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Hepatitis B virus promotes its own replication by enhancing RAB5A-mediated dual activation of endosomal and autophagic vesicle pathways

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ABSTRACT

Chronic hepatitis B virus (HBV) infection remains one of the major global public health concerns, and it develop into liver fibrosis, cirrhosis, and hepatocellular carcinoma. Recent evidence suggests that endosomal and autophagic vesicles are beneficial for HBV replication. However, it has not been well elucidated how HBV exploits such intracellular vesicle systems for its replication. RAB5A, a member of small GTPase family, plays crucial roles in early endosome biogenesis and autophagy initiation. We observed that RAB5A mRNA and protein levels were significantly increased in HBV-expressing hepatoma cell lines as well as in liver tissue samples from chronic HBV-infected patients. Moreover, RAB5A silencing inhibited HBV replication and subviral particle (SVP) expression significantly in HBV-transfected and -infected hepatoma cells, whereas RAB5A overexpression increased them. Mechanistically, RAB5A increases HBV replication through enhancement of early endosome (EE) – late endosome (LE) activation by interacting with EEA1, as well as enhancing autophagy induction by interacting with VPS34. Additionally, HBV infection enhances RAB5A-mediated dual activation of EE-LE system and autophagy. Collectively, our findings highlight that HBV utilizes RAB5A-mediated dual activation of endosomal and autophagic vesicle pathways for its own replication and persistence. Therefore, RAB5A is a potential target for chronic HBV infection treatment.

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KEYWORDS Hepatitis B virus; RAB5A; autophagy; endosome; VPS34

Introduction

Chronic hepatitis B virus (HBV) infection remains a major global public health concern, and it can develop into liver fibrosis, cirrhosis, and hepatocellular carcinoma [1]. HBV, one of the smallest enveloped DNA viruses, is composed of an inner icosahedral nucleocapsid and the outer envelope protein [2]. In HBV-infected cells, HBV mainly exists in the form of infectious virus particles, naked nucleocapsid (NC) and non-infectious subviral particles (SVPs) [3]. Extensive studies on the pathogenesis of chronic HBV infection are required for the identification of novel treatment strategies for HBV infection.

Previous studies have revealed that both the endosomal vesicle system and autophagy are closely related to different steps of the HBV life-cycle [4–6]. The endosomal vesicle system, including early endosome (EE) and late endosome (LE)/multivesicular body (MVB), plays a central role in regulation of cargo trafficking, vesicle secretion, signal transmission, and pathogen invasion [7,8]. Accumulating evidence supports that EE-LE vesicle pathway is closely related to viral endocytosis, DNA replication, and virion secretion in the HBV life-cycle [4–6]. HBV entry is mediated by its receptor on the surface of hepatocytes and then the viral envelope is fused with EEs to complete its endocytosis [9]. According to our recent study, coiled-coil domain containing 88A (CCDC88A/GIV)-mediated EE-LE trafficking plays an important role in promoting HBV DNA replication [4]. Moreover, other researchers and the authors of the present study have demonstrated that both mature HBV particles and subviral filaments are secreted from hepatocytes via an endosomal sorting complex required for transport (ESCRT)-dependent LE pathway [3–6].

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Therefore, HBV entry, trafficking, and progeny secretion are involved with the EE-LE system.

Macroautophagy/Autophagy is a conserved catabolic process that degrades long-lived proteins, damaged organelles, and invaded pathogens to maintain cellular homeostasis in eukaryotic cells [10]. There are close interactions between cellular autophagy and HBV infection [11]. Accumulating evidence supports that autophagy plays a vital role in promoting HBV DNA replication *in vitro* and *in vivo* [12–17]. On the one hand, HBV manipulates autophagic vesicles and autophagosomes for DNA replication. Doring *et al.* have revealed that ATG5-12-16L elongation complex provides a physical scaffold for HBV replication and maturation, by the intrinsically disordered region of ATG12 directly interacting with the viral core protein to mediate the trafficking of core proteins to NC assembly/envelop generation sites [18]. On the other hand, HBV infection can directly or indirectly induce autophagy formation or interfere with autophagic flux to promote viral DNA replication. Previous studies have revealed that HBV proteins, including HBx or SHBs, induce autophagosome formation and block lysosomal degradation [12,15,19,20]. Although the relationship between cellular autophagy and HBV replication has been gradually elucidated, their definite mechanisms require further investigation.

RAB is a class of critical regulators of membrane trafficking and fusion events [21–23]. We have reported that a part of HBsAg and HBV virions is degraded following the fusion of autophagosomes and lysosomes controlled by the RAB7A complex. RAB5A plays a crucial role in regulating vesicle endocytosis, endosomal membrane transport, and autophagy formation [21,24]. Notably, RAB5A expression is increased significantly in patients with chronic HBV infection when compared with in healthy controls from intrahepatic transcriptomes of chronic hepatitis B patients [25]. Moreover, our recent study further suggested that RAB5A deficiency decreases HBV production and HBsAg expression [4]. To date, the definite molecular mechanism via which RAB5A promotes HBV replication remains unclear.

Herein, we investigated the effect of RAB5A on HBV replication in HBV-transfected and -infected hepatoma cells, and elucidated its potential mechanism. Moreover, we further evaluated how HBV infection enhances RAB5A-mediated dual activation of endosomal and autophagic vesicle systems to promote its own replication.

Materials and methods

Cell culture and transfection

Human hepatoma Huh7, HepG2, and HepG2-NTCP cells were maintained in Dulbecco's modified Eagle's medium (Gibco, 8121118) supplemented with 10% fetal bovine serum, 1× Minimum Essential Medium (MEM) non-essential amino acid (NEAA) solution, and 100 U/ml penicillin–streptomycin. HepG2.2.15 cells harbouring integrated dimers of the HBV genome (GenBank accession number, U95551) were cultured in RPMI-1640 medium (Gibco, 8120429) with 10% FBS, 1×MEM NEAA solution, 100 U/ml penicillin–streptomycin, and 500 μg/ml G418 (MedChemExpress, HY-17561). Primary human hepatocytes (PHHs) purchased from ScienCell Research Laboratories were cultured in primary hepatocyte maintenance medium containing 2% dimethylsulfoxide (Solarbio, D8371) [16]. All the cells were cultured in a humidified incubator at 37 °C with 5% CO_2. All plasmid and small interfering RNAs were transfected into cells by Lipofectamine^{TM} 3000 (Invitrogen, L3000015).

Plasmids and reagents

Plasmids pHBV1.3, HA-HBc, HA-HBe, HA-HBx, HA-SHBs, HA-MHBs, HA-LHBs, HA-Pol, and pXJ40-HA were kindly provided by Dr. Xinwen Chen (University of Chinese Academy of Sciences, China). The plasmids GFP-LC3 and mCherry-GFP-LC3 were kept in our laboratory as previously described [26]. Plasmids Flag-RAB5A, GFP-EEA1, GFP-CD63, Flag-VPS34, and GFP-RAB5A were purchased from Addgene (Cambridge, MA, USA). The siRNAs against RAB5A (SI00301588 and SI02655037) were purchased from Qiagen (Hilden, Germany). The chemical inhibitors were used in the present study as follows: 3-methyladenine (3-MA; Sigma, M928), chloroquine (CQ; Sigma, C6628), dynasore (TargetMol, T1848), U18666A (MedChemExpress, HY-107433), and rapamycin (Sigma, R8781).

