Cell death-inducing DFFA-like effector b is required for hepatitis C virus entry into hepatocytes

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The molecular mechanism of the hepatic tropism of hepatitis C virus (HCV) remains incompletely defined. In vitro hepatic differentiation of pluripotent stem cells produces hepatocyte-like cells (HLCs) permissive for HCV infection, providing an opportunity for studying liver development and host determinants of HCV susceptibility. We have previously identified the transition stage of HCV permissiveness and now investigate whether a host protein whose expression is induced during this transition stage is important for HCV infection. We suppressed the expression of a liver-specific protein, cell death-inducing DFFA-like effector b (CIDEB), and performed hepatocyte function and HCV infection assays. We also used a variety of cell-based assays to dissect the specific step of the HCV life cycle that potentially requires CIDEB function. We found CIDEB to be an essential cofactor for HCV entry into hepatocytes. Genetic interference with CIDEB in stem cells followed by hepatic differentiation leads to HLCs that are refractory to HCV infection; and infection time-course experiments revealed that CIDEB functions in a late step of HCV entry, possibly to facilitate membrane fusion. The role of CIDEB in mediating HCV entry is distinct from those of the well-established receptors as it is not required for HCV pseudoparticle entry. Finally, HCV infection effectively down-regulates CIDEB protein through a post-transcriptional mechanism.

This study identifies a HCV entry cofactor that is required for HCV infection of hepatocytes and potentially facilitates membrane fusion between viral and host
membranes. CIDEB and its interaction with HCV may open up new avenues of investigation of lipid droplets and viral entry.
Viruses depend on host factors to gain entry into host cells, and the interaction between viral glycoproteins and cellular entry factors is important for this process and contributes to viral tropism. Of the two glycoproteins (E1 and E2) encoded by hepatitis C virus, E2 is a major target for neutralizing antibodies with well-defined epitopes, both linear and conformational (reviewed in (1)); two of the HCV receptors, CD81 and scavenger receptor BI (SRB1), were identified through a direct interaction with E2 (2, 3); and the crystal structure of a core domain of E2, has been recently solved (4). The structure and function of E1 is less well-understood, but it may facilitate the correct folding (5, 6) and receptor binding of E2 (7). It has also been reported to interact with cell surface proteins (8, 9).

Following attachment and receptor-binding, HCV enters the cell via endocytosis with the help of additional entry cofactors (10-14). Details of the membrane fusion process of HCV entry remains poorly defined. Both E1 and E2 proteins contain putative fusion peptides (15-17) and may participate in membrane fusion and the crystal structure of HCV E2 suggests that HCV glycoproteins may use a fusion mechanism that is distinct from related positive-strand RNA viruses including flaviviruses (4). In addition, HCV may require an additional post-binding trigger to complete membrane fusion under low pH conditions in the endosomes (18). Although it is not clear whether cellular proteins directly participate in the membrane fusion process, it has been proposed that...
removal of cholesterol from the virion by Niemann-Pick C1-like 1 (NPC1L1) is necessary before fusion can occur (14).

The cell death-inducing DFFA-like effector (CIDE) family proteins, CIDEA, CIDEB, and CIDE/C fat-specific protein 27 (Fsp27), were identified based on their homology to the N-terminal domain of DNA fragmentation factors (DFF) (reviewed in (19)). Although these proteins induce cell death when overexpressed, the physiological function of the CIDE proteins is related to energy expenditure and lipid metabolism in vivo (20-23). All three CIDE proteins associate with lipid droplets (LDs), and CIDE/Fsp27 in particular plays a role in the growth of lipid droplets by facilitating the fusion of the lipid monolayers of two contacting droplets (24, 25). Of the three CIDE proteins, CIDEB expression is enriched in liver tissues and cell lines of liver origin (26, 27). In addition, CIDEB has been reported to interact with the nonstructural protein 2 (NS2) of HCV in a yeast-two hybrid system (28), although this interaction was not detectable in HCV infected cells (29).

We and others recently developed a new HCV cell culture model by converting pluripotent stem cells into differentiated human hepatocyte-like (DHH or HLC) cultures (30-32). We also identified a critical transition stage during the hepatic differentiation process when the DHH/HLCs become permissive for HCV infection (30). Here we identify human CIDEB as a protein whose expression correlates with the transition stage and is required for HCV entry. CIDEB knockdown inhibited membrane fusion of
HCV particles produced in cell culture (HCVcc) (33-36) without affecting the entry of HIV-HCV pseudotyped particles (HCVpp) (37, 38).

MATERIALS AND METHODS

Stem cells and hepatic differentiation. Human ESC line WA09 (H9) cells were obtained from WiCell Research Institute and differentiated into hepatocyte-like cells using a previously published protocol (30). Huh-7.5 cells were kindly provided by Charles Rice (Rockefeller University) and Apath LLC.

Antibodies and inhibitors. Anti-ApoE antibody (Mab33) was kindly provided by Guangxiang Luo (University of Alabama at Birmingham). The following antibodies were purchased: anti-JFH Core, anti-NS3, anti-NS5A for HCV (BioFront Technologies Inc., FL), anti-CIDEβ, anti-HA, anti-ApoB, anti-GAPDH (Santa Cruz Biotechnology, TX), anti-CLDN1 (Invitrogen, NY), anti-CD81 (BD Pharmingen, NJ), anti-Rab5 (BD Transduction Laboratories, NJ) and anti-dsRNA (English & Scientific Consulting, Szirak, Hungary). FITC and TRITC conjugated anti-rabbit and anti-mouse immunoglobulins (IgG) were purchased from Sigma Aldrich and Alexa Fluor 647 conjugated anti-mouse IgG was purchased from Invitrogen. ITX-5061 was a gift from Dr. Flossie Wong-Staal of iTherX Inc. (San Diego, CA).

