data demonstrate the value of the genetically humanized mouse as the first immunocompetent small animal model amenable to HCV challenge.

This study represents an important step forward in developing a robust small animal model for HCV infection and immunity. To our knowledge, this is the first time that any step in the viral life cycle has been recapitulated in a rodent simply by the expression of human genes. Previously developed liver chimaeric mice are susceptible to

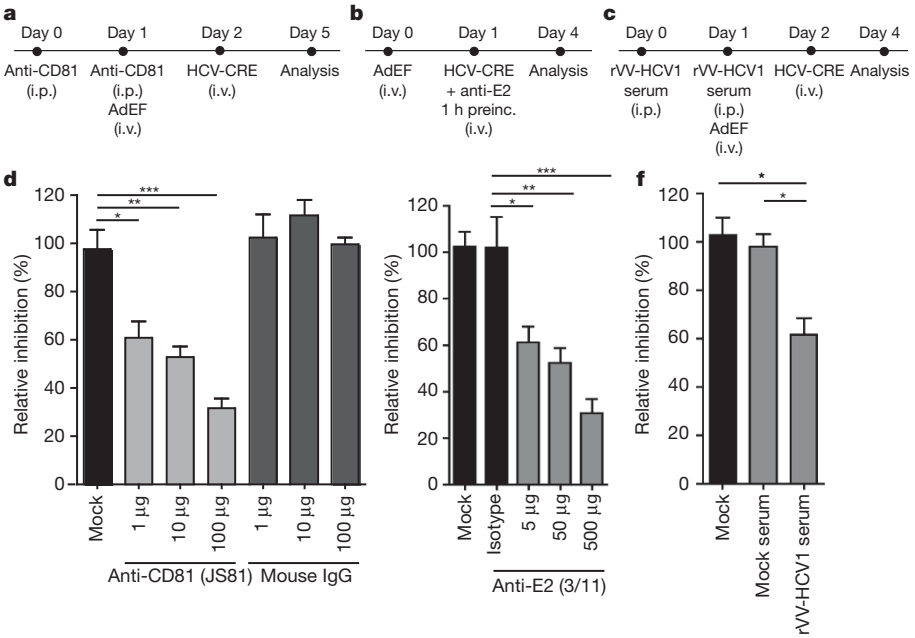

Figure 2 | HCV entry into murine hepatocytes *in vivo* can be blocked by antibodies or passive transfer of vaccine-induced antiserum. **a**–**c**, Experimental layout of CD81 blocking (**a**), virus pre-neutralization with anti-E2 (3/11) (**b**), and *in vivo* neutralization with pooled sera from immunized mice (**c**). i.p., intraperitoneal; preinc., preincubation. **d**, Rosa26-Fluc mice were injected with anti-CD81 antibodies (two doses of 1, 10 or 100 µg per animal at 24 h before and with injection of HCV-CRE; $n = 4$) or isotype control ($n = 3$). **e**, HCV-CRE was incubated with 5, 50 or 500 µg of anti-HCV E2 (clone 3/11) for 1 h before injection into Rosa26-Fluc mice expressing all four human entry factors ($n = 3$). **f**, Rosa26-Fluc mice were injected with 200 µl pooled serum from wild-type (FVB/NJ) mice immunized with rVV-HCV1 or naive control (two doses 24 h before and with injection of HCV-CRE; $n = 4$). Data shown are mean ± s.d. Statistical significance was calculated by Kruskal–Wallis one-way analysis of variance (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

