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Apolipoprotein E interacts with hepatitis C virus non-structural protein NS5A and determines assembly of infectious particles

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Abbreviations: HCV: hepatitis C virus; HCVcc: cell culture-derived HCV; HCVpp: HCV pseudotype particles; HDL: high-density lipoprotein; LDL: low-density lipoprotein; MOI: multiplicity of infection; VLDL: very low density lipoprotein; siRNA: small interfering RNA; TCID₅₀: 50% tissue culture infectious dose

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ABSTRACT

Chronic hepatitis C virus (HCV) infection is a major cause of liver disease worldwide. Restriction of HCV infection to human hepatocytes suggests that liver-specific host factors play a role in the viral life cycle. Using a yeast-two-hybrid system, we identified apolipoprotein E (apoE) as a liver-derived host factor specifically interacting with HCV non-structural protein 5A (NS5A) but not with other viral proteins. The relevance of apoE-NS5A interaction for viral infection was confirmed by co-immunoprecipitation and co-localization studies of apoE and NS5A in an infectious HCV cell culture model system. Silencing apoE expression resulted in marked inhibition of infectious particle production without affecting viral entry and replication. Analysis of particle production in liver-derived cells with silenced apoE expression showed impairment of infectious particle assembly and release. The functional relevance of apoE-NS5A interaction for production of viral particles was confirmed by loss or decrease of apoE-NS5A binding in assembly-defective viral mutants. In conclusion, these results suggest that recruitment of apoE by NS5A is required for viral assembly and release of infectious viral particles. These findings have important implications for understanding the HCV life cycle and the development of novel antiviral strategies targeting HCV-lipoprotein interaction.

Introduction

Hepatitis C virus (HCV) is a major cause of liver disease including liver cirrhosis and hepatocellular carcinoma (1). Current treatment by interferon-alpha and ribavirin is limited by resistance, toxicity and high costs (1, 2). Novel treatment approaches are therefore urgently needed. HCV is an enveloped single stranded RNA virus of positive polarity which is a member of the genus *Hepacivirus* within the family *Flaviviridae* (3, 4). The HCV RNA genome encodes a unique polyprotein of about 3000 amino acids, and is flanked at its 5' and 3' ends by two highly conserved untranslated regions (UTR) involved in the translation and replication processes of the virus, respectively. The virus enters the cell through interaction of the viral glycoproteins with cellular co-factors (3, 4). Following viral entry, viral translation and replication occurs in a cell compartment termed the "membranous web", which is followed by viral assembly and particle egress (3, 4).

An important feature of the hepatocyte is its key role in lipid metabolism. Increasing evidence suggests that the HCV life cycle and hepatocyte lipid metabolism pathways are closely linked. Indeed, HCV replication takes place, as noted above, at specialized rearranged intracellular membranes termed "membranous web" (4). Membrane vesicles containing the HCV replication complex have been shown to be highly enriched in proteins required for very low density lipoprotein (VLDL) assembly, including apolipoprotein B (apoB), apolipoprotein E (apoE), and microsomal triglyceride transfer protein (5). Furthermore, the VLDL pathway has been shown to play a role in the assembly and maturation of infectious viral particles (5, 6). Moreover, a hepatocyte organelle storing lipids - the lipid droplet - has been shown to be important for the production of infectious virus particles (7,

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3 8). Miyamura and colleagues (8) demonstrated that the viral capsid protein core
4 recruits viral non-structural proteins and replication complexes to lipid droplet
5 associated membranes, and that this recruitment is critical for producing infectious
6 viruses. Furthermore, HCV core protein has been shown to induce lipid droplet
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12 5 redistribution (9) and different HCV genotype core sequences have been shown to
13 induce morphological changes in intracellular lipid droplets (10). Finally, Chang et al.
14 (11) have shown that HCV infectious virions purified from low density fractions of cell
15 culture supernatants are assembled as apoE-enriched lipoprotein particles and can
16 be specifically precipitated by anti-apoE and anti-E2 monoclonal antibodies (11).
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24 10 These findings indicate that apoE is required for HCV virion infectivity and
25 production. However, how apoE is recruited to the infectious particle and which viral
26 factors are implicated in that interaction process remains unclear.
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34 Recent evidence suggests that the viral non-structural protein NS5A plays an
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36 15 important role in HCV virion production. NS5A was found to be recruited by core-
37 associated lipid droplets in replicating Huh7.5 cells for production of infectious
38 particles (8, 12). The introduction of specific mutations into the NS5A showed that
39 mutations in NS5A C-terminal domain III abolished core-NS5A co-localization in the
40 HCV replicating cells and hampered virion production (8, 13). These data identify
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48 20 NS5A as a viral factor for assembly of infectious viral particles (8, 13). However, the
49 functional link between NS5A and virus production is unknown.
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Materials and Methods

Plasmids. Yeast two-hybrid system expression plasmids have been described (14). The full-length apoE cDNA cloned in pOTB7 vector was purchased from Open Biosystems and contains an apoE consensus sequence. Following digestion of the plasmid by EcoRI-XhoI apoE cDNA was inserted in the pGADT7 EcoRI-XhoI sites. Plasmids pFK-Jc1 (Jc1), pFK-Luc-Jc1 (Luc-Jc1) and J6/JFH1 constructs have been described (15-19). JFH1 or H77 NS5A encoding regions were PCR amplified using Jc1 or p90/HCV FL-long pU (20) as templates and inserted in the pGBKT7 EcoRI-Sall sites (14). Alanine triplet substitutions at position 99 to 101 and 102 to 104 in the JFH1 NS5A coding region as described by Miyanari et al. (8) and "domain III" deletion mutant described by Appel et al. (13), were obtained as described recently (21).

Yeast and bacteria strains. The apoE clone (Clontech, Cat #638802) was transformed in the *S. cerevisiae* strain Y187 [MAT α , ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4 Δ , met-, gal80 Δ , MEL1, URA3:GAL1_{UAS}-GAL1_{TATA}-LacZ]. The pGBKT7 plasmids expressing the HCV NS proteins were transformed in the *S. cerevisiae* strain AH109 (MAT α , trp1-901, leu2-3, 112, ura3-52, his3-200, gal4 Δ , gal80 Δ , LYS2:GAL1_{UAS}-GAL1_{TATA}-HIS3, GAL2_{UAS}-GAL2_{TATA}-ADE2, URA3:MEL1_{UAS}-MEL1_{TATA}-lacZ). The library vectors were amplified in *E. coli* KC8 cells (CLONTECH Laboratories, Inc.) and those carrying the hybrid construct in *E. coli* XL1 blue cells (Stratagene).

Antibodies. Monoclonal mouse anti-NS5A was obtained from Virostat, rabbit polyclonal anti-NS5A (22), and monoclonal anti-apoE (Ab23, Ab33) have been

