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1 **Lysophosphatidylcholine acyltransferase 1 is downregulated by hepatitis C virus:**
2 **impact on production of lipo-viro-particles**

3

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primary human hepatocyte; very low density lipoprotein.

List of abbreviations Apo, apolipoprotein; BSA, bovine serum albumin; ffu, focus-forming
units; HCV, hepatitis C virus; LD, lipid droplet; LPCAT, lysophosphatidylcholine
acyltransferase; LVP, lipo-viro-particle; PHH, primary human hepatocytes; PL, phospholipid;
RT-qPCR, quantitative reverse-transcription real-time polymerase chain reaction; TAG,
triacylglycerol; SEM, standard error of the mean; VLDL, very-low-density-lipoprotein.

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1 **ABSTRACT**

2 **Objective** Hepatitis C virus (HCV) is intimately linked with the liver lipid metabolism,
3 devoted to the efflux of triacylglycerols stored in lipid droplets (LDs) in the form of
4 triacylglycerol-rich very-low-density lipoproteins (VLDL): (i) the most infectious HCV
5 particles are those of lowest density due to association with triacylglycerol-rich lipoproteins;
6 (ii) HCV-infected patients frequently develop hepatic steatosis (increased triacylglycerol
7 storage). The recent identification of lysophosphatidylcholine acyltransferase 1 (LPCAT1) as
8 an LD phospholipid-remodelling enzyme prompted us to investigate its role in liver lipid
9 metabolism and HCV infectious cycle.

10 **Design** Huh-7.5.1 cells and primary human hepatocytes (PHH) were infected with JFH1-
11 HCV. LPCAT1 depletion was achieved by RNA interference. Cells were monitored for
12 LPCAT1 expression, lipid metabolism and HCV production and infectivity. The density of
13 viral particles was assessed by isopycnic ultracentrifugation.

14 **Results** Upon HCV infection, both Huh-7.5.1 cells and PHH had decreased levels of
15 LPCAT1 transcript and protein, consistent with transcriptional downregulation. LPCAT1
16 depletion in either naïve or infected Huh-7.5.1 cells resulted in altered lipid metabolism
17 characterized by LD remodelling, increased triacylglycerol storage and increased secretion of
18 VLDL. In infected Huh-7.5.1 cells or PHH, LPCAT1 depletion increased production of the
19 viral particles of lowest density and highest infectivity.

20 **Conclusion** We have identified LPCAT1 as a modulator of liver lipid metabolism
21 downregulated by HCV, which appears as a viral strategy to increase the triacylglycerol
22 content and hence infectivity of viral particles. Targeting this metabolic pathway may
23 represent an attractive therapeutic approach to reduce both the viral titre and hepatic steatosis.

24

1 **SIGNIFICANCE OF THIS STUDY**

2 **What is already known on this subject?**

- 3 - The most infectious HCV particles circulate as hybrid lipo-viro-particles (LVPs) consisting
4 of viral and very-low-density lipoprotein (VLDL) components.
- 5 - The human hepatocyte is both the cell specialized in the biogenesis of VLDLs and the
6 primary replication site of HCV, but whether the VLDL pathway is usurped for LVP
7 morphogenesis is still a matter of debate.
- 8 - Besides the existence of LVPs another clinical feature that links HCV infection with the host
9 lipid metabolism is the high prevalence of liver steatosis, but whether these facts are related is
10 unknown.

11 **What are the new findings?**

- 12 - Lysophosphatidylcholine acyltransferase 1 (LPCAT1) is identified as a modulator of the
13 liver lipid homeostasis downregulated by HCV.
- 14 - LPCAT1 depletion increases LVP production, supporting the idea that HCV indeed hijacks
15 the hepatocellular lipid metabolism for the benefit of highly infectious LVP morphogenesis.
- 16 - LPCAT1 depletion also increases VLDL secretion, suggesting that at least some of the
17 mechanisms of LVP morphogenesis and VLDL biogenesis are shared.
- 18 - LPCAT1 depletion also causes steatosis, illustrating a possible link between LVP
19 morphogenesis and liver steatosis.

20 **How might it impact on clinical practice in the foreseeable future?**

- 21 - Our findings designate the LPCAT1-regulated lipid metabolism pathway as an attractive
22 host target for antiviral therapy in most difficult-to-treat patients.
- 23 - Compared to other lipid-modulating antiviral approaches expected to aggravate liver
24 steatosis, targeting the LPCAT1-regulated pathway would reduce liver steatosis besides
25 reducing HCV infectious titre.

1 INTRODUCTION

2 The hepatitis C virus (HCV) is a leading cause of chronic liver disease worldwide. Unique to
3 this human hepatotropic virus is its tight link with the host lipid metabolism.¹⁻³ Clinical
4 evidence in chronically infected patients has long recognized the high prevalence of liver
5 steatosis, defined as an accumulation of lipids, essentially accounted for by triacylglycerols
6 (TAGs), in cytosolic lipid droplets (LDs) of hepatocytes. Moreover, HCV particles are
7 heterogeneous in the circulating blood of patients and the most infectious viral particles are
8 those of exceptionally low buoyant density due to their association with apolipoprotein (Apo)
9 B-containing TAG-rich lipoproteins in original structures referred to as lipo-viro-particles
10 (LVPs).^{4 5} As hepatocytes are specialized in the assembly and secretion of the ApoB-
11 containing TAG-rich lipoproteins known as very-low-density lipoproteins (VLDLs), a
12 working hypothesis is that LVPs are formed originally within infected cells, the
13 hepatocellular lipid metabolism being hijacked by HCV for the benefit of their
14 morphogenesis.⁶⁻⁸

15 LDs consist of a core of TAGs and cholesterol esters surrounded by a monolayer of
16 phospholipids (PLs) and cholesterol.⁹ Importantly, TAGs stored in cytosolic LDs of
17 hepatocytes provide most of the TAGs present in VLDLs through a mechanism of hydrolysis-
18 reesterification.^{10 11} Many proteins interact with the LD surface, as shown by proteomic
19 characterization of LDs isolated from hepatic cells.^{12 13} The LD proteome includes structural
20 proteins, perilipins, and proteins that control several cellular pathways including lipid
21 metabolism (e.g., lipases, acyltransferases), membrane trafficking, signalling, etc.^{14 15} The
22 interaction of these proteins with the LD surface can be modulated through mechanisms,
23 probably of multiple causes, that are only just starting to be clarified.^{16 17} Because of the large
24 variety of PLs conferring different physicochemical properties to the LD surface, the nature of

1 PLs is a factor that can modulate the strength of protein association.^{18 19} The most abundant
2 PL of the LD surface is phosphatidylcholine.²⁰ The lysophosphatidylcholine acyltransferase
3 (LPCAT) family consists of PL-remodelling enzymes²¹, among which LPCAT1 and LPCAT2
4 localise partially to the LD surface where they can synthesize phosphatidylcholine.²²
5 Moreover, LPCAT1 and LPCAT3, both expressed in hepatocytes, were recently reported to
6 influence lipoprotein secretion from these cells.^{23 24}

7 These considerations prompted us to examine the hypothesis of a potential hijacking of
8 LPCAT-mediated metabolism by HCV. Indeed, LPCAT1 is shown here to be downregulated
9 by HCV. LPCAT1 depletion, in turn, was found to increase the virus infectivity.

10

11 **MATERIALS AND METHODS**

12 **Cell culture, infection and gene silencing**

13 Huh-7.5.1 cells and primary human hepatocytes (PHH) were inoculated with JFH1-HCV at a
14 multiplicity of infection of 0.2 and 2 focus-forming units (ffu)/cell, respectively, as described
15 previously.^{25 26} Further details for infection and for transfection with HCV subgenomic
16 replicon or core-encoding plasmid are given in the supplementary material. ShRNA- and
17 siRNA-mediated gene silencing was used to achieve LPCAT1 depletion in Huh-7.5.1 cells
18 and PHH, respectively (see supplementary material).

19 **Gene expression and virological analyses**

20 For details of quantitative reverse-transcription real-time polymerase chain reaction (RT-
21 qPCR), western blotting, confocal microscopy, Apo quantification, and virological
22 analyses,²⁵⁻²⁷ see the supplementary material. All analyses were done at day 3 post-

1 inoculation except for the quantification of negative-strand HCV RNA, which was done at
2 day 1 post-inoculation. Indeed, at later time points multiple rounds of infection are expected
3 to cause a bias in the assessment of viral genome replication per se if production of viral
4 particles is affected.

5 **Lipid analyses**

6 The TAG mass was quantified using the PAP150TG kit (BioMérieux) as described in the
7 supplementary material. For analysis of lipid secretion capacity, cells were incubated for the
8 last 24 h of culture with oleic acid (0.6 mM) in complex with bovine serum albumin (oleic
9 acid/BSA) containing 1 $\mu\text{Ci/ml}$ [$1\text{-}^{14}\text{C}$] oleic acid, then lipids were extracted from cells and
10 media and fractionated by thin layer chromatography followed by quantification of
11 radioactivity as described previously²⁸ and in the supplementary material.

12 **Statistical analyses**

13 Results were expressed as the mean \pm standard error of the mean (SEM) of at least three
14 independent experiments. Statistical significance was evaluated using Student's *t* test for
15 unpaired two-sided data (* for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$) after a Shapiro-
16 Wilk test was done to confirm that all data follow a normal distribution.

17

18 **RESULTS**

19 **LPCAT1 is downregulated by HCV**

20 As a starting point we investigated whether HCV infection affects the expression of any
21 member of the LPCAT family in Huh-7.5.1 cells (supplementary figure S1). Only LPCAT1
22 showed significantly reduced mRNA amounts upon JFH1-HCV inoculation (0.47 ± 0.05),

1 correlating with reduced LPCAT1 protein amounts as assessed by western blot analysis (0.61
2 ± 0.09) (figure 1A). Most importantly, confocal microscopy analysis confirmed that at the
3 single-cell level, those cells that were actually infected upon HCV inoculation showed a clear
4 decrease in LPCAT1 signal intensity compared to non-infected cells (figure 1B). We
5 concluded that HCV infection induces downregulation of LPCAT1 expression occurring at
6 least partly at the transcriptional level.

7 As approaches to unravelling mechanisms of HCV-induced downregulation of LPCAT1, we
8 transfected Huh-7.5.1 cells with either a subgenomic JFH1-HCV replicon or a plasmid
9 encoding JFH1-HCV core protein (figure 2). LPCAT1 expression was unchanged in cells
10 harbouring the subgenomic replicon, suggesting that the downregulation observed upon HCV
11 infection involves neither the virus non-structural proteins nor the viral genome replication
12 step (figure 2A-B). By contrast, cells transfected with core-encoding plasmid had
13 significantly reduced LPCAT1 mRNA amounts at the population level (figure 2C), and most
14 strikingly at the single-cell level those cells that actually expressed HCV core protein had
15 reduced LPCAT1 protein signal intensity (figure 2D). Thus, the sole expression of core
16 protein was sufficient to recapitulate HCV infection-induced downregulation of LPCAT1.

