Research Article
Viral Hepatitis

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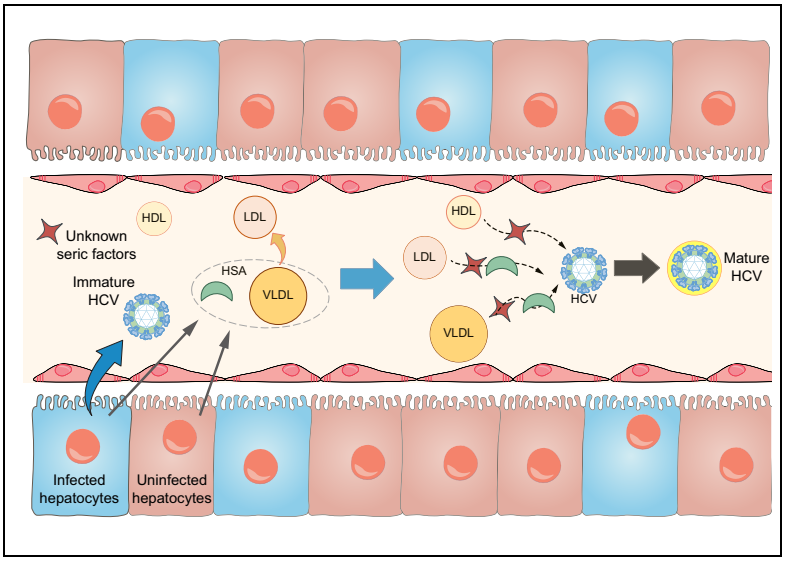
A serum protein factor mediates maturation and apoB-association of HCV particles in the extracellular milieu

Graphical abstract



Highlights

- After cell egress, HCV particles may associate with apoB and acquire neutral lipids, and hence, low-buoyant density.
- The hypervariable region 1 (HVR1) is a major viral determinant of E2 that controls this process.
- Besides lipoproteins, specific serum factors including albumin promote extracellular maturation of HCV virions.
- Simple culture conditions enable production of infectious HCV particles resembling those of infected patients.

Authors

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Lay summary

Hepatitis C virus (HCV) particles may associate with apoB and acquire neutral lipids after exiting cells, giving them low-buoyant density. The hypervariable region 1 (HVR1) is a majorviral determinant of E2 that controls this process. Besides lipoproteins, specific serum factors including albumin promote extracellular maturation of HCV virions. HCV particle production *in vitro*, with media of defined serum conditions, enables production of infectious particles resembling those of chronically infected patients.

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A serum protein factor mediates maturation and apoB-association of HCV particles in the extracellular milieu

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Background & Aims: In the sera of infected patients, hepatitis C virus (HCV) particles display heterogeneous forms with low-buoyant densities (<1.08), underscoring their lipidation via association with apoB-containing lipoproteins, which was proposed to occur during assembly or secretion from infected hepatocytes. However, the mechanisms inducing this association remain poorly-defined and most cell culture grown HCV (HCVcc) particles exhibit higher density (>1.08) and poor/no association with apoB. We aimed to elucidate the mechanisms of lipidation and to produce HCVcc particles resembling those in infected sera.

Methods: We produced HCVcc particles of Jc1 or H77 strains from Huh-7.5 hepatoma cells cultured in standard conditions (10%-fetal calf serum) *vs.* in serum-free or human serum conditions before comparing their density profiles to patient-derived virus. We also characterized wild-type and Jc1/H77 hypervariable region 1 (HVR1)-swapped mutant HCVcc particles produced in serum-free media and incubated with different serum types or with purified lipoproteins.

Results: Compared to serum-free or fetal calf serum conditions, production with human serum redistributed most HCVcc infectious particles to low density (<1.08) or very-low density (<1.04) ranges. In addition, short-time incubation with human serum was sufficient to shift HCVcc physical particles to low-density fractions, in time- and dose-dependent manners, which increased their specific infectivity, promoted apoB-association and induced neutralization-resistance. Moreover, compared to Jc1, we detected higher levels of H77 HCVcc infectious particles in very-low-density fractions, which could unambiguously be attributed to strain-specific features of the HVR1 sequence. Finally, all 3 lipoprotein classes, *i.e.*, very-low-density, low-density and high-density lipoproteins, could synergistically induce low-density shift of HCV particles; yet, this required additional non-lipid serum factor(s) that include albumin.

Conclusions: The association of HCV particles with lipids may occur in the extracellular milieu. The lipidation level depends on serum composition as well as on HVR1-specific properties. These simple culture conditions allow production of infectious HCV particles resembling those of chronically-infected patients.

Lay summary: Hepatitis C virus (HCV) particles may associate with apoB and acquire neutral lipids after exiting cells, giving them low-buoyant density. The hypervariable region 1 (HVR1) is a majorviral determinant of E2 that controls this process. Besides lipoproteins, specific serum factors including albumin promote extracellular maturation of HCV virions. HCV particle production *in vitro*, with media of defined serum conditions, enables production of infectious particles resembling those of chronically infected patients.

Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver diseases worldwide. Although direct-acting antivirals (DAAs) can now cure most patients, there remain major challenges in basic, translational and clinical research.[1] As DAAs are only curative, the development of a protective vaccine remains an important goal; yet, this requires deeper knowledge of the HCV particle's structure. Indeed, the HCV virion has unusually heterogenous morphology, size and properties.[2] Immunocapture of its surface proteins revealed particles of 50–80 nm without symmetrical arrangement.[3–6] HCV particles harbor 2 envelope glycoproteins, E1 and E2, inserted on a membranous envelope that surrounds a nucleocapsid, composed of a core protein multimer and RNA+ viral genome; yet, the organization of the virion surface remains elusive and there is currently no clear model of the HCV particle's topology.

A remarkable feature of HCV is the particularly low-buoyant density of virions, coined lipo-viro-particles (LVP).[6] In sera from infected patients and experimentally-infected chimpanzees, most HCV particles are found in densities between 1.00–1.10,[6–8] owing to their particular lipid composition, distinct from other enveloped viruses. Indeed, patient-derived HCV particles contain neutral lipids such as triglycerides or cholesteryl esters and are associated with circulating apolipoproteins such as apoC-I, apoE and apoB;[6,7,9,10] the latter being the major structural and non-exchangeable component of low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL). Importantly, injection of HCV particles into experimentally-infected animals revealed that the low-density inoculum is the most infectious.[11]

Keywords: Hepatitis C virus; Lipoprotein; Lipidation; Serum; Infectivity; Density.
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A major bottleneck of HCV characterization is the lack of *in vitro*-infectivity of patient-derived particles,[1,12] which has prevented researchers from unraveling the properties of virus/lipid interaction. To overcome this, several experimental cellular models have been designed, including cell culture-grown HCV (HCVcc) particles that are usually produced from Huh-7.5 hepatoma cells.[8,13] While HCVcc particles have a heterogeneous density profile, from 1.00 to >1.17, their specific infectivity in low-density fractions is poor,[5,8,14,15] which reflects incomplete virion lipidation. Indeed, while HCVcc particles are associated with apoC-I[16] and apoE,[17] their association with apoB is variably detected.[4,5,12,17–19]

The lipidation difference between HCVcc and patient-derived particles has been attributed to a defective lipoprotein-metabolism pathway in Huh-7 cells, preventing the formation of fully lipidated VLDLs.[12,20–22] HCVcc particles grown in primary human hepatocytes (PHH), which produce normal VLDLs, have higher specific infectivity owing to the viral RNA peak that coincides with the fractions of highest infectivity at densities of 1.10–1.11,[20] which are above those of patient-derived HCV. Likewise, HCV particles grown in PHH-xenograft mouse models that release normal human apoB and apoE levels display coincident infectivity and viral RNA peaks at 1.06–1.11,[8,21] which, again, are higher than for patient-derived HCV.

While HCVcc studies have tremendously advanced the knowledge of HCV and host-virus interactions, culminating in new DAA regimens that cure most patients, many aspects of HCV biology remain ill-defined because of the lack of models fully mimicking the conformation of authentic HCV particles. Furthermore, as lipoprotein association is thought to shape their surface and induce neutralization resistance,[4,6,7,14,23] elucidating their structure would improve the design of rational vaccine candidates.

Here, by studying HCVcc production *in vitro* with media of defined serum conditions, we show how HCVcc particles can be fully converted to apoB-containing, low- or very-low-density particles, which augments their specific infectivity and inhibits their neutralization by antibodies. We reveal that besides lipoproteins, specific serum factors are required to allow extracellular maturation of HCV virions, hence indicating that a major determinant that imprints lipidation of particles is serum itself, likely after cell egress.

Materials and methods

Human serum, lipoprotein-deficient serum and lipoproteins

The origin and characteristics of normal, consisting in pools of >4 specimen, or patient-derived human serum (HS) samples are detailed in the Supplementary Material and Methods.