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3 described (11), polyclonal rabbit anti-apoE were from Epitomics and Abgent and
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6 polyclonal rabbit anti-apoA1 was from Santa Cruz.
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10 **Yeast two-hybrid assays.** Yeast two-hybrid assays were performed as described by
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13 5 Dimitrova et al. (14). The pGBKT7- and pGADT7-derived constructs encoding HCV
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15 NS proteins were co-transformed into AH109 yeast cells. Co-transformants were
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17 grown for about 24 h in –LEU –TRP medium and then spotted onto – LEU –TRP
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19 culture plates to select for co-transformants and onto –TRP–LEU–HIS–ADE culture
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21 plates to allow for selection of interactants.
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27 **RNA interference assay.** Commercially available siRNA pools targeting apoE and
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29 apoA1 as well as control non-targeting siRNAs were purchased from Dharmacon and
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31 transfected into Huh7.5.1 cells using DharmaFect™ solution following the
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33 manufacturer's protocol. Silencing of protein expression was assessed by
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35 immunoblotting (19) for apoE or immunofluorescence (14) for apoA1.
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41 **Production of HCV pseudotype particles and infection of Huh7.5.1 cells.**
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43 Production and infection of Huh7.5.1 cells using MLV-based HCVpp (strain H77)
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45 were performed as described (19, 23, 24).
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51 **Analysis of HCV infection and replication.** For the study of the viral life cycle in
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53 cells with silenced apoE expression, siRNAs were either co-electroporated with HCV
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55 Luc-Jc1 RNA (16, 19) (protocol A) or transfected 3 days before electroporation of
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57 HCV RNA (protocol B). HCV replication was analyzed by luciferase activity as
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59 described (15, 19). For infection assays, Huh7.5.1 cells were incubated with Luc-Jc1
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3 HCVcc (TCID₅₀ of 10³, 10⁴ and 10⁵/mL corresponding to a MOI of 0.1, 1 and 10). 48 h
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5 later, infection was analyzed by quantitation of protein expression using luciferase
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7 activity (15, 16, 19).
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12 **5 Purification of intracellular or extracellular HCV virions using sucrose gradient**
13 **ultracentrifugation.** HCV virions were partially purified by pelleting lysates or culture
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15 supernatants through a sucrose cushion (20% sucrose in TN buffer) using a SW55Ti
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17 rotor (30,000 rpm for 4 h at 4 °C) and a Beckman L8-80M preparative ultracentrifuge.
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19 In addition, HCV RNA of cell lysates and supernatants were purified on iodixanol
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21 gradients as described by Gastaminza et al. (25). Viral RNA content was determined
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23 by quantitative RT-PCR and infectivity was assessed by incubation of pelleted HCV
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25 or iodixanol gradient fractions with naïve Huh7.5.1 cells and subsequent detection of
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27 infection (72 h post-incubation) by luciferase activity.
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36 **15 Co-immunoprecipitation of apoE and NS5A in HCV replicating cells.** Co-
37 immunoprecipitation experiments were performed in Huh7 cells containing replicating
38 Jc1 as described (14) using monoclonal anti-NS5A (Virostat) or an anti-c-myc-tag
39 isotype control antibody (Sigma). Immunoprecipitated proteins were subjected to
40 immunoblotting using monoclonal anti-apoE (11). In a second approach, co-
41 immunoprecipitation was performed in lysates of Huh7 cells transfected with HCV
42 J6/JFH1 RNA (17) or Huh9-13 cells containing the HCV Con-1 replicon (26) using
43 rabbit anti-NS5A (22), anti-apoE (Abgent) or an anti-HBc control antibody (27) and a
44 previously described co-immunoprecipitation protocol (28). Immunoprecipitated
45 proteins were analyzed by immunoblotting using peroxidase conjugated apoE and
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60 **25 anti-NS5A (Peroxidase conjugation kit, Pierce).**

Co-localization of apoE and NS5A in HCV replicating and infected cells.

Huh7.5.1 cells containing Jc1 HCV RNA were fixed with 3% paraformaldehyde/PBS and permeabilized with 0.1% Triton X-100 in PBS. Proteins were stained using anti-apoE (Epitomics), anti-NS5A (Virostat) and Alexa Fluor[®] 488-conjugated goat anti-rabbit IgG and Alexa Fluor[®] 568-conjugated goat anti-mouse IgG (Molecular Probes) and a Zeiss Axiovert microscope (Zeiss). Immunofluorescence and confocal laser scanning microscopy of Huh7.5.1 cells infected with HCV JFH-1 or Jc1 was performed as described (29).

Results

ApoE interacts with the HCV NS5A protein in a yeast two-hybrid assay. To

investigate apoE-HCV interactions, we first studied the interaction of apoE with HCV proteins using a yeast two-hybrid system. A yeast two-hybrid assay was constructed using HCV proteins fused to Gal4 DNA binding domain as bait and a full-length apoE fused to the Gal4 activation domain as a prey. Following viral protein-apoE interaction, reporter gene His3 is activated allowing selection on defined media (Fig 1A). As shown in Fig.1, only yeast co-transformed with apoE and HCV NS5A protein was able to grow on selective media (Fig. 1B). The specificity of this interaction for NS5A was confirmed by the absence growth in co-transformation experiments using the HCV core and non-structural proteins NS2, NS3, NS4A, NS4B and NS5B and non-related non viral proteins (Fig. 1B). Interestingly, an apoE interaction was not observed with C-terminally truncated envelope glycoproteins E1 (aa 170-311) and E2 (aa 371-661) (data not shown). The binding of apoE to NS5A appeared to be conserved among different genotypes since both NS5A of strain H77 as well as NS5A derived from strain JFH1 bound to apoE in the yeast two-hybrid system (Fig. 8A). The validity of the yeast-two hybrid system for the study of viral protein-protein interactions was further confirmed by core-NS5A interaction of both strains H77 and JFH1 (Fig. 1C) which has been shown to be required for viral assembly (8, 13). These results demonstrate that HCV NS5A interacts specifically with full-length apoE in a yeast two-hybrid assay.

ApoE and NS5A co-immunoprecipitate and co-localize in Huh7.5.1 cells containing replicating HCV. To confirm binding of host factor apoE and viral protein

NS5A during the HCV life cycle, we performed co-immunoprecipitation experiments

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3 in Huh7.5.1 cells containing replicating HCV. Anti-NS5A antibody specifically co-
4 immunoprecipitated viral protein NS5A together with apoE (Fig. 2). Detection of
5 apoE-NS5A binding was dependent on the concentration of anti-NS5A antibody,
6 since low concentrations of anti-NS5A antibody did not allow detection of apoE-NS5A
7 binding. The validity of the results was further confirmed by absence of
8 immunoprecipitation of apoE using anti-NS5A antibody in non HCV replicating
9 Huh7.5.1 cells (Fig. 2). ApoE-NS5A interaction was further confirmed by reciprocal
10 co-immunoprecipitation using anti-apoE (Fig. 2B, C). Furthermore, apoE-NS5A
11 interaction appeared to be genotype-independent since it was also easily observed
12 for NS5A of the HCV genotype 1b Con-1 replicon (Fig. 2B, C). These results clearly
13 demonstrate that HCV protein NS5A binds to host cell apoE in target cells with
14 replicating HCV. It is of interest to note that the detection of apoE-NS5A binding by
15 co-immunoprecipitation did not require over-expression of apoE or NS5A using cDNA
16 expression constructs but was easily detectable with endogenous apoE present in
17 physiological levels in hepatoma cells and NS5A in expression levels present during
18 viral replication.

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To further confirm apoE-NS5A binding during viral infection, we performed co-
localization studies in HCV replicating and infected cells. As shown in Fig. 3A,
Huh7.5.1 expressed apoE at various levels including cells with high-level expression
and cells where expression of apoE was virtually absent. Confirming the interaction
of NS5A with apoE in living cells, NS5A partially co-localized with apoE in Huh7.5.1
cells containing replicating HCV (Fig. 3A). This partial co-localization between apoE
and NS5A was also observed at distinct “dot”-like structures in HCV-infected cells
(Fig. 3B).

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3 **ApoE is required for production of infectious HCV particles.** To investigate the
4 role of apoE and the impact of apoE-NS5A interaction on the HCV life cycle, we
5 silenced apoE expression in Huh7.5.1 cells using apoE-specific siRNAs. As shown in
6 immunoblot analyses, transfection of apoE-specific siRNA (siApoE) reproducibly and
7 specifically silenced apoE expression (Fig. 4A). Using this protocol, we investigated
8 whether silencing apoE had any impact on production of infectious viral particles. As
9 shown in Fig. 4B, supernatants obtained from donor cells containing replicating HCV
10 Jc1 with silenced apoE expression (siApoE) showed markedly reduced or total lack
11 of infectivity. To confirm these results, we investigated the impact of apoE silencing in
12 single cycle infection experiments in a protocol similar to previous studies analyzing
13 the impact of apoB on HCV production (6). Again, apoE silencing resulted in marked
14 inhibition of infectivity of supernatants of infected cells (Fig. 4C). Inhibition of virus
15 production was observed for different MOIs (0.1, 1 and 10), with the most
16 pronounced inhibition occurring at the lowest MOI (Fig. 4C). These single-cycle
17 experiments using infectious recombinant HCV at different MOIs confirm that apoE is
18 required for production of infectious virions.

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44 A previous report has described an interaction of NS5A with apoA1 using co-
45 immunoprecipitation experiments (30). As shown in Fig. 4B, silencing of apoA1 by a
46 pool of validated siRNAs did not result in detectable decrease of virus production.