17 **LPCAT1 depletion increases production of HCV particles with high infectivity and low** 18 **density**

19 Next we wanted to determine whether LPCAT1 downregulation has an impact on HCV
20 infectious cycle. To address this question, we used a lentiviral vector encoding shRNA
21 against LPCAT1 (shLPCAT1). In Huh-7.5.1 cells, this indeed resulted in a strong decrease of
22 LPCAT1 protein and mRNA amounts (0.34 ± 0.1 for both) compared to control cells treated
23 with lentivirus encoding shRNA against luciferase (shLuc) (supplementary figure S2A-B).
24 This depletion was specific to LPCAT1, as the mRNA amount of LPCAT3, the main LPCAT

1 in hepatocytes,²⁹ remained unchanged (supplementary figure S2C). An even stronger decrease
2 of LPCAT1 protein and mRNA amounts was observed in shLPCAT1-transduced Huh-7.5.1
3 cells that were inoculated with HCV (0.17 ± 0.07 and 0.20 ± 0.04 , respectively), suggesting
4 an additive effect of HCV infection and shLPCAT1 on LPCAT1 depletion (supplementary
5 figure S2D-E). We also verified that shLPCAT1 transduction had no cytotoxic effect
6 (supplementary figure S3A).

7 To examine the impact of LPCAT1 depletion on HCV infectious cycle, we first used an
8 infectivity assay to evaluate the production of infectious virus in the culture supernatant. The
9 infectivity titre was significantly increased in LPCAT1-depleted Huh-7.5.1 cells (5.84 ± 2.08)
10 (figure 3A). However, this effect could not be attributed to increased replication of viral
11 genome because upon LPCAT1 depletion the amount of negative-strand HCV RNA was
12 modified neither in these HCV-infected cells (figure 3A) nor in cells harbouring a
13 subgenomic HCV replicon (supplementary figure S4). These results pointed to viral
14 morphogenesis as the step of HCV infectious cycle affected by LPCAT1 depletion.
15 Nevertheless, LPCAT1 depletion had only a slight, not significant tendency to increase the
16 viral load, which reflects the production of physical viral particles irrespective of their
17 infectivity. Accordingly, the specific infectivity (ratio of infectivity titre to viral load) was
18 significantly increased in LPCAT1-depleted cells (3.83 ± 1.69). To exclude putative off-target
19 effects of shLPCAT1, we transfected shLPCAT1-transduced Huh-7.5.1 cells with expression
20 plasmids carrying wild-type or an shLPCAT1-resistant version of LPCAT1 gene
21 (supplementary figure S5A), which led to increasing restoration of LPCAT1 protein
22 expression (supplementary figure S5B). This indeed restrained the shLPCAT1-induced gain
23 in both infectivity titre and specific infectivity in a dose-dependent manner (supplementary
24 figure S5C), with a good linear inverse relationship between LPCAT1 protein amount and

1 HCV infectivity titre (supplementary figure S5D). Collectively, the data suggested that
2 LPCAT1 depletion favours the morphogenesis of highly infectious virus.

3 Finally, because HCV infectivity is inversely correlated with the buoyant density of viral
4 particles,^{25 30} we subjected culture supernatants from HCV-infected Huh-7.5.1 cells depleted
5 or not for LPCAT1 to isopycnic ultracentrifugation through iodixanol gradients (see a
6 representative experiment in figure 3B-E). As previously reported for HCV grown in Huh-7
7 cell line,^{25 30} the majority of viral RNA peaked between 1.09 and 1.14 g/ml (figure 3B).
8 However, LPCAT1 depletion led to a shift of HCV RNA towards fractions of lower buoyant
9 density: the percentage of total viral load present below 1.08 g/ml was $22.60 \pm 1.65\%$ for
10 LPCAT1-depleted cells as compared to $12.59 \pm 1.99\%$ for control cells. The excess of highly
11 infectious particles produced upon LPCAT1 depletion was also found in these fractions of
12 lower density (figure 3C). We concluded that LPCAT1 depletion increases the production of
13 HCV particles of high specific infectivity coinciding with low buoyant density.

14 **LPCAT1 depletion leads to LD remodelling, increased TAG storage and increased** 15 **secretion of TAG-rich lipoproteins**

16 The lower buoyant density of HCV produced upon LPCAT1 depletion was consistent with a
17 TAG enrichment of viral particles, prompting us to examine the impact of LPCAT1 depletion
18 on the hepatocellular lipid metabolism. Confocal microscopy of Huh-7.5.1 cells labelled with
19 BODIPY 493/503 did not show any gross modification of the intracellular LD distribution
20 upon LPCAT1 depletion (figure 4A). However, the mean number of LDs was significantly
21 reduced in LPCAT1-depleted cells compared to control cells (153.20 ± 37.89 and $352.12 \pm$
22 78.14 LDs/cell, respectively) (figure 4B). Furthermore, measurement of LD size showed a
23 shift of the LD diameter distribution towards larger LDs upon LPCAT1 depletion (figure 4C).
24 Besides these modifications that might reflect fusion of small LDs into larger ones, the net

1 intracellular TAG amount was increased in the steady state in LPCAT1-depleted cells ($1.72 \pm$
2 0.07) (figure 4D). Because hepatic cells produce TAG-rich lipoproteins, this increased
3 intracellular TAG content could result from inhibition of their secretion. Unexpectedly,
4 however, the amount of ApoB, the constitutive apolipoprotein of TAG-rich lipoproteins, was
5 increased in the culture supernatant of LPCAT1-depleted compared to control cells ($423.28 \pm$
6 84.19 and 175.77 ± 34.35 $\mu\text{g/dl}$, respectively) (figure 4E), indicating the secretion of a greater
7 number of lipoprotein particles. An impaired capacity to enrich lipoproteins with TAGs could
8 also lead to an increased intracellular TAG content. To test this hypothesis, Huh-7.5.1 cells
9 were challenged with oleic acid containing [$1\text{-}^{14}\text{C}$] oleic acid as a tracer then radiolabelled
10 PLs and TAGs were quantified in cells and media (figure 5). The intracellular amounts of
11 newly synthesized PLs and TAGs were similar in LPCAT1-depleted and control cells,
12 indicating similar uptake and lipid synthesis capacity. However, the secretion of newly
13 synthesized TAGs was significantly, albeit slightly increased in LPCAT1-depleted cells (1.24
14 ± 0.15 fold) while PL secretion was not modified. This indicates that LPCAT1-depleted cells
15 have a slightly higher TAG secretion capacity compared to control. We concluded that
16 LPCAT1 depletion in Huh-7.5.1 cells results in an altered lipid metabolism characterized by
17 LD remodelling, increased intracellular TAG content, increased ApoB-containing lipoprotein
18 secretion and increased TAG secretion capacity.

19 Next we examined the impact of LPCAT1 depletion on the hepatocellular lipid metabolism in
20 the context of HCV infection. For all characteristics studied, similar effects were observed in
21 LPCAT1-depleted Huh-7.5.1 cells irrespective of whether they were infected or not. Indeed,
22 in infected Huh-7.5.1 cells LPCAT1 depletion led to a reduction in the mean number of LDs
23 accompanied by a shift of the LD diameter distribution towards larger LDs (figure 6A-C), and
24 increased both the intracellular amount of TAGs and the amount of secreted ApoB (figure
25 6D-E). Upon isopycnic iodixanol gradient ultracentrifugation of culture supernatant of these

1 cells, ApoB peaked at a higher buoyant density than the very low density (<1.006 g/ml)
2 expected for VLDLs (figure 6F), consistent with the reported inability of the Huh-7 cell line
3 to secrete genuine VLDLs.²⁵ Nevertheless, ApoB and ApoE, another important component of
4 VLDLs, were both found in fractions with a density below 1.08 g/ml (figure 6F-G), i.e., in the
5 density range of the excess of highly infectious HCV particles produced upon LPCAT1
6 depletion (figure 3D-E). Taken together, the results suggest that LPCAT1 depletion increases
7 the secretion of TAG-rich lipoproteins and HCV particles associated with TAG-rich
8 lipoproteins.

9 **Validation of data in physiologically relevant PHH**

10 Huh-7 sublines provide a most convenient and efficient HCV culture system, yet differ
11 significantly from normal, quiescent, highly differentiated human adult hepatocytes and
12 especially do not fully reproduce VLDL biogenesis.²⁵ In order to validate the data in a patho-
13 physiologically relevant model, we used our HCV culture system in PHH, which produce
14 genuine VLDLs and HCV particles whose properties mimic those of infectious HCV
15 produced in vivo.²⁵ We first sought to determine whether HCV infection downregulates
16 LPCAT1 expression in PHH as is the case in Huh-7.5.1 cells. Because PHH are primary cells,
17 the infectivity titres reached upon JFH1-HCV inoculation were generally lower than those
18 obtained in Huh-7.5.1 cells and varied from one donor of hepatocytes to another. When viral
19 titres were below $5 \cdot 10^4$ ffu/ml, LPCAT1 mRNA amounts did not appear to be modified in
20 PHH inoculated with HCV compared to naïve PHH (figure 7A). When higher titres were
21 reached, however, a reduction of LPCAT1 mRNA amounts was observed, and a strong linear
22 inverse relationship was found between LPCAT1 expression levels and HCV infectivity titres
23 (figure 7B), consistent with the occurrence of HCV-induced transcriptional downregulation of
24 LPCAT1 in PHH.

1 Next we investigated the impact of LPCAT1 depletion on HCV infectious cycle in PHH. For
2 this, PHH were transfected with siRNA against LPCAT1 (siLPCAT1) versus GFP (siGFP),
3 then inoculated with HCV. A significant decrease in LPCAT1 protein amount was observed
4 in PHH transfected with siLPCAT1 compared to control (0.37 ± 0.05) (supplementary figure
5 S2F), in the absence of cytotoxic effect (supplementary figure S3B). LPCAT1 depletion in
6 PHH had no impact on HCV genome replication but increased the infectivity titre by almost 1
7 log (9.27 ± 5.60) (figure 7C), indicating an effect on virus morphogenesis. However, the viral
8 load was not significantly modified, therefore the specific infectivity of viral particles
9 produced was increased in LPCAT1-depleted PHH (7.87 ± 5.14). Upon isopycnic iodixanol
10 gradient ultracentrifugation, the proportion of viral RNA-containing particles with a buoyant
11 density below 1.08 g/ml was greater for HCV grown in PHH than for HCV grown in Huh-
12 7.5.1 cells (compare siGFP in figure 7D with shLuc in figure 3B), as previously reported.²⁵
13 Nevertheless, a further shift of HCV RNA towards low buoyant density fractions was
14 observed upon LPCAT1 depletion in PHH: the percentage of total viral load present below
15 1.09 g/ml was $47.01 \pm 0.73\%$ for LPCAT1-depleted cells as compared to $35.91 \pm 0.68\%$ for
16 control cells (figure 7D). These low-density fractions contained the excess of highly
17 infectious particles produced upon LPCAT1 depletion (figure 7E), also coinciding with an
18 excess of ApoB (figure 7F). Thus, in PHH as in Huh-7.5.1 cells but even more strikingly,
19 LPCAT1 depletion appeared to favour the morphogenesis of HCV particles of high specific
20 infectivity correlating with low buoyant density.

21

22 **DISCUSSION**

23 The human hepatocyte is both the cell type specialized in the biogenesis of VLDLs, which
24 permit export of TAGs into the circulation, and the primary replication site of HCV, which

1 circulates in the form of TAG-rich LVPs. Using two cell-culture-based models of human
2 hepatocytes, we have here identified LPCAT1 as a host cell factor downregulated upon HCV
3 infection. Depletion of this PL-remodelling enzyme was further found to affect TAG
4 metabolism, leading to increased intracellular TAG content and eventually increased capacity
5 of TAG secretion in the form of ApoB-containing lipoproteins. In infected hepatocytes this
6 also resulted in increased production of HCV particles of lowest buoyant density, i.e.,
7 enriched in TAGs, which are those of highest infectivity. Thus, LPCAT1 downregulation by
8 HCV appears as an original viral strategy to increase the virus specific infectivity. These data
9 support the idea that HCV hijacks the hepatocellular lipid metabolism, which is devoted in
10 particular to the efflux of TAGs in the form of VLDLs, for the benefit of LVP morphogenesis.

