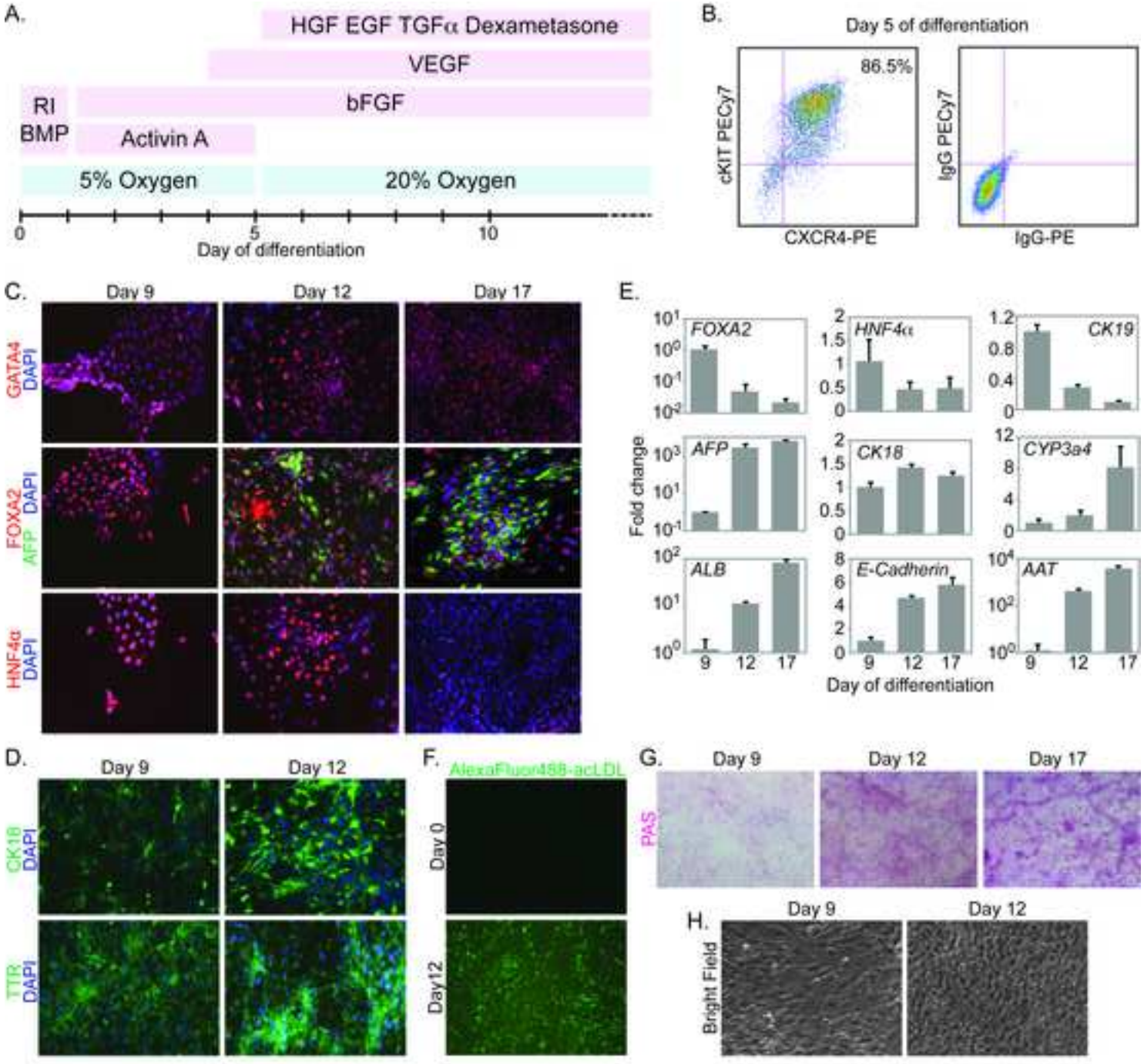