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53 **ApoE is a host factor required for a late stage in the viral life cycle.** Next, we
54 mapped the stage of the viral life cycle requiring apoE as a host cell factor. It is
55 conceivable that apoE could be involved in the early infection stages such as entry or
56 replication as well as late stages of the viral life cycle such as assembly or release of
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3 viral particles. To exclude an effect of apoE silencing on viral entry we infected
4 Huh7.5.1 cells with HCVpp and HCVcc. As shown in Fig. 5, apoE silencing had no
5 effect on HCVpp entry or HCV infection. In contrast, silencing the HCV entry factor
6 CD81 resulted in marked inhibition of HCVpp entry (Fig. 5A) and HCVcc infection
7 (Fig. 5B). These data demonstrate that host cell apoE is not essential for viral entry.
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18 To investigate a potential effect of apoE silencing on viral replication, Huh7.5.1
19 cells were co-electroporated with HCV Luc-Jc1 RNA and apoE siRNA. As shown in
20 Fig. 5C, silencing of apoE expression did not significantly alter HCV replication. In
21 contrast, an antiviral siRNA (siHCV331) targeting the 5'NTR of the viral genome (21)
22 markedly inhibited viral replication (Fig. 5C). These results demonstrate that silencing
23 of apoE expression did not modulate HCV replication. This finding is further
24 supported by the results of co-localization studies (Fig. 3) where HCV NS5A protein
25 was also detected in cells with low or absent apoE expression.
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39 **ApoE is involved in assembly and release of infectious HCV virions.** Since apoE
40 was required to produce infectious viral particles, but did not interfere with viral
41 replication, it is likely that apoE is involved in a post-replication stage of the viral life
42 cycle. Post-replication virus-host interaction stages include viral assembly and
43 release of viral particles from the infected hepatocytes. To address whether apoE
44 was required for assembly and release, we partially purified intracellular infectious
45 viral particles from cellular lysates or supernatants from Luc-Jc1 replicating cells.
46 Infectivity of the particles present in cell culture lysates or supernatants was analyzed
47 following incubation with naïve Huh7.5.1 cells. Two experimental conditions were
48 used for apoE silencing: protocol A, where siRNAs targeting apoE or control siRNA
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3 were transfected 72 hours before electroporation of cells with HCV RNA and protocol
4 B, in which siRNAs were co-electroporated with HCV RNA (Fig. 6A). ApoE silencing
5 was analyzed by immunoblot 72h after HCV RNA electroporation (Fig. 6B). Protocol
6 A resulted in less efficient apoE silencing than protocol B at the time of HCVcc
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12 5 harvesting (Fig. 6B). In both protocols, apoE silencing resulted in a marked decrease
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14 in the infectivity of extracellular virions confirming a functional role for apoE in virion
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16 production (Fig. 6C). When siRNAs and HCV RNA were co-transfected (protocol B,
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18 Fig. 6A), silencing of apoE resulted in a marked decrease in the infectivity of virions
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20 purified from intracellular lysates as well as virions present in supernatants from
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24 10 transfected cells (Fig. 6C, right panel). A dual effect on both intracellular and
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26 released infectious particles suggests that apoE is a co-factor for viral assembly.
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28 When siRNAs were transfected prior to electroporation of HCV RNA (protocol A, Fig.
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30 6A), a similar decrease in the infectivity of cell culture supernatants was observed. In
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32 contrast, the infectivity of particles in cellular lysates was almost unchanged 72 h
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35 15 after HCV RNA electroporation (Fig. 6C). The marked inhibition of released infectious
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37 particles (present in cell culture supernatants) without a concomitant decrease of
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39 intracellular particles (present in cellular lysates) suggests an additional effect of
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41 apoE silencing on release of viral particles. The minor effect of apoE silencing on
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43 assembly in this experiment may be due to the fact that low-level apoE silencing may
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47 20 still allow particle assembly. Furthermore, the impairment in release may have
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49 resulted in an accumulation of intracellular virions.
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55 To further address the impact of apoE silencing on viral assembly and egress,
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57 we performed time course experiments following apoE silencing in HCV replicating
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59 cells. As shown in Fig. 7, apoE silencing resulted in an early effect on released
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3 infectious particles and a late effect on the assembly of intracellular infectious virions.
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5 These findings suggest that apoE is a co-factor for two distinct steps in the assembly-
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7 egress process. The delayed effect of apoE silencing on assembly may be due to the
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9 impairment of viral release resulting in an accumulation of intracellular virions as well
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11 as the presence of a pool of pre-assembled particles which are not affected by apoE
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13 silencing during early time points.
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20 As shown in Fig. 6D, apoE silencing did not markedly modify the biophysical
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22 properties of released infectious viral particles (Fig. 6D).
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27 **ApoE-NS5A interaction is lost in viral mutants with a defect in viral assembly.**

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29 To further confirm the functional impact of apoE-NS5A interaction for viral assembly,
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31 we studied apoE-NS5A interaction using mutant NS5A from viral variants with a
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33 defect in virus production. Using site-directed mutagenesis we introduced an alanine
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35 triplet substitution at position 99 to 101 or 102 to 104 of the JFH1 NS5A protein as
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37 15 described by Miyanari et al. (8). These mutations have been shown to result in a
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39 defect of virion production in an infectious cell culture model (1, 8). As shown in Fig.
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41 8A, NS5A mutants containing these mutations lost their ability to interact with apoE in
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43 a yeast-two hybrid system. A marked reduction in apoE-NS5A interaction (Fig. 8B)
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45 was also observed for NS5A of a viral variant containing a deletion of the NS5A
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47 20 domain III and a marked impairment of viral assembly (1, 8). This functional
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49 correlation between apoE-NS5A binding (Fig. 8) and assembly/production phenotype
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51 (1, 8) further confirms the functional relevance of apoE-NS5A interaction in the
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53 production of infectious viral particles.
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Discussion

Using an infectious HCV cell culture system, we demonstrate that apoE binds to HCV non-structural protein NS5A. This conclusion is supported by a specific and easily detectable apoE-NS5A interaction in co-immunoprecipitation and co-localization studies in human hepatoma cells containing replicating infectious HCV as well as a yeast two-hybrid assay (Fig. 1-3).

The HCV NS5A protein is anchored in the endoplasmic reticulum (ER) via its N-terminal part (31) and forms 'claw-like' dimers presumably facing away from the membrane where it could accommodate either single- or double-stranded RNA and interact with viral and cellular proteins and membranes (32, 33). Having identified an interaction between apoE and NS5A in HCV replicating cells, it is conceivable that the apoE-NS5A interaction takes place in the vicinity or within the replication complex and that this interaction recruits apoE to the viral assembly modules which results in the production of viral particles released from the infected hepatocytes. The hypothesis of apoE-NS5A interaction within the membranous web is supported by the vesicular membranous structures harbouring replicating virus being enriched with lipoproteins including apoE (5, 6). This hypothesis is also supported by recent studies demonstrating an important role of NS5A in HCV virion production: NS5A has been shown to be recruited by core-associated lipid droplets in replicating Huh7.5 cells to allow robust infectious particle production (8, 12). Using reverse genetics, the NS5A domain III has been identified as an important viral determinant for virion production (1, 8). The relevance of apoE-NS5A interaction for virus production is further supported by the loss of apoE-NS5A binding from viral variants (8, 13) with a defect in virus production in the yeast-two-hybrid system (Fig. 8). Thus, our results identify

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3 apoE-NS5A binding as a novel virus-host interaction and point to apoE-NS5A binding
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5 having a crucial function in the production of infectious particles. Further studies are
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7 underway to fine-map the structural determinants of NS5A-apoE interactions and its
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9 impact on the viral phenotype.
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15 The functional characterization in an infectious cell culture system suggests
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17 that apoE is involved in assembly and release of viral infectious particles. This
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19 conclusion is supported by the finding that apoE silencing results in a marked
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21 decrease in viral particles in cell lysates and supernatants of Huh7.5.1 cells
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24 10 electroporated with HCV RNA (Protocol B; Fig. 6C). Interestingly, when apoE
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26 silencing was less pronounced at the time of HCVcc harvest (protocol A; Fig. 6B),
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28 apoE silencing still resulted in a decrease in released infectious particles with
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30 concomitant retention of infectious viral particles in the host cell (Fig. 6C). It seems
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32 likely, therefore, that efficient silencing of apoE at the time of HCVcc harvest results
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34
35 15 in impairment of viral assembly and release, whereas low-level silencing may allow
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37 assembly of viral particles but results in impairment of viral release. Time course
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39 experiments (Fig. 7) showed an early effect of apoE silencing on released infectious
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41 particles and a late effect on the assembly of intracellular infectious virions. These
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43 data further support a model for apoE as a co-factor for two distinct steps of the
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46 20 assembly-egress process of the viral life cycle.
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53 Recent studies have provided evidence that the very low density lipoprotein
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55 (VLDL) pathway appears to play a role in HCV assembly, maturation and egress.
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57 Indeed, the membrane vesicles in which HCV replicates are highly enriched in
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59 25 proteins required for VLDL assembly, including apolipoprotein B (apoB), apoE, and
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microsomal triglyceride transfer protein (5, 6). Moreover, Gastaminza et al. (6) demonstrated that HCV assembly and maturation occur in the ER and post-ER compartments, respectively, in a manner that parallels the formation of VLDL. In addition, it was demonstrated that only low-density particles are efficiently secreted and that immature particles are actively degraded (6). Gastaminza and colleagues postulated that by coopting the VLDL assembly, maturation, degradation, and secretory machinery of the cell, HCV acquires its hepatocyte tropism (6). Efficient HCV spread has been shown to be impaired in cells expressing reduced apoB levels (6). Furthermore, reduced apoB secretion by shRNA has been shown to reduce viral particle assembly and secretion without interfering with infection efficiency or HCV RNA replication (6). Complementing these studies, Chang et al. (11) demonstrated that HCV infectious virions purified from low density fractions from cell culture supernatants are assembled as apoE-enriched lipoprotein particles and could be precipitated specifically by anti-apoE- and anti-E2 antibodies. Collectively, our results identify NS5A as a viral factor providing a link between virus production and the VLDL pathway.