HBV replication, transcription, and gene expression analysis

HBV replication intermediates (RIs) from intracellular core particles were extracted from hepatoma cell lines and detected by Southern blotting according to published protocols [27]. HBV progeny DNA was extracted from culture supernatants using the DNA Blood Mini Kit (Qiagen, 51106), and real-time quantitative PCR (Takara, RR820A) was performed using primers specific for quantifying HBV DNA (Table S1). For HBV transcriptional analysis, total RNA was extracted with TRIzol (Invitrogen, 15596-018), followed by digestion with DNase set (Roche, 10104159001). HBV RNA in cells was detected through Northern blotting and real-time reverse transcription (RT)-PCR assays (Takara, RR086A) using primers specific for HBV RNA (Table S1), as described previously [27]. Intracellular HBsAg and secreted HBsAg and HBeAg levels in culture supernatants

were determined with HBsAg or HBeAg ELISA Kit (Kehua Bio-engineering), according to the manufacturer's instructions.

Immunoprecipitation and western blotting

Co-immunoprecipitation (Co-IP) was conducted to detect interaction between proteins. After 48 h transfection, Huh7 cells were lysed using lysis buffer (20 mM Tris-HCl, pH 7.4; 150 mM NaCl; 10 mM EDTA; 1% NP-40). Lysates were incubated with the indicated antibodies overnight at 4°C. Dynabeads™ Protein G beads (Invitrogen, 91216624) were added to the sample and incubated for 2 h at 4°C. Subsequently, the beads were washed with RIP buffer five times. Finally, the products were eluted by protein loading buffer and resolved by SDS-PAGE. Western blotting analysis was performed as described previously [26]. Information about the antibodies is presented in Table S2.

Statistical analyses

Statistical analyses were performed using GraphPad Prism software version 5.1 (La Jolla, CA, USA). Data are presented as the mean ± standard deviation of at least three independent experiments. Analysis of variance (ANOVA) with two-tailed Student's t-test or one- or two-factor ANOVA was used to determine significant differences. Differences were considered statistically significant at $P < 0.05$. All experiments were repeated independently at least three times.

Results

HBV infection increases RAB5A expression

Our intrahepatic transcriptomes of chronic hepatitis B patients by microarray analysis of liver biopsies [25] showed that RAB5A mRNA expression was increased significantly in patients with chronic HBV infection when compared with in healthy controls (Figure S1A), indicating that RAB5A gene expression would be positively correlated with chronic HBV infection. Subsequently, we confirmed whether chronic HBV infection increased RAB5A expression using Realtime RT-qPCR analysis, immunohistochemical staining assay, and western blotting analysis in the human liver tissue samples collected from the patients with chronic HBV infections through liver biopsy and excess parts from the liver transplant donors, as healthy controls. We found that the levels of RAB5A mRNA (Figure 1A) and protein (Figure 1B,C) expression were elevated significantly in the patients with chronic HBV infection when compared with healthy controls. Moreover, we further investigated whether HBV infection increased RAB5A expression in HBV-expressing hepatoma cell lines. We compared RAB5A mRNA and protein expression between HepG2 cells without HBV production and HepG2.2.15 cells with stable HBV replication, Huh7 cells transiently transfected with the HBV plasmid pHBV1.3 or empty vector pUC19 (Figure S1B), and HepG2-NTCP cells with or without HBV virion infection (Figure S1C). Realtime RT-qPCR analysis revealed that RAB5A mRNA levels in hepatoma cells with HBV infection were elevated significantly when compared with non-HBV cells (Figure 1D,E; S1D). Consistently, the results from immunofluorescence staining assays (Figure 1F,G; S1E) and western blotting analysis (Figure 1H,I; S1F) showed that RAB5A protein levels in hepatoma cells with HBV infection were increased markedly when compared with the levels in non-HBV cells. The results support that chronic HBV infection increases RAB5A mRNA and protein expression in human liver tissues.

To identify which HBV protein is responsible for RAB5A elevation, and how they influenced RAB5A expression, the overexpression of seven HBV proteins, including viral core protein (HBc), x protein (HBx), e protein (HBe), polymerase (Pol), small surface protein (SHBs), medium surface protein (MHBs), and large surface protein (LHBs) were investigated. Our western blotting analysis showed that overexpression of the HBV proteins did not change RAB5A protein levels significantly (Figures S2A and S2B), suggesting that only one viral protein cannot induce RAB5A elevation. To further test whether HBV virions inside the cells cause RAB5A elevation, HepG2.2.15 cells were treated with 1 μg/ml Entecavir (ETV) for 72 h. We found that ETV treatment effectively reduced the intracellular HBV DNA levels (Figure 1J) and decreased RAB5A mRNA and protein expression (Figure 1K,L), whereas it had no significant inhibitory effect on secreted HBsAg expression (Figure S2C). The above results indicate that HBV virions and/or two or more viral proteins may contribute to the elevation of RAB5A transcription, which needs further investigation in the following study. Collectively, HBV infection contributes to the elevation of RAB5A mRNA and protein expression in human liver tissues and *in vitro*.

RAB5A promotes HBV replication and SVP production in HBV-transfected hepatoma cells

Our recent study has revealed that RAB5A deficiency negatively regulates HBV production and efficiently blocks the positive effect of CCDC88A on HBsAg expression *in vitro* [4]. To confirm the promoting effect of RAB5A on HBV replication and SVP production, the effect of RAB5A silencing and RAB5A overexpression was investigated on intracellular HBsAg, HBcAg, HBV RNA, and encapsidated HBV replicative intermediates in HBV-transfected Huh7