Immunofluorescence analysis (IFA). Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 10 min and blocked and
permeabilized with PBTG (PBS containing 0.1% Triton X-100, 10% normal goat serum, and 1% BSA) at room temperature for 2 h. WNV infected cells were fixed as described above and then permeabilized with PBS containing 0.1% Triton X-100 for 10 min, washed three times with PBS and then blocked with PBS containing 5% horse serum. Cells were incubated with primary antibodies (anti-Rab5 or anti-dsRNA) at 4°C overnight or at room temperature for 2 h. Isotype mouse or rabbit IgGs were used as negative controls. After four washes with PBS, a FITC-, TRITC- or Alexa Fluor-conjugated secondary antibody was added and incubated at room temperature for 1 hour. Coverslips were then mounted with VECTASHIELD (H-1200, Vector Labs, CA) or Prolong Gold antifade reagent (Invitrogen). For LD staining, cells were similarly fixed and stained using Bodipy 530/550 (1:2000 diluted in PBS) at RT for 15 min before mounting and observation with confocal microscopy.

**Periodic acid-Schiff (PAS) staining and albumin ELISA.** PAS staining was done on day-16 and day-18 control (Ctrl) and CIDEBKD DHH/HLCs using a commercial kit (Sigma-Aldrich, MO) per the instructions provided by the manufacturer. Albumin ELISA was performed with a human albumin ELISA kit (Bethyl Laboratories, TX), according to manufacturer’s instructions.

**RNA interference and cDNA rescue.** A HIV-based lentiviral vector was used to express short hairpin RNAs (shRNAs). The mRNA target sequences are as follows: sh-CIDEA, 5′- AAC ACG CAU UUC AUG AUC UUG - 3′; sh-CIDEB: 5′- AAA GUA CUC AGG GAG CUC CUU- 3′. RNA duplex si-CIDEB: 5′- GAU UCA CCU UUG ACG UGU A-3′.
Stable cell lines expressing shRNAs were obtained by selection with 1.2 μg/ml (for Huh-7.5 cells) or 0.6 μg/ml (for WA09 cells) of puromycin for 3 weeks. The sh-CIDEB-resistant cDNA contained the following sequences at the siRNA target site: 5′- AAA GTc CTg cGc GAa CTC CTT - 3′.

**Electroporation of viral RNAs.** Viral J6/JFH-1/G-Luc RNAs were *in vitro* transcribed using a MEGAscript T7 kit (Ambion, TX) and purified by phenol-chloroform extraction. RNA (5-10 μg) was electroporated into 4×10^6 cells in a volume of 400 μl using a Gene Pulser Xcell Electroporation System (Bio-Rad, CA). For replication kinetics analyses, the culture medium was changed at 4 h post electroporation, and at the indicated times, 100 μl of supernatant was collected for assessment of Gaussia luciferase activity using a luciferase assay system (Promega, WI).

**HCVcc and HCVser infections.** A JFH-1-based HCVcc [(JFH1/Ad16) was kindly provided by Guangxiang Luo and a high-titer stock was produced in Huh-7.5 cells as previously described (39). HCV genotype 1b serum with an RNA titer of 1.8 × 10^6 copies/ml was obtained from a commercial supplier (Teragenix, FL). Infection of DHH/HLCs and Huh-7.5 cells was performed as previously described (30). For the infection time course, cells were incubated with virus at 4°C for 2 h with gentle shaking, and then shifted to 37°C after thorough but gentle washing with PBS. At the indicated times (except for the 0 hour), cells were trypsinized and then washed by PBS to remove surface-bound virions. Cell pellets were then subjected to RNA extraction or western
9 blot analysis. For the 0 hour sample, cells were washed with PBS, collected with Trizol reagent and cell RNA was extracted.

**GFP-VSV and WNV infection.** WNV (strain NY-99) was obtained from Robert Tesh (University of Texas Medical Branch) and a stock virus pool was produced in BHK WI2 cells. Infection was detected by western blot using goat anti-WNV NS3 antibody (R&D System). GFP-VSV was kindly provided by Fanxiu Zhu (Florida State University) and amplified in HeLa cells. Infection was assessed by fluorescent microscopy.

**HCV pseudoparticle (HCVpp) production and infection.** HCVpp were produced in 293-FT cells as previously reported (38). Cells were pre-treated with 10 μg/ml of anti-CD81 antibody for 4 h and then infected with HCVpp supplemented with the same antibody at a final concentration of 10 μg/ml. Infectivity titers were determined at 72 h post infection using a firefly luciferase assay system (Promega, WI).

**Membrane fusion assay using DiD-labeled JFH-1 HCVcc.** The HCV fusion assay was performed as previously described (14). Briefly, high titer HCVcc was labeled with DiD (Invitrogen, NY) according to the manufacturer’s instructions. DiD-HCVcc particles were then purified by density gradient centrifugation before being used for an infection. Cells grown of coverslips in 12-well cell culture plates were infected with DiD-HCVcc. Fusion spots were counted from multiple representative fields under a Zeiss 510 confocal fluorescence microscope.
Generation of CIDEB knockout 7.5 cells (CIDEB\textsuperscript{KO}) by TALEN. TALEN constructs were purchased from Cellectis (Paris, France) and designed to target exon 3 of the human CIDEB genomic locus, which contains the start codon. The sequences of the DNA binding regions of the TALEN constructs are available upon request. Huh-7.5 cells in 6-well plates were co-transfected with the TALEN and GFP expressing plasmids according to the manufacturer’s instructions. Cells were maintained at 37°C and 5% CO\textsubscript{2} and then collected 48 h post-transfection and resuspended in PBS containing 5% FBS. GFP-positive cells were enriched by fluorescence-activated cell sorting, followed by a recovery period in media containing 20% FBS. Single cell clones were generated by serial dilution in 96-well plates, and analyzed for CIDEB expression by western blot.