Several studies in HCV-infected patients have shown a statistically significant association between the presence of allelic isoforms of apoE and the severity of the HCV-induced liver disease (34, 35). The apoE- ϵ 4 allele was associated with a poor sustained viral response to interferon-alpha-based treatment (36). Taken together, these observations underline the impact of apoE as a host factor playing an important role in HCV-host interactions and the pathogenesis of disease.

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3 Since our results demonstrate that silencing of intracellular apoE results in
4 impaired particle assembly and release, it is conceivable that the interaction of HCV
5 with components of the VLDL export pathway are novel target for antiviral therapies.
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8 This concept is supported by a recent study demonstrating that stimulation of
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12 5 infected hepatocytes with the flavonoid naringenin significantly inhibits HCV secretion
13 (37). Thus, drug-induced decrease of apoE in the liver of the HCV infected patient
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15 may represent a novel approach for control of viral infection.
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23
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35 15 Dubuisson and R. Bartenschlager for helpful discussions.
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Figure Legends

Fig. 1. Interaction of apoE with HCV NS5A protein in a yeast two-hybrid assay.

(A) Principle of the yeast two-hybrid screen: in this system, interaction between two proteins is indicated by the activation of the reporter gene HIS3, which allow growth on histidine depleted (HIS-) plates. **(B)** AH109 yeasts (matchmaker Clontech) were co-transformed with a plasmid encoding a fusion of the apoE coding region and the activation domain of Gal 4 and plasmids encoding the fusion of the DNA binding domain of Gal4 and HCV H77 strain proteins (core, NS2, NS3, NS4B, NS5A and NS5B) or control protein laminin (Lam) or the Gal4 DNA activation domain only (pGBKT7). After two weeks at 30°C on selective His (-) media, yeast growth was analyzed. **(C)** AH109 yeasts (matchmaker Clontech) were co-transformed with a plasmid encoding a fusion of HCV core protein and the activation domain of Gal4 and plasmids encoding the fusion of DNA binding domain of Gal4 and NS5A from H77 or JFH1 strains or control protein Lam.

Fig. 2. Co-immunoprecipitation of apoE and HCV NS5A protein in HCV replicating Huh7.5.1 cells.

(A) Full-length replication-competent HCV RNA derived from isolate HCV Luc-Jc1 was transfected into the hepatoma cell line Huh7.5.1. Seventy-two hours post-transfection Huh7.5.1 cells containing replicating HCV were lysed and subjected to immunoprecipitation using a monoclonal anti-NS5A or an unrelated control monoclonal anti-myc antibody as described in Materials and Methods. As a positive control (PC), apoE expression was analyzed in Huh7.5.1 lysates shown in lane 1. Proteins immunoprecipitated by anti-NS5A or anti-myc control antibody (“++” corresponds to an antibody concentration of 15 µg/ml, “+” to 7.5 µg/ml) were analyzed by immunoblot using anti-apoE antibody 33 (1/500) and

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2
3 horseradish peroxidase-conjugated anti-mouse secondary antibody. **(B, C)**
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5 Reciprocal co-immunoprecipitation of NS5A of genotype 1 and 2 in HCV replicating
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7 cells. Huh7 cells containing replicating HCV Con1 (genotype 1b) or JFH1/J6
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9 (genotype 2a) were lysed and subjected to immunoprecipitation using anti-NS5A,
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11 anti-apoE or anti-HBc as an unrelated control antibody. Immunoprecipitated proteins
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13 were analyzed by immunoblot using peroxidase conjugated anti-NS5A and anti-apoE
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15 antibodies. Peroxidase-conjugation of anti-apoE and anti-NS5A antibodies in
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17 immunoblot analysis shown in panels (B) and (C) eliminated detection of cross-
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19 reacting light and heavy chains of the immunoprecipitating antibodies. PC - positive
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21 control.
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Fig. 3. Co-localization of apoE with HCV NS5A in Huh7.5.1 cells containing replicating HCV. (A) Co-localization of apoE and NS5A in Jc1 replicating cells. Full-length replication-competent HCV RNA derived from isolate Jc1 was transfected into the hepatoma cell line Huh7.5.1 cells as described in Materials and Methods. Seventy-two hours later, cells were fixed and stained for apoE and NS5A expression using rabbit anti-apoE antibody and Alexa Fluor[®] 488-conjugated goat anti-rabbit IgG (green fluorescence) and anti-NS5A antibody and Alexa Fluor[®] 568-conjugated goat anti-mouse IgG (red fluorescence) as described in Materials and Methods. Nuclei were stained in blue (DAPI; left upper panel). Co-localization of stained proteins is shown in the right lower panel. **(B)** Confocal laser scanning microscopy of Huh7.5.1 cells infected with HCV JFH-1 (TCID₅₀ 10⁴/ml). Infection and staining of apoE and NS5A was performed as described in Materials and Methods.

Fig. 4. Silencing of apoE expression in Huh7.5.1 cells results in inhibition of

HCV particle production. (A) Silencing of apoE expression in Huh7.5.1 cells.

Huh7.5.1 cells were transfected with siRNAs as described in Materials and Methods.

Lysates of control naïve Huh7.5.1 (Mock), PBS or control siRNA transfected cells

5 (siCTRL) were subjected to immunoblotting using rabbit anti-apoE and anti-beta-actin

monoclonal antibodies and HRP-conjugated secondary antibodies. ApoE and beta-

actin are indicated on the left and molecular weight (MW) markers (kDa) are

indicated on the right. **(B)** Inhibition of HCV particle production in Huh7.5.1 cells with

silenced apoE expression. Huh7.5.1 cells were transfected with siRNAs and HCV

10 Luc-Jc1 RNA. Cell culture supernatants of non transfected cells (Mock), PBS,

siCTRL, siApoE, siApoA1 and HCV Luc-Jc1 co-transfected Huh7.5.1 cells were

concentrated 50-fold 72 hours after transfection of HCV Luc-Jc1 RNA. Concentrated

supernatants (150 µl) were then used to infect 6×10^4 naïve Huh7.5.1 cells. Infectivity

of supernatants from Mock, PBS, siCTRL or siApoE treated cells was quantified by

15 measuring of luciferase activity in Huh7.5.1 lysates 72 hours after infection (mean \pm

SD; n = 4). **(C)** Impact of apoE silencing on HCV production in single cycle infection

41 experiments. Huh7.5.1 cells were transfected with control (CTRL) siRNA or apoE

43 siRNA. Transfected cells were then infected with recombinant Luc-Jc1 HCV at

45 different multiplicities of infection (MOI) using virus stocks with TCID₅₀ of 10³/ml,

47 10⁴/ml and 10⁵/ml corresponding to MOIs of 0.1, 1 and 10. Seventy-two hours after

49 infection, cell supernatants containing infectious virions were used to infect new

51 naïve Huh7.5.1 cells. Infectivity of supernatants was assessed 48 hours later as

53 described above. The results are expressed as a percentage of infection of cells

55 treated with CTRL siRNA. A representative experiment of two experiments performed

57 in triplicate is shown.

Fig. 5. Impact of apoE silencing on entry and replication of HCV Luc-Jc1.

