

Hepatitis B virus proteome analysis identifies apolipoprotein C1 facilitating particle production and virus entry

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List of Abbreviations:

ApoC1, apolipoprotein C1; BLT, block lipid transport; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; fSVP, filamentous subviral particle; GE, genome equivalent;

HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HDL, high-density lipoprotein; HDV, hepatitis D virus; HSC70, heat shock cognate protein 70; HSP90AB1, heat shock protein 90 alpha family class B member 1; HSPGs, heparan sulfate proteoglycans; MOI, multiplicity of infection; NTCP, sodium taurocholate co-transporting polypeptide; PCA, principal component analysis; pgRNA, pregenomic RNA; PHHs, primary human hepatocytes; PPIA, cyclophilin A; rcDNA, relaxed circular DNA; siNT, non-targeting siRNA; SR-B1, scavenger receptor class B type 1; sSVP, spherical subviral particle.

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Conflict of interest

Stephan Urban consults for, owns stock in, and owns intellectual property rights with Gilead. He is a co-inventor and an applicant on patents protecting HBV preS1-derived lipopeptides (Myrcludex B/bulevirtide/Hepcludex). The remaining authors have no conflicts to report.

Author contributions

Conceived and designed the experiments: SY, FN, SS, RB. Performed the experiments: SY, FN, MP, PS. Analyzed and interpreted the data: SY, FN, PS, RB. Contributed reagents/materials/analysis tools: YL, AP, PS, SN, SU, SS, RB. Drafted the manuscript: SY, FN, RB.

Graphical Abstract

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Abstract

Background & Aims: Antiviral therapies targeting hepatitis B virus (HBV) suppress viral replication, but rarely achieve functional cure. Understanding HBV-host cell interaction is crucial for developing novel therapeutic approaches. Here, we report host cell proteins associated with HBV virions and filamentous subviral particles (fSVPs) and characterize one host cell protein, apolipoprotein C1 (ApoC1), mechanistically.

Methods: Highly purified HBV virions and fSVPs were obtained by sequential use of several biophysical methods. Particles were analyzed by mass spectrometry and associated proteins were evaluated phenotypically using an HBV infection model. The top hit, ApoC1 was characterized in detail.

Results: Alongside the established HBV-associated chaperones HSP90AB1 and HSC70, we identified several apolipoprotein-related factors associated with virions and fSVPs. RNAi-based phenotypic validation identified strongest effects for ApoC1, likely due to two complementary effects. First, ApoC1 depletion reduced intracellular cholesterol level impairing HBV infection and SVP production, which was compensated by exogenous cholesterol addition. Second, ApoC1 that is mainly enriched in high-density lipoprotein (HDL), associates with HBV virions and fSVPs and increases HBV infectivity. The same was found for hepatitis D virus (HDV), a satellite virus utilizing HBV envelopes.

Supplementation of exogenous HDL enhanced infection via scavenger receptor class B type 1 (SR-B1), the natural HDL receptor. Consistently, inhibition of SR-B1 suppressed HBV and

HDV infection.

Conclusions: We established a method for obtaining highly purified HBV virions and fSVPs and identified the HDL component ApoC1 to associate with both particle types. ApoC1 promotes HBV and HDV infection most likely via SR-B1 and facilitates viral entry.

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Introduction

Chronic hepatitis B virus (HBV) infection is a major public health burden affecting an estimated 254 million people worldwide¹. They have a high risk to develop severe liver diseases, including liver cirrhosis and hepatocellular carcinoma (HCC), accounting for a total of 1.1 million deaths per year¹. Antiviral therapies suppress viral replication but do not eliminate the covalently closed circular (ccc) DNA, the viral persistence reservoir residing in the nucleus of infected cells². In addition, excessive amounts of non-infectious subviral particles (SVPs) are released from infected hepatocytes, causing dysfunction of the immune response targeting the major viral antigen, the hepatitis B surface antigen (HBsAg). Therefore, to achieve curative therapy, novel approaches are needed.

HBV particles are enveloped and contain 3 viral glycoproteins called S, M and L (small, medium and large, respectively). They share the same C-terminal S-region but have additional N-terminal extensions: the preS2 region of M and the preS1 region of L, the latter binding to the viral entry receptor NTCP^{3,4}. Initial attachment of HBV to hepatocytes is facilitated by heparan sulfate proteoglycans (HSPGs) interacting with the viral surface proteins⁵. Subsequently, the preS1 region binds to NTCP, followed by virion entry. Upon transport into the nucleus, the viral genome is converted into the cccDNA that forms a stable episome from which all HBV RNAs are transcribed⁶. Transcription requires the action of the HBx protein preventing the epigenetic silencing of the cccDNA template⁷. Infected cells release an array of HBV-derived particles, including infectious virions (Dane particles), DNA lacking (empty) virions⁸, non-enveloped nucleocapsids with and without viral RNA and excessive quantities of non-infectious SVPs. Depending on the L-M-S ratio, SVPs can form: highly abundant spherical SVPs (sSVPs), 20-25 nm in diameter and predominantly composed of S; less abundant filamentous SVPs (fSVPs) containing S and also L

Several studies reported the incorporation of host cell proteins into HBV nucleocapsids or virus particles. Early work demonstrated that the interaction between HBV polymerase and pregenomic (pg) RNA requires the heat shock protein HSP90, detected in purified nucleocapsids together with the chaperone HSP70⁹. In addition, cyclophilin A (PPIA) and apolipoprotein E (ApoE) associate with secreted particles^{10,11}, the latter enabling HBV to use the reverse cholesterol transport pathway through hepatic macrophages to facilitate virion uptake by hepatocytes¹².

Despite these advances, we still do not know the host cell proteins carried along within HBV virions and delivered into the infected cell. Here, we established a particle purification pipeline enabling the proteomic analysis of HBV virions and fSVPs with high specificity and sensitivity. We identified apolipoprotein C1 (ApoC1), a component of high-density lipoprotein (HDL), as a critical host cell dependency factor and reveal its contribution to HBV infection and particle production most likely via a dual-mode of action.

Materials and methods

Cell culture

Huh7, HepG2-NTCP-A3, HepG2-HB2.7, HEK293T and HepAD38 cells were cultured as described in Supplementary Materials and Methods, <https://links.lww.com/HEP/K558> . To suppress HBV production, 1 µg/ml doxycycline (Sigma-Aldrich, USA) was added to the HepAD38 medium for general maintenance of the cells.

Cryopreserved primary human hepatocytes (PHHs) were purchased from Thermo Fisher Scientific and cultured according to the manufacturer's instructions.

HBV particles production and purification

HepAD38 cells containing an overlenght HBV genome under the control of a tetracycline-inducible promoter were cultured as previously described¹³. HBV production was induced by omitting doxycycline from the culture medium. Culture supernatants were collected every four days, sterile filtered and stored at 4°C until further processing. HBV particles were purified as described in the Results section and Supplementary Materials and Methods, <https://links.lww.com/HEP/K558> .

Ethics statement

Use of human clinical samples was approved by the Ethics Committee of the Medical Faculty of Heidelberg University (approval no. S-522/2024) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients whose samples were collected prospectively. For retrospectively collected samples, the requirement for informed consent was waived by the ethics committee.

For all further details, see Supplementary Materials and Methods,

<https://links.lww.com/HEP/K558> .

Results

Purification of HBV virions and filamentous SVPs

To obtain HBV virions and fSVPs, we used two HBV-containing HepG2-derived cell lines (Fig. 1A): HepG2-HB2.7 cells containing only a partial 2.7 kb HBV genome encoding the envelope proteins and the HBx protein and supporting only SVP production; HepAD38 cells harboring a 1.1-overlength HBV genome under a tet-off inducible promoter and releasing both HBV virions and SVPs upon tetracycline removal from the culture medium¹³. The parental HepG2 cell line was used as control to determine contaminating proteins in the proteomics analysis (Fig. 1A). To reduce contamination of the preparations by fetal bovine serum (FBS) components, we generated 5 independent biological replicates; 3 from cells cultured with 10% FBS (replicates 1-3) and 2 cultured with 5% FBS (replicates 4-5).