Single cell clones negative for CIDEB expression were analyzed by DNA sequencing to confirm insertions or deletions in the TALEN targeted region of the CIDEB gene.

Quantitative Real-time PCR. For cellular mRNAs, cDNA was produced from 500-1000 ng total RNA, using poly-dT and Invitrogen superscript \textsuperscript{III} first strand kit according to the manufacturer’s instructions. The same procedure was used for HCV RNAs, except that gene-specific primers were used (HCV NS3-RT: GGGTCCAGGCTGAAGTCGAC). Quantitative PCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR System, with Invitrogen SYBR Green PCR Master Mix and gene-specific primers (NS3-Rev: CGGGATGGGGGGTTGTCACTG and NS3-Fwd: CTACCTCCATTCTGGCATCGG; GAPDH-Rev: GGATGACCTTGCCCACAGC and GAPDH-Fwd: TC ACTGCCCACAGAAGACTG) at 0.5 μM each in a 20μl reaction. 60°C -95°C melt curve analysis following PCR was
performed using default settings. For CIDEB mRNAs, relative quantitation was performed using the ddCt method with GAPDH as the endogenous control. For HCV RNA quantitation, similar method was used and relative folds change was calculated by normalizing to control, uninfected cells.

**Statistical analysis.** qRT-PCR data were calculated into relative fold change based on Ct (cycle threshold) values. Data were presented as means ± standard deviation (SD), and are either the average of data from three independent experiments, or as indicated in a figure legend. The Student’s t test was used for statistical analysis of the data. A P value of <0.05 was considered significant and indicated by “*”. P values of <0.01 and <0.001 were considered highly significant and indicated by “**” and “***”, respectively.

**RESULTS**

**CIDEB expression is induced during hepatic differentiation and required for HCV infection of hepatocyte-like cells.** We recently identified a transition stage during the hepatic differentiation process (days 7-11 post differentiation) when the cells became permissive for HCV infection (30). A liver-specific gene, CIDEB, was among the genes that were up-regulated at the RNA level during the differentiation process (data not shown). CIDEB has been recently reported to be upregulated by human serum treatment of hepatoma cells (40), which may promote the differentiation of these cells. In addition, CIDEB is associated with lipid droplets and may interact with HCV NS2. We
chose CIDEB for further studies based on these considerations. Consistent with the observation by Phan et al, we were unable to detect an interaction between CIDEB and NS2 in HCV-infected Huh-7.5 cells (data not shown). We did, however, observe a steady increase of the expression of CIDEB during the differentiation process. CIDEB protein became detectable by western blot around day 7 and approached the level in the hepatoma cell line Huh-7.5 cells around day 11 (Fig. 1A). We then analyzed whether induction of CIDEB was required for the transition to HCV permissiveness. A lentivirus small-hairpin RNA (shRNA) directed at CIDEB mRNA was transduced into the human pluripotent stem cell line WA09 to produce a stable cell line which, upon differentiation, produced DHH/HLCs with reduced CIDEB expression (DHH/CIDEB\textsuperscript{KD}) (Fig. 1B). We subjected the day 11 DHH/HLCs to infection by JFH-1-based HCVcc or virions derived from a HCV genotype 1b patient serum. Knockdown of CIDEB effectively inhibited infection of DHH/HLCs by both HCVcc and serum-derived HCV (Fig. 1B and C). The CIDEB knockdown cells retained their ability to store glycogen (Fig. 1D) and to secrete albumin (Fig. 1E), which are two representative indicators of hepatic functions. These results indicate that the inability of these DHH/HLCs to support HCV infection was due to CIDEB knockdown rather than to general defects due to hepatocyte differentiation or in hepatic functions.

The C-terminal domain of CIDEB is required for HCV infection \textit{in vitro}. To facilitate mechanistic studies, we next analyzed whether CIDEB is required for HCV infection of Huh-7.5 cells. Transient knock-down of CIDEB by shRNA produced Huh-7.5 cells that were less permissive to HCV infection, in accordance with results obtained
with the DHH/HLCs. In contrast, knockdown of transglutaminase 2 (TGM-2), another gene detected by our microarray analysis as being highly upregulated at the transition stage (30), had no effect on HCV infection, nor did an shRNA directed at CIDEA mRNA (Fig. 2A). In addition, the inhibitory effect by CIDEB shRNA in Huh-7.5 cells was recapitulated by siRNA duplexes that targeted a different region of the CIDEB mRNA (Fig. 2B). To further rule out possible off-target effects of the CIDEB shRNA, we investigated whether viral infection could be rescued in Huh-7.5/CIDEB KD cells by restoration of CIDEB expression. Co-expression of a full-length CIDEB cDNA containing silent mutations in the shRNA-targeted site but not of a truncation mutant with a C-terminal domain deletion, partially rescued HCV infection (Fig. 2C). Inhibition of HCV infection was also observed in two independently generated stable Huh-7.5/CIDEB KD cell lines (Fig. 2D). Collectively, these results indicate that CIDEB is required for HCV infection of both stem cell-derived DHH/HLCs and Huh-7.5 cells and the C-terminal domain of CIDEB, which differentiates the CIDE proteins from the DFF family of proteins, is required for CIDEB’s proviral function.