11 High-throughput profiling studies in the Huh-7/JFH1 model have suggested that HCV
12 regulates the expression of host genes involved in a variety of hepatocellular processes,
13 including liver lipid metabolism, at the transcriptional, translational and post-translational
14 levels.³¹⁻³⁴ Our study reveals a decrease in the amounts of both LPCAT1 mRNA and protein
15 in JFH1-infected Huh-7.5.1 cells, consistent with HCV-induced downregulation of LPCAT1
16 expression occurring at least partly at the transcriptional level. The PHH model is less suitable
17 for such studies owing to lower levels of HCV infection and to the notorious autofluorescence
18 of these cells that precludes in situ immunofluorescence analysis.²⁵ Our finding of a strong
19 linear correlation between HCV infectivity titre and LPCAT1 mRNA amount is all the more
20 striking in view of these limitations, supporting the occurrence of transcriptional
21 downregulation of LPCAT1 by HCV in a patho-physiologically relevant model. Whereas
22 LPCAT1 expression was unchanged in cells replicating an HCV subgenomic replicon, the
23 sole expression of HCV core protein recapitulated the downregulation of LPCAT1 upon HCV
24 infection. Although we cannot exclude an additional contribution of other HCV components,
25 the virus core protein thus appears to play a major role in LPCAT1 downregulation. In fact

1 this is not surprising, as this multifunctional protein has been reported to regulate the
2 expression of many host genes aside from its role as a structural component of the virion.^{35 36}
3 Furthermore, expression of HCV core protein alone is sufficient to alter the LD-associated
4 proteome in various cell models.^{37 38} Interestingly, HCV-induced changes in LD composition
5 may involve different molecular mechanisms, including displacing of LD-associated proteins
6 owing to the own localisation of HCV core protein to LDs, as reported for adipose
7 differentiation-related protein,³⁹ and direct or indirect regulation of gene expression, as
8 suggested here for LPCAT1.

9 In hepatic cells, an increased intracellular TAG content can result from increased fatty acid
10 uptake, increased TAG synthesis, decreased fatty acid oxidation and/or decreased TAG-rich
11 lipoprotein secretion. The latter mechanism cannot account for the increased intracellular
12 TAG amount observed in LPCAT1-depleted cells because the secretion of newly synthesized
13 TAGs was slightly increased in these cells. Another mechanism is suggested by the
14 localisation of LPCAT1 to LDs. Like HCV core protein,³⁹ LPCAT1 may displace LD-
15 associated proteins involved in lipid metabolism by its mere presence at the LD surface.⁴⁰
16 Furthermore, LPCAT1 is a PL-remodelling enzyme that catalyses the acylation of
17 lysophosphatidylcholine into phosphatidylcholine,^{21 41} the reverse reaction being performed
18 by phospholipases A2 that may also be present at the LD surface.²⁰ Lysophosphatidylcholine
19 and phosphatidylcholine have a different shape, and the alteration of the surface lipid
20 composition may lead to changes in LD stability (e.g., LD fusion, LD interaction with
21 membranes) and in protein binding.¹⁹ Theoretically, LPCAT1 depletion should lead to an
22 increased amount of lysophosphatidylcholine at the LD surface, a situation that does not
23 favour LD fusion.¹⁹ In fact, we observed fewer LDs of larger size, which may reflect LD
24 fusion, in agreement with Moessinger et al.^{22 23} Nevertheless, the modification of the PL
25 monolayer composition induced by LPCAT1 depletion probably initiates a reaction cascade

1 that leads to impairment of protein interactions with the LD surface and further, possibly, with
2 other organelles such as endoplasmic reticulum or mitochondria. Indeed, the trafficking of
3 LD-associated proteins, including key enzymes of TAG metabolism, can be strongly
4 modulated and the various mechanisms are only beginning to be understood as exemplified
5 by recent reports.^{16 42 43}

6 Besides the association of HCV particles with TAG-rich lipoproteins, another clinical feature
7 that links HCV infection with the host lipid metabolism is the high prevalence of liver
8 steatosis, which is partly attributable to a direct viral effect.^{1 44} LPCAT1 depletion was shown
9 here to cause both an increase in the net intracellular TAG content, i.e., steatosis, and an
10 increase in the production of HCV particles associated with TAG-rich lipoproteins, thus
11 illustrating a possible link between liver steatosis and LVP morphogenesis. However, HCV
12 infection is not sufficient to induce steatosis in cell culture⁴⁵ and a variety of mechanisms
13 have been suggested to account for HCV-induced steatosis, including inhibition of VLDL
14 biogenesis which, unlike LPCAT1 downregulation, may explain the
15 hypobetalipoproteinaemia frequently associated with liver steatosis in HCV-infected
16 patients.^{1-3 7 44 46} Thus, HCV-induced steatosis may only partially be accounted for by
17 LPCAT1 downregulation. Furthermore, it remains uncertain whether steatosis per se has a
18 role in LVP morphogenesis. The occurrence of liver steatosis in HCV-infected patients may
19 simply represent the net result of the sum of perturbations of the host lipid metabolism that
20 HCV induces primarily for the benefit of viral replication and LVP production added to non-
21 viral steatogenic mechanisms of metabolic origin that can coexist to varying degrees in the
22 same individual.

23 Upon LPCAT1 depletion in Huh-7.5.1 cells we observed an increased intracellular TAG
24 content, an increased ApoB secretion and a slight but significant increase in the capacity of

1 secretion of newly synthesized TAGs, whereas a recent study reported no alteration of the
2 total amount of lipids and a decreased secretion of both ApoB and lipids in LPCAT1-depleted
3 Huh-7 cells.²³ This apparent discrepancy may be partly due to the different Huh-7 sublines
4 but is more likely explained by the different experimental conditions used. Since ApoB is
5 stabilized by lipids or otherwise degraded,⁴⁷ we cultured Huh-7.5.1 cells in medium
6 supplemented with foetal calf serum, which contains lipids, and provided them with a
7 physiological concentration of oleic acid (0.6 mM) to assess the TAG secretion capacity. By
8 contrast, Moessinger et al.²³ cultured Huh-7 cells in serum-free medium for 3 days before
9 lipid and ApoB analysis, and incubated them with only trace amounts of radiolabelled fatty
10 acids for TAG secretion analysis. Under these conditions, the paucity of lipids available may
11 have precluded TAG synthesis, hence ApoB stabilisation by lipidation and eventually
12 lipoprotein formation.

13 LVPs found both in the liver and in the circulating blood of HCV-infected patients contain
14 ApoB, the non-exchangeable Apo essential for VLDL scaffolding, and exchangeable Apos
15 including ApoE.^{4 5 48} The presence of ApoB and ApoE in the density range of the excess of
16 HCV particles produced upon LPCAT1 depletion is compatible with their physical
17 association but does not provide direct evidence for this hypothesis. Whereas a large body of
18 evidence has underlined the essential role of ApoE both in production and infectivity of HCV,
19 the literature contains conflicting reports as to whether ApoB is incorporated into HCV
20 particles produced from Huh-7 cells and is necessary for production of infectious virus.⁴⁹⁻⁵⁸
21 One possible source of discrepancies between published data may be the intrinsic inability of
22 Huh-7 cell line to fully lipidate ApoB and hence secrete genuine VLDLs.²⁵ Our HCV culture
23 system in PHH provides a more relevant model for readdressing these issues, yet the lower
24 viral titres make it even more difficult to purify and characterize hybrid LVPs present within a
25 large excess of non-viral lipoproteins.^{6 8 25} Nevertheless, our identification of LPCAT1

1 depletion as a single mechanism that increases both VLDL and LVP secretion suggests that
2 HCV usurps at least some of the mechanisms of VLDL biogenesis for LVP morphogenesis,
3 even if ApoB per se is not incorporated into HCV particles.

4 HCV is a leading cause of chronic liver disease ranging from steatosis to cirrhosis and
5 hepatocellular carcinoma. Treatment against HCV has entered a new era with the recent
6 development of directly acting antivirals, yet these drugs have a prohibitive price, are
7 intrinsically at risk for selection of resistant variants and may not cure a small proportion of
8 hard-to-treat patients among whom those infected with genotype 3, the HCV genotype most
9 clearly associated with virally induced steatosis.^{44 59} By contrast, host-targeting agents with
10 anti-HCV activity are expected to confer a high barrier to drug resistance and a pan-genotypic
11 coverage, and therefore may appear as a useful complement to virus-targeting agents in most
12 difficult-to-treat patients.⁶⁰ The intimate link of HCV with the host lipid metabolism
13 designates lipid-modulating agents as putative antivirals.^{3 61} Depending on their mechanism of
14 action, however, a potential side effect of drugs in this therapeutic class may be to aggravate
15 hepatic steatosis. With this in mind, targeting the LPCAT1-regulated lipid metabolism
16 pathway in the opposite direction to that usurped by HCV may represent an attractive
17 therapeutic approach because it is expected to reduce not only the viral titre but also hepatic
18 steatosis.

19

20

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5

6 **FOOTNOTES**

7

8 FB and ML or SD and ARR contributed equally to this work.

9

10 **Contributors** FB, ML, SD and ARR conceived and designed the study. FB, ML, VP and DR
11 performed the experiments. FB, ML, VP, SD and ARR analysed and interpreted the data. FB,
12 ML, SD and ARR drafted or wrote the manuscript. SD and ARR obtained funding. All
13 authors approved the final version for submission.

14

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19

20 **Competing interests** None.

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5

1 **FIGURE LEGENDS**

2 **Figure 1 HCV infection downregulates LPCAT1 in Huh-7.5.1 cells.** Cells were inoculated
3 or not with HCV, and analysed 3 days later. (A) Cells were lysed for western blot analysis.
4 (Left) A representative western blot is shown. (Right) LPCAT1 protein amounts were
5 normalised to those of actin. The means \pm SEM of three independent experiments performed
6 in triplicate are shown. (B) Cells inoculated with HCV were stained for LPCAT1 (LPCAT1),
7 HCV core protein (Core) and LDs (BODIPY), and examined by confocal microscopy. (Left)
8 A merged image is shown (Merge); white and empty arrowheads indicate infected and non-
9 infected cells, respectively. (Right) LPCAT1 signals were quantified for at least 80 cells per
10 condition within three independent experiments.

11 **Figure 2 Mechanism of HCV-induced downregulation of LPCAT1.** Huh-7.5.1 cells were
12 (A and B) electroporated or not with HCV subgenomic replicon (SGR), or (C and D)
13 transfected with HCV core-encoding plasmid (pFlag-Core2a) or empty plasmid (pFlag-) as
14 control, and analysed 2 days later. (A and C) Cells were lysed for RT-qPCR analysis.
15 Expression of LPCAT1 transcript was normalised to that of human ribosomal protein L19
16 transcript. The means \pm SEM of three independent experiments performed in triplicate are
17 shown. (B) Cells electroporated with HCV subgenomic replicon were stained for LPCAT1
18 (LPCAT1), HCV NS3 protein (NS3) and LDs (BODIPY), and examined by confocal
19 microscopy. (Left) A merged image is shown (Merge); white and empty arrowheads indicate
20 NS3-positive and -negative cells, respectively. (Right) LPCAT1 signals were quantified for at
21 least 80 cells per condition within three independent experiments. (D) Cells transfected with
22 HCV core-encoding plasmid were stained for LPCAT1 (LPCAT1), HCV core protein (Core)
23 and LDs (BODIPY), and examined by confocal microscopy. (Left) A merged image is shown
24 (Merge); white and empty arrowheads indicate core-positive and -negative cells, respectively.