Huh7.5.1 cells were transfected with siRNA targeting apoE (siApoE) and CD81 (siCD81) expression, irrelevant control RNAs (siCTRL). Forty-eight hours later Huh7.5.1 cells were infected with HCVpp (**A**) or HCVcc Luc-Jc1 (**B**). Seventy-two hours later HCV entry was assessed by quantitation of luciferase activity (mean \pm SD; n = 4). (**C**) Huh7.5.1 were co-electroporated with HCV Luc-Jc1 and PBS (dark-grey curve), control siRNA (light-grey curve), siApoE (black curve) or siRNA targeting HCV translation (dashed curve). Twenty-two to ninety-six hours later, replication was assessed by quantitation of luciferase reporter activity. The results are expressed as a percentage of entry (A) or infection (B) or RLU per μ g of protein (C).

Fig. 6. Silencing of apoE expression results in an impairment of viral assembly and release.

(**A**) Schematic outline of apoE silencing protocols. In protocol A, apoE siRNAs or CTRL siRNAs were transfected seventy-two hours prior to electroporation (EP) of cells with HCV Luc-Jc1 RNA, whereas in protocol B apoE siRNAs or CTRL siRNAs were co-electroporated with HCV Luc-Jc1 RNA. (**B**) ApoE expression following RNAi silencing. Seventy-two hours following electroporation of HCV RNA, apoE expression was analyzed by immunoblot as described in Fig. 4. (**C**) Effect of apoE silencing on the quantity of infectious virions purified from the intra- and extracellular fractions of HCV replicating Huh7.5.1 cells. Supernatants (SN) or lysates (LYS) from cells treated with CTRL siRNAs (black bars) or apoE siRNAs (grey bars) as shown in panel (A) were subjected to sucrose cushion ultracentrifugation and pelleted particles were used to infect naïve Huh7.5.1 cells. Infectivity was assessed as described in Fig. 4. The results are expressed as a

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3 percentage of infection of cells treated with CTRL siRNA. (mean \pm SD; n = 6). **(D)**
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5 Biophysical characterization of released HCV virions in cells with silenced apoE
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7 expression. Cells were transfected with siCTRL or siApoE as described in protocol A.
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9 Concentrated (50 x) cell culture supernatants were subjected to 10 to 50% iodixanol
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11 step gradients and subjected to equilibrium ultracentrifugation as described in
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13 5 Materials and Methods. Fractions obtained from gradients with siCTRL (in black) or
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15 siApoE (in grey) transfected cells were measured for RNA content by qRT-PCR
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17 quantification (lines) and tested for infectivity on naïve Huh7.5.1 cells (bars).
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19 Infectivity was measured 72 hours later by qRT-PCR quantification. RNA cell
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21 10 extraction was normalized with GAPDH quantification. Mean and SD of three
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23 independent experiments are shown. The dotted line represents the density (in g/mL)
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25 of each fraction.
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34 **Fig. 7. Time-course of apoE silencing and impact on production of infectious**

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36 15 **virions. (A)** Schematic outline of the time course of the experimental protocol. Cells
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38 **were electroporated with HCV Luc-Jc1 RNA three days (day -3) before transfection**
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40 **with apoE and control (CTRL) siRNAs (day 0). On day 0 supernatants were removed**
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42 **and replaced by fresh tissue culture medium to deplete HCVcc in the extracellular**
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44 **medium at the time of siRNA transfection. On days 1, 2 and 3 post siRNA**
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46 **transfection, supernatants or lysates from cells treated with CTRL siRNAs or apoE**
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48 20 **siRNAs were subjected to sucrose cushion ultracentrifugation and pelleted particles**
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50 **were used to infect naïve Huh7.5.1 cells. Infectivity was assessed as described in**
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52 **Fig. 4. (B)** Analysis of silenced apoE expression in cell lysates using immunoblotting
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54 **as described in Fig. 4A. (C, D)** Infectivity of supernatants (SN, shown in panel C) or
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56 **lysates (LYS, shown in panel D) from cells treated with CTRL siRNAs (black bars) or**
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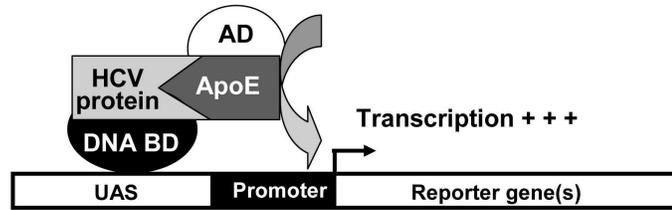
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5 cells treated with CTRL siRNA (mean \pm SD; n = 6).
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10 **Fig. 8. ApoE-NS5A interaction is absent or decreased in viral mutants with a**

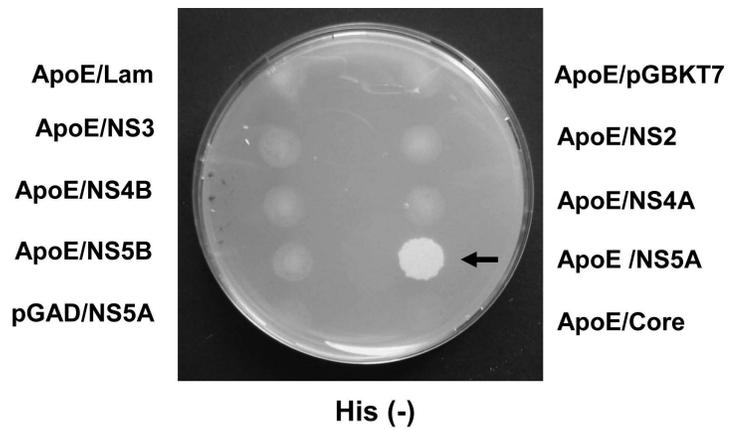
11 **defect in viral assembly.** AH109 yeasts (matchmaker Clontech) were co-
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13 5 **defect in viral assembly.** AH109 yeasts (matchmaker Clontech) were co-
14 transformed with a plasmid encoding fusion of apoE protein and the activation
15 domain of Gal4 and plasmids encoding fusions of the DNA binding domain of Gal4
16 and wild-type JFH1 NS5A or mutant JFH1 NS5A containing **(A)** an alanine triplet
17 substitution at position 99 (NS5A^{AAA99} JFH1) or 102 (NS5A^{AAA102} JFH1) (8) **(B)** a
18 deletion in domain III of NS5A resulting in abolishment of particle production
19 (“domain III deletion”) (13). Lam, pGBKT7, pGAD expressing a fusion between Gal 4
20 DNA binding domain and control protein laminin, DNA binding domain of Gal4 and
21 activation domain of Gal4 respectively, were used as controls.
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Figure 1

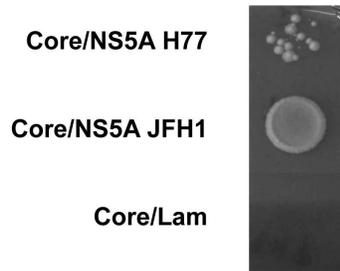
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B



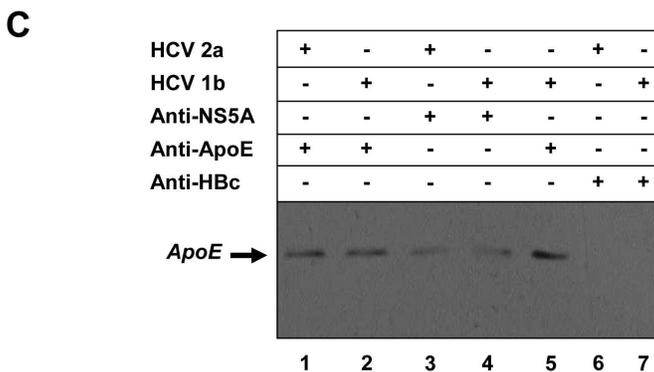
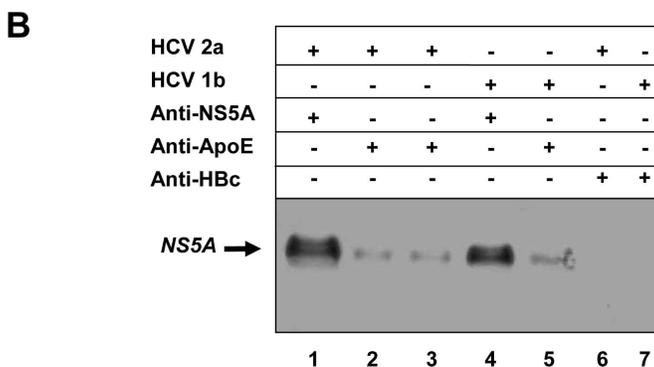
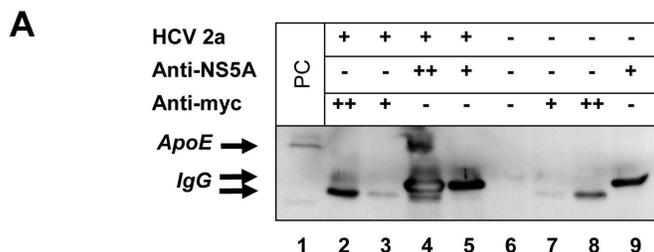
C