Lipoprotein-deficient serum (LPDS) samples, corresponding to HS fractions of density >1.24, were prepared from HS by a 2-step ultracentrifugation procedure (Supplementary Material and Methods) and exhibited over 100-fold reduced apoB levels (Fig. S1B).

Commercial sources (Supplementary CTAT Table) of purified VLDLs, LDLs, HDLs, apoE and human serum albumin (HSA) (fraction V) were used for reconstitution experiments, at amounts corresponding to that of 10% HS (Fig. S1C), *i.e.*, 0.038 mg/ml cholesterol-VLDL, 0.093 mg/ml cholesterol-LDL, 0.047 mg/ml cholesterol-HDL, 0.057 mg/ml apoE, and 4.5 mg/ml HSA, respectively.

HCVcc production and characterization

Methods for production of HCVcc from electroporated HCV RNAs (see plasmid descriptions in Supplementary Material) and characterization of viral particles were described previously.[14] Electroporated Huh-7.5 cells were grown in serum-free medium (OptiMEM, Invitrogen) only or in media supplemented with 10% fetal calf serum (FCS) or HS. For incubation experiments, HCVcc particles grown in OptiMEM were incubated with OptiMEM *vs.* with FCS, HS or serum factors at 37 °C. Intracellular infectivity was determined as described previously[14] from HCVcc producer cells following 4 freeze/thaw cycles. Buoyant-density analysis of HCV particles was performed by iodixanol density-gradient fractionation (Supplementary Materials and Methods). Neutralization assays were performed as described previously.[14]

Co-immunoprecipitation assays

Samples of 100 µl of HCVcc supernatant or patient sera were precleared for 1 h at 4 °C with protein A/G agarose beads and incubated overnight at 4 °C with 15 µl goat anti-apoB (AB742, Millipore) or appropriate control IgGs under continuous agitation. The immune complexes were incubated for 2 h at room-temperature with 40 µl of protein A/G agarose beads. The complexes were washed 3 times in PBS and were re-suspended in Tri-Reagent for RNA extraction or eluted in RIPA buffer for western-blot analysis and core quantification.

Statistical analysis

Significance values were determined by applying the non-parametric Friedman test using the GraphPad Prism-6 software. All data are represented as means ± SEM. The statistical analyses compare serum *vs.* no serum condition, unless otherwise indicated in the figure legends. p values >0.05 were considered statistically not significant (n.s.) and the following denotations were used: $^{****}p \leq 0.0001$; $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$.

Results

Serum composition of culture media dictates HCV particle density

HCV particles isolated from HCV-infected patient sera exhibit low-buoyant densities, with most viral RNAs detected at densities <1.08 (Fig. 1A), reflecting their lipidation state.[6–8] Since HCVcc is typically produced from Huh-7.5 cells grown with 10% FCS and has higher density, we hypothesized that the species origin and composition of serum used in culture media (Fig. S1A) may influence virion density. Whereas infectious HCV particles produced for 72 h in serum-free media appeared poorly lipidated (Fig. 1B), with <10% infectivity in fractions below 1.08 (see examples of raw density profiles in Fig. S2), 38% of the infectivity of HCVcc particles grown in FCS-containing media were shifted to low-density fractions, yet with <8% infectivity detected below 1.04 (Fig. 1B). Strikingly, HCVcc produced with 10% HS (from a pool of normal HS) exhibited >80% infectivity at densities <1.08 and *ca.* 60% at densities <1.04 (Fig. 1B). Importantly, the density shifts of infectivity correlated with profiles of viral RNA and core in gradients (Fig. 1C; Fig. S2A-C). Indeed, compared to serum-free HCVcc production that raised poor levels of HCV RNAs in low-density fractions (*ca.* 10% in densities <1.08), the quantities of low-density RNA were increased by *ca.* 7-fold upon virus production in 10% HS-

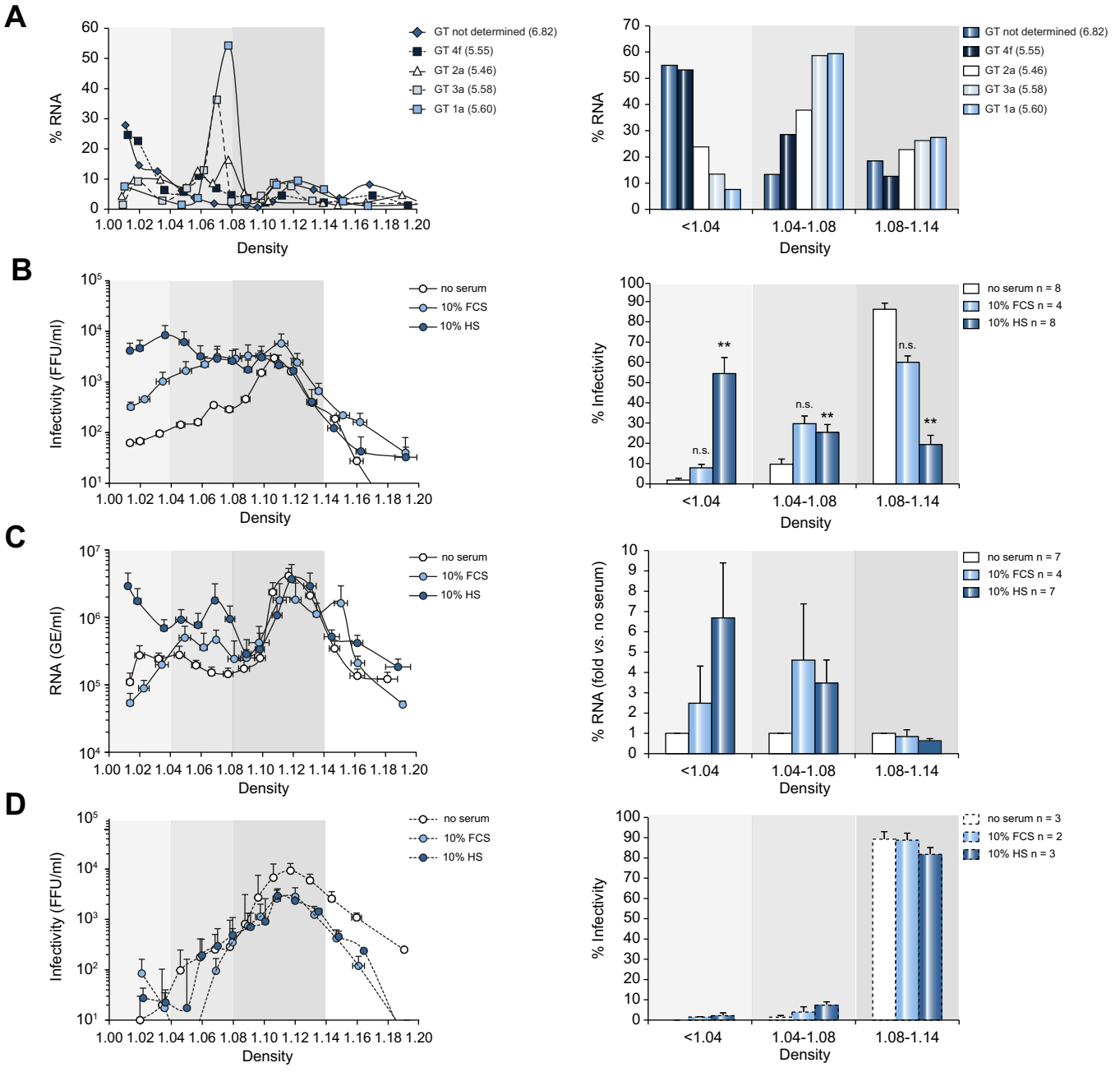

Fig. 1. Serum conditions dictate the density of extracellular HCV particles. (A) Density-gradient analysis of viral particles from HCV-infected patients displaying percentages of HCV RNAs in each fraction (left panels) or after grouping in 3 categories of density (right panels). The HCV genotypes and viral loads (log[copies]/ml) in patient sera are indicated. Density-gradient analysis of (B, C) extracellular or (D) intracellular Jc1 HCVcc viral particles produced for 72 h without serum *vs.* with FCS or HS, as indicated. The results display the mean values of infectivity or HCV RNAs in each fraction from different experiments (left panels) (see representative examples of individual experiments in Fig. S2A-C). The values from different gradients regrouped in 3 categories of density (right panels) display the mean percentages of infectivity or the fold-increase of percentages of HCV RNAs relative to serum-free conditions (see the mean percentages of infectivity or HCV RNAs in each fraction from different experiments in Fig. S3A-C). FCS, fetal calf serum; FFU, focus forming units; GE, genome equivalent; GT, genotype; HCV, hepatitis C virus; HCVcc, cell culture grown HCV; HS, human serum.

containing media (Fig. 1C). Of note, although most infectious particles shifted to low density, a large portion of HCV RNAs could still be detected in densities >1.08 (Fig. 1C); they could represent non-infectious particles such as RNA-containing exosomes.[24] Similar low-density shift of HCV particles was obtained when using different pools of normal HS or shorter production times (data not shown), suggesting that HCV virions become lipidated when produced in the presence of serum, with a major effect of HS.