CIDEB functions early during the HCV infection cycle, prior to viral protein translation and RNA replication. We next investigated the specific step during the HCV infection cycle that was inhibited by knockdown of CIDEB. Comparison of infection time courses in control cells expressing a shRNA directed at firefly luciferase (Huh-7.5/Ctrl) and stable CIDEB knockdown cells (Huh-7.5/CIDEB KD) revealed that reduction of HCV RNA signal began in the knockdown cells between 12-16 h after infection. A similar reduction time course was observed in cells with knockdown of a
known HCV entry factor claudin-1(Huh7.5/CLDN1\textsuperscript{KD}) (Fig. 3A). HCV proteins could not be detected at 8 h and 12 h post-infection of Huh-7.5/CIDEB\textsuperscript{KD} cells while readily detectable in the wildtype cells at these time points, indicating a suppression or delay of protein expression caused by CIDEB knockdown (Fig. 3B, top). The difference of time points when the RNA or proteins were affected suggested that CIDEB knockdown did not affect RNA internalization but blocked a step prior to viral protein translation.

Interestingly, HCV protein expression was not affected by CIDEB knockdown if the HCV RNA was introduced into Huh-7.5/Ctrl and Huh-7.5/CIDEB\textsuperscript{KD} cells by electroporation, for up to 24 h after transfection (Fig. 3B, bottom), indicating that the role of CIDEB in the HCV life cycle is specific to a virion-mediated early step. Consistent with the above results, the replication kinetics of a full-length J6/JFH-based genome in Huh-7.5/CIDEB\textsuperscript{KD} cells, as measured by expression of the reporter gene (Gaussia luciferase) inserted into the genome, was also comparable to that in Huh-7.5/Ctrl cells when the RNA genome was introduced by electroporation (Fig. 3C). In contrast, knockdown of cyclophilin A, a cellular cofactor required for HCV replication (41-43), efficiently suppressed replication after both infection and viral RNA electroporation. Importantly, performing a mock electroporation prior to infection did not affect the inhibition of infection by CIDEB knockdown (Fig. 3D), indicating that the electroporation process did not simply eliminate CIDEBKD cells. We conclude from these results that CIDEB is not required for the initial translation of input RNA or subsequent RNA replication.

CIDEB knockdown does not affect virion attachment, RNA replication, or virion production. To investigate whether CIDEB is involved in HCV binding to the cell
surface, we determined the effect of CIDEB knockdown on viral attachment. HCVcc particles were incubated with Huh-7.5/Ctrl and Huh-7.5/CIDEB\(^{KD}\) cells for 2 h at low temperature to allow binding but prevent entry. After extensive washing, the amount of virus bound was determined by quantitative RT-PCR with HCV-specific primers. The level of virion-binding to Huh-7.5/CIDEB\(^{KD}\) cells was comparable to that to Huh-7.5/Ctrl cells (Fig. 4A). To determine whether CIDEB is important for sustained HCV RNA replication, shRNAs directed at CIDEA, luciferase or CIDEB mRNA were introduced into a stable subgenomic replicon cell line harboring a NS5A-GFP fusion (44). Knockdown of CIDEB did not affect subgenomic RNA replication, as measured by NS5A-GFP expression (Fig. 4B). Furthermore, knockdown of CIDEB did not affect the establishment of persistent RNA replication by subgenomic RNAs of various genotypes in Huh-7.5 cells as measured by a colony formation assay (Fig. 4C). These results further confirmed that CIDEB is not important for HCV RNA replication in Huh-7.5 cells.

Because CIDEB associates with both intracellular membranes and lipid droplets (LDs) (45) and LDs have been reported to be the site of HCV assembly (46), we tested the possibility that CIDEB participates in the production of viral particles. HCV RNA was electroporated into both Huh-7.5/Ctrl and Huh-7.5/CIDEB\(^{KD}\) cells and the culture supernatants at 48 h and 96 h post transfection were collected for measurement of virus production (Fig. 4D). The total amounts of core secreted into the supernatant of the control and the KD cells were comparable (Fig. 4E). In addition, when reinfection experiments were performed, supernatants collected from Huh-7.5/CIDEB\(^{KD}\) cells showed no significant reduction in virus titers compared to those harvested from Huh-
7.5/Ctrl cells (Fig. 4F). We observed that in the Huh-7.5 cells, CIDEB knockdown reduced both the number and the mean intensity of neutral lipid staining of the LDs (Fig. 4G-H), nevertheless, neither core secretion nor infectivity appeared to be affected. Taken together, these results demonstrate that inhibition of HCV infection by the CIDEB shRNA was not due to defects in viral attachment, viral RNA translation, viral RNA replication or viral particle production.

**CIDEB is required for HCV membrane fusion.** CIDEB may participate in the HCV life cycle in a step that occurs post attachment but before initial RNA translation, such as membrane fusion. To investigate whether CIDEB is required for fusion between the HCV envelope and host membrane, we used a virus-host membrane fusion assay that takes advantage of a self-quenching, lipophilic dye, DiD (14, 47). HCVcc particles were labeled and purified as previously reported (14), and then used to infect Huh-7.5/Ctrl cells. Punctate fusion signals became detectable around 2 h post-binding and the number of puncta increased over the next 4 h (Fig. 5A). The detected punctate signals colocalized with endosome markers (Fig. 5B), where HCV membrane fusion occurs (48). Pretreatment of cells with various entry inhibitors (an anti-CD81 antibody, a small molecule inhibitor of SRBI ITX-5061, or anti-ApoE) all effectively inhibited the punctate signals at 6 h post binding (Fig. 5C). In addition, punctate signals were also significantly reduced when NH₄Cl, an inhibitor of endosomal acidification and pH-dependent membrane fusion, was added to the media of virus-bound Huh-7.5 cells prior to the temperature shift (Fig. 5C). Collectively, these results indicated that the punctate signals detected resulted from productive fusion between the endosomal membrane
and viral envelope. When DiD-labeled HCV particles were incubated with Huh-
7.5/CIDEB<sup>KD</sup> cells, the number of puncta detected at 6 h was reduced by greater than
70% compared to the number detected in Huh-7.5/Ctrl cells (Fig. 5D). As expected,
knockdown of cyclophilin A (Huh-7.5/CyPA<sup>KD</sup>) had no effect on virus fusion efficiency.
These results suggest that CIDEB is functionally important at or before the membrane
fusion step that is required for HCV RNA to escape from the endocytic vesicles and
become available as a template for translation and replication.