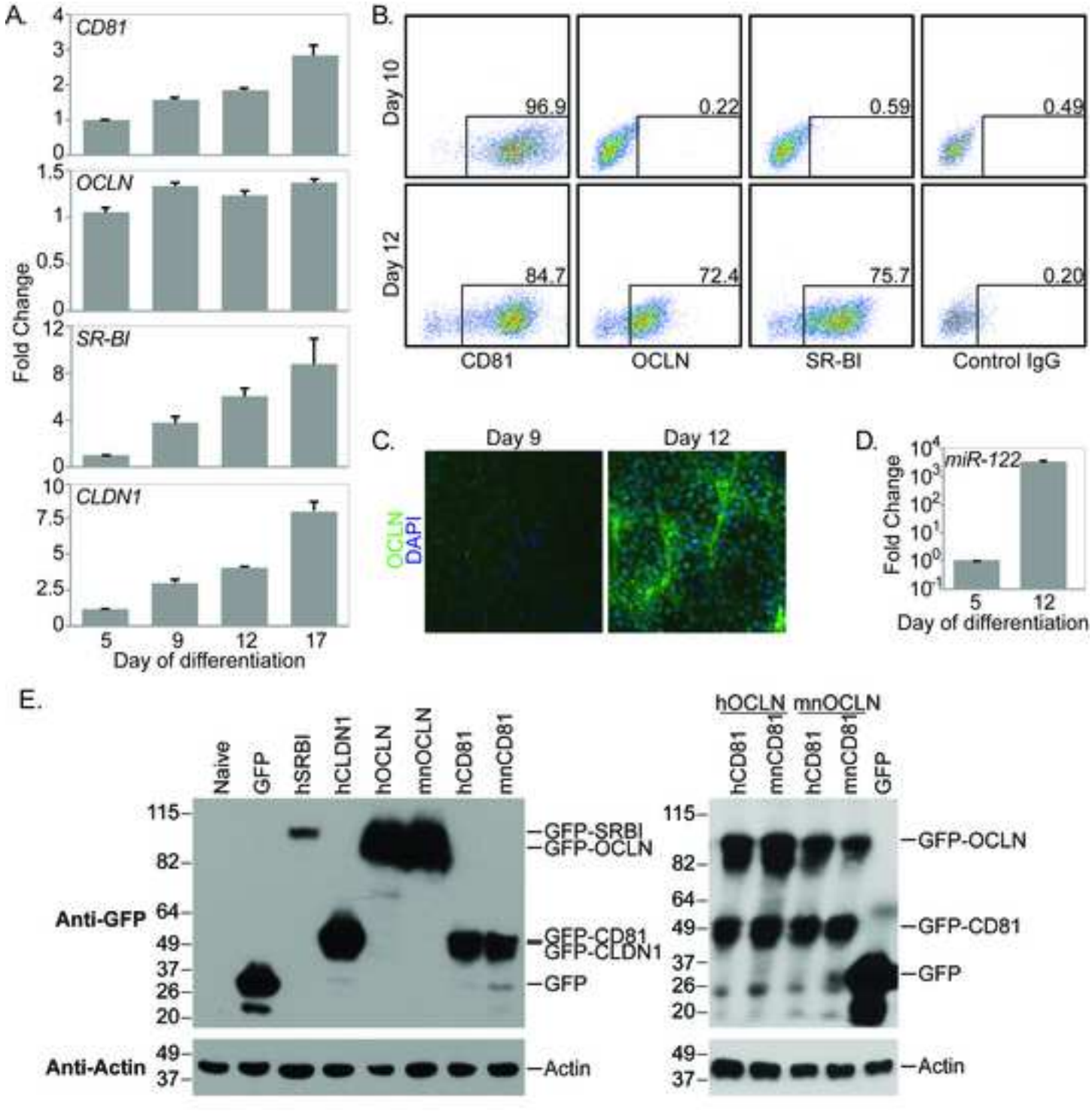