We extended these results obtained with genotype 2a (Jc1 strain) to H77 strain (of genotype 1a) HCVcc (Fig. 2; Fig. S2D-F). Interestingly, the infectivity of H77 HCVcc grown with serum shifted to lower densities than Jc1 particles. Indeed, compared to Jc1, H77 infectious particles were detected in higher proportions in densities <1.08 (60% [H77] *vs.* 38% [Jc1]) upon FCS production, and, strikingly, peaked at lower densities: 1.02–1.09 (H77) *vs.* 1.06–1.09 (Jc1) and 1.00–1.03 (H77) *vs.* 1.03–1.06 (Jc1) upon FCS and HS production, respectively (Figs. 1B and

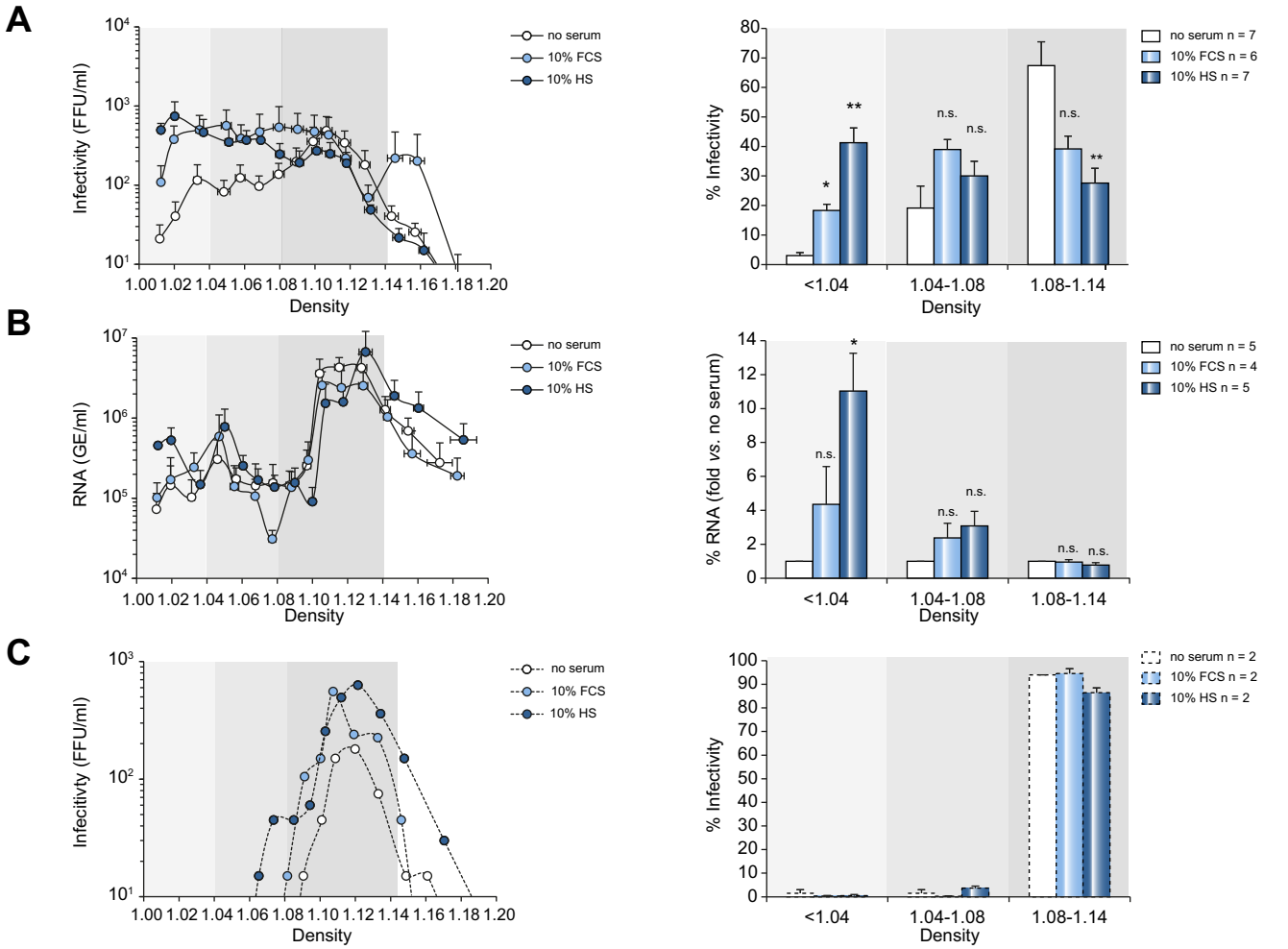

Fig. 2. Lipidation of HCV particles is HCV genotype-independent. Density-gradient analysis of (A, B) extracellular and (C) intracellular H77 HCVcc particles produced for 72 h without serum *vs.* with FCS or HS, as indicated. The results display the mean values of infectivity or HCV RNAs in each fraction from different experiments (left panels) (see representative examples of individual experiments in Fig. S2D-F). The values from different gradients regrouped in 3 categories of density (right panels) display the mean percentages of infectivity or the fold-increase of percentages of HCV RNAs relative to serum-free conditions (see the mean percentages of infectivity or HCV RNAs in each fraction from different experiments in Fig. S3G-I). FCS, fetal calf serum; FFU, focus forming units; GE, genome equivalent; HCV, hepatitis C virus; HCVcc, cell culture grown HCV; HS, human serum.

2A). Likewise, compared to serum-free production, the amounts of H77 physical particles produced in serum-containing medium increased by >10-fold in low-density fractions, with higher increases of viral RNAs and core for particles produced in HS than for FCS (Fig. 2B; Fig. S2E-F) and for H77 than for Jc1 particles (Figs. 1C and 2B). Altogether, these results suggested that HCV-specific sequences influence the lipidation level of viral particles.

Lipidation of HCV particles occurs post-egress and enhances specific infectivity

The high-density profile of HCVcc particles produced in serum-free conditions suggested that the extracellular milieu imprints virion composition. Accordingly, when we investigated Jc1 and H77 intracellular particles, we found that they had identical density profiles with almost no infectivity in fractions <1.08 whether the virus-producer culture media contained no serum or up to 50% HS (Figs. 1D and 2C; Fig. S3C,F,I). This indicated that acquisition of the low-density profile of HCV occurs at a post-assembly step, *i.e.*, during secretion, as proposed elsewhere,[15] and/or after secretion in the extracellular milieu.

To test the latter possibility, we incubated similar inputs of Jc1 and H77 physical particles (5×10^7 GE) produced in serum-free conditions in media containing 10% HS or FCS *vs.* no serum for 6 h at 37 °C (Fig. 3; Fig. S4A,D for raw data examples). We found that this incubation procedure could reproduce the patterns observed in the HCVcc production protocol (Figs. 1–3). Indeed, Jc1 or H77 viruses incubated with HS shifted >90% of infectivity in densities <1.08 (Fig. 3A,B), which correlated with a shift of physical particles in low-density fractions (Fig. 3C; Fig. S4A,B,D,E). Again, more marked shifts of both infectious and physical particles were detected for incubation in HS- rather than FCS-containing media, implying the presence of species-specific lipidation factor(s). Likewise, a stronger shift was observed for H77 compared to Jc1 particles (Fig. 3A-C), with 50% *vs.* 20% of infectivity in densities <1.04, respectively. Note that similar differences were obtained when using smaller Jc1 inputs to normalize Jc1 *vs.* H77 infectivity differences (*ca.* 6-fold) or when using HS from different individuals (Fig. S5). Finally, we found that HS-incubation of HCV particles produced from PHH could also induce low-density shifts of both infectious and physical particles (Fig. S6), suggesting that maturation of HCV may occur after egress from infected hepatocytes.