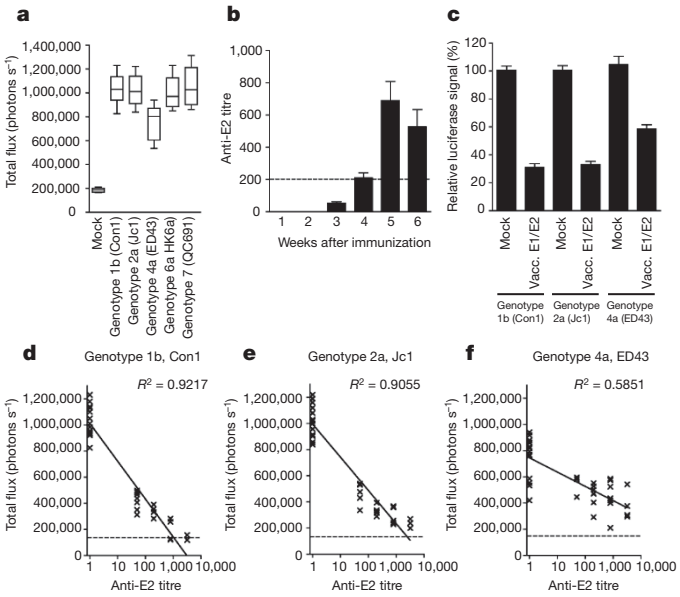

Figure 3 | Use of genetically humanized mouse model to evaluate vaccines against multiple HCV genotypes. **a**, Genetic humanization supports HCV entry mediated by structural proteins of various genotypes. Rosa26-Fluc mice expressing all four human entry factors were infected with intergenotypic chimaeras (Con1 genotype 1b, Jc1 genotype 2a, ED43 genotype 4a, HK6a genotype 6a and QC69 genotype 7a, 2×10^7 $TCID_{50}$ per animal; $n = 3$). **b**, Priming of humoral immune responses with rVV. Rosa26-Fluc mice were injected intraperitoneally with rVV encoding the HCV-1 (genotype 1a) structural genes (10^7 p.f.u. per animal; $n = 10$) and anti-HCV E2 antibody titres were determined by ELISA. **c**–**f**, Protection of rVV-immunized mice against challenge. Immunized mice were challenged with heterologous HCV strains Con1 (1b), Jc1 (2a) and ED43 (4a) and infection was quantified by bioluminescence imaging 72 h later ($n = 5$). Vacc., vaccinated. Data represent mean ± s.d.

HCV infection and support the entire viral life cycle[23–25]. Despite recent improvements[23], however, these models are hampered by low throughput, intra-experimental variability, and high costs[26]. Furthermore, the lack of a functional immune system negates their value for vaccination studies. The inbred mouse model presented here is not only susceptible to infection by diverse HCV genotypes, but it is also fully immunocompetent, providing the first small animal platform suitable for combined immunization and challenge studies. Although it may eventually be desirable to transgenically express the HCV entry factors, adenoviral expression allows rapid testing of various mutant proteins on diverse backgrounds. The genetically humanized mouse model provides proof of principle for such applications and holds promise for assessing the immunogenicity and efficacy of HCV vaccines.

METHODS SUMMARY

Mice. Gt(ROSA)26Sor$^{tm1(Luc)Kaelin}$ (ref. 11), B6;129-Gt(ROSA)26Sor^{tm1Joe}/J (ref. 27) and B6;129S2-Scarb1^{tm1Kri}/J (ref. 17) and FVB/NJ wild-type mice were obtained from The Jackson Laboratory. Mice were bred and maintained at the Comparative Bioscience Center of The Rockefeller University according to guidelines established by the Institutional Animal Committee.

Recombinant adenoviruses. Adenoviral constructs encoding human and murine homologues of the four HCV entry factors (CD81, SCARB1, CLDN1 and OCLN) and mutants thereof were created using the AdEasy Adenoviral Vector System (Agilent Technologies) according to the manufacturer's instructions.

Hepatitis C virus. Plasmids encoding chimaeric HCV genomes, including Jc1FLAG2(p7Fluc2A) and bicistronic HCV genomes expressing CRE, were linearized with XbaI and transcribed using MEGAscript T7 (Ambion). RNA was electroporated into Huh-7.5 cells using an ECM 830 electroporator (BTX Genetronics) and infectious virus was collected from supernatants 48–72 h after transfection.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions M.D., C.M.R. and A.P. designed the project, analysed results and wrote the manuscript. M.D., J.A.H., J.B.R., W.T.B., Q.F., K.M., M.T.C. and M.L. performed the experimental work, J.W.S., C.T.J. and D.R.B. provided reagents.