1 (Right) LPCAT1 signals were quantified for at least 80 cells per condition within three
2 independent experiments. Of note, the proportion of core-positive cells among the total
3 population of cells transfected with HCV core-encoding plasmid was approximately 15%.

4 **Figure 3 LPCAT1 depletion in Huh-7.5.1 cells increases production of HCV particles**
5 **with high specific infectivity and low density.** Cells were transduced with lentiviral vectors
6 encoding shLuc or shLPCAT1, and inoculated with HCV 3 days later. (A) Cells were lysed at
7 day 1 post-inoculation for quantification of negative-strand HCV RNA, and culture
8 supernatants were collected at day 3 post-inoculation for determination of infectivity titre and
9 viral load, then calculation of specific infectivity. The means \pm SEM of three to eight
10 independent experiments performed in triplicate are shown. (B-E) Culture supernatants were
11 subjected to isopycnic iodixanol gradient ultracentrifugation, and fractions were probed for
12 (B) viral load, (C) specific infectivity, (D) ApoB and (E) ApoE. All graphs (B-E) are from the
13 same representative experiment shown, and are aligned with one another (a dotted line has
14 been placed at the density of 1.08 g/ml).

15 **Figure 4 LPCAT1 depletion in Huh-7.5.1 cells alters lipid metabolism.** Cells were
16 transduced with lentiviral vectors encoding shLuc or shLPCAT1. Three days later, (A) cells
17 were stained for LDs; LDs were (B) counted and (C) analysed for size distribution; (D)
18 intracellular TAG amount and (E) secreted ApoB were quantified. The number and size of
19 LDs were analysed for at least 10 cells per condition within three independent experiments.
20 The means \pm SEM of three independent experiments performed in triplicate are shown.

21 **Figure 5 LPCAT1 depletion in Huh-7.5.1 cells increases TAG secretion capacity.** Cells
22 were transduced with lentiviral vectors encoding shLuc or shLPCAT1, and cultured for the
23 last 24 h with oleic acid/BSA containing [1-¹⁴C] oleic acid as a tracer. Lipids from cells and
24 media were extracted and fractionated by thin layer chromatography, and radioactivity in

1 TAGs and PLs was quantified. The means \pm SEM of three independent experiments
2 performed in triplicate are shown.

3 **Figure 6 LPCAT1 depletion in HCV-infected Huh-7.5.1 cells alters lipid metabolism.**

4 Cells were transduced with lentiviral vectors encoding shLuc or shLPCAT1, inoculated with
5 HCV 3 days later and cultured for 3 more days. (A) Cells were stained for HCV core protein
6 (Core) and LDs (BODIPY), and merged images are shown (Merge). LDs were (B) counted
7 and (C) analysed for size distribution. (D) Intracellular TAG amount and (E) secreted ApoB
8 were quantified. (F and G) Culture supernatants were subjected to isopycnic iodixanol
9 gradient ultracentrifugation, and fractions were analysed for (F) ApoB and (G) ApoE by
10 (bottom) western blot and (top) ELISA. The number and size of LDs were analysed for at
11 least 10 cells per condition within three independent experiments. The means \pm SEM of three
12 independent experiments performed in triplicate are shown.

13 **Figure 7 HCV infection downregulates LPCAT1 in PHH and LPCAT1 depletion in**
14 **these cells increases production of HCV particles with high specific infectivity and low**

15 **density.** (A and B) PHH were inoculated or not with HCV, and analysed 6 days later for
16 LPCAT1 mRNA amounts and infectivity titres; (B) linear regression between these two
17 variables. (C-F) PHH were transfected with siGFP or siLPCAT1, and inoculated with HCV 3
18 days later: (C) same legend as figure 3A, (D, E and F) same legend as figure 3B, 3C and 3D,
19 respectively. The means \pm SEM of three to five independent experiments (i.e., with PHH from
20 different donors) performed in triplicate are shown.

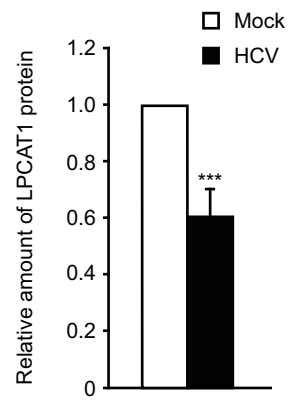
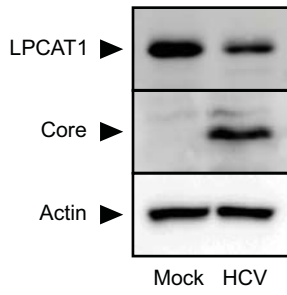
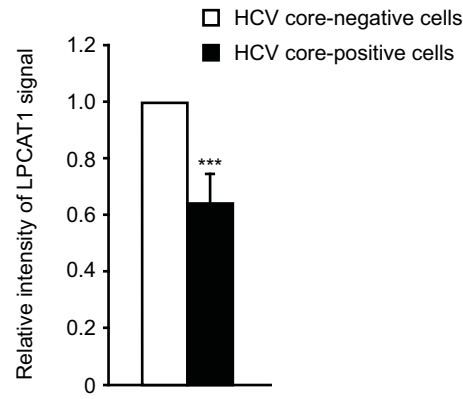
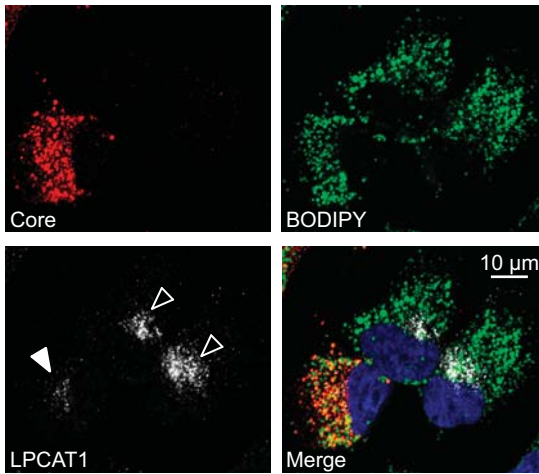
A**B**

Figure 1

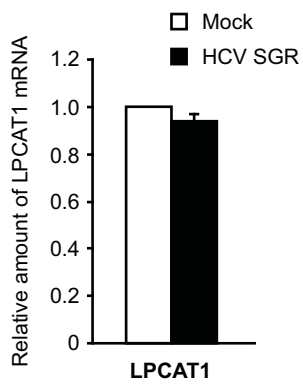
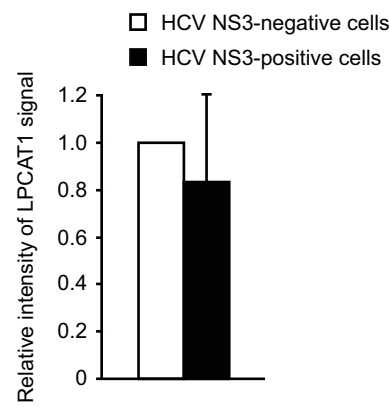
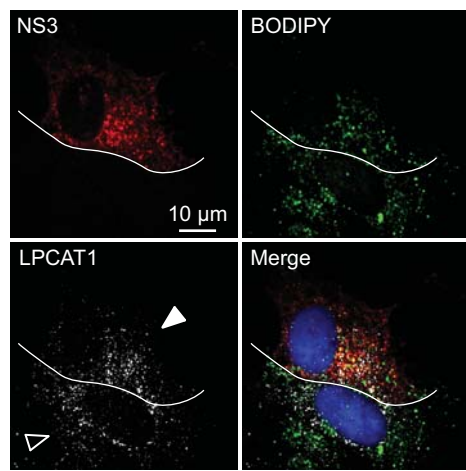
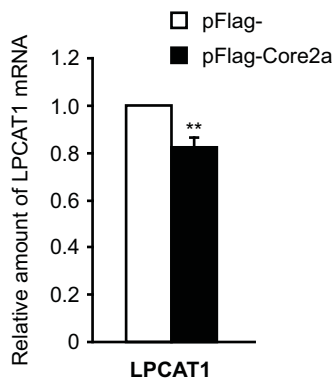
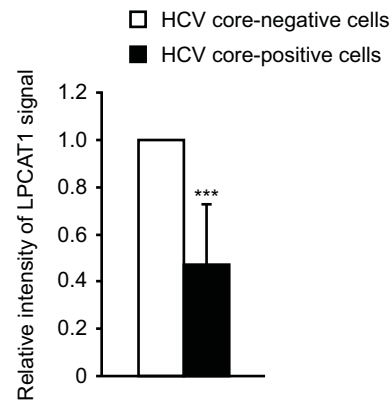
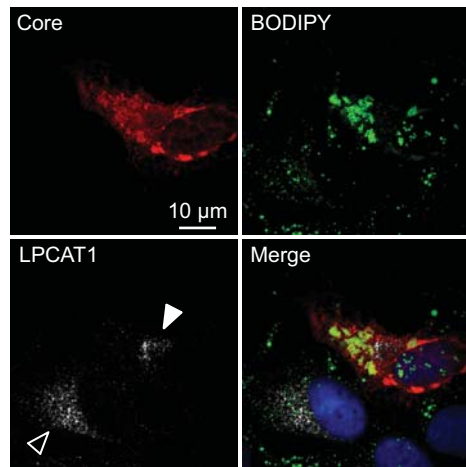
A**B****C****D**