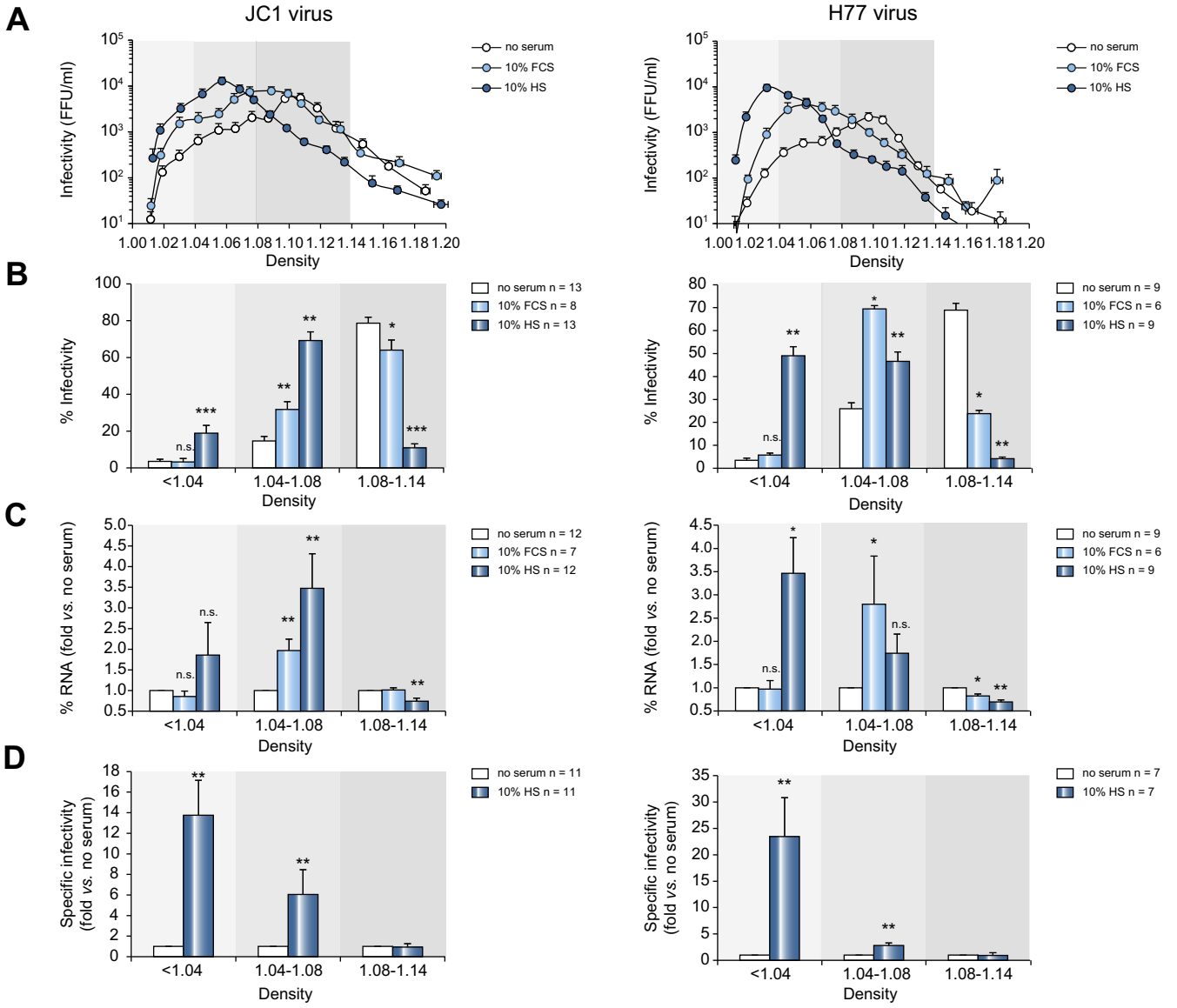

Fig. 3. Lipidation of HCV particles occurs in the extracellular medium and increases their specific infectivity. Density-gradient analysis of serum-free produced Jc1 (red) or H77 (blue) extracellular HCVcc particles (5×10^7 GE) incubated for 6 h at 37 °C with or without serum. The results display (A) the mean values of infectivity or the analysis by categories for (B) infectivity, (C) viral RNAs, and (D) specific infectivity. See representative examples of individual experiments and of the mean percentages of infectivity in each fraction from different experiments in Fig. S4A,B,D,E. FCS, fetal calf serum; FFU, focus forming units; GE, genome equivalent; HCV, hepatitis C virus; HCVcc, cell culture grown HCV; HS, human serum.

Importantly, when we incubated purified intracellular particles with serum-containing media, they readily shifted to low-density fractions, again with more marked shifts for HS rather than FCS and for H77 virus rather than Jc1 (Fig. 4A,B; Fig. S4C, F), suggesting that HCV particles are initially produced as no or poorly lipidated forms that are progressively matured during secretion[15] or, as indicated by our results, at a post-egress step. To corroborate these results, we incubated extracellular or intracellular viral particles purified from fractions of densities of 1.08–1.14 (Fig. 5A,B) with HS-containing *vs.* serum-free media before subjecting them to a second density-gradient analysis. We found that incubation with HS readily shifted infectious and physical particles to low-density fractions (Fig. 5C,D).

Altogether, these results indicated that incubation of poorly lipidated viral particles change virion structure and/or composition, inducing low-density shift and suggesting lipid acquisition after virion egress.

While displacement of HCV particles to low-density fractions upon HS-incubation resulted in strongly reduced infectivity in fractions of density 1.08–1.14 (Figs. 3–5), the levels of viral RNAs and core in these fractions remained abundant (Fig. 3C; Fig. 5C,D; Fig. S4A,D), reflecting that most viral RNAs and/or particles in these densities are not infectious. In contrast, the comparison of the shifts of infectious *vs.* physical particles in low-density fractions suggested that HS-incubation of Jc1 and H77 viruses more readily increased the infectivity than the viral RNA quantities (Fig. 3B,C). Accordingly, the ratio of infectivity to viral RNAs calculated in each fraction steadily increased once viruses acquired lower densities (Fig. S7), with respectively 14-fold and 25-fold mean augmentations for HS-incubated Jc1 and H77 particles compared to serum-free incubation in fractions

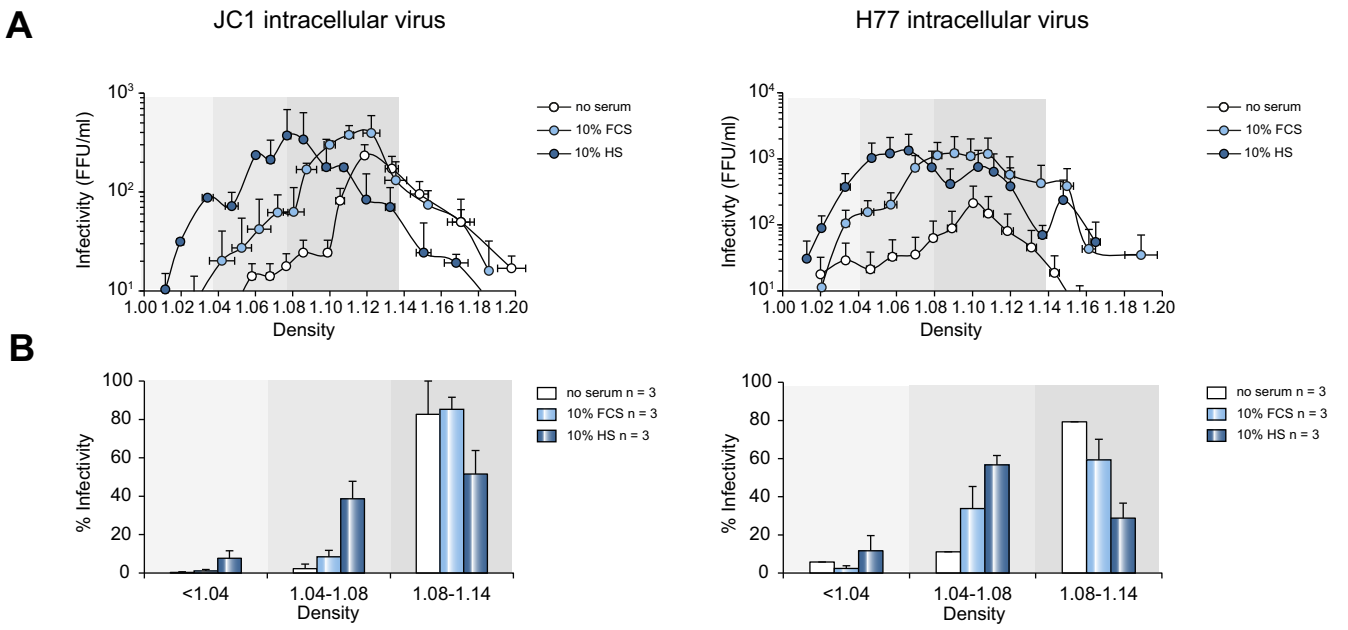

Fig. 4. Lipidation of intracellular HCV particles. Density-gradient analysis of serum-free produced Jc1 (red) or H77 (blue) intracellular HCVcc particles incubated for 6 h with or without serum. The results display (A) the mean values of infectivity or (B) the analysis by categories for infectivity. See the mean percentages of infectivity in each fraction from different experiments in Fig. S4C,F. FCS, fetal calf serum; FFU, focus forming units; HCV, hepatitis C virus; HCVcc, cell culture grown HCV; HS, human serum.

below <1.04 (Fig. 3D), and likewise for fractions of densities 1.04–1.08. This suggested that lipidation modifies the specific infectivity of HCV particles upon their shift to low densities and renders the particles more infectious.