To obtain mass spectrometry-grade HBV particles, we employed our previously reported purification protocol comprising FPLC with heparin column and size exclusion chromatography (Fig. 1A)¹⁴. To remove the majority of spherical SVPs we intentionally selected only the early fractions of the size-exclusion chromatography, containing Dane particles and fSVPs. Pooled fractions were then subjected to density gradient centrifugation. Dual fluorescence western blot analysis of the gradient fractions demonstrated distinct distribution of particles: naked capsids (density ~ 1.23 g/cm³; indicated also by HBeAg that cross-reacts in the core ELISA); virions (~ 1.20 g/cm³; indicated by HBsAg and HBV DNA) and fSVPs (density of ~ 1.13 g/cm³; indicated by high amounts of HBV envelope proteins and

low HBcAg and HBV DNA) (Fig. 1B, upper panel; Fig. S1, <https://links.lww.com/HEP/K558>). Pooled virion fractions and the selected fSVP fraction were then analyzed for total protein by using silver-stained protein gels. We observed high amounts of protein in both samples, dampening detection sensitivity of the HBV proteome (Fig. 1B, lower panel, input lanes; gradient fractions in Fig. S2A, <https://links.lww.com/HEP/K558>). The same was true for fSVPs purified from culture supernatant of HepG2-HB2.7 cells (gradient fractions in Fig. S2B, <https://links.lww.com/HEP/K558> and S3A, <https://links.lww.com/HEP/K558> , input lane). To reduce impurities, we performed HBs-specific immunoprecipitation (IP); IP with an IgG isotype served as specificity control. Protein amounts and complexities of all samples were greatly reduced after the IP (Fig. 1B, lower panel; Fig. S3A, <https://links.lww.com/HEP/K558>). Notably, core protein remained detectable in the virion fractions after IP, indicating preserved particle integrity (Fig. S3B, <https://links.lww.com/HEP/K558> , left panel), although we cannot exclude the presence of partially damaged particles or the co-IP of empty virions secreted by HepAD38 cells. As expected, re-probing the blots with HBs-specific antibodies revealed HBV envelope proteins of different isoforms in the virion fractions (Fig. S3B, <https://links.lww.com/HEP/K558> , right panel).

Proteomics analysis of purified HBV virions and filamentous SVPs

Proteins contained in IP-purified virion and fSVP samples were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). For each experimental condition, 4 or 5 biologically independent replicates of affinity-purified complexes obtained from IgG or HBs-specific immunoprecipitations were analyzed. Although abundance of each unique viral peptide varied between the different replicates, HBV envelope proteins, core- and

polymerase (P)-protein were consistently detected in all virion fractions with the best peptide coverage in replicate #5 (Fig. 1C; Table S1, <https://links.lww.com/HEP/K558>). Notably, we detected 5 different peptides derived from the HBV P-protein that is present in only one copy per particle¹⁵. In addition, we detected core- and envelope-specific peptides, whereas HBx- and HBe-specific peptides were not identified. In contrast, only some P- and much lower amounts of core-specific peptides were detected in the fSVP fraction from HepAD38 cells, demonstrating a strong depletion of virions from the fSVP fraction. Much stronger signals for HBV envelope proteins were detected in fSVP fractions derived from HepG2-HB2.7 cell supernatants, consistent with their higher SVPs production rate, whereas P- and core-specific peptide signals were absent (Table S1, <https://links.lww.com/HEP/K558>). No or only background levels of HBV-specific peptides were identified in the IgG control IP or in samples derived from HepG2 cells.

Regarding non-viral proteins co-purifying with HBV virions and fSVPs, a total of 787 host cell proteins was detected (Table S2A, <https://links.lww.com/HEP/K558>). Principal component analysis (PCA) of the individual samples confirmed high correlation within biological replicates (Fig. S4A, <https://links.lww.com/HEP/K558>). For further analysis, only proteins consistently identified in at least 3 biological replicates of at least one experimental group were retained (n=266 host proteins; Table S2B, <https://links.lww.com/HEP/K558>). This list was additionally filtered to retain only proteins completely missing from all IgG controls or displaying at least a median log₂ (fold-change) ≥ 3 for each HBs-specific pull-down compared to the corresponding IgG control group. In this way, 165 host cell proteins were identified, especially in the fSVP fractions of HepG2-HB2.7 cells, consistent with their higher SVP production rate compared to HepAD38 cells (Fig. 1D, Table S2B, <https://links.lww.com/HEP/K558> and Table S3, <https://links.lww.com/HEP/K558>).

Nevertheless, a substantial subset of these proteins was also detected in virion and fSVP fractions from HepAD38 cells. Among all identified host proteins, chaperones of different families were enriched in all conditions, including HSPB1 that modulates cccDNA activity and HSC70 that binds to PreS1 and regulates L protein topology (Fig. S4B, <https://links.lww.com/HEP/K558>)^{16,17}. Three proteins, CASP14, CWF19L2 and HNRNPC were highly enriched in virion fractions with little to no signal overlap with peptides detected in the fSVP preparations (Fig. 1D; Table S2B, <https://links.lww.com/HEP/K558>). Moreover, several members of the apolipoprotein family were detected in HBV particles and fSVPs derived from both cell lines (Fig. 1D; Fig. S4B, <https://links.lww.com/HEP/K558>).

Taken together, we identified a set of previously published host proteins, along with several hitherto unknown cellular proteins selectively enriched with highly purified HBV virions and/or fSVPs.

Functional validation of virion- and fSVP-associated host proteins

To determine whether these proteins contribute to the HBV replication cycle, 48 candidates were prioritized based on their consistent identification across HBV virions or fSVPs samples and validated by RNAi-based screening in an HBV infection assay (Fig. 2A). Two days after reverse transfection of siRNAs, HepG2-NTCP-A3 cells were infected with HBV and the amounts of secreted HBeAg, HBsAg, intracellular core protein (HBcAg) and viral RNAs were measured 5 days after infection (Fig. 2B-E; Fig. S5A-B, <https://links.lww.com/HEP/K558>). Cytotoxicity assays identified only one knock-down as cytotoxic (PCBP1; Fig. S5C, <https://links.lww.com/HEP/K558>). For each parameter, values were normalized to non-targeting siRNAs pool (siNT). Cells treated with a siRNA targeting viral transcripts (siHBV) or the peptidic entry inhibitor Bulevirtide (Myrcludex B; MyrB)

were used as positive controls; both reduced HBV parameters without cytotoxic effects (Fig. 2B-E; Fig. S5, <https://links.lww.com/HEP/K558>). Considering the dynamic range of the various HBV markers in this set-up, phenotypes were ranked based on secreted HBeAg levels, a sensitive HBV replication marker. Overall, results of the different HBV replication parameters were consistent, although changes of HBsAg levels were less pronounced while intracellular core protein measurements had higher variability than HBeAg ELISA. Focusing on host cell factors whose knockdown reduced viral parameters, ApoC1 knockdown gave the most consistent and strongest effect, decreasing all viral parameters in a very reproducible manner (Fig. 2B-E).

As noted above, 5 human apolipoproteins were identified in our proteomic analysis. Amongst these, ApoE, ApoA1 and ApoC3 were detected in virions and fSVPs whereas ApoA2 and ApoC1 were only detected in highly concentrated fSVPs derived from the high-producer HepG2-HB2.7 cells (Fig. S4B, <https://links.lww.com/HEP/K558>). While ApoE was previously reported to enhance HBV infection and production¹⁰ ApoC1 has not yet been studied. Therefore, we compared the roles of the apolipoproteins identified in our proteome for their impact on HBV infection/replication and SVP production. Knockdown efficiency measured 7 days after siRNA transfection was high (Fig. 3A). Zooming into the RNAi validation results (Fig. 2), amongst the five apolipoproteins, ApoC1 knockdown caused the strongest reduction of secreted HBsAg and HBeAg (Fig. 3B), intracellular HBV transcripts and pgRNA (Fig. 3C) as well as intracellular core protein (Fig. 3D), all determined 5 days after infection. Notably, also in the context of the HepG2-HB2.7 cell line, extracellular HBsAg was reduced most by ApoC1 knockdown (Fig. 3E).

ApoC1 is a proviral host protein promoting the HBV replication cycle

To assess whether the 5 apolipoproteins affect HBV replication at a post-entry stage, we employed HepG2.2.15 cells containing two head-to-tail HBV genome dimers¹⁸. In line with our finding in HepG2-HB2.7 cells, ApoC1 knockdown reduced HBsAg levels (SVP production), while HBeAg and HBV DNA levels did not decrease, but rather increased (Fig. S6A, <https://links.lww.com/HEP/K558>). Complementing these data, we depleted apolipoproteins in the HBV infection system just several days after infection to avoid any effect on HBV entry (Fig. S6B, <https://links.lww.com/HEP/K558>). Although knockdown efficiency was limited compared to reverse siRNA transfection used for all other experiments, siHBV treatment significantly decreased measured HBV parameters, demonstrating that we could detect antiviral effects even under these conditions. No changes of HBV parameters were observed in ApoC1 knockdown cells, arguing against a post-entry role of ApoC1 in HBV infection.