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METHODS

Animals and cell lines. Gt(ROSA)26Sor$^{tm1(Luc)Kaelin}$ (ref. 11) (Rosa26-Fluc), B6;129-Gt(ROSA)26Sor^{tm1Joe}/J (ref. 27) (Rosa26-GNZ), B6;129S2-Scarb1^{tm1Kri}/J (ref. 17) (SCARB1$^{-/-}$) and FVB/NJ (wild-type) mice were obtained from The Jackson Laboratory. Rosa26-Fluc mice contain the firefly luciferase (luc) gene inserted into the Gt(ROSA)26Sor locus. Expression of the luciferase gene is blocked by a loxP-flanked STOP fragment placed between the luc sequence and the Gt(ROSA)26Sor promoter. CRE recombinase mediated excision of the transcriptional stop cassette results in luciferase expression in Cre-expressing tissues. Rosa26-GNZ knock-in mice have widespread expression of a nuclear-localized green fluorescent protein/β-galactosidase fusion protein (GFP-NLS-GNZ) once an upstream loxP-flanked STOP sequence is removed. When CRE recombinase is introduced into cells the resulting GNZ fusion protein expression allows for enhanced (single-cell level) visualization. Mice were bred and maintained at the Comparative Bioscience Center of the Rockefeller University according to guidelines established by the Institutional Animal Committee. Huh-7.5, Huh-7.5.1, 293T, HepG2 and HEK293 were maintained in DMEM with 10% fetal bovine serum (FBS) and 1% nonessential amino acids (NEAA) and 786-O cells were maintained in RPMI with 10% FBS and 1% NEAA.

Antibodies. Blocking antibodies against CD81 (JS81) and IgG1 control antibodies were obtained from BD Biosciences. Antibodies against NS5A[28] and E2 (clone 3/11)[29] have been described previously. Antibodies for the detection of human CD81 were purchased from BD Biosciences, OCLN from BD Biosciences (for histology) and from Invitrogen (for western blotting), CLDN1 from Invitrogen (for western blotting) and Abcam (for histology), and SCARB1 from Genetex (for histology) and from BD Biosciences (for western blotting). Antibodies for *in vivo* depletion of T cells (anti-mouse CD4 and anti-mouse CD8) were obtained from Biolegend and the antibody for depletion of NK cells (anti-mouse Asialo GM1) was purchased from Cedarlane. Fluorochrome-conjugated antibodies against mouse CD3, CD4, CD8, CD49b, Ly-6G were obtained from BD Biosciences and the IRDye700-conjugated anti-Luciferase antibody was acquired from Acris antibodies and Bethyl Laboratories.

Adenovirus constructs. Adenoviral constructs encoding human and murine homologues of the four HCV entry factors (CD81, SCARB1, CLDN1 and OCLN) and fluorescently tagged human SCARB1, CLDN1 and OCLN were created using the AdEasy Adenoviral Vector System (Agilent Technologies) according to the manufacturer's instructions. Briefly, human and murine entry factor cDNA was PCR-amplified from TRIP-based constructs[4] and inserted into the pShuttle-CMV using KpnI/XhoI sites (KpnI/NotI for human SCARB1). Mouse Kate-SCARB1, Cerulean-CLDN1, and Venus-OCLN fusions were cut from existing TRIP-based constructs and inserted into pShuttle-CMV using compatible restriction sites. Recombinant pShuttle-CMV plasmids were linearized with PmeI and ligated to pAdEasy by homologous recombination followed by electroporation into BJ5183 cells (Agilent). Recombinant pShuttle-pAdEasy constructs were identified by PacI restriction analysis. All plasmid constructs were verified by DNA sequencing.