CIDEB is not required for infection of Huh-7.5 cells by West Nile virus (WNV)
or vesicular stomatitis virus (VSV). We next investigated whether CIDEB is also
important for infection by other RNA viruses. WNV and VSV, both of which efficiently
infect Huh-7.5 cells, were studied. Expression of WNV NS3 was comparable in Huh-
7.5/CIDEB<sup>KD</sup> cells and in Huh-7.5 parental cells following infection (Fig. 6A). Yields of
extracellular WNV were also not significantly affected by CIDEB knockdown (Fig. 6B).
Similar results were obtained for VSV (Fig. 6C) and a HIV pseudotype bearing VSV
glycoprotein (VSV-G) (Fig. 6D, right panels). Interestingly, the entry of HIV particles
pseudotyped with HCV glycoproteins (HCVpp) was also not inhibited by CIDEB
knockdown (Fig. 6D, left panels), while blocking CD81 or suppression of CLDN1
expression inhibited HCVpp entry as expected.

CIDEB knockout blocks HCV infection in vitro. As RNA interference only
suppresses but does not abolish gene expression, we used transcription activator-like
effector nucleases (TALEN) to create CIDEB knockout cell lines to further demonstrate
The functional requirement of CIDEB for HCV infection. We designed a pair of TALENs targeting the first coding exon (exon 3) of the CIDEB gene (Fig. 7A) and then delivered the TALEN-expressing plasmids into Huh-7.5 cells, followed by single cell cloning using published procedures (49, 50). Western blot was used to identify CIDEB knockout clones. Four out of sixteen clones analyzed had no detectable CIDEB signal on western blots, indicating complete gene knockout (Fig. 7B). We selected clone #3 for infection with HCVcc. DNA sequencing revealed that there were three copies of the CIDEB gene in these Huh-7.5-based cells and all three contained an insertion that, albeit different in each copy, created frame-shifts and introduced premature stop codons (data not shown). HCV infection was blocked in these cells (Fig. 7C). Inhibition of infection in the knockout cells was first detected between 12-16 h post-infection (Fig. 7D), the same time observed in the knockdown cells (Fig. 3A). As expected, the extent of inhibition of HCV infection in the knockout cells was greater than that in the knockdown cells (Fig. 7D versus Fig. 3A). In contrast, neither VSV nor WNV infection was significantly inhibited in CIDEB knockout cells (Fig. 7E and F). These data demonstrate that CIDEB is dispensable for cell survival but specifically required for HCV infection.

**HCV infection downregulates CIDEB at the protein level.** We observed a significant reduction in intracellular CIDEB protein levels in HCV infected cells starting approximately 24 h post infection (Fig. 8A), whereas CIDEB mRNA was actually slightly upregulated in infected cells (Fig. 8B), indicating that downregulation of CIDEB occurs at the post-transcriptional level. To investigate whether infection per se is required for the downregulation, we also electroporated Huh-7.5 cells with HCV genomic RNA and
then determine CIDEB protein levels 24hr or 48hr later. Two wildtype genomes (JFH-1 and wt G-Luc) expressed HCV proteins (NS3 shown) and decreased CIDEB protein levels as early as 24 h (Fig. 8C). The polymerase mutant GND, which carries a defective replicase, was not able to replicate or express any detectable HCV protein and did not affect CIDEB protein levels.

DISCUSSION

Studying the molecular determinants of the transition of cells into a permissive state for viral infections during directed differentiation is a novel approach for identifying host factors contributing to viral tropism. The identification of a new entry cofactor for HCV in the present study represents a proof-of-concept for this approach. Our demonstration that HCV-resistant DHH/HLCs could be produced in vitro by coupling genetic modification of stem cells with hepatic differentiation has implications for the clinical utility of stem cell-based therapy.

Although the mechanism by which CIDEB facilitates HCV entry remains unclear at this time, it appears to be distinct from those of many of the previously reported entry cofactors. Overall our data point to a late step of the viral entry process at which CIDEB functions. The RNA time course data suggest that internalization is not affected, but we cannot rule out other steps of the endocytic pathway such as trafficking of the endosomes to the proper site for membrane fusion and uncoating. The membrane topology of CIDEB (i.e. it is associated with the cytosolic side of the intracellular
vesicular membranes) argues against a direct interaction between CIDEB and the HCV glycoproteins displayed on the incoming virions. On the other hand, the observation that CIDEC/Fsp27 locates to junctions between connecting LDs and facilitates fusion of the LD membranes of (24, 25) suggests the intriguing possibility that CIDEB can participate in a similar hemi-fusion event that is part of a virus uncoating process. In any case, since we did not detect any redistribution of CIDEB to plasma or endosomal membranes upon HCV infection, the presence of another transmembrane protein that bridges an interaction between CIDEB and HCV glycoproteins would be necessary. The effect of CIDEB on HCV entry could also be indirect. Note that CIDEB is not required for the entry of HCVpp, a surrogate system that largely recapitulates the HCV entry requirement of glycoproteins and cellular cofactors. The NPC1L1 protein is another HCV entry cofactor that is not required for HCVpp entry (14), and given the association between lipids and both CIDEB and NPC1L1, it is possible that these proteins act through altering the lipid content of either viral or cellular membranes. We have indeed observed an inhibitory effect of CIDEB knockdown on the number and size of LDs in Huh-7.5 cells and will follow up the intriguing possibility that this organelle may participate in virus entry.