Figure 2

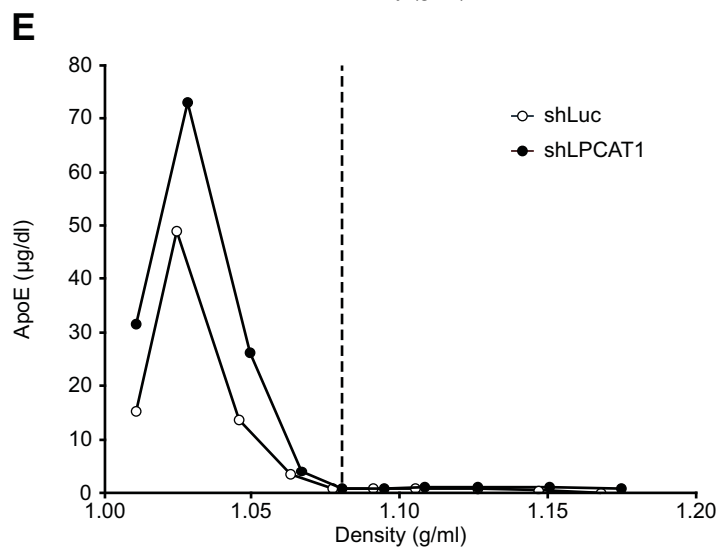
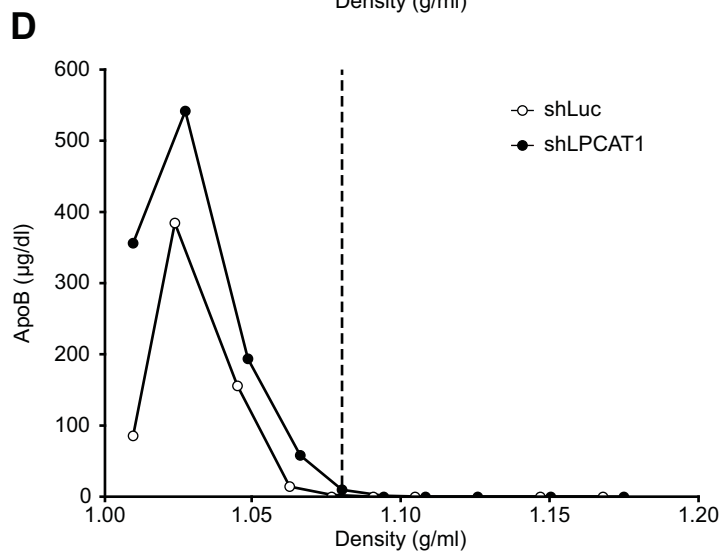
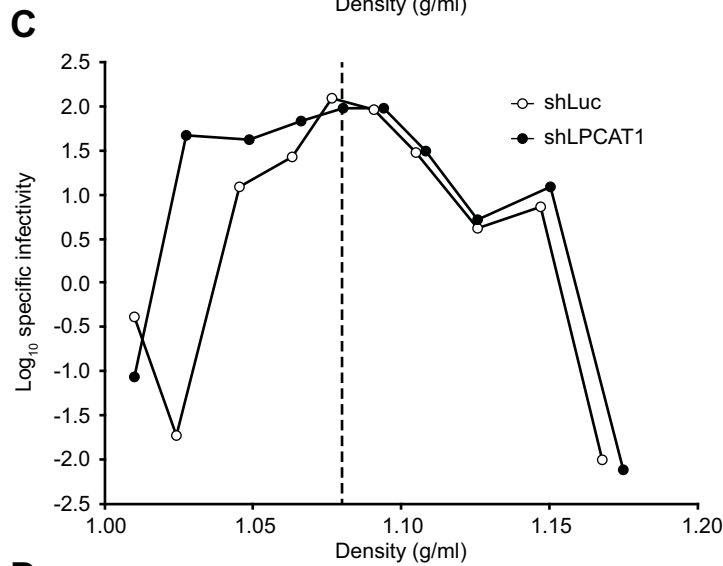
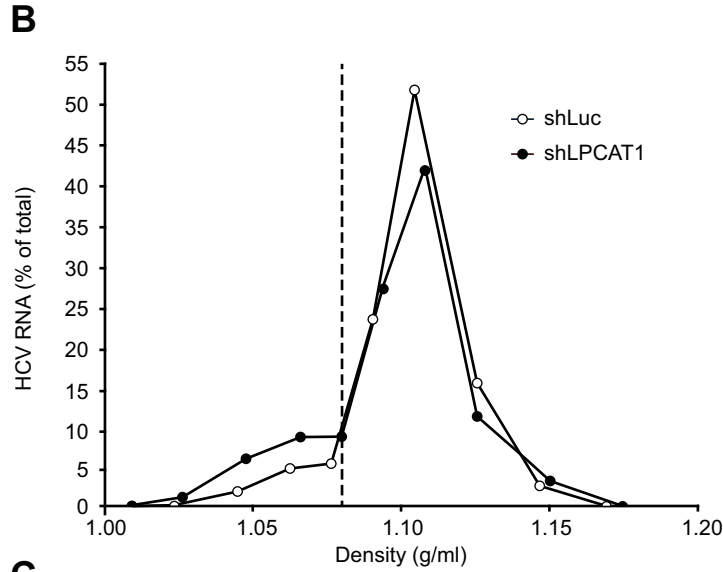
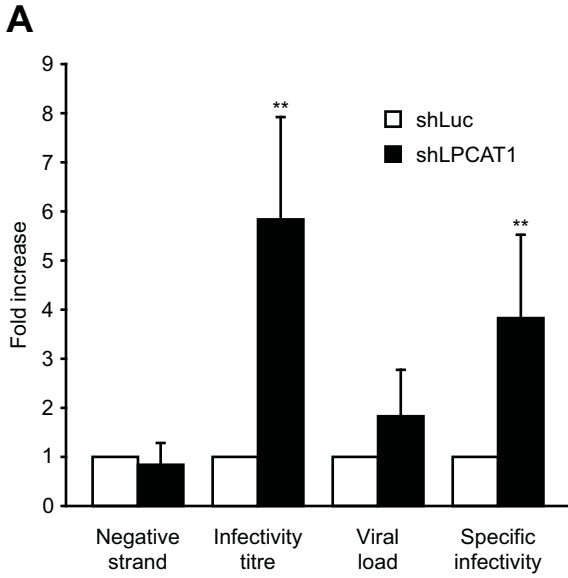


Figure 3

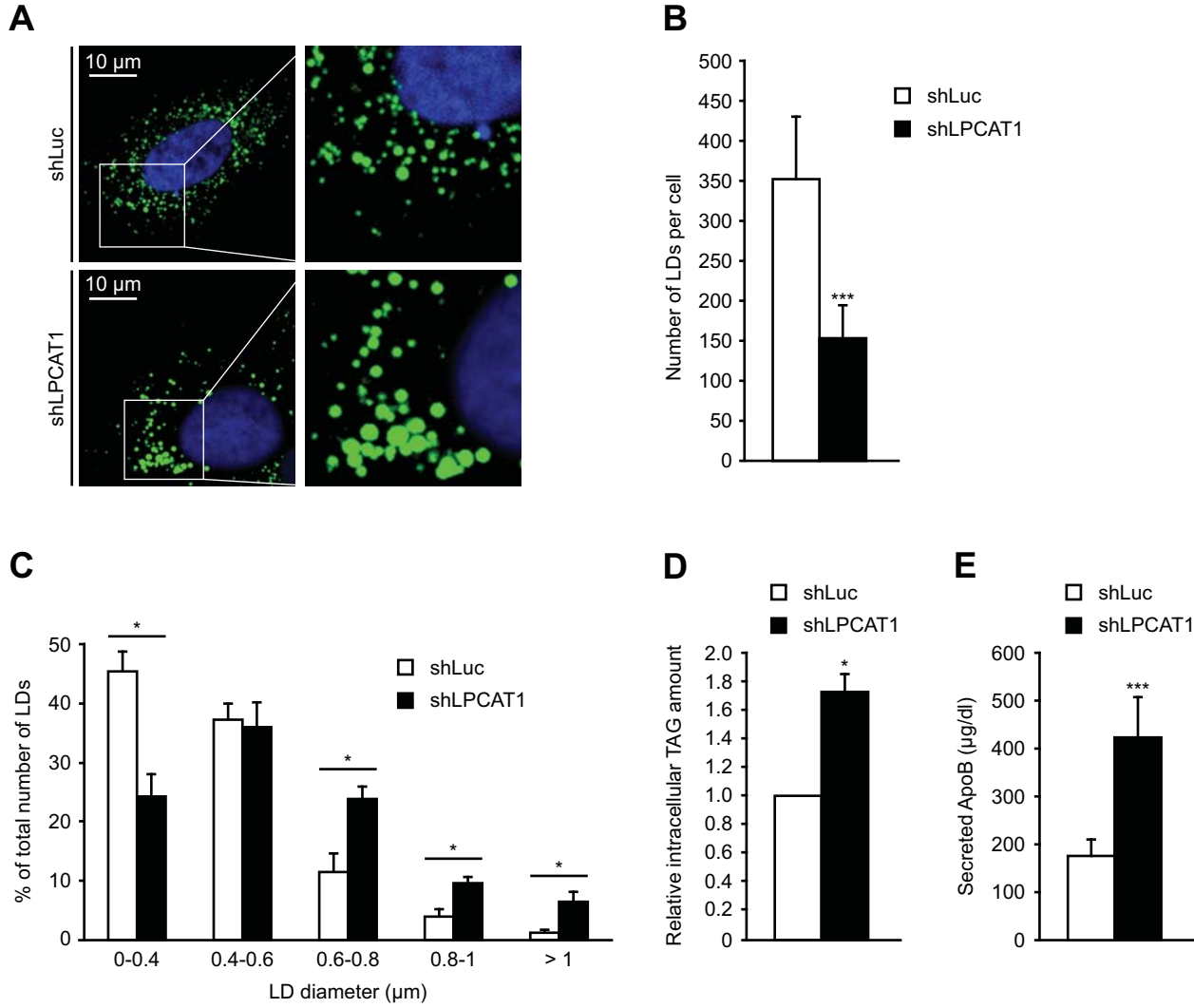


Figure 4

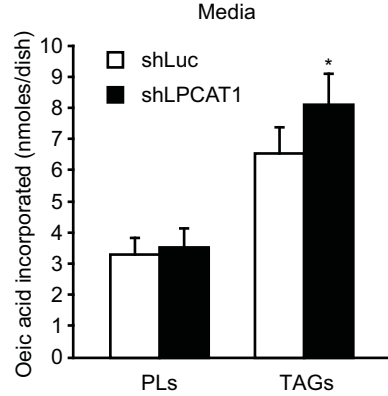
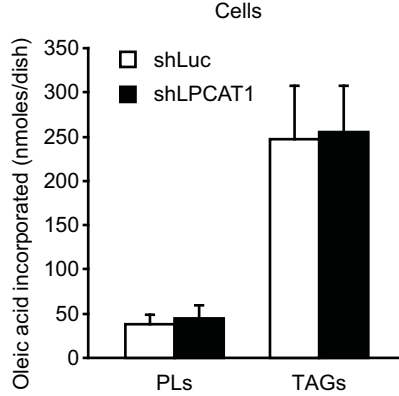
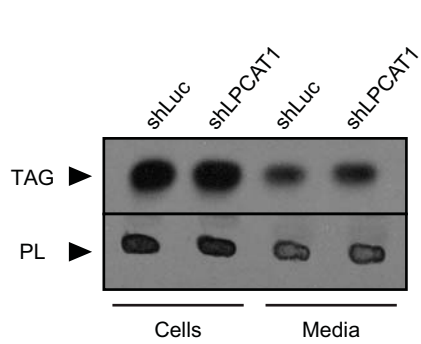