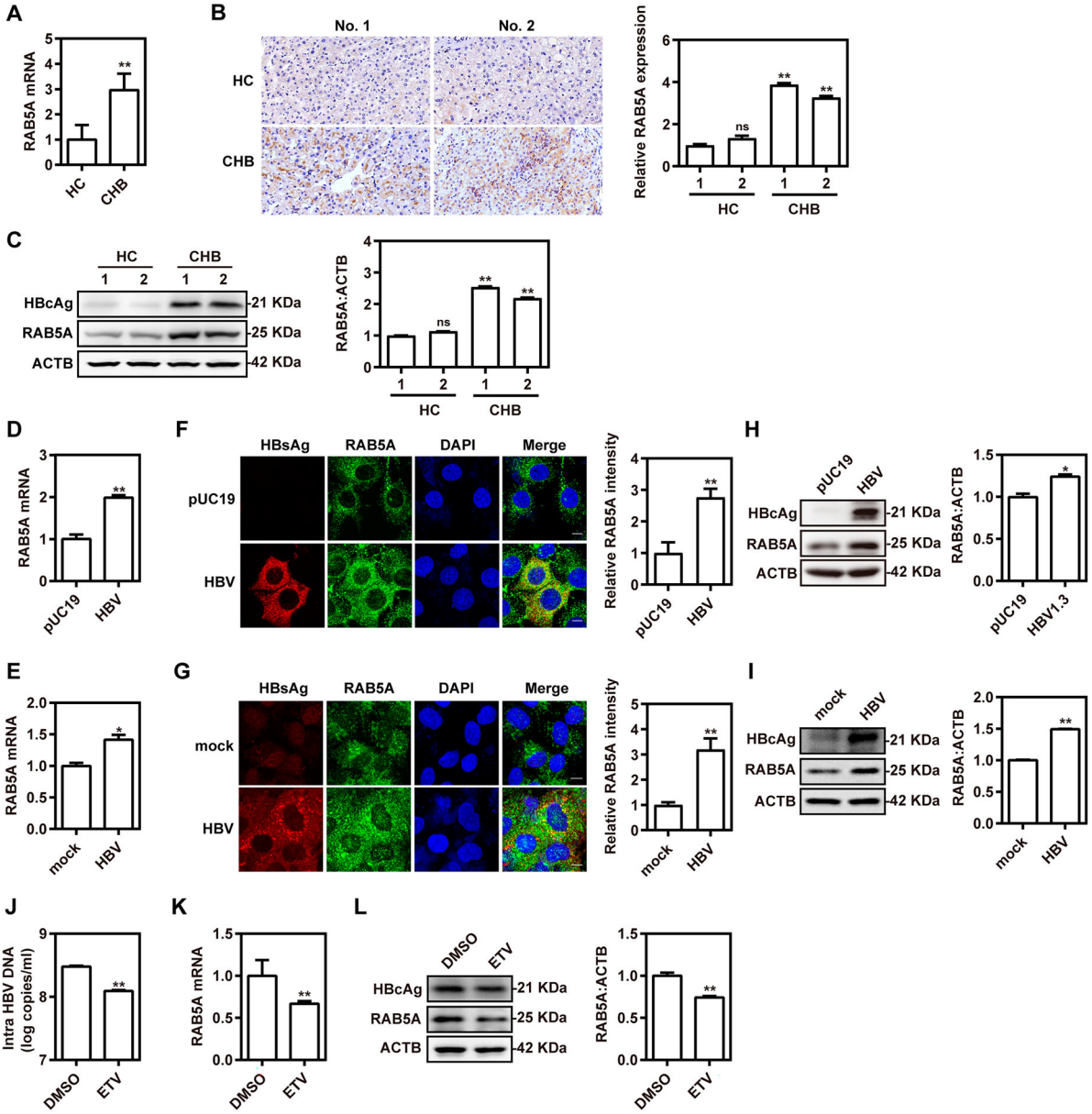

Figure 1. HBV infection increases RAB5A expression. (A–C) Liver tissue samples were collected from patients with chronic hepatitis B (CHB, 6 cases) or healthy controls (HCs, 6 cases). RAB5A mRNA levels were determined by real-time quantitative RT-PCR. Intracellular RAB5A protein expression inside the cells from two liver tissue samples was measured by immunohistochemical staining assay. Magnification, 40×; scale bar: 20 μm. Total RAB5A protein expression from the lysis of two liver tissue samples was measured by western blotting analysis. (D-I) Huh7 cells transfected with HBV plasmid pHBV1.3 or vector control pUC19 and HepG2-NTCP cells with or without HBV infection (MOI = 1000) were harvested at 72 h post HBV transfection or infection. (D–E) RAB5A mRNA levels were determined by quantitative RT-PCR. (F–G) The treated cells were incubated with horse anti-HBsAg and rabbit anti-RAB5A primary antibodies for immunofluorescence staining and finally imaged under confocal microscopy. Scale bar, 10 μm. (H–I) RAB5A protein levels were measured by western blotting. (J–L) HepG2.2.15 cells were treated with 1 μg/ml Entecavir (ETV) for 72 h. Intracellular encapsidated HBV replicative intermediates were isolated and detected by real-time quantitative PCR. RAB5A mRNA and protein levels were determined by real-time quantitative RT-PCR and western blot analysis, respectively. *$P < 0.05$; **$P < 0.01$; ns, not significant.

cells and HBV-steadily produced HepG2.2.15 cells. We found that RAB5A silencing reduced the amounts of secreted and intracellular HBsAg in HBV-transfected Huh7 cells and HepG2.2.15 cells significantly (Figure 2A; S3A), in addition to decreasing intracellular HBcAg expression measured by immunofluorescence staining and western blotting, respectively (Figure 2B,C; S3B and S3C). Moreover, RAB5A silencing had a significant inhibitory effect on HBV DNA replication measured by real-time qPCR and Southern blotting, respectively (Figure 2D,E; S3D and S3E). Consistent with our previous findings [4], RAB5A silencing did not significantly alter the levels of secreted HBeAg and HBV RNA (Figures S4A and S4B), indicating that RAB5A had no effect on HBV transcription. In contrast, we observed that RAB5A overexpression increased HBsAg production (Figure 2A), HBcAg expression (Figure 2B,C), and