Primer sequences were as follows. Human *CD81*, forward primer, CGCGGT ACCCCACCATGGGAGTGGAGGGCTGCAC; reverse primer, ACGCTCGAG TCAGTACACGGAGCTGTTCC. Mouse *Cd81*, forward, GCATACAGGGTAC CGCCACCATGGGAGTGGAGGGCTGCACCAAA; reverse, GCATACAGCT CGAGTCAGTACACGGAGCTGTTCCGGAT. Human *SCARB1*, forward, CGC GGTACCCCACCATGGGCTGCTCCGCCAAAGC; reverse, CAGCGCTGCG GCCGCCTACAGTTTTGCTTCCTGCA. Mouse *Scarb1*, forward, GCATACAG GGTACCGCCACCATGGGCGGCAGCTCCAGGGCGCGC; reverse, GCATAC AGCTCGAGCTATAGCTTGGCTTCTTGCAGCAC. Human *CLDN1*, forward, CGCGGTACCCCACCATGGCCAACGCGGGGCTGCA; reverse, ACGCTCGA GTCACACGTAGTCTTTCCCGC. Mouse *Cldn1*, forward, GCATACAGGGTA CCGCCACCATGGCCAACGCGGGGCTGCAGCTG; reverse, GCATACAGC TCGAGTCACACATAGTCTTTCCCACTAGA. Human *OCLN*, forward, CGCG GTACCCCACCATGTCATCCAGGCCTCTTGA; reverse, ACGCTCGAGCTA TGTTTTCTGTCTATCAT. Mouse *Ocln*, forward, GCATACAGGGTACCGCC ACCATGTCCGTGAGGCCTTTTGAAAGT; reverse, GCATACAGCTCGAGC TAAGGTTTCCGTCTGTCATAATC.

Production of recombinant adenoviruses. Adenoviral stocks were generated as previously described[30]. Briefly, adenoviral constructs were transfected into HEK293 cells (American Type Culture Collection) using the calcium-phosphate method. Transfected cultures were maintained until cells exhibited full cytopathic effect (CPE), then harvested and freeze-thawed. Supernatants were serially passaged two more times with harvest at full CPE and freeze thawed. For virus purification, cell pellets were resuspended in 0.01 M sodium phosphate buffer pH 7.2 and lysed in 5% sodium-deoxycholate, followed by DNase I digestion. Lysates were centrifuged and the supernatant was layered onto a 1.2–1.46 g ml^{-1} CsCl gradient, then spun at 95,389*g* on a Beckman Optima 100K-Ultra centrifuge using an SW28 spinning-bucket rotor (Beckman-Coulter). Adenovirus bands were isolated and further purified on a second CsCl gradient using an SW41.Ti spinning-bucket rotor. Resulting purified adenoviral bands were isolated using a 18.5G needle and twice-dialysed against 4% sucrose. Adenovirus concentrations were measured at 10^{12} times the OD_{260} reading on a FLUOstar Omega plate reader (BMG Labtech). Adenovirus stocks were aliquoted and stored at −80 °C.

Production of recombinant vaccinia virus. rVV expressing HCV-1 C-NS2 (rVV E12 C/B from Chiron Corporation) was obtained through the National Institutes of Health AIDS Research and Reference Reagent Program[31,32]. rVV was amplified by infecting HeLa S3 cells in suspension culture at a multiplicity of infection of 0.5 for 48 h. Intracellular virus was released by three freeze–thaw cycles and sonication. The virus was pelleted over a 36% sucrose cushion and resuspended in PBS[33].