174x237mm (300 x 300 DPI)

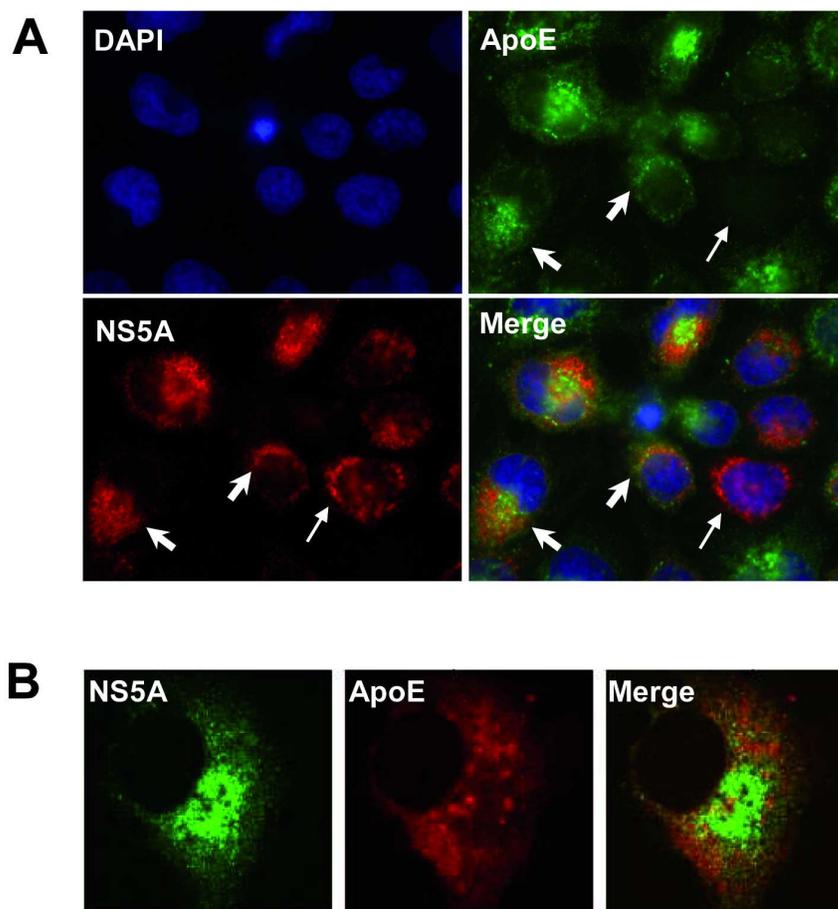
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Figure 2



136x239mm (300 x 300 DPI)

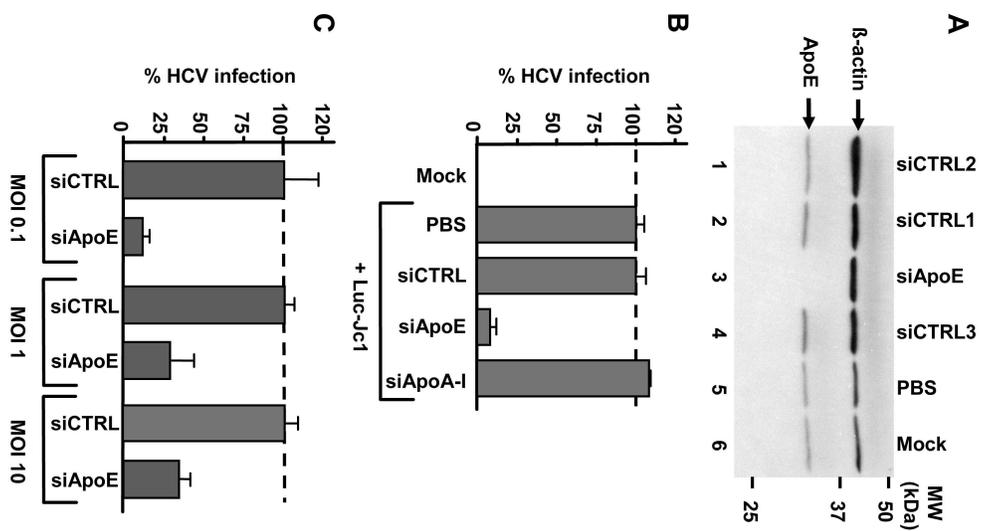
Figure 3



142x152mm (300 x 300 DPI)

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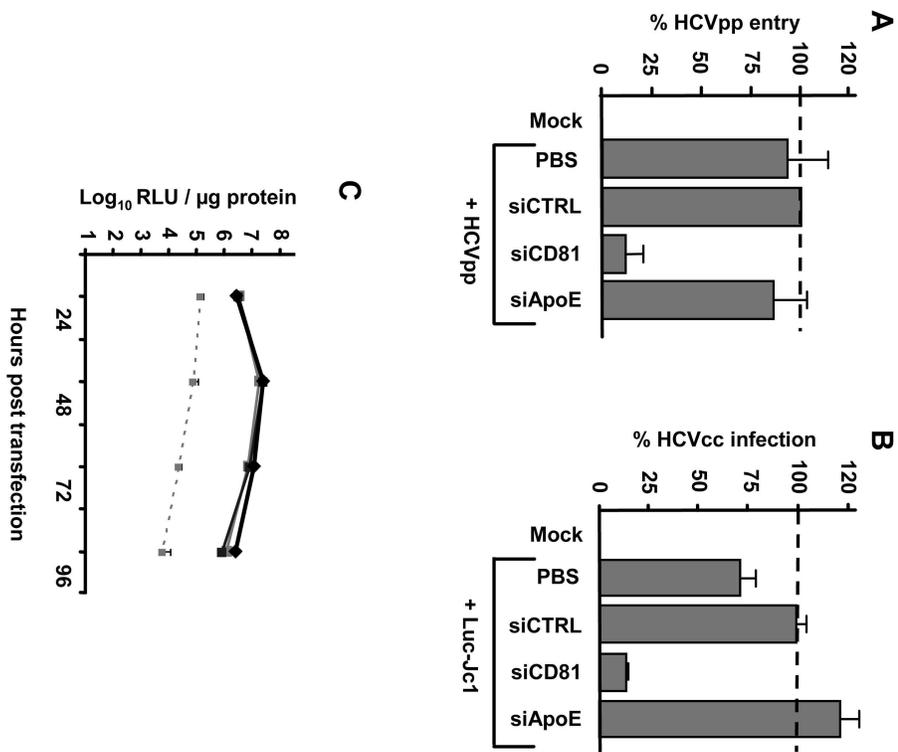
Figure 4



249x177mm (300 x 300 DPI)