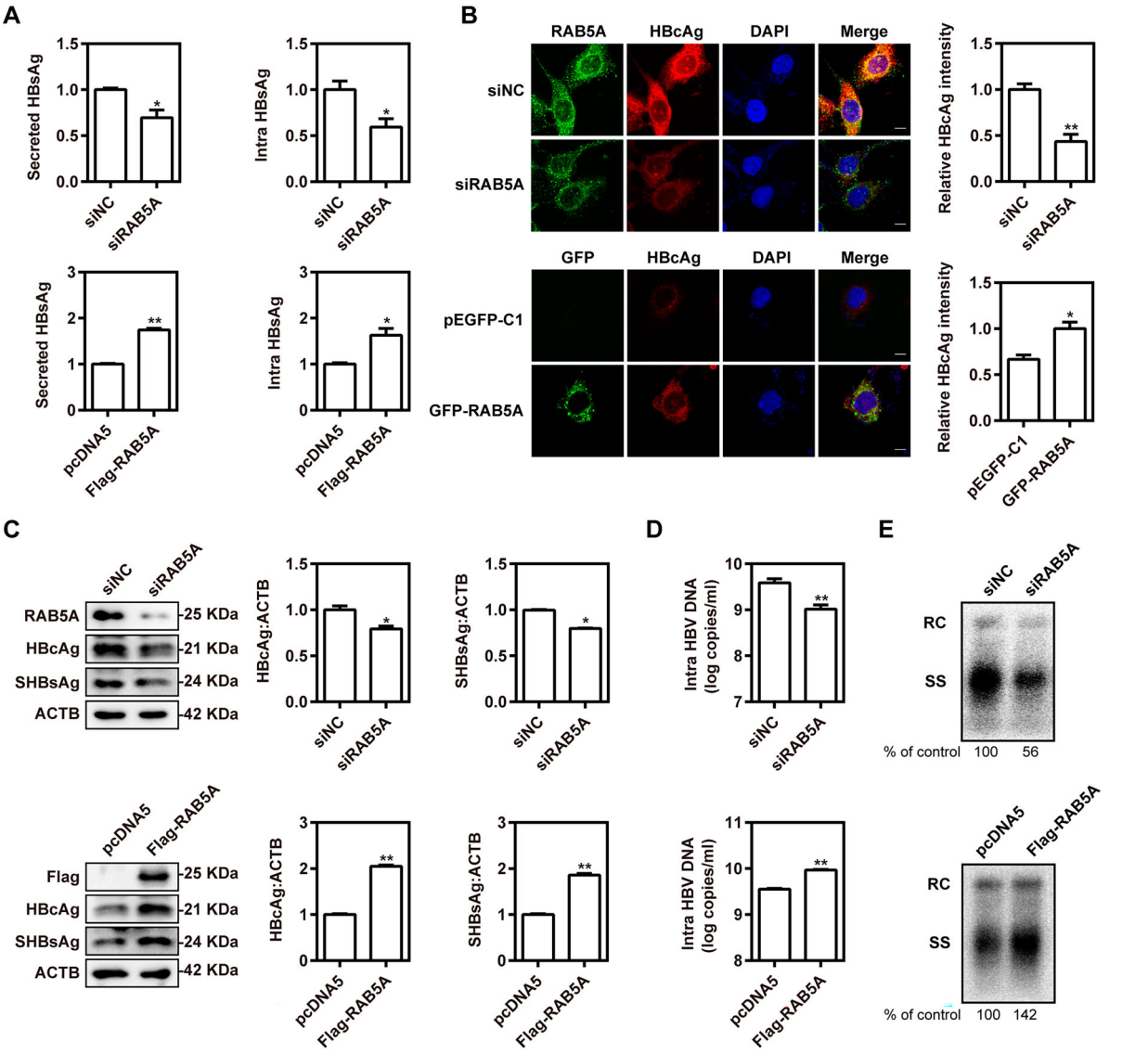

Figure 2. RAB5A promotes HBV replication and viral gene expression in HBV-transfected hepatoma cells. Huh7 cells were co-transfected with HBV plasmid pHBV1.3 and 40 nM siRNAs against RAB5A (siRAB5A) or siRNA negative control (siNC) or Flag-RAB5A or control vector pcDNA5 for 72 h. (A) The levels of secreted HBsAg in culture supernatants and intracellular HBsAg from cell lysates were determined using ELISA. (B) HBcAg expression was measured by immunofluorescence staining using anti-HBcAg antibody. Scale bar, 10 μm. (C) The levels of RAB5A, HBcAg, and SHBsAg were measured by western blotting analysis. (D–E) Intracellular encapsidated HBV replicative intermediates were isolated and detected by real-time quantitative PCR and Southern blot analysis, respectively. *$P < 0.05$; **$P < 0.01$; ns, not significant.

viral DNA replication (Figure 2D,E) in HBV-transfected Huh7 cells significantly. Collectively, RAB5A silencing inhibited HBV replication and SVP expression significantly, whereas RAB5A overexpression increased them in different HBV cell culture systems, indicating that RAB5A positively modulates HBV replication and SVP production *in vitro*.

RAB5A promotes HBV replication and SVP production in HBV-infected hepatoma cells and PHHs

To further confirm the promoting effect of RAB5A on HBV replication and SVP production in HBV-infected cell systems, the effect of RAB5A silencing or overexpression was assessed in HBV-infected HepG2-NTCP cells. The cell culture supernatants were collected at three indicated time points, at 3, 5 and 7 days after infection (dpi), followed by quantifying of HBsAg and HBeAg expression using ELISA [26]. RAB5A silencing effectively reduced the levels of secreted and intracellular HBsAg (Figure 3A,B) but did not decrease the levels of secreted HBeAg and intracellular HBV total RNA and pgRNA significantly (Figures S5A and S5B). Moreover, RAB5A silencing decreased intracellular HBcAg expression (Figure 3C,D) and HBV DNA replication (Figure 3E) significantly. In contrast, RAB5A overexpression increased HBsAg and HBcAg expression and viral DNA replication (Figure 3A–E) significantly in HBV-infected HepG2-NTCP cells. Interestingly, RAB5A overexpression did not decrease the levels of

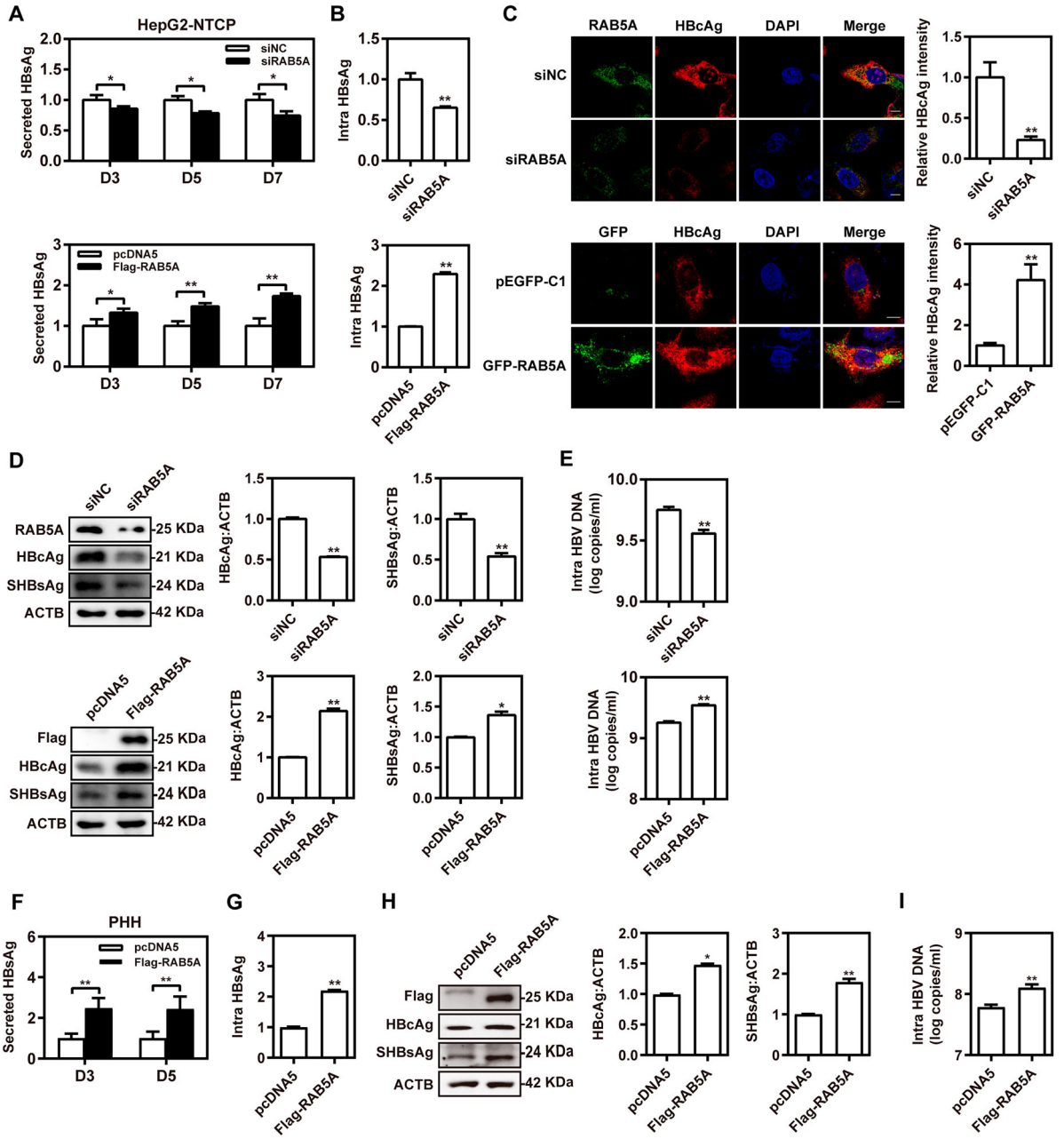

Figure 3. RAB5A promotes HBV replication and viral gene expression in HBV-infected HepG2-NTCP cells and PHHs. (A-E) HepG2-NTCP cells were infected with HBV virions (MOI = 1000) for 24 h, followed by transfecting with 40 nM siRNAs against RAB5A (siRAB5A) or siRNA negative control (siNC) or plasmid Flag-RAB5A or vector control pcDNA5. The culture supernatants were collected at days 3, 5, and 7 post-HBV infection. (A–B) The levels of secreted HBsAg in culture supernatants and intracellular HBsAg from cell lysates were determined using ELISA. (C) HBcAg expression was imaged by confocal microscopy. Scale bar, 10 μm. (D) The levels of RAB5A, HBcAg, and SHBsAg were measured by western blotting analysis. (E) Encapsidated HBV replicative intermediates were isolated and measured by real-time quantitative PCR. (F–I) PHHs were infected with HBV virions (MOI = 1000) for 24 h, followed by transfecting with Flag-RAB5A or pcDNA5. The culture supernatants were collected at days 3 and 5 post-HBV infection. (F–I) The levels of secreted HBsAg and intracellular HBsAg, HBcAg, and encapsidated HBV replicative intermediates were separately measured as mentioned above. *$P < 0.05$; **$P < 0.01$; ns, not significant.

secreted HBeAg and intracellular HBV total RNA and pgRNA significantly (Figures S5A and S5B). Moreover, we further revealed that RAB5A overexpression also increased the levels of secreted and intracellular HBsAg and HBcAg expression and HBV DNA replication in HBV-infected PHHs (Figure 3F–I). Collectively, the results indicate that RAB5A increases HBV replication and SVP production in different HBV-infected cell models.