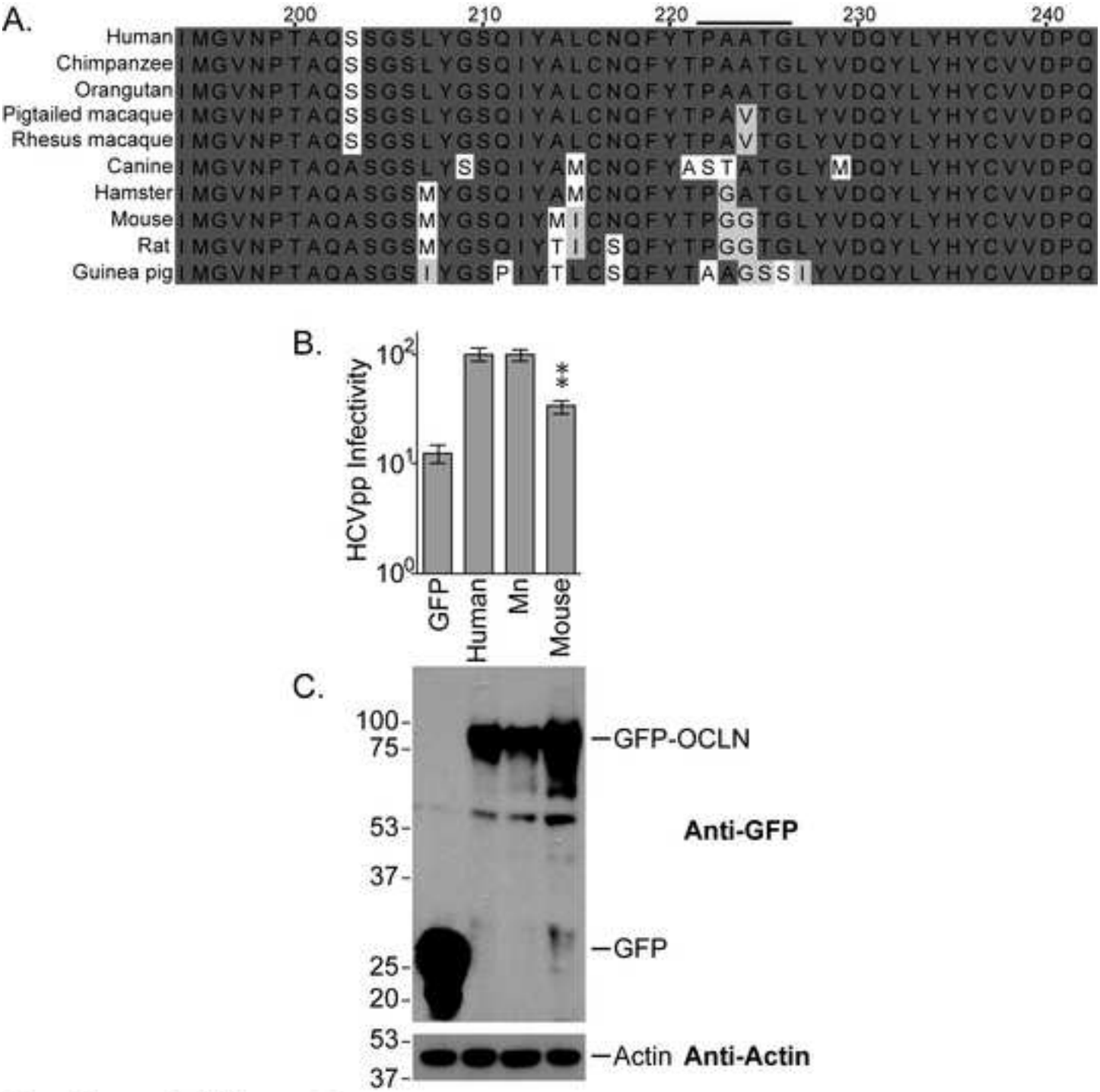
Supporting Fig 1