ApoC1 is the smallest, exchangeable, and most positively charged apolipoprotein. It regulates the activity of several lipid metabolizing enzymes and the function of lipoprotein receptors, including SR-B1¹⁹. Since ApoC1 was identified as top hit from the phenotypic screen, we further validated its relevance for the HBV replication cycle by time-course experiment. ApoC1 depleted HepG2-NTCP cells were infected with HBV and the kinetics of secreted HBsAg, HBeAg, and HBV DNA were evaluated 5, 7 and 9 days post-infection. ApoC1 knockdown significantly reduced HBsAg and HBeAg, comparable to NTCP control knockdown at all time points (Fig. 4A). Extracellular HBV DNA, a marker for virus particle production, detected 5 days post-infection was derived from the inoculum, as levels were equivalent amongst all conditions, including cells treated with the entry inhibitor MyrB (Fig. 4A, lower panel). However, at later time points when most of the input had decayed, HBV DNA was significantly reduced upon ApoC1 knockdown relative to siNT-transfected control

cells, with the reduction being comparable or even stronger to NTCP depletion.

Quantification of infected cells number and cccDNA levels confirmed the ApoC1 knockdown phenotype (Fig. 4B, C, respectively). The analogous result was found when using HBV infection in the absence of polyethylene glycol (PEG), commonly used to enhance virion binding and internalization (Fig. S7, <https://links.lww.com/HEP/K558>).

Since previous studies reported functional redundancy of ApoC1 and ApoE²⁰, we investigated possible additive or even synergistic effects of combined ApoE/ApoC1 depletion. However, secreted HBeAg levels remained comparable between HBV-infected ApoC1/ApoE double and ApoC1 single knockdown cells (Fig. S8, <https://links.lww.com/HEP/K558>).

These results suggest that ApoC1 plays a role at an early step of the HBV life cycle, and, at least to some extent, in the production or release of SVPs from integrated viral sequences.

ApoC1 regulates intracellular cholesterol level affecting HBV and HDV entry

Cholesterol plays an important role in the HBV replication cycle²¹. Moreover, ApoC1 has been reported to regulate intracellular cholesterol levels²². Therefore, we quantified cholesterol amounts in HepG2-NTCP cells depleted for ApoC1 or any of the other 4 different apolipoproteins identified in our proteome (Fig. 4D, left panel). Only ApoC1 knockdown caused a small, yet significant, reduction of cholesterol level. To determine whether intracellular cholesterol amounts affect the HBV replication cycle, we conducted rescue experiments by replenishing ApoC1 knockdown cells with different concentrations of water-soluble cholesterol added to the cell culture medium during HBV infection in the absence of PEG (Fig. 4D, right panel). Although we observed an increase of the HBV replication marker HBeAg in ApoC1 knockdown cells by exogenously added cholesterol (around 2-fold at high

cholesterol concentration), an analogous increase was found in cells transfected with siNT. This result indicated that cholesterol is a more generally limiting factor in the HBV replication cycle and not specific to ApoC1 depletion. Nevertheless, the reduction of cholesterol we observed in ApoC1 knockdown cells could explain, at least in part, the impairment of the HBV replication cycle observed in these cells. Notably, cholesterol addition restored HBV replication close to the level detected in siNT-transfected control cells (Fig. 4D, right panel). Moreover, we found that cholesterol substitution also boosted SVP production in the context of HepG2-HB2.7 cells (Fig. 4E), regardless of whether FBS was absent or present (Fig. S9, <https://links.lww.com/HEP/K558>). These results suggest that cholesterol supports several steps of the HBV replication cycle, including SVP production.

Association of ApoC1 with HBV virions and filamentous SVPs

In addition to regulating intracellular cholesterol levels, ApoC1 appeared to promote an early HBV replication cycle step (Fig. S6, <https://links.lww.com/HEP/K558>). Therefore, we analyzed two independent heparin-purified cell culture-derived HBV stocks by HBs- or ApoC1-specific IP and quantified captured particles (Fig. 5A). We observed co-IP of ApoC1, HBc protein and HBV DNA when capturing the particles with an HBs-specific antibody; vice versa, co-IP of HBc protein and viral DNA was found when we captured the particles with an ApoC1-specific antibody (Fig. 5A). The analogous result was found with particles derived from Huh7 cells, excluding confounders due to HepG2 cells or heparin purification (Fig. 5B; Fig. S10A, <https://links.lww.com/HEP/K558>). Notably, analysis of HBV particles contained in sera of three patients with CHB corroborated the association of ApoC1 with HBV particles (Fig. 5C; Fig. S10B, <https://links.lww.com/HEP/K558>).

To confirm this association, HepAD38-derived and purified HBV virions or fSVPs were

analyzed by immuno-EM using ApoC1- or HBs-specific primary antibodies that were detected with Protein A-coupled to 10 nm gold particles. HBs proteins were present on the surface of virions and fSVPs (Fig. 5D; Fig. S10C) , <https://links.lww.com/HEP/K558> .

Notably, ApoC1 was also detected in association with virions and fSVPs. Quantification of gold-labeled particles revealed ~10% of HBV particles being ApoC1 positive (Fig. 5D).

Interestingly, ApoC1 gold labeling was observed both in direct proximity to HBV virions and fSVPs, and adjacent to HDL-like structures residing next to these particles. Thus, ApoC1 may associate with HBV particles both directly and indirectly via HDL-containing lipoprotein particles (Fig. 5D; Fig. S10C, <https://links.lww.com/HEP/K558>).

These results confirm ApoC1 association with HBV virions and fSVPs and they suggest that at least a fraction of ApoC1 associates with these particles in the form of HDL.

ApoC1 regulates HBV and HDV entry

To study the functional role of ApoC1 in HBV uptake into liver cells, we performed ApoC1 antibody-mediated neutralization and ApoC1 protein competition assays in the context of HBV infection. An ApoC1-specific antibody dose-dependently reduced all HBV parameters (Fig. 6A). The analogous result was obtained using recombinant ApoC1 protein (Fig. S11, <https://links.lww.com/HEP/K558>), suggesting that viral entry is facilitated by HBV-associated ApoC1.

HBV and its satellite virus HDV share the same envelope and thus, the early steps of hepatocyte infection. Consistently, we found that ApoC1 knockdown impaired HDV infection (Fig. S12, <https://links.lww.com/HEP/K558>). Therefore, we used HDV as a tractable surrogate to determine how ApoC1 depletion affects virion infectivity. We employed Huh7-

END cells²³ stably releasing HDV and depleted these cells for ApoC1 (Fig. 6B). HDV released from these cells and from siNT-transfected Huh7-END cells was quantified for viral genomes and equal amounts of genome equivalents were used to infect permissive Huh7-NTCP cells. ApoC1-depleted HDV had significantly lower specific infectivity as compared to wildtype HDV virions (Fig. 6B). Moreover, infection by ApoC1-free HDV produced in HEK 293T cells that do not express endogenous ApoC1²⁴, could be enhanced by adding low amounts of exogenous ApoC1. High ApoC1 amounts diminished HDV infection in this setting (Fig. 6C), most likely due to competition, similar to the competition we observed for HBV (Fig. S11, <https://links.lww.com/HEP/K558>). These results suggest that ApoC1 associated with HBV or HDV particles increases their infectivity.

Both HBV and HDV use NTCP as entry receptor. To assess whether ApoC1 knockdown affects cell surface availability of NTCP, we employed a fluorophore-conjugated preS1-peptide³. Using HepG2 or Huh7 cells stably expressing NTCP, ApoC1 depletion had no effect on NTCP cell surface abundance (Fig. 6D, E).

Impairment of HBV infection by pharmacological inhibition of SR-B1

Since ApoC1 is mainly associated with HDL (Fig. 7A), the role of HDL in HBV infection was investigated. Pretreatment of purified HBV stocks with exogenous HDL increased HBsAg and HBeAg secreted from infected cells, indicating enhanced viral infectivity by HDL (Fig. 7B). Since SR-B1 is a natural receptor of HDL, we next studied the possible contribution of SR-B1 to HBV infection. Cells depleted of either SR-B1 or ApoC1 were infected in the presence of HDL. Depletion of either protein substantially attenuated the HDL-induced increase of HBV infection (Fig. 7B), indicating HDL facilitated HBV infection through a SR-B1 and ApoC1 dependent mechanism.