RAB5A enhances EE-LE system activation by interacting with EEA1

RAB5A plays a critical role in activating endocytic vesicle pathway and promoting the transition from EE to LE by recruiting RAB5A effectors [28,29]. RAB5A silencing decreased the levels of early endosome antigen (EEA1, EE marker) and CD63 (LE marker) by immunofluorescence staining in Huh7 cells

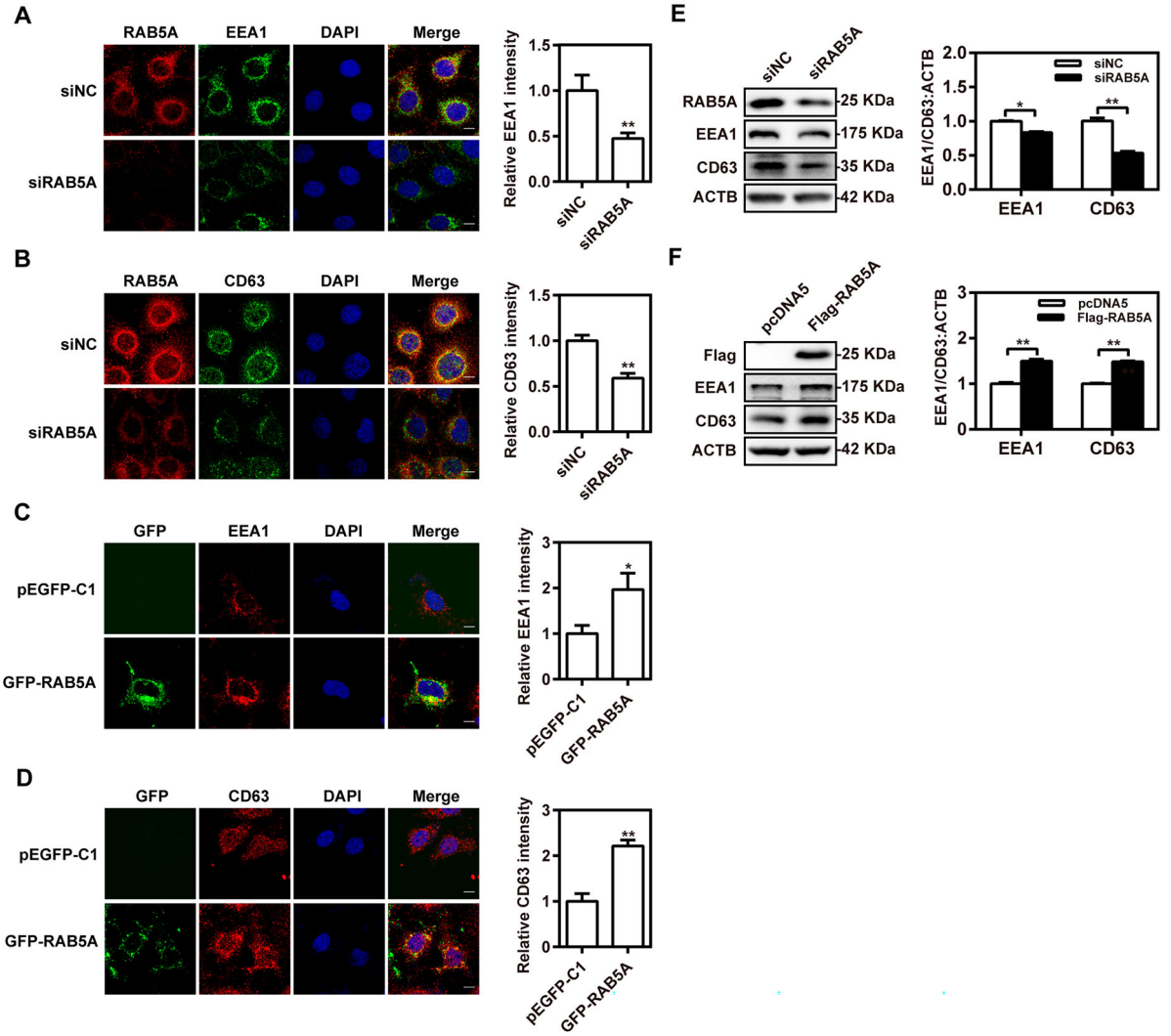

Figure 4. RAB5A enhances EE–LE system activation. (A–B) Huh7 cells were transfected with 40 nM siRNAs against RAB5A (siRAB5A) or siRNA negative control (siNC). (C-D) Huh7 cells were transfected with an expression vector carrying RAB5A (GFP-RAB5A) or control vector pEGFP-C1. At 48 h post transfection, EEA1 and CD63 expression inside the cells was imaged under confocal microscopy. Scale bar, 10 μm. (E-F) At 72 h post transfection, the levels of EEA1 and CD63 were measured by western blotting analysis. *$P < 0.05$; **$P < 0.01$; ns, not significant.

dramatically (Figure 4A,B), whereas RAB5A overexpression increased them obviously (Figure 4C,D). Moreover, we confirmed that RAB5A increased the levels of EEA1 and CD63, by western blotting analysis (Figure 4E,F). These results indicate that RAB5A activation promotes the transition from EE to LE. It has been previously reported that RAB5A effector EEA1 is involved in mediation of endosome docking and membrane fusion, and endosome biogenesis [30,31]. To verify the interaction of RAB5A and EEA1 in an HBV-infected cell model, endogenous and exogenous Co-IP assays were performed in HBV-transfected Huh7 cells. The results showed that RAB5A has an interaction with EEA1 (Figure S6), indicating that RAB5A activates EE-LE vesicle system by recruiting EEA1, leading to enhances EE biogenesis and promotes the transition from EE to LE. Collectively, the above results support that RAB5A enhances EE–LE vesicle system activation by interacting with EEA1.