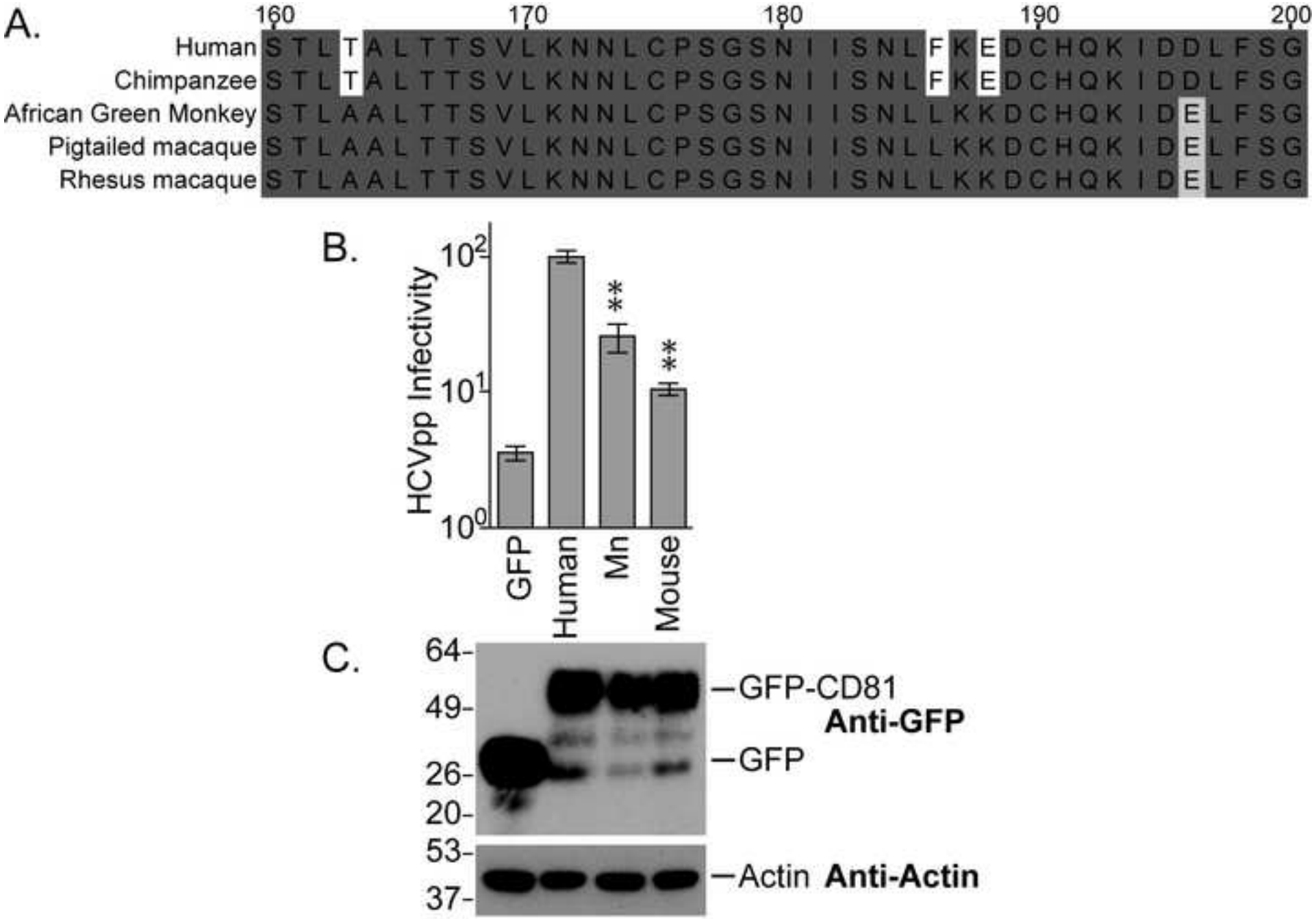

Supplemental Figure 1




Supplemental Figure 2




Supplemental Figure 3




Supplemental Figure 4

## Supplemental Materials and Methods

### Macaque iPSC cell Culture

The derivation and validation of MniPSC line 3 has been previously described[1]. Prior to differentiation studies, the normal karyotype and the phenotype were confirmed by flow cytometry. Cells were maintained on irradiated mouse embryonic fibroblast feeder layers in mTESR media (Gibco) supplemented with 20ng/ml of basic fibroblast growth factor (bFGF) (R&D systems).

### Hepatic Differentiation

MniPSCs were induced toward definitive endoderm and then specified toward the hepatic lineage using a modified version of a protocol for human ESCs that has been described[2] (Supplementary Figure 1A). Briefly, the day prior to differentiation, MniPSCs were harvested using accutase (Life Technologies) and passaged on matrigel (BD Biosciences) to exclude the MEFs. On day 0 of differentiation, MniPSCs were harvested using accutase and cultured in low cluster plates (Costar) to allow embryoid bodies (EB) formation in the presence of BMP4 (3 ng/ml) and Y-27632 rock inhibitor (5 µM, Calbiochem) in serum-free differentiation (SFD) media as previously described[2]. On day 1 of differentiation, endoderm program was induced with Activin-A (100 ng/ml) in SFD media supplemented with bFGF (2.5 ng/ml) and BMP4 (0.5 ng/ml). On day 4, the medium was changed to SFD media supplemented with Activin-A (100ng/ml), bFGF (2.5 ng/ml) and VEGF (10 ng/ml). On day 5, EBs were dissociated with trypsin/EDTA 0.25% (Cellgro) and subsequently plated on gelatin-coated dishes (30,000 cells per well of 48 well-plate) in defined hepatic media as previously described[2]. All cytokines were purchased from R&D Systems.

### RNA extraction and quantitative real time-PCR (qRT-PCR)

Total RNA was prepared with the RNeasyMicro Kit (Qiagen). RNA was reverse transcribed into cDNA using the Superscript III First-strand Synthesis System kit (Life Technologies). All experiments were done in triplicate using the Roche SYBR Green master mix and the LightCycler 480 I Real Time PCR System (Roche). Relative quantification was calculated using the comparative threshold cycle (CT) method and was normalized against the ΔCT of the housekeeping gene β-actin. Melting curves for each gene were used to confirm homogeneity of the DNA product. The following primer sequences were used:

SRBI F 5'TCCTCGAGTACCGCACCTTCCA, R 5'AGTCAACCTTGCTCAGCCCGTT

OCLN F 5'AGACCCAAGAGCAGCAAAGGGC, 5'ACAATGGCAATGGCCTCCTGGG

CD81 F 5'ATGACCCGCAGACCACCAACCT, R 5'TCCTTGGCGATCTGGTCCTTGT

AFP F 5'CTACCTGCCTTTCTGGAAGAACTTTG, R 5'GATCGATGCTGGAGTGGGCTTT

FOXA2 F 5'AAGTGGGGGTCGAGACTTTG, R 5'CTGCAACAACAGCAATGGAG

CK19 F 5'CCGCGACTACAGCCACTACT, R 5'GAGCCTGTTCCGTCTCAAAC

ALB F 5'GTGAAACACAAGCCCAAGGCAACA, R 5'TCCTCGGCAAAGCAGGTCTC

CK18 F 5'ATCTTGGTGATGCCTTGGAC, R 5'CCTCAGAACTTTGGTGTCATTG

CYP3a4 F 5'GTGACCAAATCAGTGTGAGGAGGTA, R

5'AGGAGGAGTTAATGGTGCTAACTGG

HNF4α F 5'CATCAGAAGGCACCAACCTCAACG, R 5'ATACTGGCGGTCGTTGATGTAGTCC

AAT F 5'AGGGCCTGAAGCTAGTGGATAAGT, R 5'TCTGTTTCTTGGCCTCTTCGGTGT

ACTIN F 5'TTTTTGGCTTGACTCAGGATTT, R 5'GCAAGGGACTTCCTGTAACAAC

CDH1 F 5'GGCCTGAAGTGACTCGTAACG, R 5'TCAGACTAGCAGCTTCGGAACC

miR122 F5'AGCAGAGCTGTGGAGTGTGAC, R 5'AGTAGCTATTTAGTGTGATAA

CLDN1 F 5' GGGTTGCTTGCAATGTGCTGCTC, R 5' TCTCTGCCTTCTGCACCTGCC

For quantifying HCV RNA, qRT-PCR was performed on 20 ng of total RNA or 2 µl of extracted RNA from supernatant, prepared with the Qiamp Viral RNA kit (Qiagen), with the HCV 5'UTR Taqman assay kit (Pa03453408_s1, Applied Biosystems) and the LightCycler 480

Master Hydrolysis Probes kit (Roche) using the LightCycler 480 II Real Time PCR System (Roche).

**Flow cytometry**

Day 5 EBs or MnHep cells at day 10 and 12 were dissociated with trypsin 0.25%. Cells were stained with CXCR4-PE (R&D systems), cKIT-PECy7 (BD Bioscience), CD81 (BD Bioscience), OCLN (Invitrogen), CLA-1/SR-BI (BD Bioscience) or IgG control (BD Bioscience) in PBS with BSA 0.1% at 4°C for 20 minutes, with 0.05% saponin (Sigma) for OCLN and CLA-1/SR-BI. Cells were then incubated with a donkey anti-rabbit IgG-AlexaFluor488 or donkey anti mouse IgG- AlexaFluor488 (Invitrogen). Cells were analyzed using a LSRII flow cytometer (Becton Dickinson).

**Immunocytochemistry**

Cells were fixed in 4% paraformaldehyde/PBS for 15 minutes at room temperature and rinsed with 1X PBS. For intracellular staining, cells were permeabilized with 0.1% Triton X100 in PBS for 10 minutes at room temperature. Cells were incubated overnight with control isotype (mouse IgG or rabbit IgG, Jackson Immunoresearch Laboratories) or with monoclonal antibodies anti-AFP (Dako), anti-FOXA2 (Novus), anti-GATA4 (Santa Cruz), anti-HNF4$\alpha$ (Santa Cruz) anti-CK18 (Sigma), anti-TTR (Abbiotec), anti-CD81 (BD Bioscience) and OCLN (Invitrogen). Cells were than incubated with secondary antibodies donkey anti-rabbit IgG-Cy5, donkey anti-mouse IgG-Cy3 or donkey anti-goat IgG-Cy3 (Jackson Immunoresearch Laboratories) for 1 hour at room temperature and counterstained with 40,6-diamidino-2-phenylindole (DAPI). Stained cells were visualized using a confocal or regular fluorescent microscope (Leica) and images captured using the Leica software. For HCV E2 staining, cells were permeabilized with 0.05% saponin for 10 minutes at room temperature, incubated 1 hour with anti-E2 monoclonal antibody (clone AR3A)[11], provided by Mansun Law (Scripps Research

Institute), then incubated with goat anti-mouse IgG-AlexaFluor568 (Invitrogen), and finally counterstained with Hoechst (Invitrogen).

**Hepatic Functional Assays**

For LDL-acetylated uptake, cells were incubated 6 hours at 37C with AlexaFluor488-acLDL (Invitrogen). Stained cells were visualized using a fluorescent microscope (Leica).

For Peridic Acid Shiff (PAS) staining, cells were fixed in 4% paraformaldehyde/PBS for 15 minutes at room temperature prior to performing the PAS assay as per manufacturer instruction (Sigma).