Earlier studies reported facilitated HBV infection by ApoE, which binds to LDL-receptors²⁵. We therefore compared the impact of combined SR-B1 - LDL or -VLDL depletion on HBV infection. Single knockdown of SR-B1 or LDL-receptor significantly reduced HBV infection whereas knockdown of the VLDL-receptor had no effect (Fig. S13A, <https://links.lww.com/HEP/K558>). Combination of SR-B1 and LDL-receptor knockdown had no additive effect. Analogous results were obtained with HDV, consistent with the same entry route used by both viruses (Fig. S13B, <https://links.lww.com/HEP/K558>).

SR-B1 mediates bidirectional lipid transport between HDL and cells by facilitating the selective uptake of cholesterol esters from HDL into liver cells for bile acid synthesis and the efflux of free cholesterol to HDL²⁶. We explored this SR-B1 function for HBV infection using the pharmacological inhibitors BLT-1 and BLT-4. They bind to SR-B1 in an irreversible or reversible manner, respectively, and inhibit SR-B1-mediated lipid transfer activity between HDL and cells^{27,28}. HepG2-NTCP-A3 cells were infected with HBV for 4 h in the presence of BLT compounds and viral replication was measured 5 days later. Both BLT compounds significantly reduced HBV replication, indicating that SR-B1 is a potential co-factor for HBV entry (Fig. 7C). To investigate whether SR-B1 inhibition exerts effects beyond the viral entry step, we treated the cells with BLT-1 at later stages of infection. BLT-1 treatment three days after inoculum removal no longer reduced HBeAg levels, whereas treatment during the infection period reduced HBeAg levels by approximately 30% (Fig. S14, <https://links.lww.com/HEP/K558>), consistent with the SR-B1 knockdown phenotype. These findings suggest that SR-B1 does not affect HBV at a post-entry step. To corroborate this conclusion in a more relevant cell system, the effect of BLT-1, the more potent and specific inhibitor^{27,29}, was evaluated in HBV-infected primary human hepatocytes (PHHs) derived

from two different donors (Fig. 7D). The reduction of extracellular HBsAg and HBeAg observed at non-cytotoxic concentrations of BLT-1 (Fig. S15, <https://links.lww.com/HEP/K558>) supported our conclusion on the important role of SR-B1 in HBV entry. Consistently, HDV infection was also reduced by both SR-B1 inhibitors (Fig. 7E).

These results suggest that HDL enhanced HBV and HDV infection in an ApoC1- and SR-B1-dependent manner. ApoC1, probably in part via HDL, targets virions to SR-B1 that might act as co-factor to facilitate infection. This would explain why supplementation of exogenous HDL enhanced HBV infection while pharmacological inhibition of SR-B1 suppressed the infection.

Discussion

Here, we comprehensively determined the first proteome composition of HBV particles allowing the detection of very low abundance proteins such as the HBV P-protein that is present at approximately one copy per DNA-containing virion¹⁵. Consistent with earlier studies, the HBx protein was not detected³⁰. Regarding host cell proteins, a recent cryo-EM study of pgRNA-containing nucleocapsids revealed an electron-dense region within the capsid interior, proposed to contain entrapped host factors³¹. Consistently, we identified three cellular proteins (CASP14, CWF19L2 and HNRNPC) highly enriched in the virion/fSVP preparation derived from HepAD38 cells, but not in fSVPs from HepG2-HB2.7 cells.

Whether these proteins play a role in HBV biology remains to be determined. We also detected several chaperones in the virion and fSVP fractions, including HSP90AB1 earlier shown to facilitate HBV polymerase-RNA interactions and assembly of the reverse transcription initiation complex³².

Additional chaperones we identified associated with viral envelope proteins included HSC70 and its interaction partner, the cytosolic anchorage determinant binding protein. These proteins inhibit co-translational translocation of the PreS1 domain through the viral envelope enabling the dual topology of the L protein¹⁷. Similarly, PPIA has been reported to interact with HBsAg¹¹. In our fSVP proteome, we also detected the HSP40 chaperone DNAJB12, an interaction partner of the nucleic acid polymer REP-2139 interfering with assembly and release of HBV SVPs and currently in clinical trials for HBV and HDV infections³³. These findings underscore the superior properties of our particle purification method.

We also identified HDL-enriched apolipoproteins, 5 co-purifying with fSVPs and 3 also with virions. HDL mediates cholesterol transport from peripheral tissues to the liver by carrying free cholesterol and cholesteryl esters as part of the reverse cholesterol transport pathway. Recently, macrophages were shown to internalize lipoprotein-associated HBV particles into recycling endosomes enriched in cholesterol, which are subsequently trafficked back to the cell surface to facilitate hepatocyte infection¹². The co-accumulation of HDL apolipoproteins with cholesterol in the HBV lipid envelope and host endosomal membranes might confer favorable biophysical properties promoting virus entry. Cholesterol influences membrane fusion in several enveloped viruses, including Ebola virus, HIV, Zika virus and SARS-CoV-2^{34,35}. Consistently, cholesterol-depleted HBV virions have reduced particle diameter and lower infectivity, both parameters being restored upon cholesterol replenishment²¹. In our study, HDL supplementation during infection enhanced viral entry, supporting a role for lipoproteins during HBV infection (Fig. 7B). Moreover, pharmacological inhibition of cholesterol and cholesteryl ester transport via SR-B1 reduced HBV and HDV infection (Fig. 7C-E). While these results suggest that SR-B1 might act as auxiliary HBV receptor,

consistent with an earlier study³⁶, we note that SR-B1 splice variants localize to distinct subcellular compartments, including plasma membrane and endosomes³⁷. This localization may extend the role of SR-B1 beyond its interaction with HBV particles at the cell surface. Moreover, SR-B1 association with lipoproteins is not limited to HDL as interactions with LDL and VLDL have also been reported^{37,38}. Therefore, we cannot exclude the involvement of other lipoproteins in HBV entry, which might explain the earlier report of LDLR being an important host cell factor for HBV infection²⁵.

Comparing the 5 different apolipoproteins identified in our proteome, ApoC1 depletion had the most prominent effect on HBV. The impact was even stronger than the one of ApoE previously reported to enhance HBV cell surface attachment by binding to the HSPG syndecan-2 and the LDLR^{25,39}. Since the bovine genome lacks an ApoC1 gene⁴⁰, we can exclude the possibility that bovine ApoC1 contributes to any of the observed effects. However, ApoC1 released from HepG2-hNTCP cells could potentially associate with virus particles and facilitate infection. This might explain why depletion of ApoC1 in infected cells reduces HBV and HDV infection by limiting ApoC1 association with viral particles, in addition to ApoC1's role in maintaining cellular cholesterol homeostasis.

Attempts to produce ApoC1-depleted HBV particles encountered technical limitations, especially the presence of naked capsids and background signals from plasmid DNA in transfection-based systems, both of which confounded viral DNA quantification. Therefore, we employed HDV that has the same envelope glycoproteins as HBV, but is a more tractable model for infectivity analyses. The lower infectivity of ApoC1-depleted HDV suggests that virion associated ApoC1 enhances viral entry (Fig. 6B), although we cannot exclude that ApoC1 depletion alters other lipid-related properties of the virus particles such as their

cholesterol content or impaired association with lipoproteins. In contrast, excess ApoC1 may interfere with particle integrity or compete with virion-associated ApoC1 and/or HDL for cell-surface binding, possibly accounting for reduced infection (Fig. 6C). Interestingly, a role for ApoC1 in viral membrane fusion was demonstrated for HCV⁴¹ and a similar mechanism might operate during HBV and HDV infection. However, the lack of a robust HBV fusion assay and the limited efficiency of current in vitro infection systems preclude studying this hypothesis experimentally.