Time and serum dose-dependent HCV virion lipidation induces apoB-association and neutralization escape

The higher infectivity detected in low-density fractions for viral particles produced for 72 h with HS-containing media compared to viruses incubated for 6 h with HS suggested that virion lipidation is a time-dependent process (Figs. 1–3). Upon incubation with HS for different times, we found that Jc1 and H77 particles (Fig. 6A,B; Fig. S8 for examples of raw data of infectivity, core and RNA) progressively shifted to densities of 1.04–1.08, reaching a transient maximum at *ca.* 4–6 h incubation, after which the infectious particles in these densities decreased and steadily accumulated in the very-low-density fractions (<1.04). This suggested that HCV lipidation is a continuous process that may progressively occur or proceed after secretion from producer cells.

Next, we determined whether serum concentration influences the lipidation levels of HCVcc. As shown in Fig. 6C-E (Fig. S9 for raw data), incubation of HCVcc particles with increasing HS doses gradually shifted infectivity and physical particles, reaching nearly 100% infectivity in low-density fractions (<1.08) with 50% HS. These results suggested that lipid acquisition depends on serum concentration and underscored a limiting serum factor(s) responsible for lipidation of HCV particles at a post-egress step.

Since HS-incubated HCVcc particles reached densities close to those of HCV-infected patients (Fig. 1A), we wondered if the former viruses were associated with apoB, as observed *in vivo*.[6,7,9,10] When we quantified viral components after co-immunoprecipitation assays with apoB antibodies, we found a strong enrichment of viral RNAs (Fig. 6F) or core (Fig. 6G) for Jc1 and H77 particles incubated with HS. Note that the levels of co-immunoprecipitated HCVcc RNAs were comparable to those of patient-derived virus (Fig. 6F). Conversely, E2 immunoprecipitation of HS-incubated HCV particles, *via* a FLAG tag inserted at E2 amino-terminus (Jc1 FLAG-E2 virus[5,25]), allowed apoB co-immunoprecipitation (Fig. S10). Altogether, this indicated that the low-density profile of HCVcc incubated with HS reflects their association with apoB, suggesting that upon HS-incubation, virus particles interact with apoB-containing lipoproteins in a manner similar to patient-derived HCV.

Finally, we found that while Jc1 and H77 particles produced in serum-free conditions were readily inhibited by the 3/11[26] and AR3A[27] E2 neutralizing antibodies, with IC_{70} of *ca.* 2 μg/ml, they were strongly resistant to neutralization after HS treatment (Fig. 6H). Specifically, such antibodies neutralized less than 40–70% of HCVcc particles, even for antibody concentrations higher than 20 μg/ml. This indicated that acquisition of low-density shift correlates with HCV neutralization escape.

HVR1 sequence of HCV E2 glycoprotein determines lipidation of viral particles

Since Jc1 and H77 viruses displayed different lipidation levels and rates (Fig. 6A,B), we sought to investigate which viral determinant could influence virion lipidation. We found that swapping the H77 hypervariable region 1 (HVR1) sequence in Jc1 virus (Fig. 7A) induced stronger low-density shift of the resulting Jc1_HVR1_H77 recombinant particles compared to parental Jc1 virus, with an increase of over 5–6-fold of both infectious (Fig. 7B,C [left panels]; Fig. S11B) and physical (Fig. 7D [left panel]; Fig. S11C) particles detected in densities <1.04 upon HS-incubation. Interestingly, the low-density shift of this recombinant virus was higher than that of parental H77 virus itself, with >2-fold more infectious particles detected in such fractions (80% *vs.* 40%; Fig. 7B,C; Fig. S11B,E), correlating with the stronger shift of physical particles (6–7-fold *vs.* 2.5-fold; Fig. 7D; Fig. S11C,F). Conversely, compared to parental H77 virus, swapping the Jc1 HVR1 sequence decreased the low-density shift of the resulting H77_HVR1_Jc1 recombinant

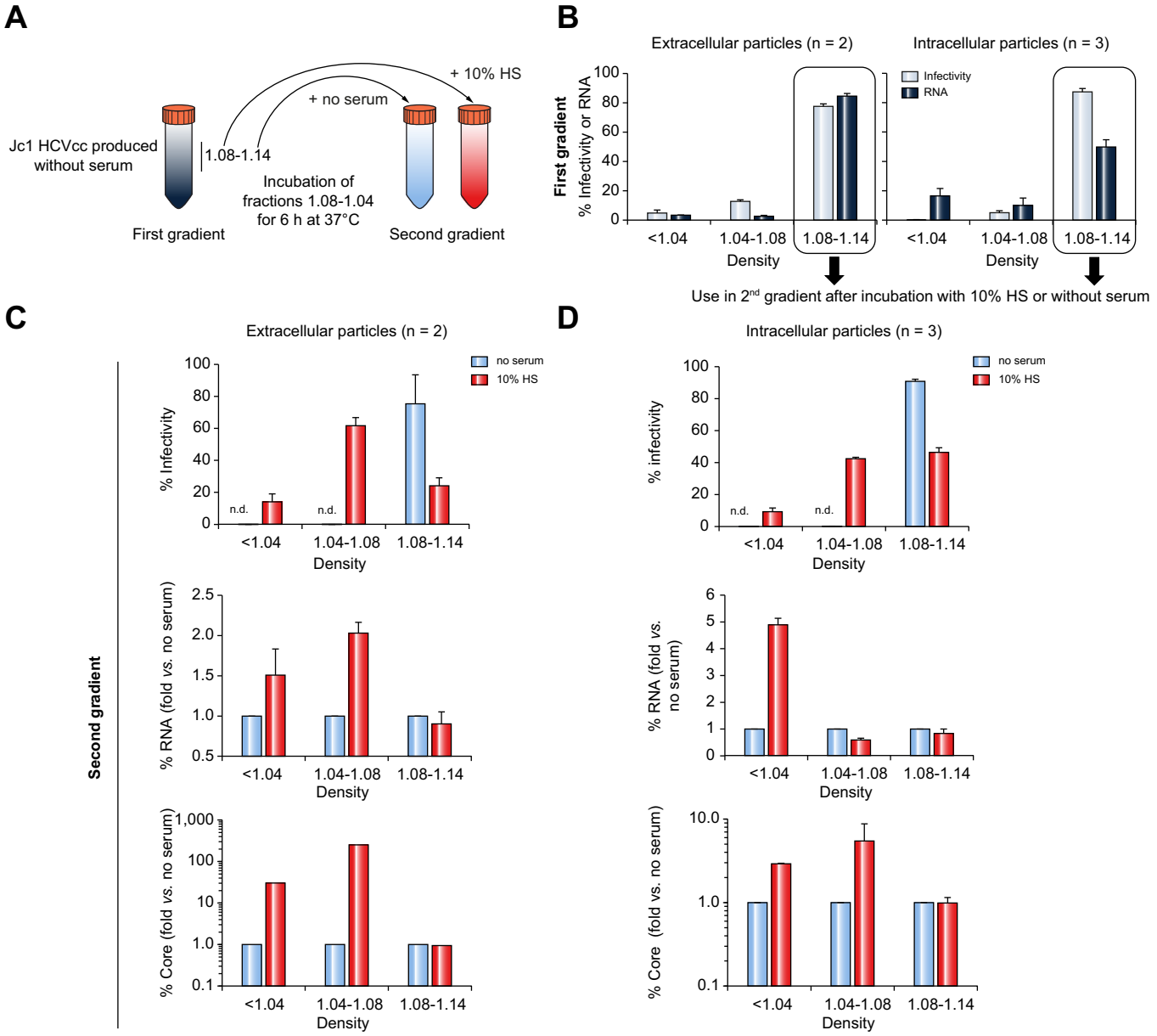

Fig. 5. High-density HCV particles shift to low densities upon lipidation. (A, B) Serum-free produced Jc1 extracellular (left panels) or intracellular (right panels) HCVcc particles were layered on a first density-gradient. Fractions of densities 1.08–1.14, containing *ca.* 80% of infectivity, were pooled and incubated for 6 h with or without serum before being layered on secondary density-gradients and performing infectivity analyses. (C, D) Infectivity analyses displayed by categories of density for infectivity, HCV RNA, and core, as indicated. HCV, hepatitis C virus; HCVcc, cell culture grown HCV; HS, human serum; nd, not detectable.

particles upon HS-incubation, as shown by a *ca.* 40-fold reduction in the percentage of infectious particles detected in fractions of densities <1.04 (Fig. 7B,C [right panels]; Fig. S11E). Note that similar inputs of mutant and parental physical particles were used in these assays (Fig. S11A). Altogether, these results suggested that HVR1 is a key viral determinant regulating the lipidation level of viral particles in an HCV strain-dependent manner.