**Virus generation and infection.**

HCV and VSVG pseudoparticles production was performed as previously described[4, 5] by cotransfection of three plasmids encoding (i) a provirus containing the desired reporter (V1-GLuc), (ii) HIV Gag-Pol, and (iii) the necessary envelope glycoprotein(s) (HCV H77 1a E1E2 or VSVG). Briefly, 293-T cells were seeded at $7 \times 10^6$ cells/well into a poly-L-lysine (Sigma, St. Louis, MO)-coated 10 mm diameter plate. Transfection was performed the next day with TransIT-LT1 Transfection Reagent (Mirus, Madison, WI). Supernatants were collected 2 and 3 days post-transfection and filtered (0.45 µm pore size). HCV pseudoparticles were concentrated 200-times by centrifugation through a 20% sucrose cushion for 2h at 4°C and 34K rpm. All infection assays using pseudoparticles were performed in the presence of 4 µg/ml polybrene (Sigma) and 0.01 M HEPES (Gibco).

To produce HCVcc, two HCV genome configurations were used. Both encoded the structural proteins and a portion of NS2 from the HC-J6 isolate cloned in the context of the rest of the genome from the JFH-1 isolate. This genome, termed Jc1, was used as it produced a high level of infectious virus in cell culture[6]. One configuration represented the normal HCV genomic organization, while the other was a reporter virus that expressed the *Gaussia principes*

luciferase protein (GLuc)[7] off the HCV internal ribosome entry site (IRES) with the IRES from the encephalomyocarditis virus directing translation of the HCV polypeptide. Plasmids encoding both HCVcc configurations were provided by Charles Rice (Rockefeller University). HCVcc stocks were produced as previously described[5]. Briefly, supernatants from Huh-7.5 cells transfected by electroporation with in vitro transcribed HCV genomic RNA were collected at 2, 3, and 4 days post-transfection and filtered (0.45μm pore size). HCVcc stocks were concentrated 200-times by centrifugation through a 20% sucrose cushion for 2 hours at 4°C and 34,000 rpm. Depending on the efficiency of virus concnetration, nonreporter HCV was used at an approximate multiplicity of infection (M.O.I.) of 1 to 10, and GLuc expressing HCVcc was used at an M.O.I. of 0.01 and 0.1, as determined by titration on Huh-7.5 cells.

Infections were performed, in triplicate, on cells seeded on 48 well plates with 10 µl of HCVpp or HCVcc, or 0.01 µl of VSVGpp added in 0.2 ml fresh media. For pseudoparticle infections media was supplemented with plus 4 µg/ml polybrene (Sigma) and 50 mM Hepes (Gibco). MnHep were infected at day 12 or day 13 of differentiation. Twenty-four hours post-infection, cells were washed three times with fresh media to remove Gluc protein present in the inoculum. Supernatants were harvested two days post infection in 25 µl Renilla luciferase assay lysis buffer (Promega, Madison, WI) and the expression of the luciferase reporter was measured as previously described[8].

Virus titration was performed by limiting dilution assay as previously described[9]. Briefly, rows of Huh-7.5 cells seeded in poly-L-lysine coated 96-well plates at 3 x $10^4$ cells/well were infected with serial dilutions of virus. Three days post infection, infected cells were fixed and immunostained for NS5A as described in using the clone 9E10 anti-NS5A antibody, provided by Charles Rice (Rockefeller University) and detection was performed with the ImmPRESS peroxidase anti-mouse conjugated antibody (Vector Laboratories, Burlingame, CA). Wells that expressed at least one NS5A-expressing cell were counted as positive, and the TCID50 was calculated according to the method of Reed and Muench[10].

To neutralize HCVpp infection, the anti-E2 monoclonal antibody (clone AR3A)[11], provided by Mansun Law (Scripps Research Institute), was used at a concentration of 10 µg/ml.The HCV polymerase inhibitor 2'C-methyl-adenosine (2'CMA)[12] was provided by Timothy Tellinghuisen (Scripps Research Institute) and used at 6 µM to inhibit HCVcc intracellular RNA replication.

**Rescue and activity evaluation of HCV cell entry factors.**

As the coding sequences for Mn OCLN and CD81 have not been previously reported, we rescued their ORF sequences by RT-PCR with oligos designed of highly conserved regions of each. To ensure that the correct sequences were identified, multiple independent cDNA synthesis and PCR reactions were used to assemble consensus sequences. Reverse transcription was performed using the Superscript III First-strand Synthesis System (Invitrogen), according to manufacturer's instructions, with an oligo dT primer and total RNA prepared from day 13 post differentiation MnHep cells as template. The Mn OCLN sequence was PCR amplified from this cDNA with forward (5' GAAGATCAGCTGACCATTGACA) and reverse (5' AAAATTCTTAATTGGAGTGTTCAGCCCAGT), which anneal just before and after the protein coding sequence. This sequence has been deposited in Genbank as accession number KF188431. For cell culture expression, oligos 5'- A GAC ACC GAC TCT AG A GGA TCT AGA ATG TCA TCC AGG CCT CTT GAA AGT and 5'- TTGCTCACCATGTTTAAACCCGGTGGGGATCTTGTTTTCTGTCTATCATAGTCTCC were used to precisely amplify the coding sequence. A 'sequence- and ligation-independent cloning' method[13] was used to clone this product into the TRIP plasmid[14, 15], a self-inactivating lentiviral provirus that expresses no HIV proteins, but instead employs an internal CMV promoter to express cloned genes. For vector, TRIP-hOCLN-PmeIGFP, which is a TRIP vector encoding the human OCLN orf fused to the amino-terminus of GFP and has been previously described[8], was