An interaction between CIDEB and HCV NS2 identified by a yeast two-hybrid screen was previously reported (28). However, a more recent study by Phan et al. (29) could not detect this interaction in infected cells. Our results are consistent with those of Phan et al. since we also could not detect any interaction between NS2 and CIDEB in either 293FT or Huh-7.5 cells co-transfected with these proteins (data not shown). It
was somewhat surprising that CIDEB knockdown had no effect on HCV particle production, given the association of CIDEB with LDs, which have been reported to be the sites of HCV virion assembly (46). However, the localization of HCV core on the surface of LDs is not universally required for producing high-titer HCVcc (51) and a potential role of LDs in viral entry was suggested by a recent study which demonstrated that depletion of the sterol regulatory element binding protein (SREBP)-1 and SREBP-2 could inhibit both the formation of LDs and HCV infection in Huh-7.5 cells (52). The inhibition of HCV infection observed extended beyond suppression of viral assembly and suggested a reduction in viral entry as well.

Because shRNAs do not completely abrogate the expression of a gene, a gene knockout system offers a more definitive answer about a host factor’s function both in normal physiology and during viral infections. Meganucleases that combine the power of sequence-specific DNA binding with the ability to generate double-stranded breaks has emerged as an enabling technology of genome editing in human cells. These include zinc finger nuclease (50, 53, 54), TALENs (55-59) and the CRISPR-Cas9 system (60, 61). We used TALEN technology to generate CIDEB-knockout Huh-7.5 cells and confirmed the inhibition to HCV infection and the lack of a significant effect on WNV or VSV infections in these cells. Interestingly, a very low level of HCV infection was still detectable in the knockout cells after prolonged infection periods. The reason for this phenomenon is currently unknown but may be related to alternative infection cell entry routes such as the one recently reported for HCV that is mediated by exosomes secreted by infected cells (62).
HCV entry into (upon exogenous expression of claudin-1) and virus RNA replication in nonhepatic 293T cells have been reported (10, 63). Recently, the combination of expression of HCV receptors, microRNA-122, and ApoE has made these cells permissive for the full life cycle of HCV infection (64). Using antibodies that readily detected CIDEB in Huh-7.5 cells and DHH/HLCs derived from hepatic differentiation, we did not detect CIDEB expression in either parental 293T cells or the modified 293T cells that expressed miR-122 (64) by western blot (data not shown). In addition to the possibility that the entry pathway used by HCV is different in non-liver cells and does not require CIDEB, it is also possible that another related protein(s) functions in place of the liver-specific CIDEB.

Given the strong apoptotic effect of CIDEB protein when expressed at high levels, downregulation of CIDEB after infection may promote the survival of infected cells and contribute to the establishment of chronic infections. In this regard, it is interesting to note that the expression of HCV transgenes in mice also contributed to CIDEB down-regulation by an adenovirus infection (28). The mode of action for the downregulation of CIDEB in the infected cells appears to be post-transcriptional although the precise mechanism remains to be determined. Examples may include translational silencing and accelerated protein degradation.

In summary, we have identified a new HCV entry cofactor the upregulation of which contributes to, although is not sufficient for, the hepatic tropism of HCV. CIDEB
acts at a late step of viral entry and may, among other possibilities, facilitate fusion
between HCV and endosome membranes. The identification of CIDEB adds to the list
of cellular factors important for productive entry of HCV into hepatocytes and provides a
starting point to investigate the potential role of LDs in viral entry and fusion.

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Author names in bold designate shared co-first authorship.
FIGURE LEGENDS

Figure 1. CIDEB is required for HCV infection of stem cell-differentiated human hepatocyte-like cells (DHHs). (A) Expression of CIDEB protein increased during the hepatic differentiation process. (B-C) Knockdown of CIDEB in DHHs suppressed infection by HCVcc (B) and by HCV patient serum (C). Modified WA09 cells (sh-Ctrl and sh-CIDEB) were subjected to hepatic differentiation and the cells at day 11 post-differentiation were infected by HCVcc (multiplicity of infection =1) or HCVser (MOI=0.5 RNA copy) for 4-6 h and cultured for another 48 h before being collected for detection of infection by western blot or qRT-PCR. Values represent mean ± SD, n = 2 independent experiments. **: p<0.01 (D) Glycogen storage in DHH/Ctrl and DHH/CIDEB\textsuperscript{KD} cells. DHHs collected at day 16 post-differentiation were fixed for Periodic acid-Schiff staining and cell images were taken with a 10X objective. (E) Albumin secretion by DHH/Ctrl and DHH/CIDEB\textsuperscript{KD} cells. Cell culture media were harvested from day 16 and day 18 DHHs and analyzed by albumin ELISA. Data were normalized to DHH/Ctrl cells at day 16, and values represent mean ± SD, n = 3 independent experiments.

Figure 2. CIDEB knockdown inhibits HCVcc infection of Huh-7.5 cells. (A) Transduction of Huh-7.5 cells with a CIDEB-directed shRNA suppressed CIDEB expression and HCV infection. Huh-7.5 cells were transduced with lentiviral vectors targeting luciferase, CIDEA, CIDEB, or TGM2. Four days after transduction, cells were challenged with HCVcc and viral infections were analyzed by western blot at 24 h post-infection. (B) A synthetic siRNA duplex that targets a different site on CIDEB mRNA
than the shRNA inhibited infection. Huh-7.5 cells were transfected with duplex siRNA and then infected with HCVcc at 48 h post transfection. Infected cells were then harvested for detection of HCV NS3 and CIDEB by western blot at 24 h post infection.