By using immuno-EM, we observed ApoC1 positivity in only ~10% of cell culture-derived highly purified HBV particles. This rather low percentage may underestimate the real ApoC1 association with HBV particles for several reasons. First, neutralization with an ApoC1-specific antibody reduced HBV infection by 30-50% (Fig. 6A), which is more than can be expected if only 10% of the particles would be associated with ApoC1. Second, ApoC1 concentrations are substantially higher in human serum than in standard cell culture supernatants, indicating that in vivo more HBV particles might be associated with ApoC1 and/or HDL (Table S4, <https://links.lww.com/HEP/K558>). Whether this difference accounts for the observed difference of HBV infection efficiency of cell lines in vitro vs. animals in vivo^{42,43} remains to be determined. Third, ApoC1 association with HBV particles might be highly dynamic and influenced by the harsh sample preparation and fixation conditions we had to use for the EM-based analysis. Fourth, the concentration of FBS used for virus production may influence the composition of cell culture-derived HBV particles. SVPs isolated from patient serum contain ~15% cholesterol and ~14% cholesteryl esters⁴⁴, whereas HBsAg particles produced under serum-free or low-serum (1% FCS) cell culture conditions have only ~3% cholesterol and ~1.5% cholesteryl ester^{45,46}. Since cholesteryl esters are predominantly transported within circulating lipoproteins, the low cholesteryl ester content of

cell culture-derived HBsAg particles is consistent with reduced lipoprotein association under in vitro conditions. Consequently, differences in lipoprotein association, including ApoC1, may contribute to the altered composition and reduced infectivity of cell culture-derived HBV particles. Overall, the proteome and lipid composition of cell culture-derived HBV particles might not fully recapitulate those of virions produced in vivo, which is an inherent limitation of cell culture-based HBV proteomic studies. Nevertheless, we also detected association between ApoC1 and HBV virions or SVPs in sera from patients with CHB, indicating that HBV can hijack circulating lipoproteins in vivo possibly to facilitate its transport to the liver²³. Given these findings, combining current HBV therapies with FDA-approved serum lipid-modulating drugs may represent a novel antiviral strategy.

Data Availability

All data supporting the findings of this study are available within this paper and its supplementary information, <https://links.lww.com/HEP/K558> . The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE⁴⁷ partner repository with the dataset identifier PXD076462. Source data are provided with this paper (Table S1 and S2, <https://links.lww.com/HEP/K558>).

Acknowledgements

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Fig. 1. Purification of HBV virions and filamentous SVPs. (A) Purification pipeline of HBV virions and fSVPs from HepG2-HB2.7 and HepAD38 culture supernatants. Yellow particles represent subviral particles and empty virions; cyan particles represent Dane particles. (B) Upper panel: Sucrose fractions of one representative equilibrium density gradient were analyzed by dual-fluorescence western blot detecting HBV envelopes (green) and core proteins (red), with densities specified above each lane. Fractions enriched for virions (9-10) or fSVPs (14) were used for HBsAg-specific pull-down. Lower panel: Pull-down samples and corresponding inputs were analyzed by SDS-PAGE and silver staining. Arrowheads indicate glycosylated (orange) and non-glycosylated (green) small HBs proteins; stars indicate IgG heavy (blue) and light (gold) chains. (C) Viral peptides identified by AP-LC-MS/MS across all 5 biological replicates are categorized according to viral proteins. Individual values represent spectral intensities ($\log_2$) of unique peptides from pull-down samples across independent biological replicates. (White circles = not identified). (D) Proteomic analysis of affinity-purified particles by AP-LC-MS/MS ($n \geq 4$). The heatmap represents intensity-based quantification (iBAQ) values of significantly enriched proteins in virion or fSVPs pull-downs ($\log_2$). Each column corresponds to one pull-down sample from one independent biological replicate. N.I.= not identified.

Figure 1

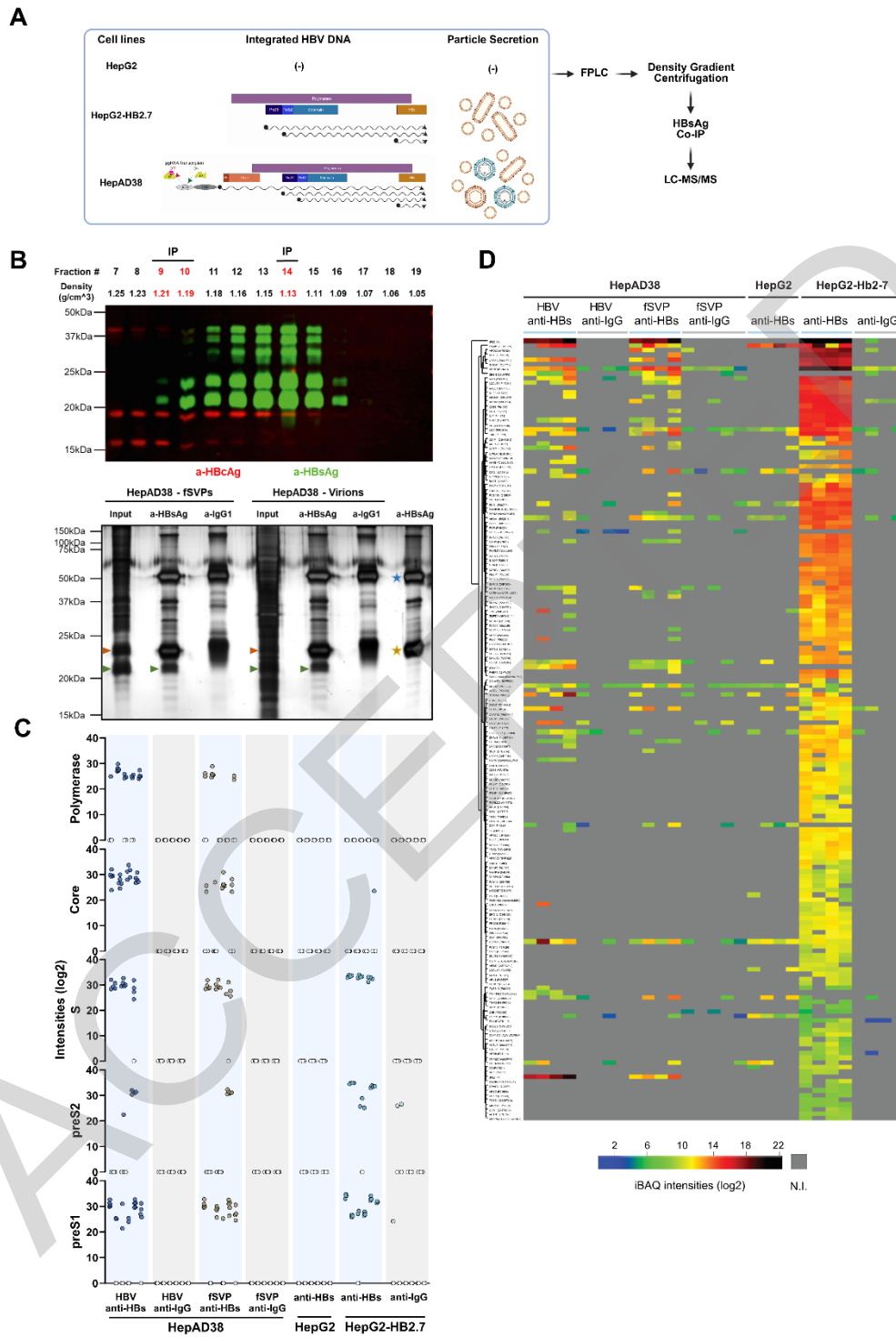


Fig. 2. siRNA-based screen identifies novel HBV host-dependency factors. (A) Schematic of the experimental approach. HepG2-NTCP-A3 cells were reverse-transfected with gene-specific siRNAs pool for 48 h and subsequently infected with HBV (500 genome equivalents (GE)/cell with 4% PEG8000). After 24 h, the inoculum was removed, cells were washed and cultured for an additional 5 days. Cells treated with the entry inhibitor Bulevirtide (MyrB) or transfected with siRNA targeting HBV transcripts (siHBV) served as positive controls. (B-C) HBV infection was evaluated by measuring secreted HBeAg and HBsAg at 5 days post-infection (dpi) by ELISA. (D) Intracellular HBcAg was detected by western blot and quantified using ImageLab. (E) HBV total RNAs were quantified by RT-qPCR. Values were normalized to cells transfected with non-targeting siRNA (siNT), set to 1 (green bar). Data are presented as mean $\pm$ SEM from at least two independent experiments performed in triplicate. Orange bar, flawed result because of cytotoxicity (see Fig. S5C, <https://links.lww.com/HEP/K558>).