digested with the restriction enzymes XbaI and PmeI (New England Biolabs). This plasmid was designated TRIP-MnOCLN-GFP.

The Mn CD81 orf was amplified from the above cDNA with forward (5' GCT AGC ATG GGA GTG GAG GGC TGC ACC) and reverse oligos (5' ACT AGT GTA CAC GGA GCT GTT CCG GAT), and the rescued sequence was deposited in Genbank as accession number KF188430. To clone the coding sequence into the TRIP plasmid, this product was digested with NheI and SpeI and ligated into like digest TRIP-GFP-hCD81-linker[16], which encodes the human CD81 protein fused to the carboxyl-terminus of GFP, to generate TRIP-GFP-MnCD81-linker.

VSVGpp lentiviral pseudoparticles encapsidating either the above TRIP vectors, or TRIP-GFP[14, 15], TRIP-GFP-hSRBI-linker[16], which encodes GFP fused to the human SR-BI protein, and TRIP-GFP-hCLDN1-linker[16], which encodes GFP fused to the human CLDN1 protein, were generated as described above. To generate MnHep cells expressing these proteins, the pseudoparticles were concentrated by ultracentrifugation for 2h at 4°C and 34K rpm. All transductions were performed in the presence of 4 µg/ml polybrene (Sigma) and 0.01 M HEPES (Gibco). Transduction efficiency was monitored by GFP expression and immunoblotting confirmed transgene expression.

To test OCLN and CD81 related HCV cell entry functions, human 786-O or HepG2 cells, which are deficient in endogenous OCLN and CD81, respectively, were transduced with unconcentrated VSVGpp supernatants. Transduction efficiency was monitored by GFP expression and immunoblotting confirmed transgene expression.

**Immunoblot Analysis**.

For immunoblot analysis of GFP expression, transduced MnHep were lysed in a volume of 1x SDS-PAGE sample buffer plus dithiothreitol (DTT) that was proportional to the approximate cell confluency. Cell lysates were passed through a 22-gauge needle several times and heated 5 minutes at 95ºC. Equivalent volumes of lysate were immunoblotted with rabbit

anti-GFP (ab290; Abcam, Cambridge, MA) and mouse anti-β-actin antibodies (AC-15; Sigma, St. Louis, MO), to ensure analysis of comparable protein concentrations. For both antibodies, horseradish peroxidase (HRP) conjugated goat anti-mouse and goat anti-rabbit secondary antibody (GE Healthcare Lifescience) was used and detection was performed with Immobilon Chemiluminescent HRP (Millipore, Billerica, MA).

**Statistical analysis**. Data were analyzed for statistical significance using Prism software (GraphPad Software) using the Mann-Whitney test. A p-value ≤0.05 was considered significant.

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**Supplemental Figure 1. Generation of hepatocytes from macaque iPSCs.** (A) Illustration of the hepatic differentiation protocol used to generate MnHep cells from MniPSC cells, as described in the Supplemental Materials and Methods. (B) By day five of endoderm differentiation, the majority of cells (86.5%) had developed into definitive endoderm-enriched populations, as gauged by the flow cytometry analysis of co-expression of two endodermal markers CXCR4 and cKIT. On day five, endoderm-enriched populations cells were plated onto gelatin and specified toward the hepatic lineage. On the indicated days of differentiation, cells were fixed and immunostained for (C) the endoderm markers FOXA2 and GATA4, and (D) the hepatic markers HNF4□, alphafetoprotein (AFP), and Transthyretin (TTR), and the epithelial marker cytokeratin 18 (CK18). Nuclei of cells were also counterstained with DAPI. Note that virtually all cells express GATA4 and FOXA2, and that most cells are positive for AFP, CK18 and TTR, indicative of an efficient endoderm induction and hepatic specification. (200-fold