(C) Rescue of HCV infection by full-length CIDEB cDNA that contained silent mutations in the shRNA-targeted site. Huh-7.5 cells were first transduced with lentiviral vectors targeting luciferase, TGM2 or CIDEB for four days and then transfected with an expression plasmid containing the indicated cDNA. At 16 h post transfection, cells were infected with HCVcc and infected cells were collected 20 h later for the detection of viral infection. The C-terminal truncated cDNA of CIDEB also lacks the shRNA target site (CIDEB 517-537). (D) Huh-7.5 cell lines with stable CIDEB knockdown were less susceptible to HCV infection. Huh-7.5 cells were transduced with lentiviral vectors targeting CIDEB followed by puromycin selection for two weeks. Two independently generated cell lines were infected with HCVcc for 24 h and then harvested for the detection of viral infection by western blot. All HCVcc infections were done with a MOI of 5.

**Figure 3. CIDEB acts at an early step of the HCV infection cycle.** (A) Time course analysis of HCV infection in control, CIDEB<sup>KD</sup>, and CLDN1<sup>KD</sup> cells. The viral inoculum was added to cells at -2 h at 4°C and the cells were shifted to 37°C at 0 h after extensive washing. At the indicated times (with the exception of the 0 h samples), cells were trypsinized and then washed to remove surface-bound virions. For the 0 h samples, cells were collected without trypsinization for the detection of virions bound to the cell surface prior to entry. Data were normalized to the value obtained for the control
cells at -2 h, and values represent mean ± SD, n = 3 independent experiments. **: p<0.01; ***: p<0.001. (B) Core expression from viral RNA delivered by infection or transfection in control and CIDEB\textsuperscript{KD} cells. Cells were either infected with HCVcc or electroporated with JFH1-RNA. Total cell lysates were collected at the indicated times after the addition of virus (top) or electroporation (bottom) and subjected to western blot. (C) Intracellular replication of transfected HCV RNA in control, CIDEB\textsuperscript{KD}, and CyPA\textsuperscript{KD} cells. At the indicated times after electroporation with WT or replication deficient (GND) Jc1 G-Luc RNA, cell culture media were collected for luciferase assay. Values represent mean ± SD, n = 3 independent experiments. All HCVcc infections were done with a MOI of 30. (D) Infection of CIDEB\textsuperscript{KD} cells were still suppressed after a round of mock electroporation. Ctrl and CIDEBKD cells were mock electroporated and cultured for 40hr before HCVcc was used to infect the recovered cells. Detection of NS3, GAPDH, and CIDEB was done at 8 and 12 h post-infection.

Figure 4. CIDEB knockdown does not affect virion attachment, RNA replication and virion production. (A) HCV attachment is not affected by CIDEB knockdown. Control and CIDEB\textsuperscript{KD} cells were exposed to HCVcc (MOI=30) at 4°C for 2 h with gentle shaking and then harvested for analysis of viral RNA using qRT-PCR. Data were normalized to the value obtained for control cells without HCVcc exposure. Heparin (75 μg/ml) treated cells were used as a positive control for inhibition of virus binding (65), Values represent mean ± SD, n = 2 independent experiments (B) CIDEB is not required for RNA replication of HCV replicons. A genotype 1b NS5A-GFP replicon GS5 was transduced with lentiviral vectors targeting luciferase, CIDEA or CIDEB and four days...
later cells were fixed and analyzed by fluorescence microscopy. (C) Stable CIDEB\(^{KD}\) cells supported efficient colony formation by subgenomic replicons of genotype 1a (H77), 1b (Con1), and 2a (JFH-1). (D) A diagram of experimental design. Jc1 G-Luc RNA (a full-length HCV genomic RNA containing the Gaussia luciferase gene) was electroporated into both Huh-7.5/Ctrl and Huh-7.5/CIDEB\(^{KD}\) cells and supernatants collected from the electroporated cells at the indicated times post electroporation were labeled as Ctrl and CIDEB\(^{KD}\) viruses, respectively. The amounts of core proteins in the supernatants were determined by a HCV Core ELISA kit (OrthoDiagnostics) (E) and were used to normalize the inoculums used to infect naïve Huh-7.5 cells in (F). (F) G-luciferase activity was measured in the infected cells 48 h after infection. 0h point supernatant was collected immediately after PBS washes, and the data represents the residual signal from the original inoculum. Values plotted were mean ± SD, n = 3 independent experiments. (G and H) LD staining and quantification in Huh-7.5/Ctrl and Huh-7.5/CIDEB\(^{KD}\) cells. Representative images of Bodipy 530/550 staining were shown and the size and the mean fluorescence intensity of LDs were quantified through “particles analyzing” function of ImageJ software (NIH).