Figure 2

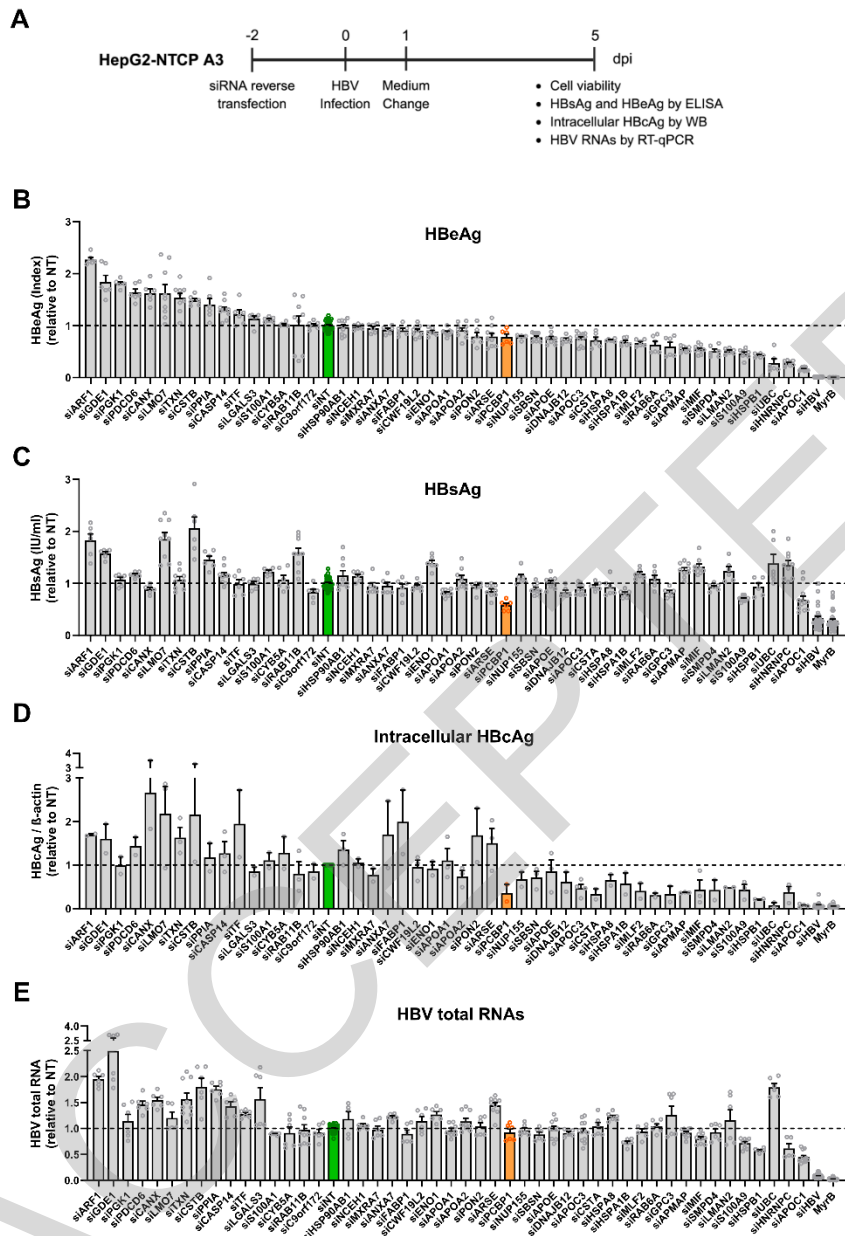


Fig. 3. Comparative analysis of the impact of apolipoprotein depletion on HBV infection and HBsAg production. (A-D) Detailed view of apolipoprotein knockdown results from Fig. 2. (A) Knockdown efficiency was assessed by RT-qPCR. (B) Secreted HBsAg and HBeAg were measured by ELISA. (C) HBV total RNAs and pgRNA were quantified by RT-qPCR. (D) Intracellular HBcAg was detected by western blot and quantified using Image Lab. One out of at least three independent experiments is shown. (E) HepG2-HB2.7 cells were reverse-transfected with siRNAs; medium was refreshed after 48 h. Cells were harvested 6 days post-transfection. Knockdown efficiency and secreted HBsAg were measured by RT-qPCR and ELISA, respectively. Values were normalized to siNT, set to 1 (green bar). Data represent mean $\pm$ SEM from at least three independent experiments. B, C, D, E, One-way ANOVA; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns-not significant ($p > 0.05$). NC, no infection control; MyrB, Bulevirtide-treated cells.

Figure 3

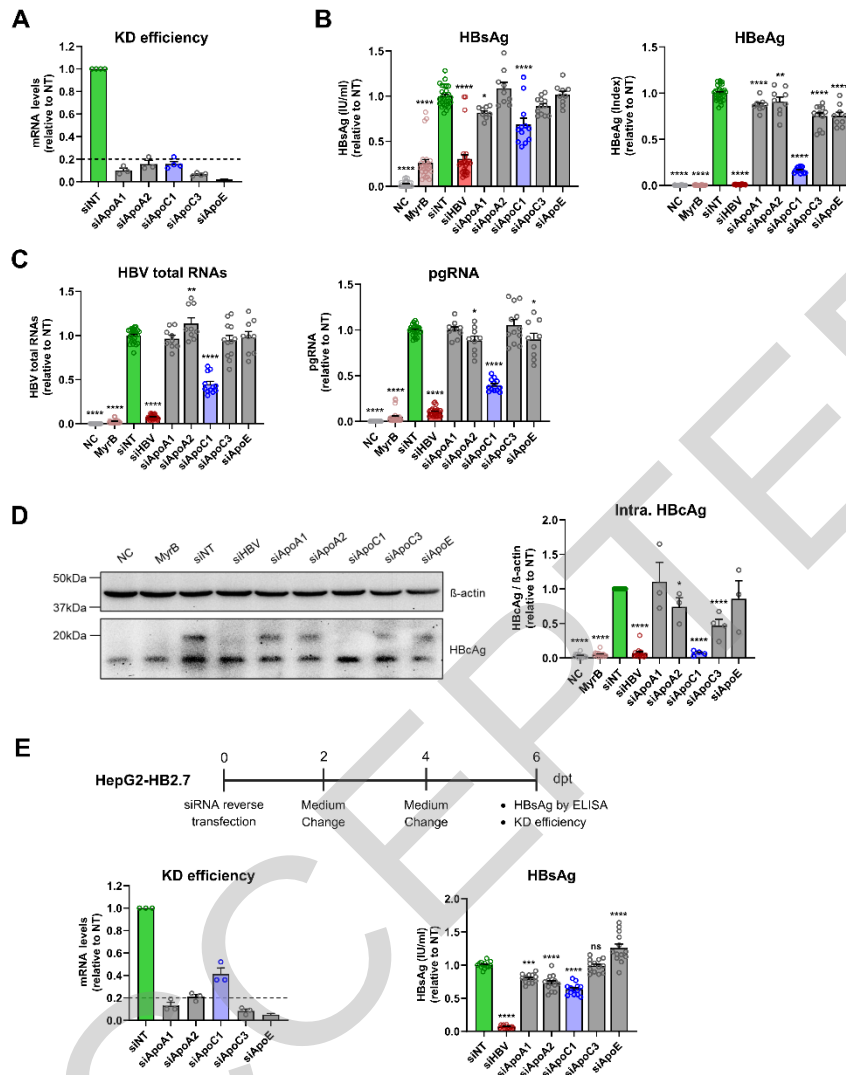


Fig. 4. Role of ApoC1 for HBV infection and intracellular cholesterol level. (A-C) HepG2-NTCP-A3 cells were reverse-transfected with siRNAs for 48 h and then infected with HBV (500 GE/cell; 4% PEG8000). After 24 h, the inoculum was removed, cells were washed and harvested at different time points. (A) Secreted HBsAg, HBeAg and HBV DNA were quantified by ELISA and qPCR at 5, 7 and 9 dpi, respectively. (B) Intracellular HBc and HBs proteins were assessed by immunofluorescence at 5 dpi. Red, green and blue signals depict HBc protein, HBs protein and nuclei, respectively. Scale bar: 100 μ m. (C) HBV cccDNA was detected by qPCR at 5 dpi. (D) HepG2-NTCP-A3 cells were reverse-transfected with siRNAs, and medium was replaced by FBS-free medium after 48 h. Left: Intracellular cholesterol levels, measured one day later. Right: Cholesterol supplementation during HBV infection in the absence of PEG8000 and FBS; secreted HBeAg was measured at 5 dpi. (E) HepG2-HB2.7 cells were reverse-transfected with siRNAs and 48 h later medium was refreshed. Cells were treated with water-soluble cholesterol between day 5 and 6 and secreted HBsAg was quantified. Data represent mean $\pm$ SEM from at least three independent experiments. A, D (right panel), E, Two-way ANOVA; C, D (left panel), One-way ANOVA; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, ns-not significant ($p > 0.05$).