Review

Figure 5

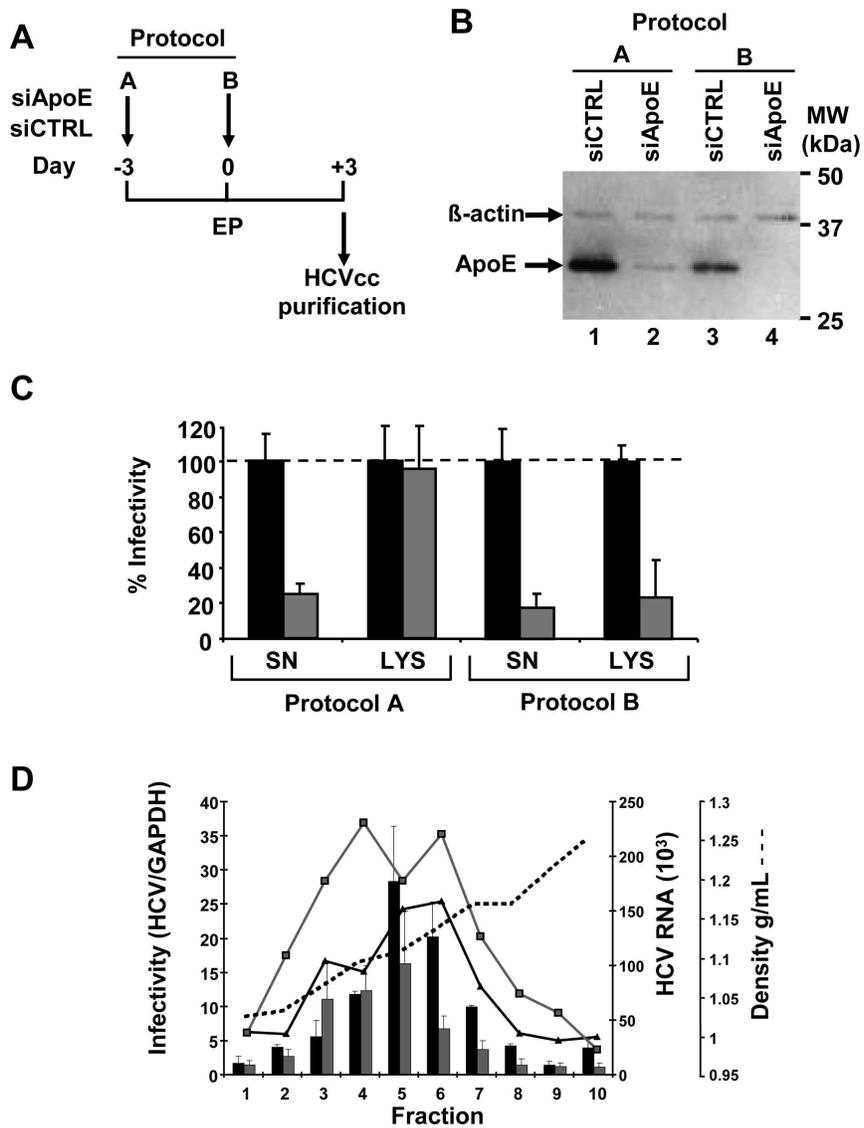


235x181mm (300 x 300 DPI)

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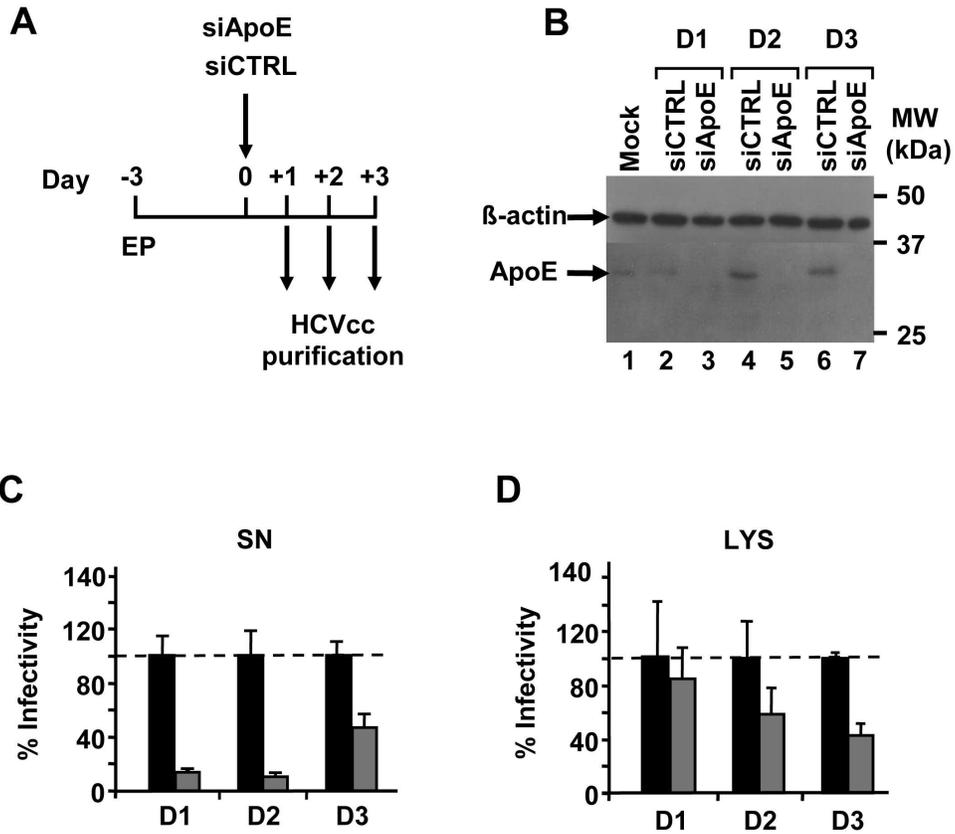
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Figure 6



189x249mm (300 x 300 DPI)

Figure 7

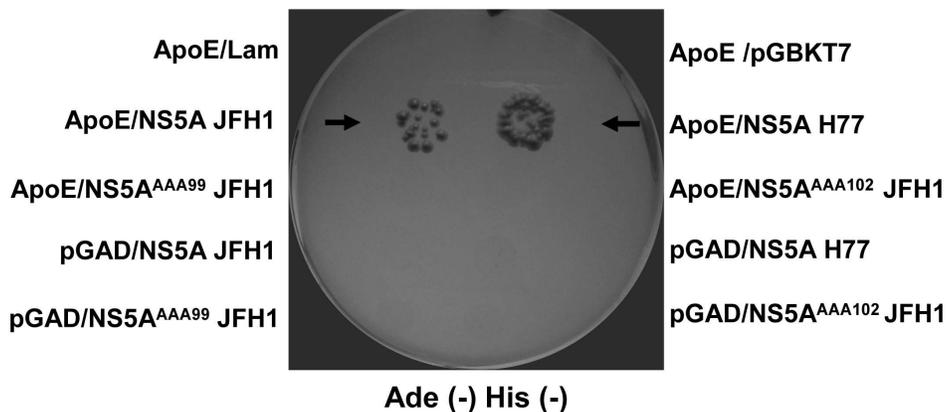


186x179mm (300 x 300 DPI)

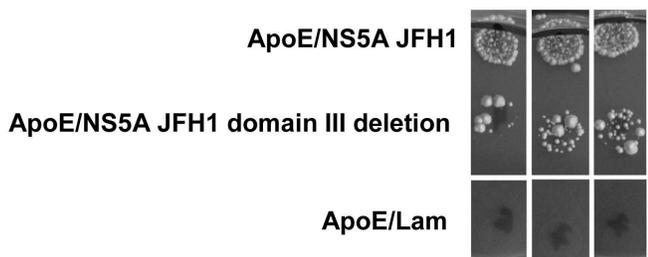
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Figure 8

A



B



192x200mm (300 x 300 DPI)

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3 Thomas F. Baumert, MD
4 Inserm Unit 748
5 Service d'Hépatogastroentérologie
6 Université de Strasbourg
7
8 3, rue Koeberlé
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10 F-67000 Strasbourg
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12 France
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14

16 Keith D. Lindor, MD, PhD
17 Editor-in-Chief
18 Hepatology
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23 August 20, 2009
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27

28 Dear Dr. Lindor, dear Dr. Szabo:
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30

31 please find enclosed our revised manuscript Hepatology HEP-09-0768.R1 including a point-
32 by-point-response to the reviewers' comments (acceptance with revisions). The reviewers' comments
33 were most helpful. All comments and suggestions were answered by a series of additional
34 experiments and modification of the text section. Furthermore, the manuscript text section was shorted
35 to meet the length requirements for the journal (5000 words).
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39
40 We hope that the revised manuscript (with changes underlined) is now acceptable for
41 publication in Hepatology.
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45
46 Sincerely yours,
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50
51 Thomas F. Baumert, M. D.
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Catherine Schuster, PhD

Hepatology MS# HEP-09-0768.R1**Point-by-point response to reviewer 1:**

We thank the reviewer for his or her very helpful comments, which were addressed by a series of additional experiments. The results of these experiments are shown in revised Figs. 2, 3, 4, new Fig. 7 and the revised text sections of Materials and Methods, Results and Discussion.

1. The paper is well written, focused, and concise.

We thank the reviewer for his or her positive comment.

2. Reciprocal coIP studies showing Ns5A comes down with apoE should be included.

We thank the reviewer for this helpful comment which was addressed in a series of additional experiments. As shown in revised Fig. 2B, reciprocal Co-IP studies confirm co-immunoprecipitation of NS5A with apoE.