**Figure 5. HCV entry and membrane fusion is inhibited in CIDEB\(^{KD}\) cells.** (A) The fusion spots of DiD labeled HCVcc increased over time after initiation of cell entry by the temperature shift. Control cells were exposed to DiD labeled HCVcc (MOI=20) at 4°C for 2 h then washed and shifted to 37°C to initiate internalization. At the indicated times, cells were fixed for fluorescence microscopy. The average number of spots per 100 cells was calculated and values shown represent mean ± SD, n = 3 independent
experiments. (B) Colocalization of DiD fusion signals with the endosome marker Rab5. Control cells were treated as indicated in (A). At 4 h post binding, cells were fixed and stained with anti-Rab5 antibody (green). Arrows indicate colocalization between DiD signal and Rab5. (C) The DiD HCV fusion signal was sensitive to pH perturbation and HCV entry inhibitors. The number of DiD signals at 2 and 6 h after the temperature shift were quantified. NH₄Cl was added to the medium at a final concentration of 20 mM; anti-ApoE (at a final concentration of 50 μg/ml) was incubated with virus for 2 h, and the entry inhibitors (anti-CD81 at 10 μg/ml and ITX-5061 at 1 μM) were incubated with cells for 2 h before temperature shift. The number of DiD signals were quantified and normalized to the value obtained for DMSO treated cells at 2 h. Values are relative fold change and represent mean ± SD, n = 2 independent experiments. *: p<0.05. (D) HCV fusion was inhibited in CIDEB$_{KD}$ but not in CyPA$_{KD}$ cells. Experiments were performed as indicated in (A). Medium from Huh-7.5 cultured for 48 h was processed using the same DiD labeling procedure and used as the control background fluorescence signal. The data represent the average number of spots per 100 cells and the mean ± SD, n = 3 independent experiments SD. *: p<0.05; ***: p<0.001.

Figure 6. CIDEB knockdown does not affect infection by WNV, GFP-VSV, or HIV-based pseudotyped viruses. (A) CIDEB knockdown in Huh-7.5 cells did not suppress WNV NY99 infection. Huh 7.5/Ctrl and Huh7.5/CIDEB$_{KD}$ cells in 6-well plates were infected with WNV NY-99 at a MOI of 1. At the indicated times after infection, cells were lysed with RIPA buffer (50mM Tris, pH7.4, 150mM NaCl, 0.1% SDS, and 1% NP-40) and intracellular viral protein was detected by western blot using anti-WNV NS3.
antibody. (B) CIDEB knockdown in Huh-7.5 cells did not inhibit the production of WNV. Confluent monolayers of Huh 7.5/Ctrl and Huh7.5/CIDEB\textsuperscript{KD} cells in 6-well plates were infected with WNV NY-99 strain (MOI=1) for 30 min at 37\degree C, washed 3 times with medium, and 2 ml of medium were added per well. Aliquots of culture fluid harvested from duplicate wells at 8, 10, 16, 24, 32, and 48 h after infection were assayed for infectivity by plaque assay on BHK cells (66). (C) Efficient infection of the CIDEB\textsuperscript{KD} cells by a GFP-tagged VSV. Huh 7.5/Ctrl and Huh7.5/CIDEB\textsuperscript{KD} cells were infected with GFP-VSV (MOI=1) and then fixed and processed for fluorescence microscopy at the indicated time post infection. (D) HCVpp and VSV-Gpp infection of CIDEB\textsuperscript{KD} cells. Huh7.5/Ctrl, Huh7.5/CIDEB\textsuperscript{KD}, and Huh7.5/CLDN1\textsuperscript{KD} cells were infected with HIV-luciferase particles pseudotyped with HCV E1/E2 or VSV-G. Firefly luciferase activities were measured 72 h post infection. Values of HCVpp and VSV-Gpp infection in control cells were normalized to 100\% for each of the respective pseudotypes. Anti-CD81 antibody was added at a final concentration of 10 \mu g/ml at 4 h before infection and then maintained throughout the experiment. Values are the relative percentage over that for infection in control cells and represent mean ± SD, n = 2 independent experiments. **: p<0.01; ***: p<0.001.

**Figure 7. Knockout of CIDEB blocks HCV infection in cell culture.** (A) Schematic representation of TALEN constructs targeting the genomic sequence immediately downstream of the CIDEB start codon, located in exon 3. (B) Knockout of CIDEB by TALEN. Four of the 16 clones analyzed had no detectable CIDEB western blot signal. (C) Inhibition of HCV infection by CIDEB knockout in Huh-7.5 cells. Wild-type and
CIDEBKO-003 cells were infected with HCVcc (MOI=5) and cell lysates prepared at the indicated times post infection were used to detect HCV NS3 expression by western blot. (D). Comparison of the time course of HCV infection in wild-type and CIDEBK0 cells. Experiments were performed as described in the figure 3A legend. Data were normalized to the value for wild-type cells obtained at -2 h and represent mean ± SD, n = 2 independent experiments. **: p<0.01. (E) GFP-tagged VSV efficiently infected CIDEBK0 cells. Experiments were carried out as described in the figure 6C legend. Representative images were shown. (F) Knockout of CIDEB does not affect WNV infection. Control and CIDEBK0-012 cells were infected with WNV NY-99 (MOI=1) for 24 hours, fixed, permeabilized and incubated with an anti-dsRNA antibody to detect sites of viral RNA replication. Hoechst 33342 dye (Invitrogen) was used to detect nuclei. Images were acquired with an inverted fluorescence microscope (Zeiss 510) using a 100X oil immersion objective.

Figure 8. Downregulation of CIDEB protein by HCV infection. (A) CIDEB protein levels were reduced in HCV-infected cells. Huh-7.5 cells were exposed to HCVcc (MOI=10) at 4°C for 2 h and then shifted to 37°C to initiate infection. Cell lysates were prepared at the indicated times post infection and used to detect viral infection and CIDEB expression by western blot. (B) CIDEB mRNA is not down-regulated by HCV infection. Experiments were carried out as described in (A), except that cells were subjected to RNA extraction and qRT-PCR was used to analyze CIDEB mRNA levels. (C) CIDEB was downregulated by active viral RNA replication. Huh-7.5 cells were electroporated with replication competent (JFH1 and G-Luc-WT) or defective (GND)
genomes. At the indicated times, cells were lysed and analyzed by western blot. The GND mutant contained mutations in the RNA-dependent RNA polymerase gene that abolishes replication and accumulative protein expression.