RAB5A increases HBV replication by enhancing EE-LE system activation

To investigate whether RAB5A increases HBV replication through enhancing EE/LE activation, we verified the role of RAB5A-mediated endosomal activation on HBV replication and SVP production in HBV-expressed hepatoma cells. Huh7 cells were co-transfected with plasmids pHBV1.3 and GFP-EEA1, GFP-CD63 or control vector pEGFP-C1, and cultured for 72 h. We observed that overexpression of endosomal proteins EEA1 and CD63 elevated the levels of secreted HBsAg and intracellular HBsAg, HBcAg and HBV DNA markedly, based on ELISA, western blotting, and real-time qPCR, respectively (Figure 5A–C). Subsequently, we treated HepG2.2.15 cells with early endosomal inhibitor dynasore [4] or late endosomal inhibitor U18666A [6] for 48 h, separately. U18666A, as a classic inhibitor of intracellular cholesterol transport, is also used as an inhibitor of LE

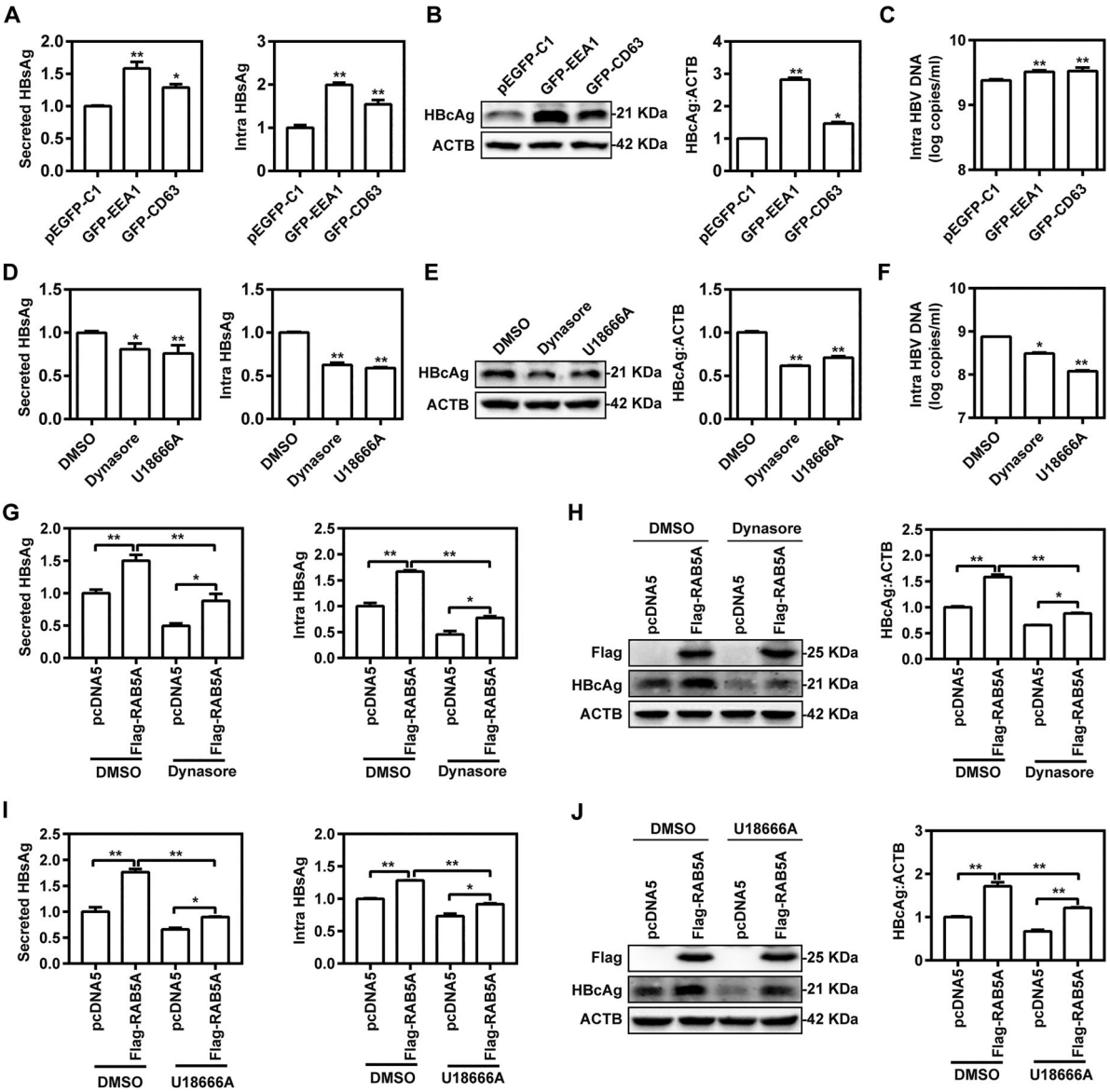

Figure 5. RAB5A promotes HBV replication through enhancement of EE–LE system activation. (A-C) Huh7 cells were co-transfected with HBV plasmid pHBV1.3 and GFP-EEA1 or GFP-CD63 or control vector pEGFP-C1 for 72 h. (D-F) HepG2.2.15 cells were treated with 10 μM dynasore or 2 μM U18666A or DMSO for 48 h. Huh7 cells transiently transfected with plasmids pHBV1.3 and Flag-RAB5A or empty vector pcDNA5. At 24 h post-transfection, the cells were treated with 80 μM dynasore (G-H) or 4 μM U18666A (I–J) or DMSO for 48 h. The levels of secreted HBsAg in culture supernatants and intracellular HBsAg from cell lysates were determined using ELISA. HBcAg level was measured by western blotting analysis. *$P < 0.05$; **$P < 0.01$; ns, not significant.

biogenesis to proving an inhibitory effect of endosomal sorting complex required for transport I machinery on HBV and hepatitis C virus release [6,32,33]. Inversely, both early and late endosome inhibitors had inhibitory effects on HBV replication and SVP production (Figure 5D–F). To address whether RAB5A promotes HBV replication through activating EE/LE vesicle system, Huh7 cells were transfected with Flag-RAB5A plasmids, followed by treatment with endosomal inhibitors, dynasore and U18666A. Notably, we observed that dynasore (Figure 5G,H) or U18666A (Figure 5I,J) treatment largely attenuated the positive effect of RAB5A overexpression on SVP and HBcAg expression, whereas they cannot completely inhibit this. Therefore, the results support that RAB5A increases HBV replication and SVP production through activating the EE-LE vesicle pathway. However, our data suggest that RAB5A-mediated EE-LE vesicle system activation is not the sole pathway of promotion of HBV replication and SVP production.

RAB5A enhances autophagy formation

Previous studies have revealed that RAB5A not only modulates EE-LE vesicle pathways but is also a key regulator of autophagy initiation in mammalian cells [24]. Confocal microscopic analysis showed that RAB5A silencing decreased the number of LC3 puncta as well as intracellular HBsAg expression significantly (Figure 6A). RAB5A silencing reduced the levels of