magnification) (E) Relative transcript levels, as determined by qRT-PCR at the indicated days of differentiation, of early hepatic endoderm markers *FOXA2*, *HNF4*, and *CK19* decreased over time, while markers for specification and maturation *AFP*, *CK18*, *CYP3a4*, albumin (*ALB*), 1antitrypsin (*AAT*), and E-cadherin increased over time, indicative of maturation of hepatic cells. Levels of the master regulator of hepatic cell fate, *HNF4*, remained relatively constant with time. Shown are means and standard deviations from three independent cultures. (F) MnHep cells at 12 days post differentiation, but not MnIPS cells, efficiently internalize acetylated LDL (AlexaFluor488-acLDL) (green staining). (G) Periodic Acid Schiff staining (lower panels) of day 9, 12 and 17 hepatic cultures show increasing glycogen storage (red staining) with time in hepatic cells. The bright field pictures (upper panels) demonstrate confluent monolayers in each culture.

**Supplemental Figure 2. MnHep HCV entry factor expression.** At the indicated days of hepatic differentiation, the expression levels of the cellular factors required for HCV cell entry in naïve MnHep cells were gauged by (A) qRT-PCR (means and standard deviations of n=3 analysis, normalized to *ACTIN*), (B) flow cytometry analysis for CD81, occludin, SR-B1 and IgG control (as a suitable antibody to stain MnCLDN1 was not identified, this protein was not analyzed by FACS), and (C) immunostaining for OCLN. Note that although OCLN transcript levels do not vary greatly, this protein is not detected until 12 days into the differentiation process. (D) The levels of miR-122 were quantified by qRT-PCR from total RNA samples collected at the indicated day of differentiation. (E) Immunoblot with antibodies that bind either GFP, to track transgene expression, or Actin, as a loading control of lysates from naïve MnHep cells or those transduced with lentiviruses to express a fusion protein of GFP and the indicated HCV cell entry factor. Approximate molecular weight (kDa) marker positions are indicated to the left and the predicted size of each expressed protein is marked on the right of each blot.

**Supplemental Figure 3. Comparison of OCLN ortholog HCV cell entry functions.** (A) The MnOCLN coding sequence was amplified from MnHep cDNA as described in Materials and Methods. The MnOCLN protein sequence had 97.3% identity and 98.7% similarity to human OCLN, and 99.8% identity and 99.8% similarity to the Rhesus macaque OCLN protein (only encoded a single alanine to valine change in the second transmembrane domain at amino acid position 157). Shown is an alignment of the OCLN second extracellular loop sequences of the indicated species, with amino acid numbering corresponding to the human OCLN sequence and identical and similar amino acids dark and light shaded, respectively. We previously showed that this region contains the determinants for differences in OCLN HCV cell entry factor activity between these species[8]. A line above the alignment marks the cluster of residues shown to be most important for species-specific functions. (B) To test OCLN entry factor activities, 786-O cells, which are unable to support HCV cell entry due to insufficient levels of endogenous OCLN[5], were transduced to express either GFP alone or GFP fused to OCLN from the indicated species. These cells were challenged with HCVpp as described in Materials and Methods. Luciferase reporter levels, assayed two days post infection are shown normalized to parallel infections with VSVGpp, which infect nearly all mammalian cells and thus help control for cell number variations, and set relative to infections of cells expressing human OCLN. Mean and standard error of two independent experiments, each performed in quadruplicate, are shown. (C) Immunoblots for either GFP or actin of lysates from 786-O cell populations transduced to express the indicated transgenes. Approximate molecular weight (kDa) marker positions are indicated to the left of each blot. $^{**}P < 0.01$ (Mann-Whitney test)

**Supplemental Figure 4. Comparison of CD81 ortholog HCV cell entry functions.** (A) The MnCD81 coding sequence was amplified from MnHep cDNA as described in Materials and Methods. While this sequence shared 98.3% identity and 99.2% similarity with the human CD81sequence, it has 100% identity with the Rhesus macaque and African green monkey

CD81 proteins, the latter of which was previously shown to mediate HCV cell entry less efficiently than the human version due to diminished HCV E2 glycoprotein binding[17]. Shown in this figure are the only four amino acid differences between these sequences, all occurring in the CD81 large extracellular loop. The amino acid positions are labeled above the sequence and identical and similar amino acids are dark and light shaded, respectively. (B) HCVpp infectivity (normalized to parallel VSVGpp infections and relative to infections of cells expressing human OCLN) of HepG2 cells, which do normally do not support HCV cell entry due to a lack of endogenous CD81, expressing either GFP alone or GFP fused to the CD81 protein from the indicated species. Mean and standard error of two independent experiments, each performed in quadruplicate, are shown. (C) Immunoblots for either the GFP or actin of lysates from HepG2 cell populations transduced to express the indicated transgenes. Approximate molecular weight (kDa) marker positions are indicated to the left of each blot. $^{**}P < 0.01$ (Mann-Whitney test)