HSA is a serum factor that promotes HCV particle lipidation by apoB-containing lipoproteins

That HS more efficiently shifted the density of viral particles than FCS (Figs. 1–4) suggested that there are differences in HCV-specific lipidation factor(s) between the serum types. Indeed, when we quantified the lipoprotein composition of sera used in our experiments, we found that compared to HS, FCS displayed 6–7-fold lower amounts of both HDLs and LDLs, and no VLDLs (Fig. S1A). While this difference may partially explain a better maturation of viral particles in HS, as suggested in Fig. 6C-E, we sought to characterize serum factors, including lipoproteins, that modulate HCV lipidation.

To determine which lipoprotein class induces lipidation of viral particles, we depleted lipoproteins from HS to generate a LPDS with >100-fold decreased apoB levels despite normal HSA levels (Fig. S1B), indicating effective lipoprotein removal. Next, we incubated Jc1 HCVcc produced in serum-free conditions with LPDS *vs.* different lipoprotein types, *i.e.*, VLDL, LDL or HDL, before density-gradient analyses (Fig. 8A). We found

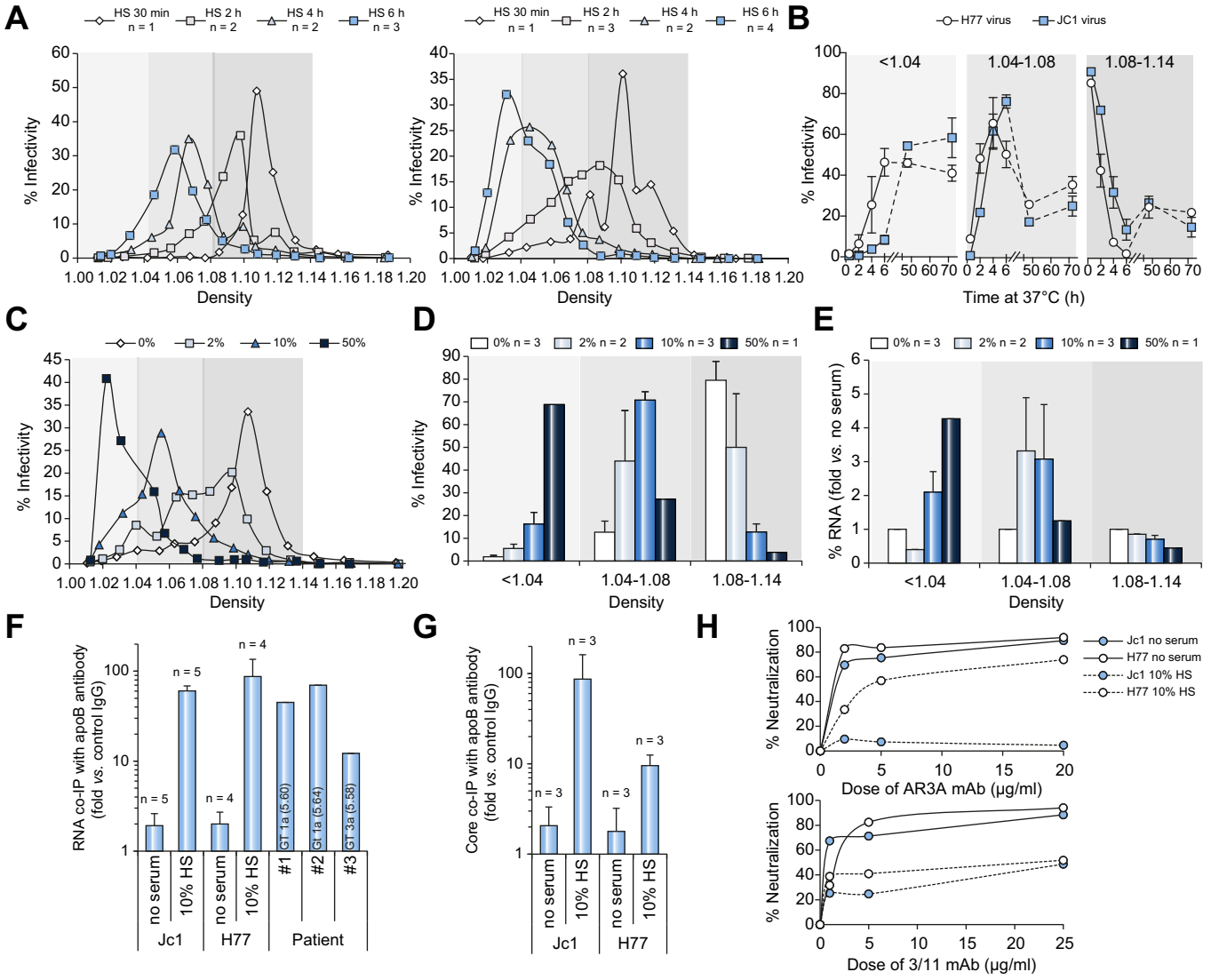

Fig. 6. Lipidation of secreted HCV particles is time- and dose-dependent, and induces apoB-association. (A, B) Serum-free produced Jc1 (red) and H77 (blue) extracellular HCVcc particles were incubated for different times with 10% HS. The results display (A) representative examples or (B) analysis by categories of densities for infectivity. The results of HCVcc production for 48 h and 72 h in HS-containing medium are included here for sake of comparison (dotted lines). See representative examples of individual experiments in Fig. S8. (C-E) Serum-free produced Jc1 extracellular HCVcc particles were incubated with different HS concentrations. The results display (C) representative examples or analysis by categories of densities for (D) infectivity and (E) viral RNAs. See representative examples of individual experiments in Fig. S9. (F, G) Co-immunoprecipitation analysis of viral particles from sera of HCV-infected patients and from serum-free produced Jc1 or H77 HCVcc particles incubated for 6 h with or without human serum. The results show the levels of (F) RNAs or (G) core protein co-immunoprecipitated with apoB relative to control antibodies. (H) Serum-free produced Jc1 (red) or H77 (blue) extracellular HCVcc particles were incubated with HS (dotted lines) or without HS (plain lines) for 6 h and were mixed with different concentrations of AR3A (top panel) or 3/11 (bottom panel) neutralizing monoclonal antibodies. The results show the mean percentages of neutralization relative to absence of antibody. GT, genotype; HCV, hepatitis C virus; HCVcc, cell culture grown HCV; HS, human serum; IP, immunoprecipitation; mAb, monoclonal antibody.

that incubation with LPDS did not grossly change the lipidation profile of viral particles (Fig. 8B,C), suggesting that serum lipoproteins are likely the source of virion lipidation. Likewise, incubation of HCVcc with purified VLDLs, LDLs or HDLs at physiological concentrations did not significantly change their lipidation profiles, as shown by peaks of infectivity that remained in the 1.10–1.11 range (Fig. 8B), underscoring the requirement for a serum protein component for virus lipidation with either lipoprotein type. Accordingly, we found that HCVcc incubation with LPDS added to lipoproteins shifted viral particles to low densities, with 55–70% of infectivity detected below 1.08 (Fig. 8B,C). Furthermore, incubation of HCVcc particles with LPDS and combinations of HDL and VLDL or LDL induced full low-density shifts, similar to those detected with HS-incubation (Fig. 8B-D). Of note, similar results were obtained with H77 HCVcc particles (data not shown).

Finally, we sought to unravel serum factors promoting HCV lipidation. We found that the blocking of serum lipoprotein-modification factors such as cholesteryl ester transfer protein (CETP) and lecithin-cholesterol acyltransferase (LCAT) did not change HS-induced virion shift to low density (Fig. S12), thus ruling out their involvement in HCV lipidation. Likewise, apoE did not allow lipidation by either of the 3 lipoprotein types, alone or in combination (Fig. S13), indicating that apoE is likely