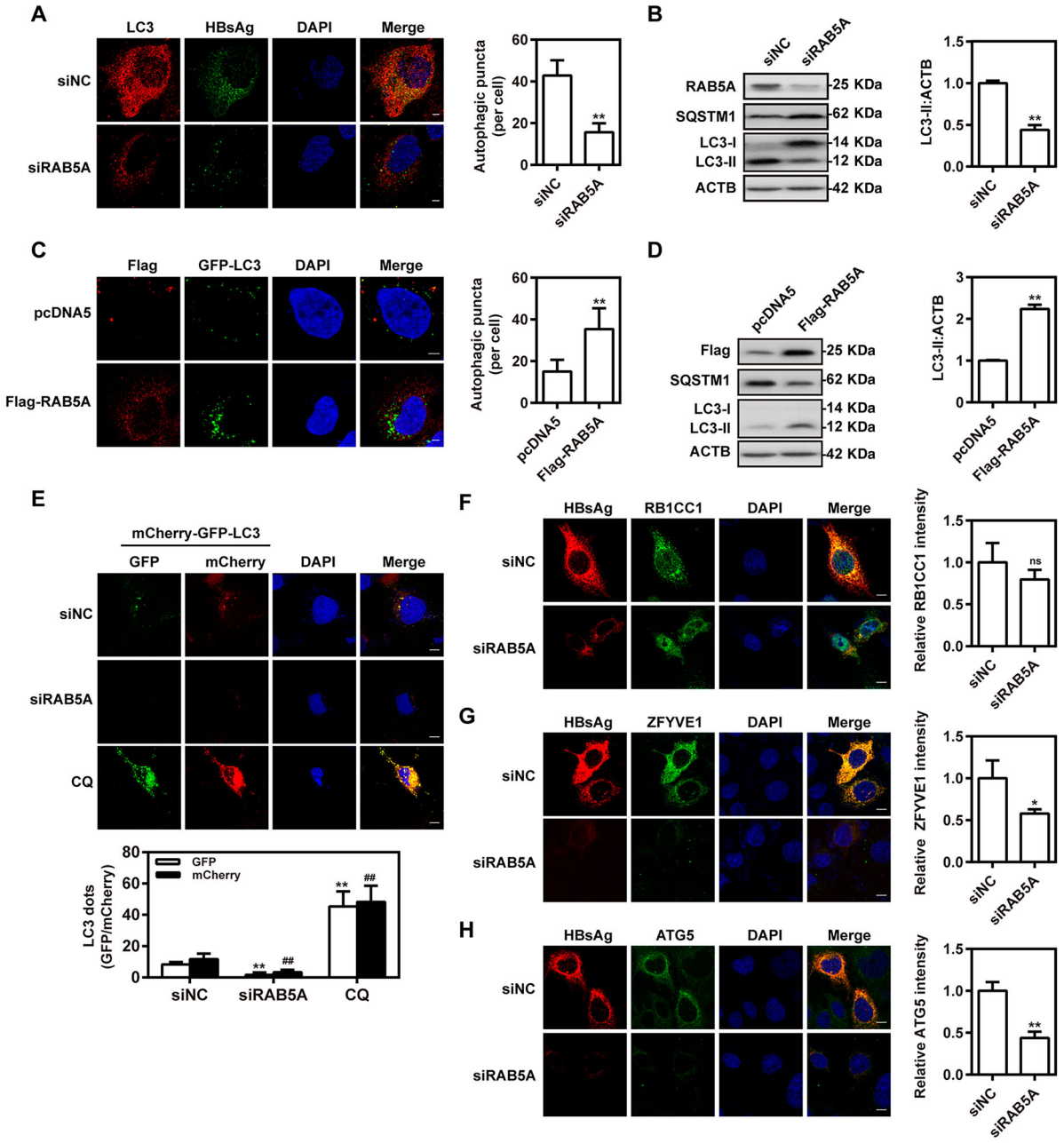

Figure 6. RAB5A enhances autophagy induction. (A-B) Huh7 cells were co-transfected with pHBV1.3 plasmid and 40 nM siRNAs against RAB5A (siRAB5A) or siRNA negative control (siNC) for 48 h. (C-D) Huh7 cells were co-transfected with plasmids GFP-LC3 and Flag-RAB5A or control vector pcDNA5 for 48 h. The puncta of LC3 or GFP-LC3 inside the cells were imaged by confocal microscopy. Scale bar, 10 μm. LC3 puncta per cell were counted and statistically analysed. The levels of LC3 and SQSTM1 were analysed by western blotting. (E) Huh7 cells were co-transfected with mCherry-GFP-LC3 plasmids and 40 nM siRAB5A or siNC for 48 h, using 10 μM chloroquine (CQ) treatment for 24 h as a positive control. The dots of GFP-LC3 and mCherry-LC3 were imaged by confocal microscopy, followed by statistical analysis. (F–H) Huh7 cells were transfected with pHBV1.3 and 40 nM siRAB5A or siNC for 48 h. The levels of RB1CC1, ZFYVE1 and ATG5 expression were imaged by immunofluorescence staining. Scale bar, 10 μm. *$P < 0.05$; **$P < 0.01$; ns, not significant.

LC3-II obviously but increased the level of autophagic cargo receptor SQSTM1 by western blotting analysis (Figure 6B). Conversely, RAB5A overexpression increased LC3 puncta number and LC3-II expression obviously but decreased SQSTM1 expression (Figure 6C,D). To further investigate how RAB5A modulates autophagic flux, Huh7 cells were co-transfected with mCherry-GFP-LC3 plasmid and siRAB5A or siRNA control for 48 h. RAB5A silencing decreased the number of GFP-LC3 and mCherry-LC3 puncta (Figure 6E), whereas chloroquine, which inhibits the acidification of the lysosomal compartment and prevents cargo degradation in lysosomes [34], caused an obvious accumulation of GFP-LC3 and mCherry-LC3 puncta inside the cells. The results indicate that RAB5A induced complete autophagy process by mediating the initiation of autophagic flux. Besides, we further investigated which step in RAB5A silencing modulated in autophagy formation by measuring the levels of different autophagic vesicle marker proteins,

RB1CC1 (a critical member of ULK1 kinase complex), ZFYVE1 (omegasome), and ATG5 (phagophore). Confocal microscopic analysis showed that RAB5A silencing reduced the levels of ZFYVE1 and ATG5 but had minimal inhibitory effects on RB1CC1 expression (Figure 6F–H), suggesting that RAB5A may induce the nucleation of autophagic membranes without changing ULK1 signalling. Therefore, we speculated that RAB5A enhances autophagy formation by promoting autophagic membrane nucleation.

RAB5A increases HBV replication through enhancing VPS34-mediated autophagy induction

To investigate whether RAB5A increases HBV replication by enhancing autophagy induction, we verified the role of RAB5A-mediated autophagy initiation on HBV replication and SVP production in HBV-expressed hepatoma cells. To test the promotion effect of RAB5A-mediated autophagy induction on HBV production, we first used ATG5-knockout (KO) Huh7 cells with ATG5 deletion to completely block the autophagy flux (Figure S7). Western blotting analysis showed that autophagy flux was completely blocked and HBcAg expression was inhibited significantly in ATG5 knockout cells. We found that ATG5 deficiency attenuated the promotion effect of RAB5A overexpression on secreted and intracellular HBsAg expression partially, based on ELISA as well as intracellular HBcAg expression, based on western blotting analysis (Figure 7A,B), whereas the RAB5A promotion effect on HBV gene expression could not be absolutely eliminated. Consistent with the above-mentioned Figure 5G–J, autophagy was not the only pathway for RAB5A to promote HBV replication and SVP production. HepG2.2.15 cells were silenced by transfection with siRAB5A, followed by treatment with the MTOR inhibitor rapamycin and VPS34 inhibitor 3-MA, successively. Rapamycin treatment