3. Why was the bulk of infection experiments performed with transfection of HCV RNA? why were there so few studies with titrated virus stock? in this regard, what is the effect of apoE silencing on infection with different multiplicities of infection?

We thank the reviewer for this helpful comment. The reviewer may not have realized that key experiments were performed in transfection and infection experiments. This is illustrated by apoE-NS5A co-localization studies in HCV-infected Huh7.5.1 (Fig. 3B) or HCV entry experiments (Fig. 5B). Furthermore, the impact of apoE silencing was investigated in single cycle infection experiments in a protocol similar to previous studies analyzing the impact of apoB on HCV production (Gastaminza P, Cheng G, Wieland S, Zhong J, Liao W, Chisari FV. Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion. J Virol 2008; 82: 2120-2129). To better illustrate these results mentioned already previously in the text section as data not shown, we now show HCV single cycle infection data in a new panel of revised Fig. 4C. Titers of viral stocks are now provided in the Materials and Methods section (p. 8, line 1) and the respective figure legends (Fig. 3 and 4). Furthermore, as suggested by the reviewer, we investigated the effect of apoE silencing on infection with different multiplicities of infection. The results of the new experiments are shown in Fig. 4C and the revised Results section (p. 12, line 10-16).

4. Ns5a also binds to apoA1. does silencing apoA1 also prevent virus assembly? what about apoB?

We thank the reviewer for this helpful comment. A previous report had demonstrated that apoA1 interacts with NS5A in pull-down and coimmunoprecipitation experiments (Shi ST, Polyak SJ, Tu H, Taylor DR, Gretch DR, Lai MM. Hepatitis C virus NS5A colocalizes with the core protein on lipid droplets and interacts with apolipoproteins. Virology 2002:198-210). As shown in revised Fig. 4B, silencing of apoA1 by using a pool of validated siRNAs did not result in detectable decrease of virus production. These data are presented in the revised Results section (p.12, lines 18-20) section and suggest that apoA1 may not essential for virus production. The impact of apoB on HCV production and assembly has been studied in detail by the Chisari lab (Gastaminza P, Cheng G, Wieland S, Zhong J, Liao W, Chisari FV. Cellular determinants of hepatitis C virus assembly, maturation, degradation, and

1
2
3 secretion. J Virol 2008; 82: 2120-2129). To avoid duplication of already published results, we did not
4 perform experiments to address the role of apoB for viral assembly in this manuscript. Instead, we cite
5 and discuss the study of Gastaminza and colleagues in more detail in the revised Discussion section
6 (p. 18, lines 7-11).
7
8
9

10 *5. It is interesting that marginal apoE silencing reduces extracellular/supernatant infectivity, but not*
11 *intracellular infectivity (fig 6). What is the "other" function of apoE in virus assembly that the authors*
12 *speculate?*
13

14 We thank the reviewer for this interesting point. To further address the impact of apoE as a co-factor
15 for viral assembly and egress, we performed time course experiments following apoE silencing in
16 HCV-replicating cells. As shown in Fig. 7, apoE silencing resulted in an early effect on released
17 infectious particles and a late effect on the assembly of intracellular infectious virions suggesting that
18 apoE is a co-factor for two different steps in the assembly-egress process. These data further support
19 a model for apoE as a co-factor for two distinct steps of the assembly-egress process of the viral life
20 cycle including a function for release of viral particles. These results are now detailed in Results
21 section (p.14, lines 18-25; p.15, lines 1-6) and Discussion section (p. 17, lines 16-20).
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27 **Point-by-point response to reviewer 2:**

28
29 We thank the reviewer for his or her very helpful comments, which were addressed by a series of
30 additional experiments shown in revised Figs. 2 and 8 as well as modification of Materials and
31 Methods, Results and Discussion sections.
32
33

34 Reviewer: 2

35
36 *The authors show that NS5A interacts with apoE in both a yeast two-hybrid system and in an HCV*
37 *infectious cell culture system. Silencing of apoE by siRNA inhibits HCV partivle production by*
38 *impairing virion assembly and release, not by affecting particle entry or viral replication. Finally, the*
39 *authors demonstrate that there is impaired NS5A-apoE interaction using NS5A mutant strains with*
40 *defects in virion assembly in a yeast two-hybrid system. These experiments show that the newly-*
41 *discovered apoE-NS5A interaction is important for virion assembly and release.*
42

43 *Overall, the experimental design and logic are excellent. Suggestions:*

44
45 We thank the reviewer for this comment.

46
47 *1) They should extend their experiment with NS5A mutant strains from the yeast two-hybrid system to*
48 *a human cell-based system, much as they did to establish the NS5A-apoE interaction. While the*
49 *mutant NS5A and apoE should still co-localize, they should not co-precipitate.*
50

51 We thank the reviewer for this interesting suggestion. To answer this question we need to engineer the
52 NS5A mutants into assembly competent HCV JFH/J6 or Jc1. However, a detailed and thorough
53 characterization of mutant strains in cell culture models, co-immunoprecipitation and imaging studies
54 requires more time than the short timeframe allowed for revision of the manuscript for Hepatology.
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59 Since results of co-IP of apoE with NS5A in the human cell-based system as well as the
60 findings in the yeast-two-hybrid system for mutant NS5A were clear and robust (as demonstrated by
reciprocal co-IP and co-IP for different isolates and genotypes in Huh7.5.1 cells shown in revised

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3 figure 2B and C), we feel that the suggested experiments are very interesting but not essential for the
4 conclusions of this study.
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8 To address the reviewer's comment, we state more clearly in our revised discussion section
9 that the study of mutant NS5A-apoE interactions was performed in the yeast-two-hybrid system. The
10 revised sentences addressing this issue reads now: "The relevance of apoE-NS5A interaction for virus
11 production is further supported by the loss of apoE-NS5A binding from viral variants with a defect in
12 virus production in the yeast-two-hybrid system." (p. 16, lines 24-25 and p. 17, line 1). Incorporating
13 the suggestion of the reviewer and addressing our ongoing experiments, we added the following
14 statement: "Further studies are underway to fine-map the structural determinants of NS5A-apoE
15 interactions and its impact on the viral phenotype." (p. 17, lines 2-4).
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21 *2) It is very important to show that this interaction is not genotype-specific. On page 10, line 4 the*
22 *authors' state that the interaction is not genotype specific, but do not show the JFH1 data. These data*
23 *should be presented in the manuscript.*

24 We agree with the reviewer that this is an extremely important issue. To further address this issue, we
25 studied the interaction of apoE with NS5A with genotypes 1a and 1b in additional co-
26 immunoprecipitation and yeast-two-hybrid system experiments. Co-immunoprecipitation experiments
27 confirmed apoE-NS5A interaction in HCV replicating cells for NS5A derived from the Con1 strain
28 (genotype 1b). The results of these new experiments are presented in revised Fig. 2 and discussed in
29 Results section (p. 11, line 8-10). Furthermore, we show that apoE binds to NS5A of strain H77
30 (genotype 1a) in the yeast two-hybrid system. The results of these experiments are shown Fig. 8A.
31 Taken together, these new experiments in a human cell-based system clearly demonstrate that the
32 interaction is easily detectable for different isolates and is not genotype-specific.
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38 ***There are some minor points with the manuscript itself:***

39
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41 *1) page 8, line 1 - "pseudotypes" should be amended to "pseudotype particles"*

42 As requested by the reviewer we replaced the term "pseudotypes" by "pseudotype particles"
43 throughout the manuscript.
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45

46
47 *2) page 16, line 29 – Please reword this sentence to more clearly state that the newly discovered*
48 *apoE-NS5A interaction is novel, not that apoE is a novel host co-factor. As the authors have*
49 *previously pointed out in the introduction, Chang et. Al. has previously demonstrated that apoE is an*
50 *important host factor for HCV.*

51 As requested by the reviewer, the sentence on page 17, line 2 was reworded. It reads now: "Thus, our
52 results identify apoE-NS5A binding as a novel virus-host interaction and point to apoE-NS5A binding
53 having a crucial function in the production of infectious particles (p. 17, line 1).
54
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56
57 *The authors have unlocked an important interaction worthy of further study. Although they do not*
58 *define the exact binding interaction between apoE and NS5A and they do not demonstrate the*
59 *mechanism by which the NS5A-apoE interaction inhibits virion assembly and release, this does not*
60 *diminish the impact of the currently presented work.*

We thank the reviewer for this appreciation of our study.