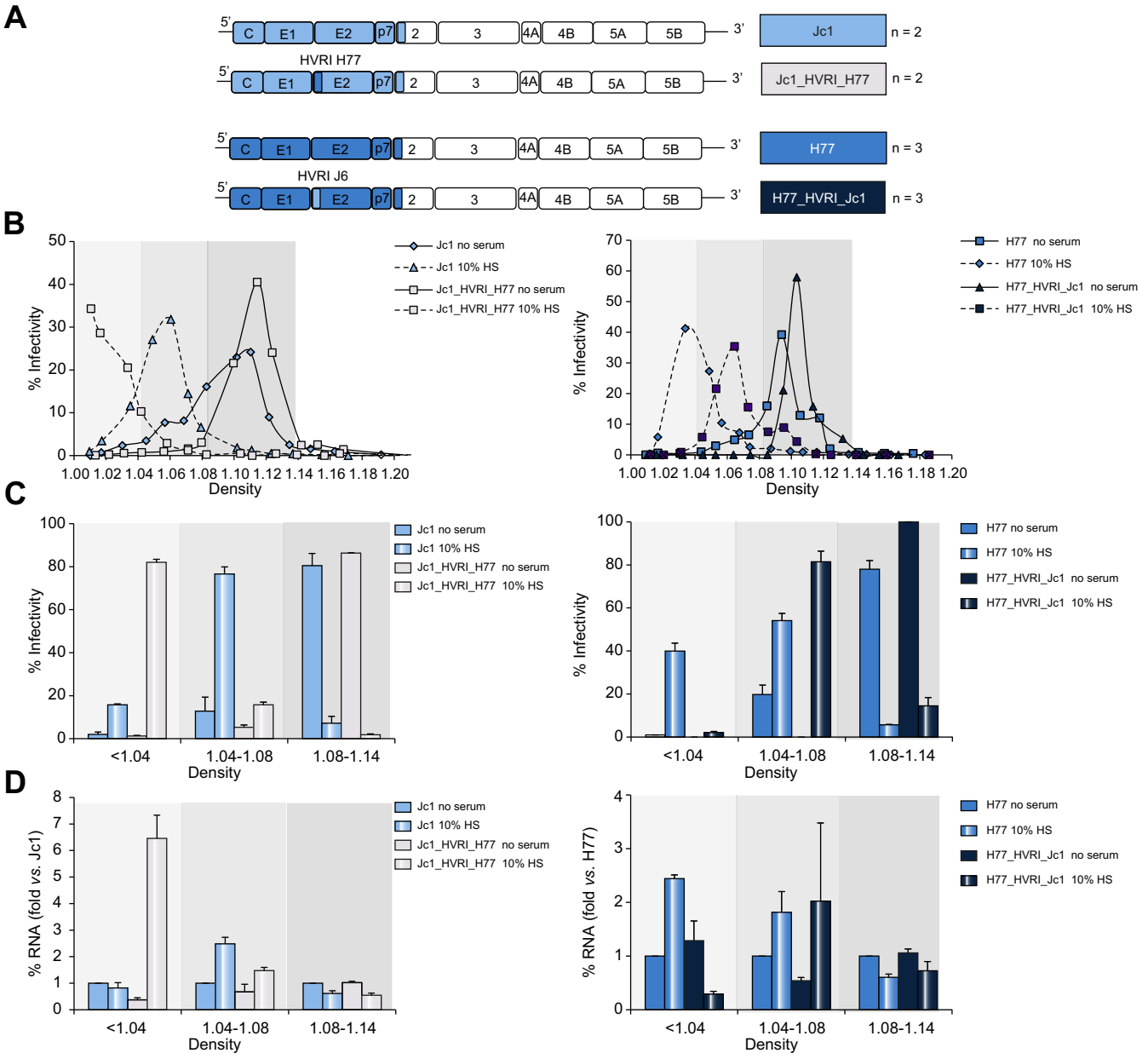

Fig. 7. An HVR1 determinant of HCV E2 regulates lipidation levels of HCV particles. (A) Schematic representation of recombinant genomes generated by swapping HVR1 domains between Jc1 and H77 HCVcc sequences. (B-D) Density-gradient analysis of serum-free produced Jc1 *vs.* Jc1_HVR1_H77 (left panels) or H77 *vs.* H77_HVR1_Jc1 (right panels) extracellular HCVcc particles (5×10^7 GE) incubated for 6 h with or without serum. The results display (B) representative examples or analysis by categories for (C) infectivity and (D) viral RNAs. See representative examples of individual experiments in Fig. S11. GE, genome equivalent; HCV, hepatitis C virus; HCVcc, cell culture grown HCV; HS, human serum; HVR1, hypervariable region 1.

not the factor of LPDS that stimulates extracellular virion lipidation. Interestingly, we found that purified HSA, a protein abundant in HS and LPDS (Fig. S1B), added to VLDL or LDL induced HCV low-density shift, though to lower extents than LPDS (Fig. 8B,C). Of note, in contrast to LPDS, HSA (Fig. 8B,C) did not allow virion lipidation by HDL.

Altogether, these results suggested that lipoproteins are the main source of HCV lipidation, which requires additional serum component(s) that include HSA.

Discussion

In vivo, HCV particles are associated to apoB, the non-exchangeable and major structural component of VLDLs and LDLs. This may explain the particularly low-buoyant density of patient-derived HCV LVPs, below 1.08 (Fig. 1A), which is significantly lower than other enveloped viruses.[2] Indeed, the lipidomics of purified HCVcc particles confirmed that their lipid content is related to that of serum lipoproteins.[5] Several studies proposed that HCV production from hepatocytes depends on cellular factors controlling VLDL-biosynthesis, suggesting that virion interaction with serum lipoproteins begins at the steps of viral assembly and secretion processes during which immature particles are converted to mature virions by lipidation and incorporation of some apolipoproteins.[4–10,12,14–19] Yet, while there is ample evidence that the exchangeable apolipoprotein apoE is necessary to promote envelopment, lipidation and/or release of viral particles, some studies suggested

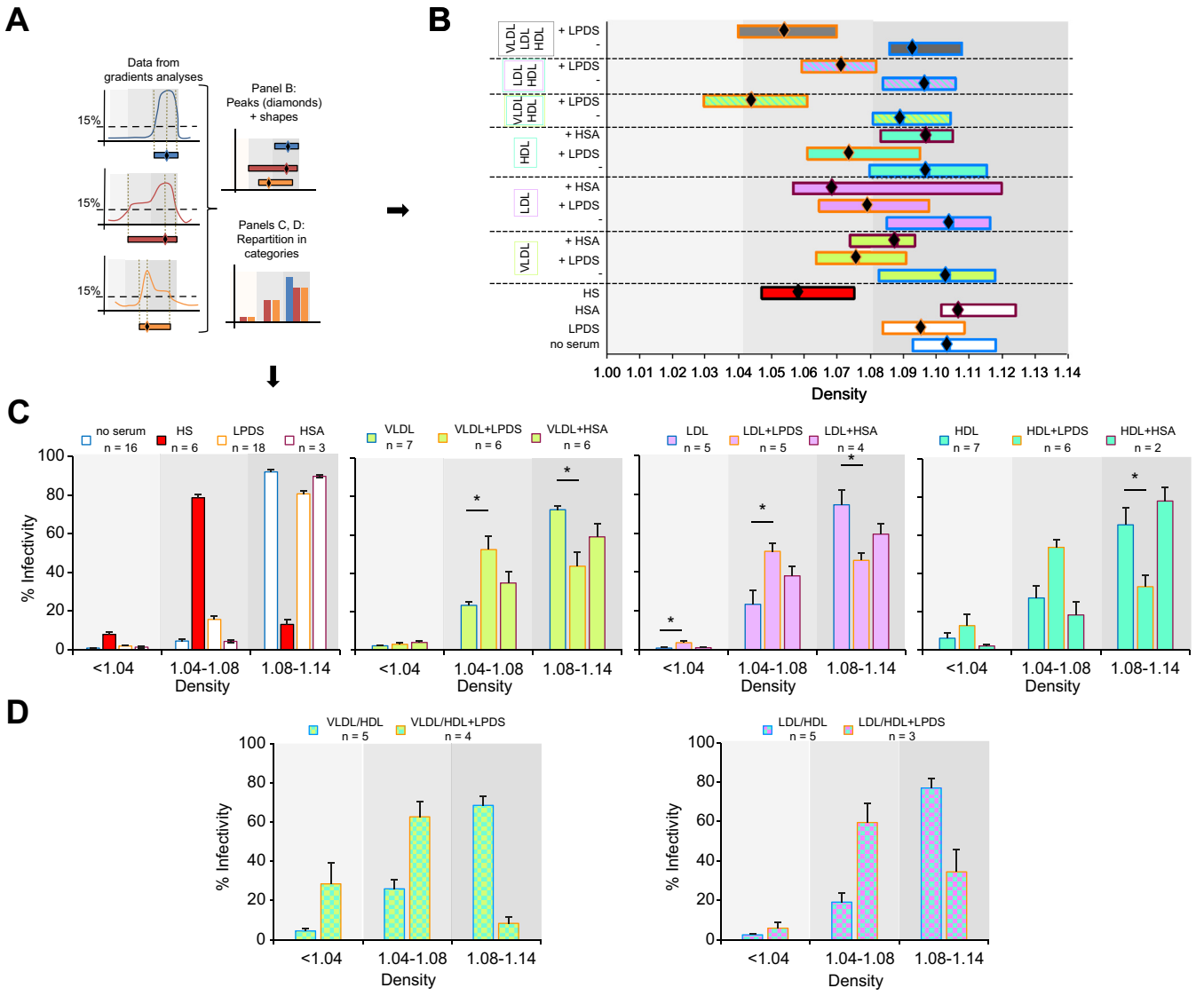

Fig. 8. Lipoproteins and serum protein(s) are required for lipidation of HCV particles. Density-gradient analysis of Jc1 extracellular HCVcc particles produced in serum free media and incubated for 6 h without serum *vs.* with the indicated serum factors added at concentrations equivalent to 10% HS. (A) Illustration of the method used to analyze and represent the data. (B) For each condition, the boxes represent the densities for which infectivity values were above 15% of total infectivity. The black diamonds in each box represent the density of the peak infectivity. (C, D) The infectivity values from different gradients are regrouped by categories of density. HCV, hepatitis C virus; HCVcc, cell culture grown HCV; HDL, high-density lipoprotein; HS, human serum; HSA, human serum albumin; LDL, low-density lipoprotein; LPDS, lipoprotein deficient serum; VLDL, very-low-density lipoprotein.