Figure 4

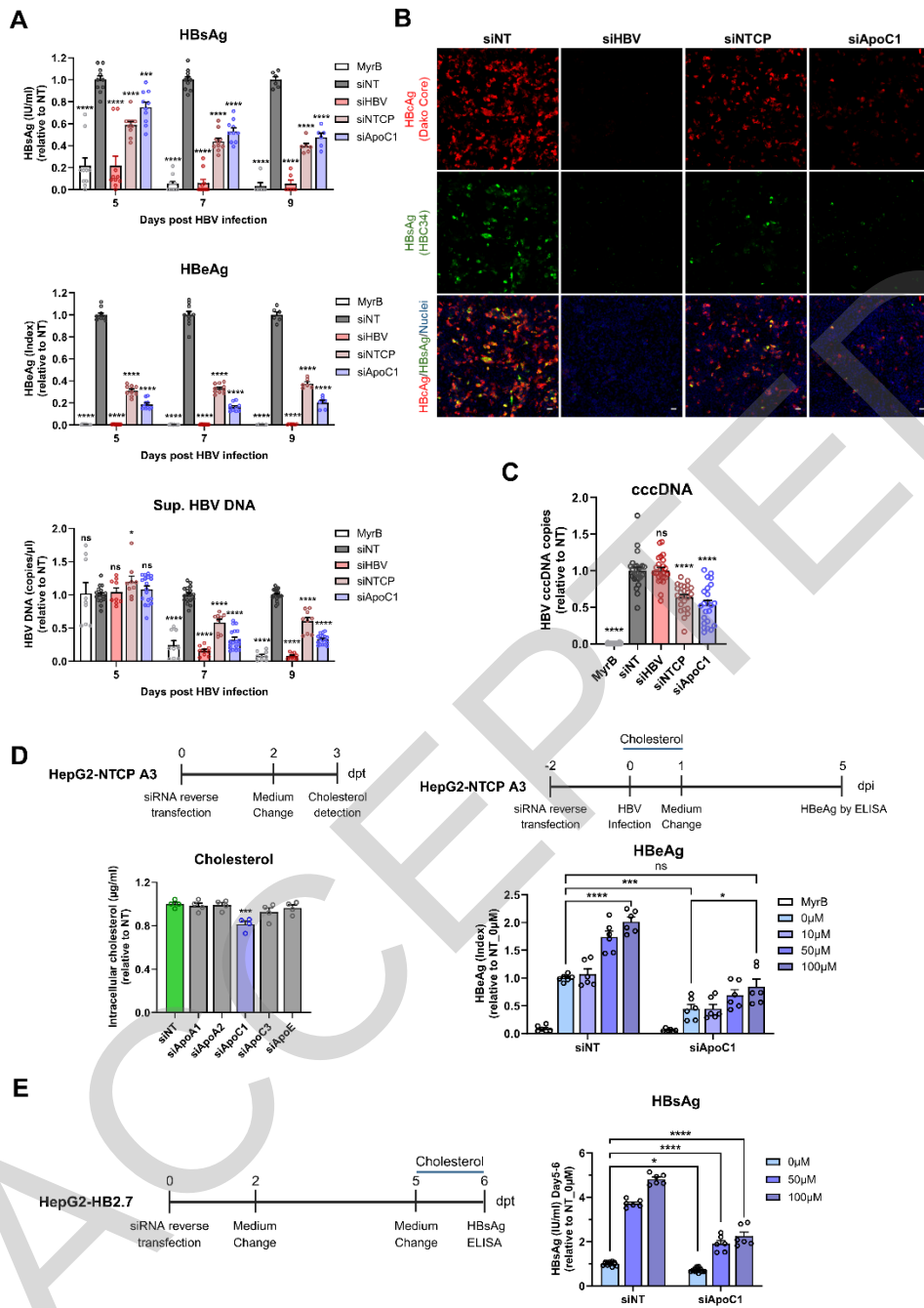


Fig. 5. ApoC1 associates with HBV virions and filamentous SVPs. (A) Two different heparin-purified HBV stocks were subjected to immunoprecipitation using HBsAg- or ApoC1-specific or IgG antibodies. Eluates were analyzed for ApoC1 and HBcAg by western blot or HBV DNA by qPCR (normalized to input). (B) Huh7 cells were transfected with a plasmid containing a 1.1-overlength HBV genome. HBV accumulating in culture supernatant between day 4 and 7 post-transfection were subjected to immunoprecipitation with HBsAg-specific or IgG antibodies. HBsAg ELISA values of input and flow-through fractions are indicated on the bottom. N.d., non-detectable. (C) HBV particles in serum of a patient with CHB were analyzed by immunoprecipitation and western blot. (D) HBV virions from HepAD38 cells purified by heparin affinity chromatography, size exclusion chromatography and sucrose gradient centrifugation were immunogold-labeled for HBsAg and ApoC1 and visualized by transmission electron microscopy. IgG antibodies (Neg. Ctrl) served as specificity controls. Quantification of gold-positive structures is shown below. Scale bar: 50 nm. Unpaired t test; ** $p < 0.01$, **** $p < 0.0001$.