Figure 5

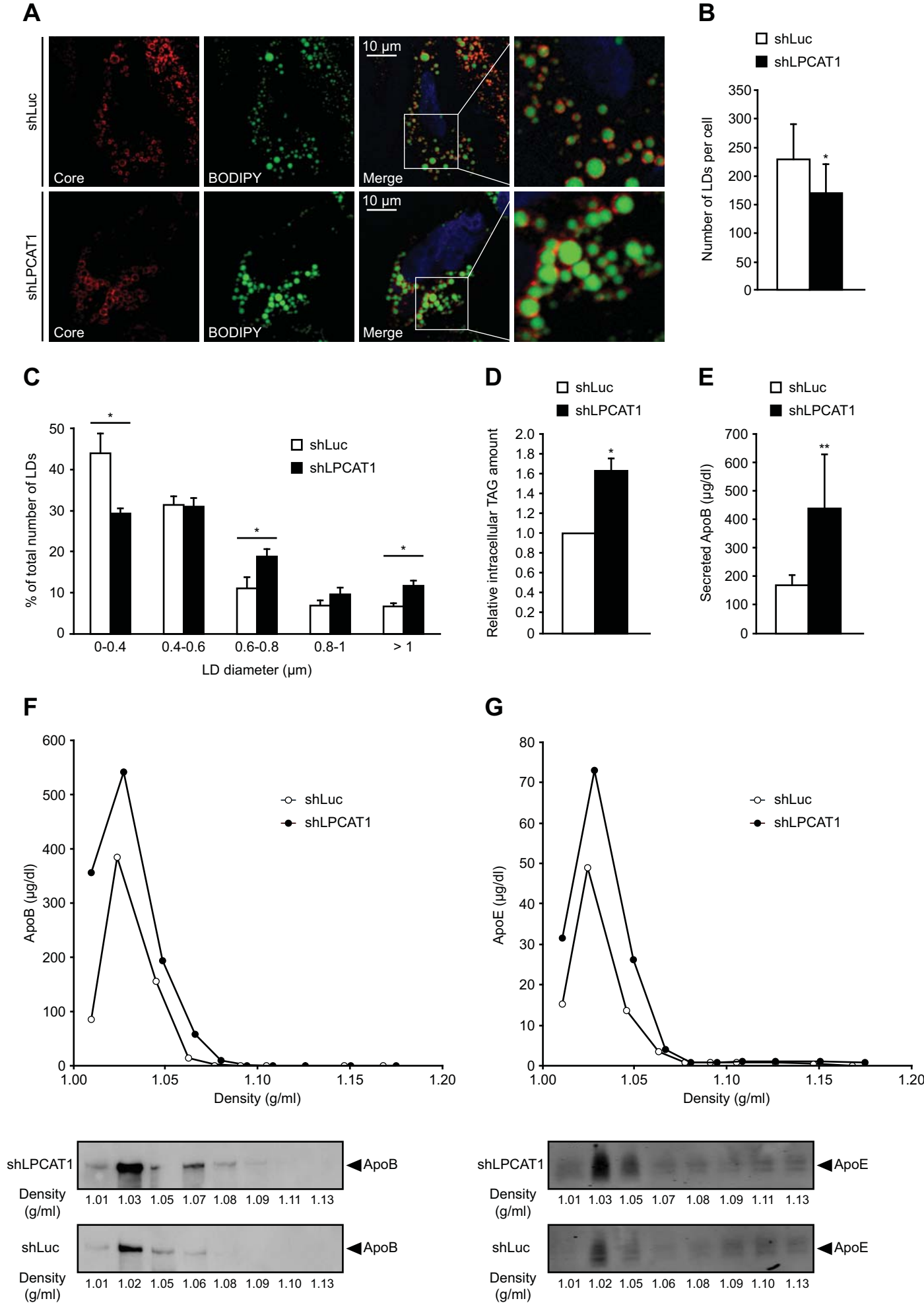


Figure 6

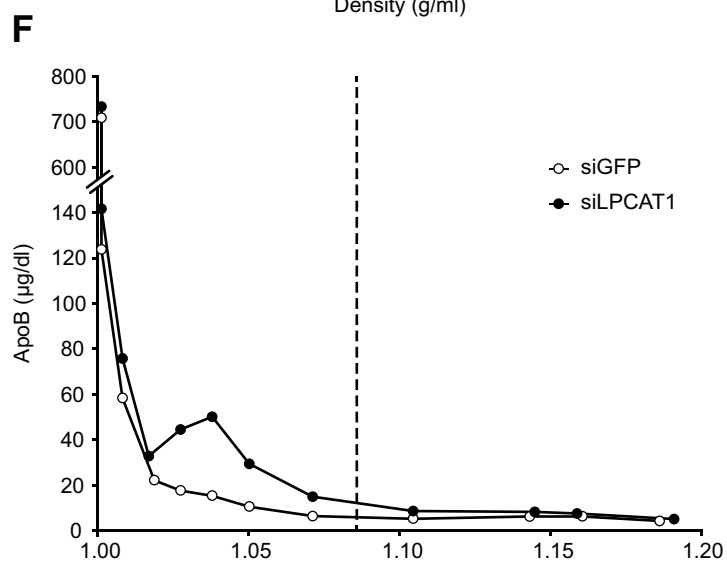
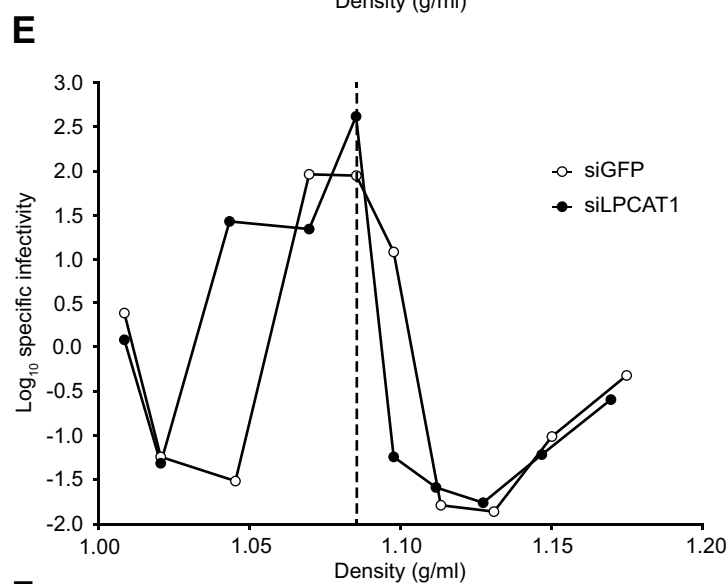
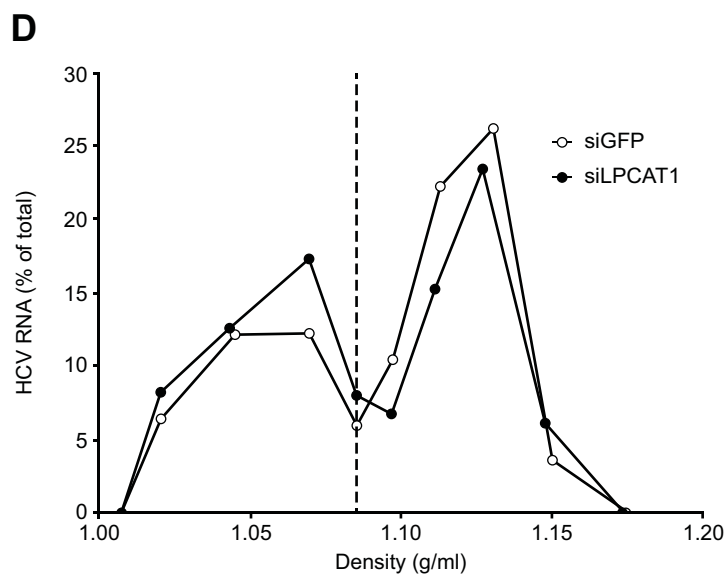
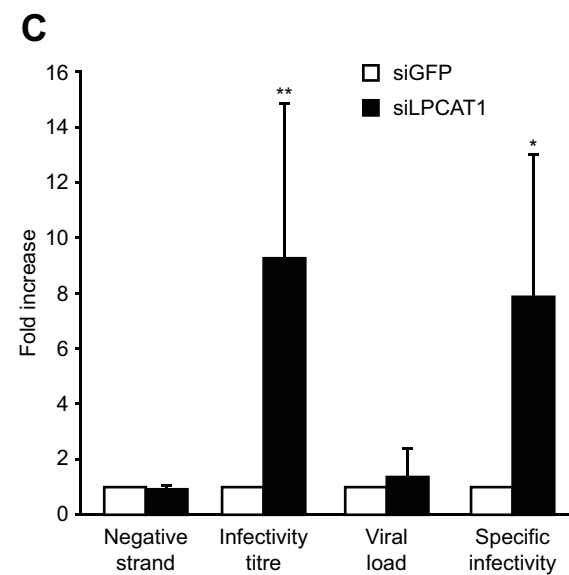
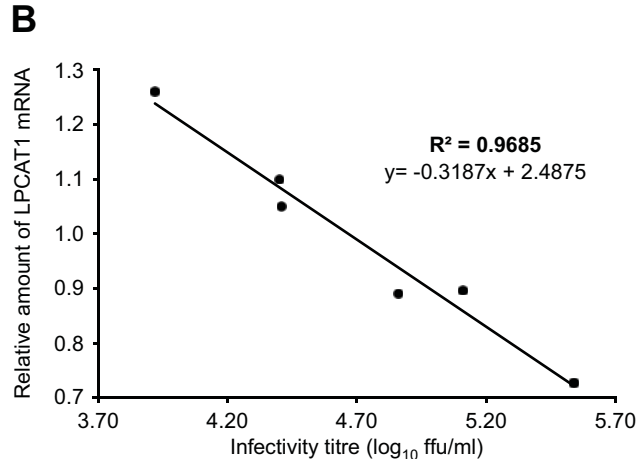
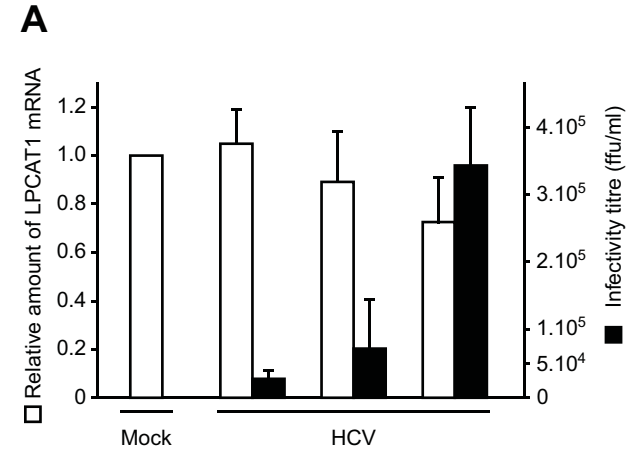


Figure 7

SUPPLEMENTARY MATERIAL TO:

**Lysophosphatidylcholine acyltransferase 1 is downregulated by hepatitis C virus:
impact on production of lipo-viro-particles**

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SUPPLEMENTARY MATERIALS AND METHODS

Antibodies

The antibodies against LPCAT1 (HPA022268) and actin (A3853) were obtained from Sigma-Aldrich (St. Louis, MO, USA), the anti-ApoE antibody (AB947) was from Millipore (Darmstadt, Germany), and the monoclonal antibodies against HCV core protein (C7-50) and NS3 protein (AB65407) used for confocal microscopy were from Abcam (Cambridge, UK). The anti-HCV core protein antibody (R308) used for western blots was generously provided by J. McLauchlan¹ and the monoclonal antibody against human ApoB (1D1) was from the Heart Institute of the University of Ottawa (Canada). Secondary antibodies were appropriate Alexa Fluor 568- and 633-conjugated antibodies and horseradish peroxidase (HRP)-conjugated IgGs (GE Healthcare, Chalfont St. Giles, UK).

Cell culture

HEK 293T cells were grown at 37°C in Dulbecco's modified Eagle medium (DMEM, Life technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated foetal calf serum (FCS, GE Healthcare).

Huh-7.5.1 cells² (a kind gift from F.V. Chisari, the Scripps Research Institute, La Jolla, CA, USA) were grown in DMEM supplemented with 10 mM hydroxyethyl piperazineethanesulfonic acid (HEPES) buffer (pH 7.3), 1% non-essential amino acids, 2 mM L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin (all Life technologies) and 10% heat-inactivated FCS, at 37°C in a humidified 5% CO₂ atmosphere.

PHH freshly isolated from HCV-seronegative adult patients were purchased from Biopredic (Rennes, France) and maintained in primary culture as described previously.³ Briefly, PHH were resuspended in complete medium consisting of Leibovitz's L-15 medium (Invitrogen,

Cergy Pontoise, France) supplemented with 26 mM NaHCO₃, 100 µg/ml streptomycin, 100 U/ml penicillin, 100 IU/l insulin (Novo Nordisk, Bagsvaerd, Denmark) and 10% heat-inactivated FCS, and seeded onto 6-well plates pre-coated with calf skin type I collagen (Sigma-Aldrich) at a density of 1.6 x 10⁵ viable cells/cm². The medium was replaced 16 h later with fresh complete medium supplemented with 1 µM hydrocortisone hemisuccinate (SERB, Paris, France), and cells were left in this medium until HCV inoculation or siRNA transfection. The cultures were maintained in complete medium at 37°C in a humidified 5% CO₂ atmosphere.