HCV genome construction. Jc1FLAG2(p7Fluc2A) is a fully infectious HCV reporter genome similar to Jc1FLAG2(p7nsGluc2A)[34]. This monocistronic genome encodes a Flag epitope, followed by a Gly-Ser-Gly-Ala linker, fused to the N terminus of E2. The Fluc reporter, in tandem with the foot and mouth disease virus autoproteolytic peptide sequence (2A), was inserted between p7 and NS2. Bi-nlsCre-JC1FLAG2 (HCV-CRE) was created by amplification of nlsCre-recombinase from a TRIP-based construct, followed by insertion into the MluI/PmeI sites of a wild-type or polymerase defective (GNN) BiYpet-JC1FLAG2 (ref. 14) genome, replacing Ypet. nlsCre was similarly inserted into bicistronic versions of intergenotypic chimaeras encoding the core-NS2 sequences of Con1 (1b), ED43 (4a), HK6a (6a) and QC69 (7a), which have been previously described[35]. All plasmid constructs were verified by DNA sequencing. Primer sequences for CRE were: forward, CCCAACGCGT ATGCCCAAGAAGAAGAGGAAGGTGTCCA; reverse, AGGGTTTAAACTTA CTTGTACAGATCGCCATCTTC.

HCV generation and infections. Huh-7.5.1 or Huh-7.5 cells were electroporated with *in vitro* transcribed full-length HCV RNA. Seventy-two hours after electroporation, the medium was replaced with DMEM containing 1.5% FBS and supernatants were harvested every six hours starting from 72 h. Pooled supernatants were clarified by centrifugation at 1,500*g*, filtered through a 0.45 μm bottle top filter (Millipore) and concentrated using a stirred cell (Millipore). Viral titres ($TCID_{50}$) were determined using Huh-7.5 cells as previously described[28].

E2 antibody ELISAs. To determine end-point titres (EPT) of mouse sera, microplates (Corning Costar 3690) were coated with *Galanthus nivalis* lectin (GNL; Sigma) at 5 μg ml^{-1} overnight at 4 °C. Microwells were washed four times with PBS containing 0.05% Tween 20 and blocked with non-fat milk (NFM, 4%; BioRad) diluted in the wash buffer. HCV glycoproteins E1 and E2 were produced by transient transfection of 293T cells with phCMV-H77 and solubilised in lysis buffer (25 mM Tris pH 7.6, 140 mM NaCl, 1% Triton X-100, 0.5% NP-40 & 0.02% sodium azide). To adsorb E1E2, transfected cell lysate was diluted 1:30 in wash buffer containing NFM (1%) and incubated in the microwells for 1.5 h at room temperature (22 °C). Following blocking and washing, serially diluted mouse sera were added to the ELISA plates and incubated at room temperature for 1 h. Microwells were washed four times and incubated with peroxidase-conjugated goat anti-mouse IgG $F(ab')_2$ (diluted 1:2,000) at room temperature for 1 h. Following detection with tetramethylbenzidine substrate (TMB; Pierce), absorbance at 450 nm was measured with a micoplate reader (Molecular Devices). The EPT of each serum was defined as the reciprocal of the dilution giving a threefold higher signal than the negative control. The positive control was a mixture of sera from 12 mice immunized with E2 and diluted at 1:2,000; the negative control was serum from non-immunized Rosa26-Fluc mice. Owing to high non-specific reactivity in mouse sera, only an EPT >200 is considered a true positive signal in this assay. Each ELISA was done in duplicate.

Histological detection of HCV entry factors. Liver and spleen of mice injected with adenoviruses encoding human entry factors were harvested 24 h after injection and fixed using 4% paraformaldehyde. Tissue sections (8 μm) were deparaffinized and subjected to antigen retrieval by boiling for 30 min in citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). Entry factors were stained with human-specific primary antibodies for 16 h at 4 °C followed by secondary antibody staining using Alexa 488 or Alexa 633-conjugated antibodies for 2 h at room temperature. For *in situ* detection of EGFP fluorescence, mouse tissue was immediately frozen in O.C.T. (Optimal Cutting Temperature) compound at −80 °C. Tissue sections (~5–6 μm) were cut on poly-L-lysine coated slides. Secondary antibodies goat anti-mouse or goat anti-rabbit Alexa 488- or rhodamine conjugates (Invitrogen; 1:1,000) were used for immunofluorescence. Nuclei were detected using DAPI in VectaShield Mounting medium (Vector Laboratories). Images were captured on an Axioplan 2 imaging fluorescence microscope (Zeiss) using Metavue Software (Molecular Devices). Images were processed using ImageJ software (NIH).