that apoB may not be required for these initial steps (reviewed in[2]). This is intriguing because apoB is essential for VLDL-biogenesis[28] and because patient-derived HCV is readily immunoprecipitated with apoB antibodies[7,9] (Fig. 6F). One orthodox explanation is that Huh-7.5 hepatoma cells that efficiently support HCVcc production[8,13] lack many properties of hepatocytes, such as the ability to produce normal VLDLs.[12,20–22] That Huh 7.5 cells produce under-lipidated, VLDL-like particles would thus explain why the HCVcc particles they produce have higher buoyant-density and lower specific infectivity than HCV produced *in vivo*[6–8] or from PHH.[8,20,21] However, when HepG2 hepatoma cells, thought to represent more mature hepatocytes than Huh-7 cells, are induced to produce normal, apoB-containing VLDLs, they yield infectious particles that are biophysically and biochemically similar to those produced from Huh-7.5 cells.[29]

Since this and other evidence[2] showed that there is no clear correlation between the ability of HCV-infected cells to secrete apoB or VLDLs and their capacity to produce LVPs, we decided to investigate whether LVP formation could derive from a post-secretion interaction between extracellular HCV particles and VLDLs or other lipoproteins. Our results indicate that while Huh-7.5 cells grown in serum-free medium produce immature HCV particles of density >1.08, their maturation to fully lipidated, apoB-containing infectious virions of low density is rapidly induced once they encounter in the extracellular milieu appropriate serum conditions with physiologic concentrations of lipoproteins. Importantly, our results do not disrepute results of others[12,15,19] proposing that, *in vivo* as well as in metabolically adapted hepatoma cells upon prolonged (*ca.* 21 day) cultures in HS, HCV maturation may occur during transit through the cell secretory pathway, where virions could associate or fuse with

apoB-containing lipoproteins precursors and/or luminal, apoE-containing lipid droplets.[28] Yet, our report shows that an alternative factor of lipid-imprinting of HCV is the immediate environment of the extracellular milieu and/or serum characteristics.

Our data suggest that HCV lipidation requires interaction(s) with defined serum lipoproteins but also involves additional, presumably non-lipid serum factor(s) such as HSA (Fig. 8). We found that all principal lipoprotein classes, *i.e.*, VLDL, LDL and HDL, induce low-density shift of HCVcc particles *in vitro*, upon short-time incubation in the presence of LPDS, with a synergistic effect when either VLDL or LDL was combined with HDL. These results are consistent with a stronger lipidation induced by HS in contrast to FCS that contains >6-fold lower concentrations of either lipoprotein type (Fig. S1A). This questions the nature of interactions between lipoproteins and viral particles, and their outcome in terms of virion morphology. As HCV LVPs incorporate different lipoprotein components, they may indeed be formed by the transfer of lipids, through spontaneous release/exchange or *via* fusion ("single-particle" model), or, alternatively, by virion association with lipoproteins ("two-particle" model).[2]

Although direct evidence by electron microscopy is missing in favor of the latter model,[3–6] perhaps owing to its transient or unstable state, our results argue for it since lipidation of cell-free viral particles upon short-time HS treatment induced their association with apoB, a non-exchangeable apolipoprotein (Fig. 6F,G; Fig. S10). Other arguments for this model are that *in vivo*, HCV particles associate with apoB48 containing chylomicrons,[10] although HCV is not produced by enterocytes, and that they exhibit rapid though transient postprandial shifts in buoyant-density upon lipid-rich diet in HCV-infected patients.[30] Yet, these facts do not exclude the nonexclusive possibility that viral particles may mimic lipoproteins and thus, exchange or uptake lipids.[31] Indeed, previous reports indicated that apoliproteins can be exchanged or transferred from cells, lipoproteins or serum itself to infectious particles.[16,32,33] Thus, as LVPs contain specific apoliprotein subsets,[4–6] this may regulate the exchange and/or transfer of lipids with/from lipoproteins, which could modify their inner neutral lipid content, as suggested by the lipidation state and infectivity of viral particles that are altered upon treatment with lipoprotein lipase and/or hepatic triglyceride lipase.[34] Hence, our HS-incubation kinetics and dose-dependence assays (Fig. 6), suggesting that lipidation of infectious particles is progressive, also argue for the "single-particle" model of the HCV virion, in which its envelope is shared with lipoprotein particle(s) and continuously remodeled upon interactions with these lipoproteins. Since HCV lipidation is induced by different lipoprotein types (Fig. 8), we surmise that the mode of lipoprotein association or lipidation varies accordingly, which would be consistent with coexistence of both HCV morphologic models.

Importantly, we reveal that HCV lipidation by lipoproteins requires additional serum factor(s), since incubation with lipoproteins could induce low-density shift of HCV virions only in the presence of LPDS. We were unable to block HS-induced lipidation of viral particles by inhibitors of CETP and LCAT that promote lipid transfer to lipoproteins and maturation (Fig. S12), hence discarding these candidate factors. Likewise, apoE could not mediate low-density shift by lipoproteins (Fig. S13). Yet, we found that HSA, added to VLDL or LDL (though not HDL), induces HCV partial lipidation. This indicates that HSA is a serum co-factor mediating LVP formation, perhaps by bridging apoB-containing lipoproteins and virions[35] or by favoring lipid efflux.[36] Of note, additional serum factors, present in LPDS (Fig. 8), seem required to induce full virion lipidation.

Our results reveal that the HVR1 domain of the E2 glycoprotein accounts for the differential lipidation sensitivity of Jc1 *vs.* H77 viruses, consistent with its previously proposed roles in the HCV lipidation process[14,25,37,38] and recruitment and/or conformation of apolipoproteins such as apoC1[16] and apoE[25] on virions. While HVR1-deletion shifts HCV particles to densities of 1.10–1.16[14,37,39] corresponding to those of the non-lipidated particles of our study, it is interesting that simple HVR1-swaps between H77 and Jc1 HCVcc reconstituted the differential density profiles of either parental virus. This suggests that HVR1 organizes the HCV lipidation process in a strain-specific manner, which raises different and fascinating scenarios that may explain why HCV is lipidated *in vivo*. First, in agreement with previous reports suggesting that lipidated viruses exhibit strain-dependent usage of different lipoprotein receptors as entry factors,[38] including SR-BI[14,21,38,39], LDLr[38] and VLDLr,[40,41] HVR1 plasticity and evolution may dictate the set of receptors used during entry steps by driving both lipidation and apolipoprotein display. Second, as shown by neutralization resistance induced by low-density shift of HS-treated HCV particles (Fig. 6H), lipidation may contribute to shielding cross-neutralizing epitopes,[4,6,7,14,23] perhaps in concert with the alternative role of HVR1 in concealing the CD81-binding site[14,42] and accessibility to antibodies that target virus-CD81 interaction.[39,43,44] Accordingly, it is possible that lipidation allows viral particles to access, following a first interaction with lipoprotein receptors, a protected environment where they can subsequently and safely interact with CD81 in a putative neutralizing antibody-free sanctuary and where further steps of cell penetration may proceed. A third scenario, consistent with the latter, is that usage of lipoprotein receptors increases HCV-specific infectivity. Indeed, lipidation of viral particles, which shift them to low/very-low densities, raised their specific infectivity (Fig. 3D). This could provide a positive selection for eliciting HVR1 variants that drive full lipidation. The varying specific infectivity according to virion density could thus reflect the differential efficiency of cell entry pathways depending on the lipidation status of the particles, which would influence binding and usage of specific lipoprotein receptors. Finally, it is also possible that lipidation increases the stability and/or infectivity of particles by protecting them from degradation.[12]

In conclusion, our results suggest that HCV particles acquire neutral lipids from the extracellular milieu, which requires both lipoproteins and serum protein factors as well as HVR1 determinants. Overall, these findings allow for the development of simple culture conditions that enable production of infectious HCV particles resembling those retrieved from chronically-infected patients, which will facilitate structural and functional investigations as well as the rationale design of a much-needed vaccine.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Study concept and design: SD, FLC. Acquisition of data: CG, NF, TB, MG, SD. Analysis and interpretation of data: SD, CG, NF, MG, FLC. Drafting of the manuscript: SD, FLC. Material support: BP.

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Supplementary data

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Author names in bold designate shared co-first authorship

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