HCV infection

A high-titre stock of JFH1-HCV⁴ was produced as described previously.³ Huh-7.5.1 cells and PHH were inoculated at a multiplicity of infection of 0.2 and 2 ffu per cell, respectively. The culture medium was replaced with the inoculum diluted in the smallest volume of fresh medium sufficient for covering the cells. The inoculum was removed after a 4-h incubation at 37°C. Cells were washed three times with phosphate-buffered saline (PBS) and maintained in culture for the indicated times post-inoculation.

Subgenomic replicon transfection

The HCV subgenomic R-Luc/JFH-1-replicon (SGR) was provided by Taka Kato (NIID, Tokyo, Japan).⁵ The plasmid was linearized at the XbaI site, purified by phenol-chloroform-isoamyl alcohol extraction and used as template for in vitro transcription by T7 RNA polymerase with the RiboMAX™ large scale RNA production system (Promega), according to the manufacturer's instructions. The resulting RNA were purified by use of TRIzol RNA isolation reagent (Life Technologies), and their quality and quantity were evaluated by electrophoresis and NanoDrop spectrophotometry (Thermo Scientific). Huh-7.5.1 cells in mid-log phase were detached with trypsin, and resuspended at 10⁷ cells/ml in Opti-MEM^R

(Invitrogen). Then 400 μ l of this suspension were mixed with 10 μ g of in vitro transcribed RNA in an electroporation cuvette (0.4-cm gap), and pulsed once at 0.28 kV, 975 μ F, in a Gene PulserR II electroporator (Bio-Rad, Marne La Coquette, France). Immediately after electroporation, cells were plated at a density of 5.10^4 cells/cm² and maintained in culture for the indicated times post-electroporation.

Plasmid cloning and transfection

The pUC/JFH-1 plasmid was provided by Takaji Wakita (NIID, Tokyo, Japan).⁴ HCV core sequence was amplified from pUC/JFH-1 plasmid using the following primers: CGAAGCTTATGAGCACAAATCCTAAACCTCAAAGAAAACC and CGGAATTCTTAAGCAGAGACCGGAACGGTG. The plasmid pFLAG-Core2a was generated by cloning the HCV core amplicon into p3xFLAG-Myc-CMV-26 expression vector (Sigma-Aldrich) between *HindIII* and *EcoRI* restriction sites.

The plasmids pcDNA3.1, pcDNA3.1-LPCAT1-WT and pcDNA3.1-LPCAT1-RESCUE were provided by Life Technologies.

All plasmid transfections were performed using Lipofectamine 2000 transfection reagent (Life Technologies), according to the manufacturer's instructions.

Gene silencing

The use of lentiviral vectors expressing small hairpin RNA (shRNA) was described previously.⁶ Briefly, HEK 293T cells were co-transfected with three plasmids: pVSV-G, pCMVDR8.91 (provided by D. Trono [<http://ww1.tronolab.com/>]) and the shRNA-encoding plasmid based on pLKO.1puro (the 19-nucleotide sequences used for silencing LPCAT1 and luciferase were GGAAGATCACATTCGCTGA and GTGCGTTGCTAGTACCAAC, respectively). The culture supernatant containing recombinant lentivirus was harvested 3 days

post-transfection and used to transduce Huh-7.5.1 cells in the presence of hexadimethrine bromide (5 µg/ml polybrene, Sigma-Aldrich). After overnight incubation, the cells were placed in selective medium containing 2 µg/ml puromycin (Sigma-Aldrich) for 1 day, then culture supernatant was changed for fresh selective medium and cells were cultured in this medium 1 additional day. Finally, culture supernatant was replaced by complete medium and the next day cells were inoculated or not with HCV.

Three days post-seeding, PHH were transfected with 20 nM siRNA (Eurogentec) using RiboCellin (BioCellChallenge) according to the manufacturer's instructions. The siRNA sequences were GGACCUGCCUAAUUACCUUtt and GAACGGCAUCAAGGUGAACTt for LPCAT1 and GFP, respectively. After 3 days, cells were washed with PBS then inoculated with HCV.

RT-qPCR

Total RNA was isolated from cells using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA), according to the manufacturer's protocol. Reverse transcription was performed with 1 µg of total RNA in a total volume of 20 µl. PCR reactions were performed in quadruplicate using a Light-cycler instrument (Roche, Basel, Switzerland). For each reaction, a 1:400 final dilution of the reverse transcription product was used with 0.4 µM (final concentration) of each primer in SYBR Green I master mixture (Roche). PCR conditions were one step of denaturation (8 min at 95 °C) followed by 45 cycles (each cycle consisting of 10 s at 95 °C, 10 s at 60 °C and 10 s at 72 °C). Expression of LPCAT1, LPCAT3 or LPCAT4 transcripts was normalised to that of human ribosomal protein L19 transcript. The following oligonucleotide primers were used (for each couple, the forward primer is listed first and the reverse primer second): L19 (AAGATCGATCGCCACATGTAT / TGCCTGCTTCCTTGGTCTTAG), LPCAT1 (CGATGTCCTCCATCGTGA /

GGCCGTATATACTGGATCAGAGTT), LPCAT3 (ACCAGGAAAGATACCAAACAGC / GGTAGAAAAGGCCCAGACTCA) and LPCAT4 (CCCTTCGTGCATGAGTTACA / ATAAAGGCCAGAAGCACTCG).

Western blotting

Proteins were resolved on 10% (5% for ApoB) sodium dodecyl sulphate-polyacrylamide gels and transferred to nitrocellulose membrane (Hybond ECL, GE Healthcare). Blots were blocked for 30 min with 5% skimmed milk in TBS-Tween consisting of 20 mM Tris-HCl (pH 7.6), 137 mM NaCl and 0.1% Tween 20, then probed with primary antibodies diluted in TBS-Tween containing 1% skimmed milk followed by appropriate peroxidase-conjugated secondary antibodies. Actin was used as a loading control. Blots were developed by enhanced chemiluminescence using ECL reagent (GE Healthcare). Bands were visualised using the Image Reader LAS-4000 (Fujifilm, Tokyo, Japan) and quantified using ImageJ software.

Confocal microscopy

Cells on glass coverslips were fixed with 4% paraformaldehyde for 10 min at room temperature and, after two washes with PBS, permeabilized with 0.03% saponin in PBS for 30 min. After incubation with antibodies against LPCAT1 and HCV core protein (C7-50) or HCV NS3 protein for 1 h at room temperature, the coverslips were washed three times with PBS and incubated with appropriate secondary antibodies (Alexa Fluor 568-conjugated anti-rabbit IgG and Alexa Fluor 633-conjugated anti-mouse IgG, respectively) for 1 additional hour. After three washes with PBS, cells were stained for neutral lipids by a 10-min incubation with 10 µg/ml BODIPY 493/503 (Life Technologies) to visualise LDs and mounted in Fluoprep (BioMérieux, Marcy l'Etoile, France) containing 1 µg/ml 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich). The samples were examined using a Zeiss LSM 710 Meta confocal microscope. ImageJ software was used for

quantification of signal intensity and for measurement of LD number and size, using data from three independent experiments and at least 10 different pictures per condition.

ApoB and ApoE quantification

ApoB and ApoE levels in filtered culture supernatants or iodixanol gradient fractions were quantified by using the total human ApoB or ApoE ELISA assay from ALerCHEK (Springvale, MA, USA), respectively, according to the manufacturer's instructions.

Virological analyses

Intracellular amounts of negative-strand HCV RNA were quantified by a RT-qPCR technique described previously.⁷ HCV RNA amounts in filtered cell culture supernatants or in iodixanol gradient fractions were quantified by the Abbott RealTime HCV viral load assay and expressed as international units (IU)/ml; infectivity titres were assessed by focus-formation assay and expressed as ffu/ml.^{3 8} Specific infectivity values were calculated as follows: $10^3 \times$ infectivity titre (ffu)/HCV RNA level (IU). The buoyant densities of viral particles were determined by isopycnic iodixanol gradient ultracentrifugation, as previously described.³

Lipid analyses

Quantification of the TAG mass was done on dried lipids extracted from cells with chloroform/methanol (2:1, v/v) using the PAP150TG kit (BioMérieux) according to the manufacturer's instructions with the following modifications: 0.5 ml of the buffer provided with the kit was added to the dried lipids and tubes were sonicated for 10 s in a water bath sonicator; then 0.5 ml of the 2-fold concentrated kit reagent was added and absorbance was measured after a 1-h incubation at 37°C.

The analysis of lipid secretion capacity was performed as described previously.⁹ Briefly [1-¹⁴C] oleic acid (Perkin Elmer Life Sciences, Waltham, MA, USA) (1 μ Ci per ml of final medium to prepare) was added to non-radioactive oleic acid (6 μ l from a 100 mM stock solution in chloroform/methanol 2:1 (v/v) per ml of final medium to prepare) before drying under a stream of nitrogen, followed by incubation with FCS (0.1 ml per ml of final medium to prepare) for 1 h at 37 °C to obtain oleic acid in complex with BSA. The mixture was adjusted to 1 ml with culture medium without serum and supplied to the cells for the last 24 h of culture. Lipids were extracted from cells and media and fractionated by thin layer chromatography (TLC). The radioactive bands of the TLC plates were excised and the radioactivity was quantified by liquid-scintillation counting to evaluate the incorporation of [1-¹⁴C] oleic acid into lipids. For normalisation, protein concentration of cell lysates was measured using the BC Assay (Interchim, Montluçon, France), according to the manufacturer's instructions.

Cytotoxicity assay

To assess the potential cytotoxicity of LPCAT1 silencing, the activity of lactate dehydrogenase released into culture supernatants was measured in triplicate using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA), according to the recommendations of the manufacturer.