Isolation of murine hepatocytes. Mice were anaesthetized by intraperitoneal injection of a mixture of 100 mg kg^{-1} ketamine and 10 mg kg^{-1} xylazine. Livers were perfused through the inferior vena cava for 5 min each with chelating buffer (0.5 mM EGTA, 0.05 M HEPES pH 7.3 in Ca/Mg-free HBSS) at a flow rate of 2 ml min^{-1} followed by collagenase solution (4.8 mM $CaCl_2$, 100 U ml^{-1} collagenase type IV,

0.05 M HEPES pH 7.3 in Ca/Mg-free HBSS). The resulting cell suspension was passed through a 100 μm cell strainer, washed twice in HBSS and was fixed in 4% paraformaldehyde. Purity of isolated hepatocytes was over 90% in all preparations as confirmed by intracellular staining for murine albumin.

Western blotting. Perfused murine liver tissue was homogenized in lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl pH 8, 150 mM NaCl, and Mini EDTA-free Protease Inhibitor Cocktail (Roche) for 30 min on ice. Fifteen micrograms of protein lysate was separated on 4–12% Bis/Tris NuPage polyacrylamide gels (Invitrogen). Proteins were transferred to nitrocellulose membranes and entry factors were detected using antibodies against CD81 (1:200), human SCARB1 (1:500), CLDN1 (1:200) or OCLN (1:200). β-Actin (1:10,000) was probed as a loading control. Following secondary antibody staining with HRP-conjugated anti-mouse IgG Fc (JIR, 1:10,000), western blots were visualized using SuperSignal West Pico (Thermo Scientific). 786O, 293T and HepG2 cell lysates, deficient in OCLN, CLDN1 and CD81, respectively, were used as negative controls. Huh-7.5 cell lysates served as positive control.

RT–PCR quantification of cellular RNAs. To quantify expression of human and murine genes (entry factors and interferon-stimulated genes, ISGs), the livers of FVB/NJ mice were harvested 24 h after adenovirus injection. Total liver RNA was isolated using RNeasy isolation kit (Qiagen) and cDNA was synthesized from 0.5 μg RNA using a SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to manufacturer's instructions. Quantitative PCR was performed with a light cycler LC480 (Roche Applied Science) using an Applied Biosystems SYBR Green PCR Master Mix and the following primer pairs. Human *CD81*, forward, TGTTCTTGAGCACTGAGGTGGTC; reverse, TGGTGGATGATGACGCCA AC. Human *SCARB1*, forward, CGGATTTGGCAGATGACAGG; reverse, GGG GGAGACTCTTCACACATTCTAC. Human *CLDN1*, forward, CACCTCAT CGTCTTCCAAGCAC; reverse, CCTGGGAGTGATAGCAATCTTTG. Human *OCLN*, forward, CGGCAATGAAACAAAAGGCAG; reverse, GGCTATGGT TATGGCTATGGCTAC. Mouse *Cd81*, forward, GGCTGTTCCTCAGTATG GTGGTAG; reverse, CCAAGGCTGTGGTGAAGACTTTC. Mouse *Scarb1*, forward, CAAAAAGCATTTCTCCTGGCTG; reverse, AATCTGTCAAGGGCAT CGGG. Mouse *Cldn1*, forward, TTATGCCCCCAATGACAGCC; reverse, ATGAGGTGCCTGGAAGATGATG. Mouse *Ocln*, forward, ACTAAGGAAG CGATGAAGCAGAAG; reverse, GCTCTTTGGAGGAAGCCTAAACTAC. Mouse *Gapdh*, forward, ACGGCCGCATCTTCTTGTGCA; reverse, ACGGCCA AATCCGTTCACACC. Mouse viperin, forward, TGCTGGCTGAGAATAG CATTAGG; reverse, GCTGAGTGCTGTTCCCATCT. Mouse *Ifi27l2a*, forward, GCTTGTTGGGAACCCTGTTTG; reverse, GGATGGCATTTGTTGATGTG GAG. Mouse *Ifi44*, forward, AACTGACTGCTCGCAATAATGT; reverse, GTAACACAGCAATGCCTCTTGT. Mouse *Mx1*, forward, GACCATAGG GGTCTTGACCAA; reverse, AGACTTGCTCTTTCTGAAAAGCC. Mouse *Eif2ak2*, forward, ATGCACGGAGTAGCCATTACG; reverse, TGACAATCCAC CTTGTTTTCGT. Mouse *Oas1*, forward, ATGGAGCACGGACTCAGGA; reverse, TCACACACGACATTGACGGC. Mouse *Ifnb1*, forward, CAGCTCCAA GAAAGGACGAAC; reverse, GGCAGTGTAACTCTTCTGCAT. Mouse *Cxcl10*, forward, CCAAGTGCTGCCGTCATTTTC; reverse, GGCTCGCAGGGATGAT TTCAA.