Figure 5

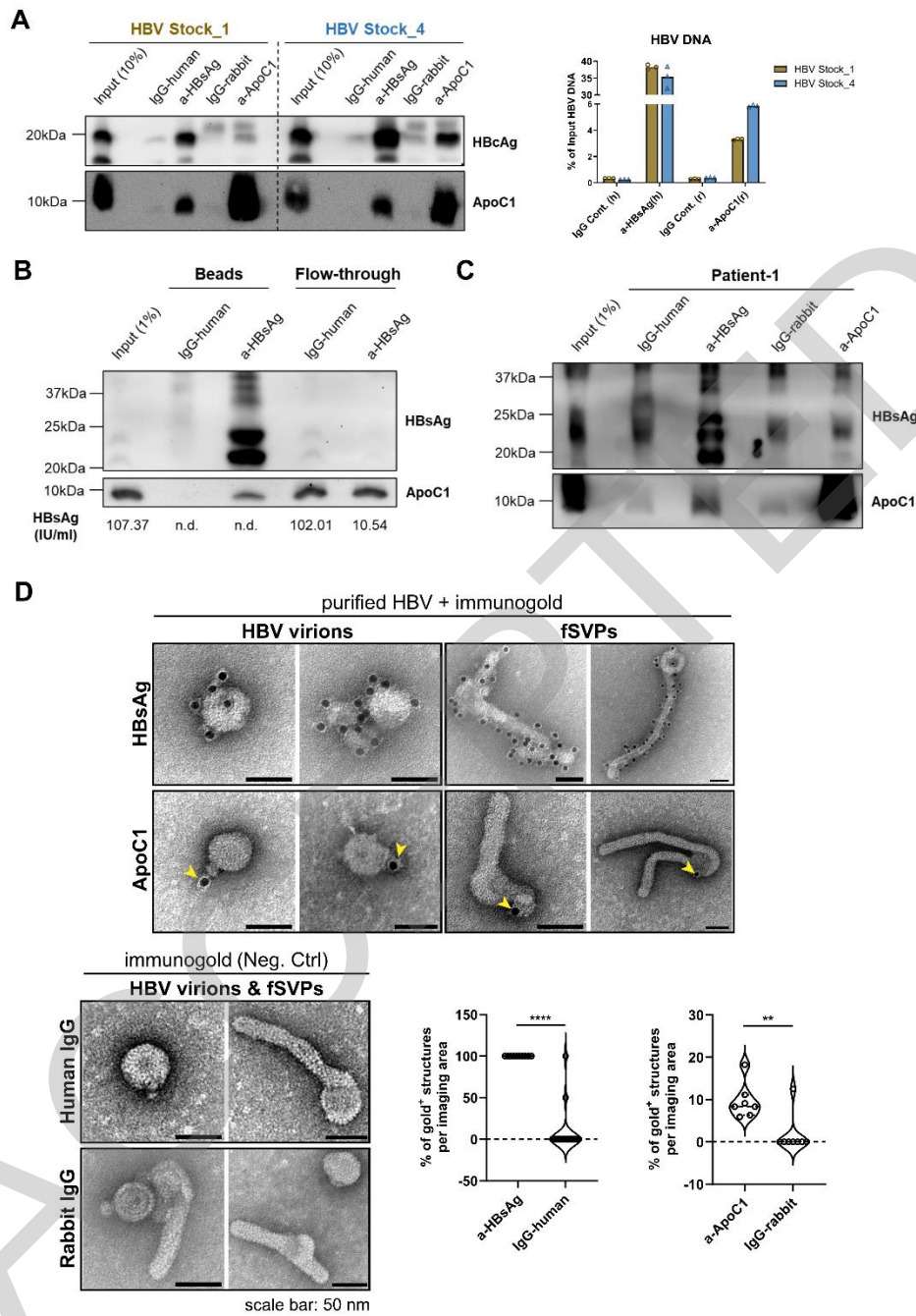


Fig. 6. ApoC1 enhances HBV and HDV infectivity. (A) HBV stocks were pre-incubated with ApoC1 antibody for 1 h at room temperature prior to infecting HepG2-NTCP-A3 cells (1,500 GE/cell) in the absence of PEG8000 and FBS for 24 h. Secreted HBsAg, HBeAg and HBV DNA were quantified by ELISA and qPCR at 7 dpi, respectively. Intracellular HBcAg (right panel) was detected by western blot at 7 dpi and quantified using Image Lab. One out of three representative experiments is shown. (B) To obtain HDV depleted for ApoC1, Huh7-END cells (schematic on the left) were transfected with siApoC1 or siNT. HBsAg levels and HDV titers were determined by ELISA and RT-qPCR, respectively. Owing to lower HDV production in ApoC1 depleted cells (upper right panels), Huh7-NTCP target cells were inoculated with the same HDV genome equivalents to evaluate the impact of ApoC1 depletion on HDV infectivity. At 5 dpi, intracellular HDV RNAs were quantified by RT-qPCR (lower right panel). Data represent mean $\pm$ SEM from three independent experiments. (C) HDV were produced in 293T cells by co-transfection of vectors encoding HBV surface proteins and a vector harboring a 1.1-fold cDNA copy of the HDV antigenome. HDV particles contained in the supernatant were pre-incubated with different concentrations of ApoC1 protein for 1 h at room temperature prior to infecting Huh7-NTCP cells. HDV infection of cells was assessed by quantification of intracellular HDV RNAs at 5 dpi, and is displayed relative to untreated control cells. The dashed line indicates HDV RNA amounts in cells treated with MyrB. Data are presented as mean $\pm$ SEM from two independent experiments. (D) Huh7-NTCP or (E) HepG2-NTCP-A3 cells were reverse-transfected with given siRNAs for 48 h and subsequently exposed to 200 nM atto488-labeled MyrB peptide (MyrB^{atto}) for 30 mins at 37°C. Green and blue signals on the left panels depict MyrB^{atto} and nuclei, respectively. Cells incubated with an excess of unconjugated MyrB peptide served as nonspecific binding control. Scale bar: 100 μ m. Data represent mean $\pm$ SEM from two independent experiments performed in duplicate. A, B (right panel), C, D, E, One-way

ANOVA; B (left and middle panels), unpaired t test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns-not significant ($p > 0.05$).

Figure 6

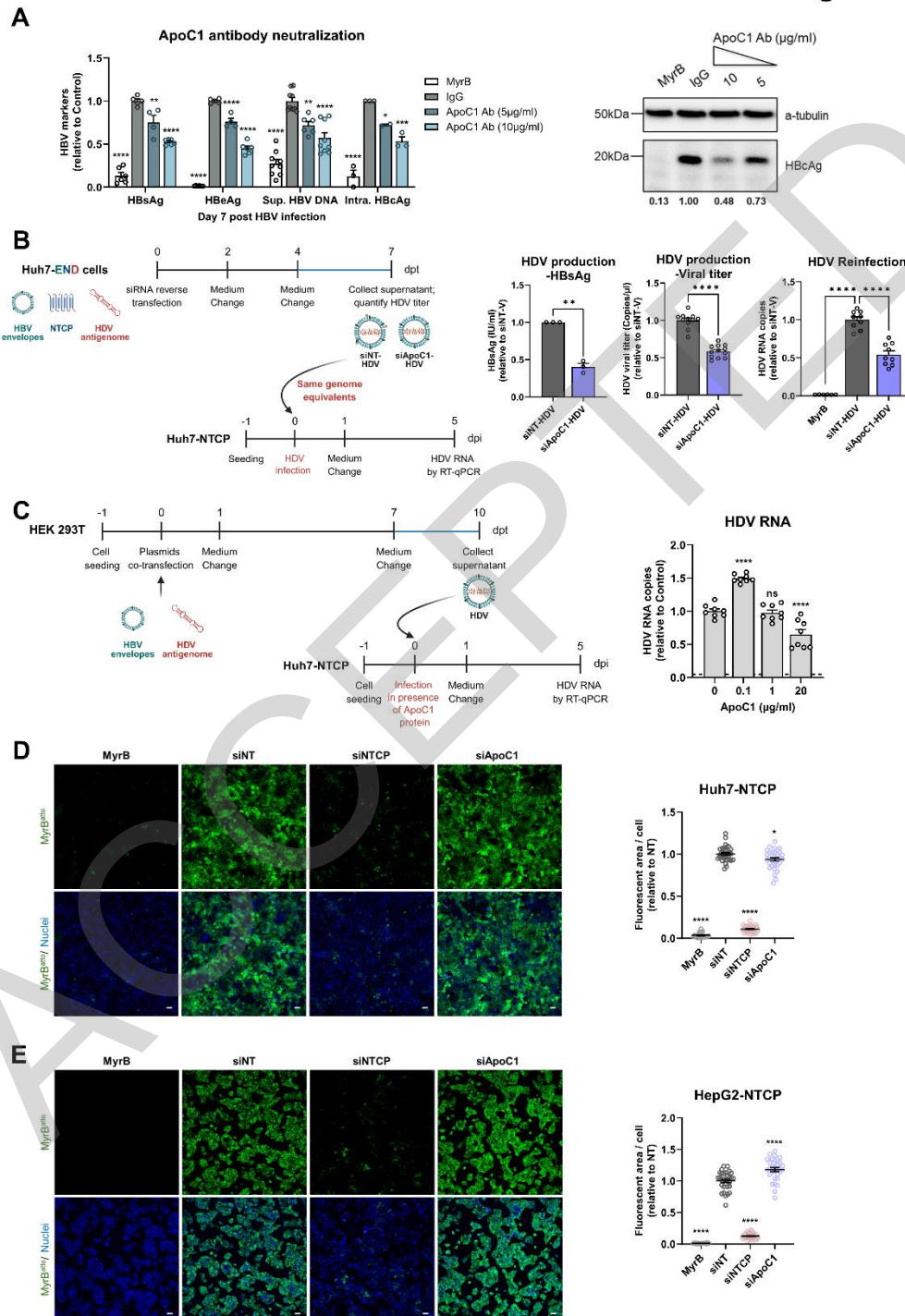


Fig. 7. Inhibition of HBV and HDV infection by pharmacological block of SR-B1. (A)

Schematic diagram of high-density lipoprotein (HDL). Created in BioRender. (B) HepG2-

NTCP-A3 cells were reverse-transfected with siRNAs specified on the bottom for 48 h and

subsequently infected with HBV or with HBV that had been pre-incubated with HDL for 30

mins at room temperature. The experiments were performed in the absence of 4% PEG8000

and cells were inoculated with 1,500 HBV GE/cell. After 24 h in FBS-free medium, cells

were washed and complete medium was added. Secreted HBsAg and HBeAg were quantified

at 7 dpi. (C) HepG2-NTCP-A3 cells or (D) PHHs were treated with SR-B1 inhibitors (BLT1

or BLT4) throughout infection with HBV (1,000 GE/cell with 4% PEG8000). Secreted

HBsAg, HBeAg, HBV total RNAs and pgRNA were quantified by ELISA and RT-qPCR at 5

dpi, respectively. (E) Huh7-NTCP cells were treated with BLT1 or BLT4, starting at 4 h prior

to and throughout infection with HDV (5 GE/cell with 4% PEG8000). After 24 h, cells were

washed and cultured in medium without SR-B1 inhibitors. Intracellular HDAg was assessed

by immunofluorescence at 5 dpi. White and blue signals depict HDAg and nuclei,

respectively. Quantification of nuclei number and HDAg-positive cells are shown. Scale bar:

100µm. Data are presented as mean ± SEM from three independent experiments. B, C, D, E,

One-way ANOVA; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, ns-not significant ($p > 0.05$).

Figure 7