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Supplementary Figures

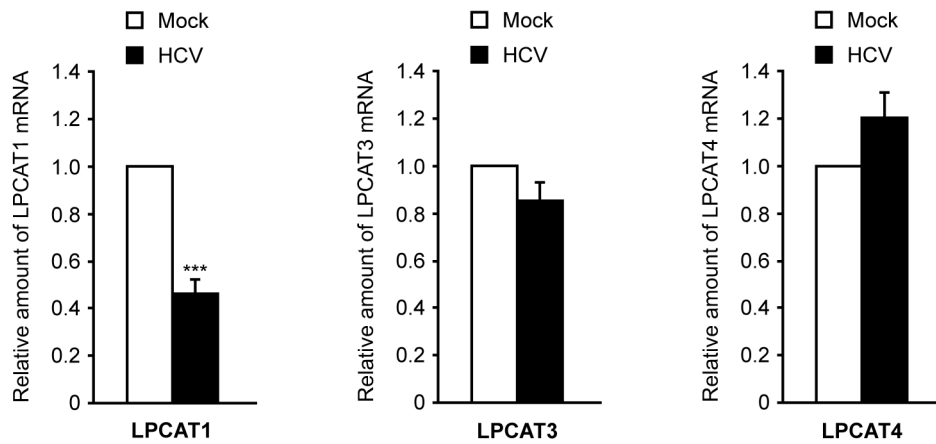


Figure S1. Effect of HCV infection on LPCAT expression. Huh-7.5.1 cells were inoculated or not with HCV, and lysed 3 days later for RT-qPCR analyses. Expression of the different LPCAT transcripts was normalised to that of human ribosomal protein L19 transcript. The means \pm SEM of three independent experiments performed in triplicate are shown. Note that LPCAT2 was not expressed in these cells, as previously reported.¹⁰

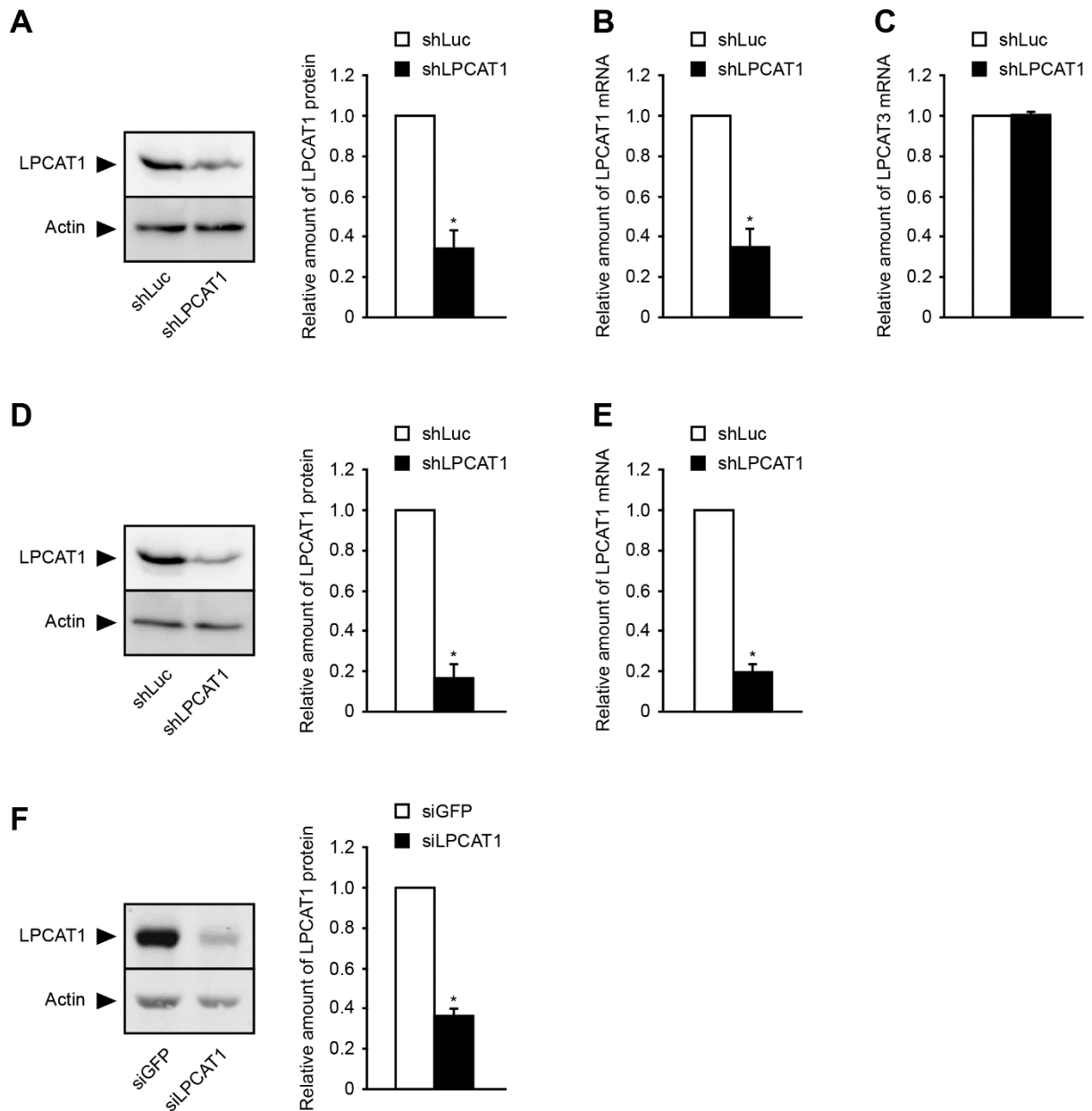


Figure S2. Efficiency of LPCAT1 silencing. (A-E) Huh-7.5.1 cells were transduced with lentiviral vectors encoding shLuc or shLPCAT1, and (A-C) left naïve or (D-E) inoculated with HCV 3 days later. (F) PHH were transfected with siGFP or siLPCAT1, and inoculated with HCV 3 days later. Three days post-inoculation, cells were lysed for (A, D and F) western blot and (B, C and E) RT-qPCR analyses. The means \pm SEM of three independent experiments performed in triplicate are shown.

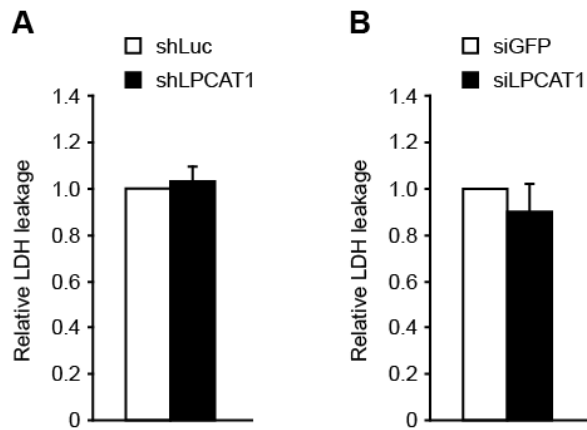


Figure S3. Absence of cytotoxicity upon LPCAT1 depletion. (A) Huh-7.5.1 cells were transduced with lentiviral vectors encoding shLuc or shLPCAT1, or (B) PHH were transfected with siGFP or siLPCAT1. Three days later, cells were inoculated with HCV, and culture supernatants were collected 3 days post-inoculation to measure lactate dehydrogenase (LDH) leakage. The means \pm SEM of three independent experiments performed in triplicate are shown.

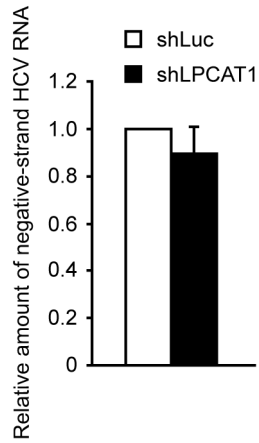


Figure S4. Absence of effect of LPCAT1 depletion on HCV genome replication. Huh-7.5.1 cells were transduced with lentiviral vectors encoding shLuc or shLPCAT1, and electroporated with HCV SGR 3 days later. Cells were lysed at day 1 post-electroporation for quantification of negative-strand HCV RNA. The means \pm SEM of three independent experiments performed in triplicate are shown.

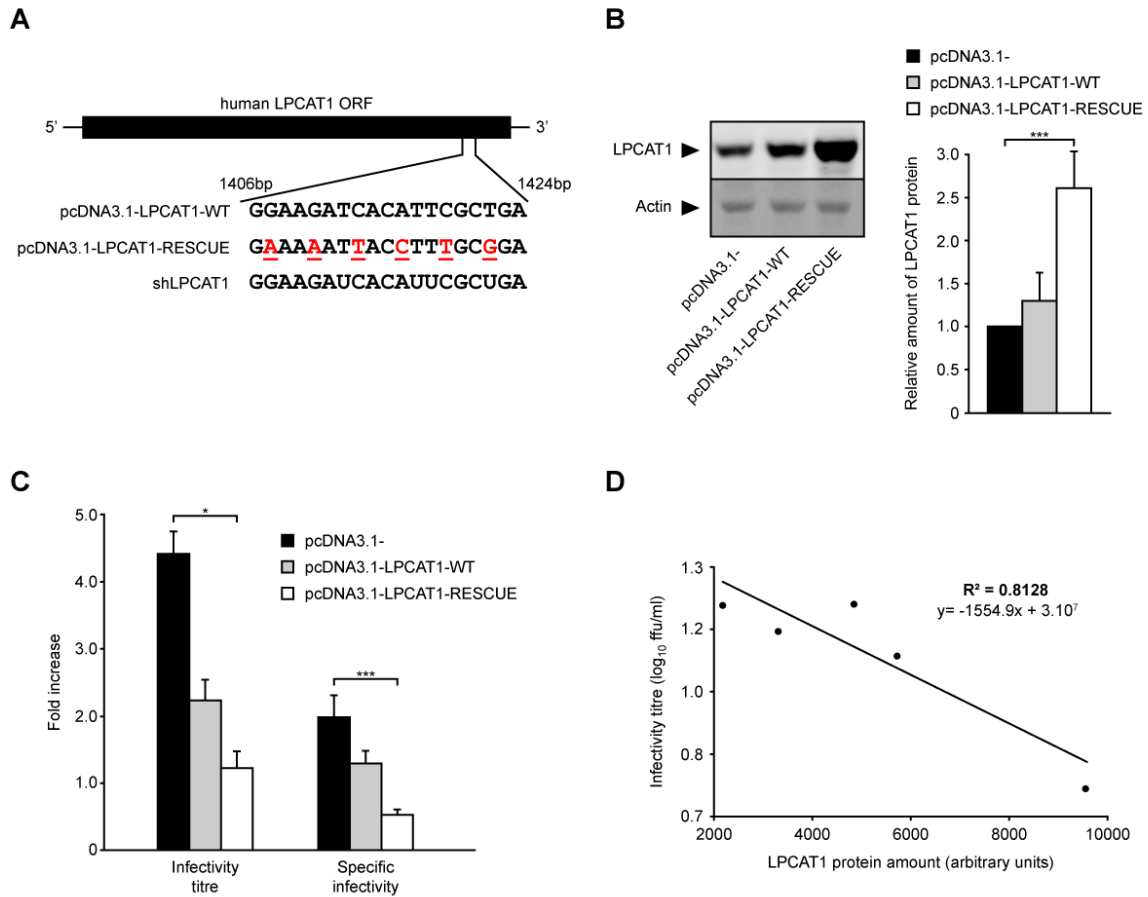


Figure S5. Effect of rescue of LPCAT1 expression in LPCAT1-depleted cells. (A) A mutant of LPCAT1 gene encoding a non-modified LPCAT1 protein but resistant to shLPCAT1 (RESCUE) was constructed. Shown are the sequences of shLPCAT1 and of the corresponding region in the plasmids carrying the wild-type (WT) or RESCUE version of LPCAT1 gene (pcDNA3.1-LPCAT1-WT and pcDNA3.1-LPCAT1-RESCUE, respectively). (B-D) Huh-7.5.1 cells were transduced with lentiviral vector encoding shLPCAT1, and inoculated with HCV 3 days later. The next day cells were transfected with empty plasmid pcDNA3.1- as control, or with pcDNA3.1-LPCAT1-WT or pcDNA3.1-LPCAT1-RESCUE, and cultured for 2 more days. (B) Cells were lysed for western blot analysis. (Left) A representative western blot is shown. (Right) LPCAT1 protein amounts were normalised to those of actin. The means \pm SEM of three independent experiments performed in triplicate are shown. (C) Culture supernatants were collected for determination of infectivity titre and calculation of specific infectivity. Shown are the fold-increase values for shLPCAT1- compared to shLuc-transduced cells. The means \pm SEM of three independent experiments performed in triplicate are shown. (D) Linear regression between LPCAT1 protein amount (as assessed by western blot quantification) and infectivity titre.