RT–PCR quantification of HCV RNA. Total RNA was isolated from mouse brain, liver and sera using the RNAeasy kit (Qiagen). HCV genome copy number was quantified by one-step RT–PCR using Multicode-RTx HCV RNA Kit, (EraGen) and a light cycler LC480 (Roche Applied Science), according to manufacturers' instructions.

Bioluminescence imaging. Unless otherwise specified, mice were injected with 10^{11} adenoviral p.f.u. 24 h before intravenous injection with 2×10^7 $TCID_{50}$ HCV-CRE. At 72 h after infection, mice were anaesthetized using ketamin/xylazine and injected intraperitoneally with 1.5 mg luciferin (Caliper Lifesciences). Bioluminescence was measured using an IVIS Lumina II platform (Caliper Lifesciences).

***In vitro* neutralization assay.** Serum of FVB/NJ mice, either mock immunized or immunized with rVV-HCV1 were collected and pooled 5 weeks after immunizations. Serial dilutions of mouse serum or monoclonal anti-HCV E2 antibody (clone 3/11) were pre-incubated with intergenotypic JFH1 chimaeras expressing the structural proteins of genotypes 1a (H77), 1b (Con1 and J4), 2a (J6), 3a (S52), 4a (ED43), 5a (SA13), 6a (HK6a) or 7a (QC69) for 1 h at 4 °C. Supernatants were used to infect naive Huh7.5 cells at a calculated multiplicity of infection of 0.1 for 6 h after which cell were washed and medium was replaced. Cells were collected 48 h after infection and were stained for expression of HCV NS5a and were analysed by flow cytometry.

Flow cytometry. Immune activation, depletion efficiency as well as the frequency of infected hepatocytes were confirmed by flow cytometry using an LSRII flow cytometer (BD Biosciences). For immune activation and depletion efficiency, peripheral blood mononuclear cells (PBMCs) and splenocytes of mice were isolated and purified via density gradient centrifugation. Cells were stained with directly fluorochrome-conjugated antibodies directed against CD3, CD4, CD8, CD11b and CD49b. For the determination of infection frequency, hepatocytes were isolated from Rosa26-GNZ mice, fixed in 4% paraformaldehyde, permeabilized in PBS + 0.01% Triton X-100 and stained with fluorochrome-conjugated antibodies against murine albumin and CD81. Data were analysed using Flowjo software (Treestar Software).

***In vivo* depletion.** Mice were injected intraperitoneally with 100 mg kg^{-1} of either anti-mouse Ly-6G, anti-mouse Asialo GM1 or a mixture of anti-mouse CD4 and CD8. Depletion of the cell populations was verified 24 h after injection by flow cytometry. Adenoviral injection of HCV entry factor constructs was initiated following confirmation of depletion.

Statistical analysis. Statistical analyses were performed using Graphpad Prism Software. Statistics were calculated using Kruskal–Wallis one-way analysis of variance. *P* values below 0.05 were considered statistically significant.

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