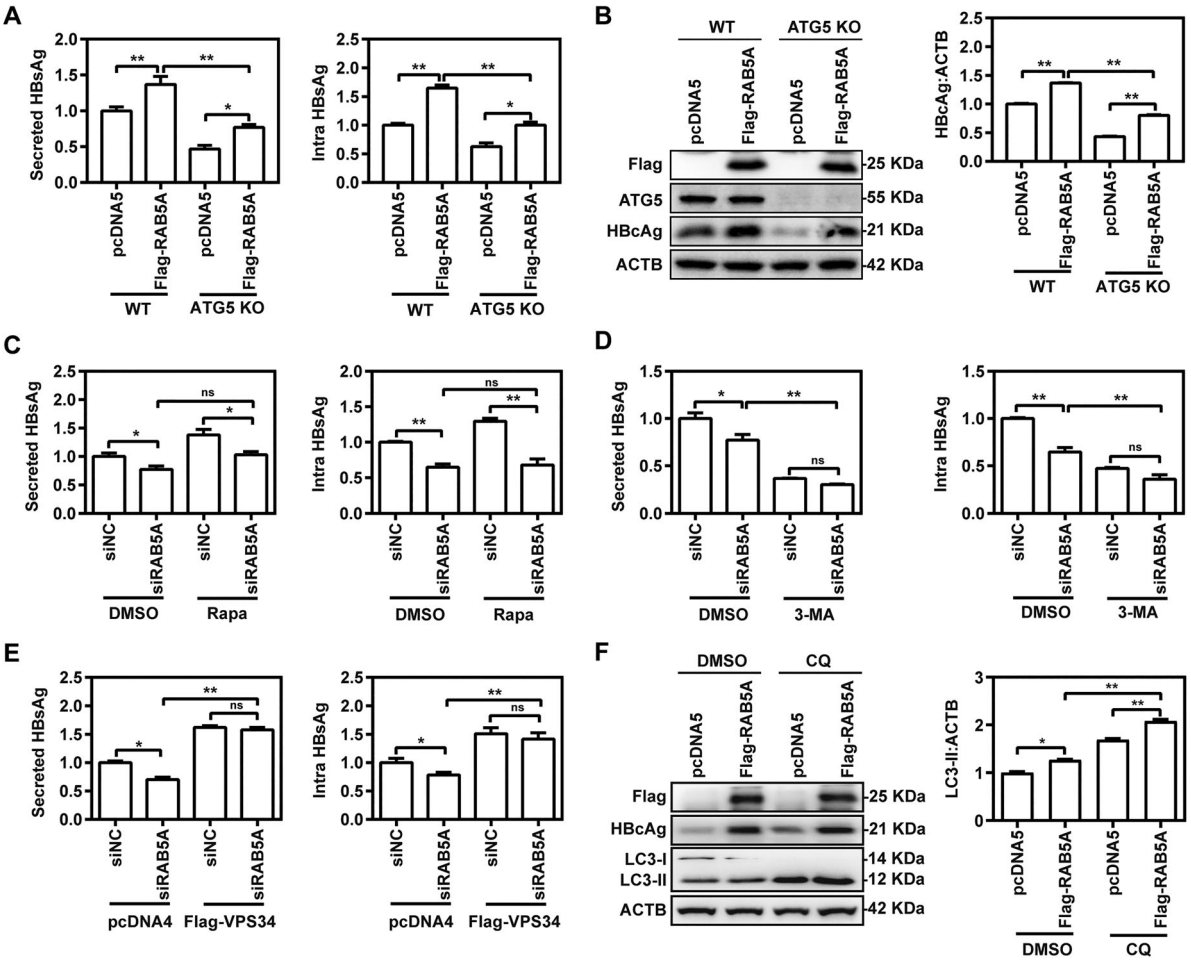

Figure 7. RAB5A promotes HBV replication through enhancement of autophagy induction. (A-B) Wild-type (WT) and ATG5 knockout (KO) Huh7 cells were co-transfected with plasmids pHBV1.3 and Flag-RAB5A or control vector pcDNA5. The levels of secreted and intracellular HBsAg were determined by ELISA. The levels of ATG5 and HBcAg were analysed by western blotting analysis. (C-D) HepG2.2.15 cells were transfected with 40 nM siRNAs against RAB5A (siRAB5A) or siRNA negative control (siNC). At 24 h post-transfection, the cells were treated with 2 μM rapamycin (Rapa), 10 mM 3-MA or DMSO for 48 h. (E) Huh7 cells were co-transfected with plasmids pHBV1.3, Flag-VPS34, and 40 nM siRAB5A or siNC for 72 h. (F) Huh7 cells were co-transfected with plasmids pHBV1.3 and Flag-RAB5A or pcDNA5 for 24 h, followed by treating with 10 μM chloroquine (CQ) or DMSO for 48 h. The levels of HBcAg and LC3 were analysed by western blotting analysis. *$P < 0.05$, **$P < 0.01$; ns, not significant.

could not reverse the inhibitory effect of RAB5A silencing (Figure 7C), whereas 3-MA treatment nearly attenuated the inhibitory effect of RAB5A silencing on SVP expression (Figure 7D). Subsequently, we further observed that VPS34 overexpression reversed the inhibitory effect of RAB5A silencing on HBsAg production (Figure 7E). The effects of RAB5A silencing and VPS34 inhibitor or overexpression were resulted from the multiple roles of VPS34, which has revealed to not only participate in autophagy initiation but also promote EE-LE transition [28,35]. To further verify the interaction of RAB5A and VPS34 in HBV-infected cell model, we further performed endogenous and exogenous Co-IP assays, respectively. The results showed that RAB5A had an interaction with VPS34 (Figures S8A and S8B), indicating that RAB5A may induce the activation of class III phosphoinositide 3-kinase (PI3KC3) complex to initiate autophagy [36,37]. To evaluate the effect of RAB5A on autophagy flux, Huh7 cells with or without RAB5A overexpression were followed by treating with lysosome inhibitor CQ. Western blotting analysis revealed that RAB5A overexpression significantly enhanced the promoting effect of CQ treatment on LC3-II expression (Figure 7F), supporting that RAB5A enhances autophagy induction in HBV-infected cell model. Additionally, considering RAB5A deletion may change mechanistic target of rapamycin complex1 (MTORC1) activation to regulate autophagy initiation [38–40], we examined the effects of RAB5A silencing or overexpression on total and phosphorylated MTOR expression in HBV-transfected Huh7 cells. Western blotting analysis revealed that RAB5A silencing increased phosphorylated MTOR level (Figure S8C), whereas RAB5A overexpression exerted the opposite effect (Figure S8D), indicating that RAB5A inhibits MTORC1 activity. Collectively, the above results support that RAB5A increases HBV replication and HBsAg production through enhancing VPS34-mediated autophagy induction.

HBV infection enhances RAB5A-mediated dual activation of EE-LE system and autophagy

To investigate whether HBV infection enhances RAB5A-mediated EE-LE system activation and autophagy induction, western blotting analysis revealed that RAB5A silencing obviously attenuated the positive effect of HBV infection on EEA1, CD63 and LC3II expression (Figure 8A), supporting that HBV infection enhances RAB5A-mediated dual activation of EE-LE system and autophagy. Confocal microscopy and western blotting analysis further showed that HBV infection obviously increased the number of $EEA1^+$, $CD63^+$, $LC3^+$ and $LC3^+$ $CD63^+$ puncta as well as the levels of EEA1, CD63 and LC3II expression (Figure 8B–E). Therefore, these results support that HBV infection enhances autophagy induction and leads to the enhanced intertwining of endosomal and autophagic vesicle systems. Moreover, we observed that RAB5A exhibited a high ratio of colocalization with EEA1, and low colocalization with the ER marker protein mCherry-ER-3 (Figure S9A), indicating that RAB5A was mainly distributed in EEs. However, HBV infection induced a much higher ratio of co-localization of RAB5A with the ER marker PDI in HBV-transfected Huh7 cells (Figure S9B), suggesting that HBV infection may induce the translocation of RAB5A from EEs to ER. We further demonstrated that HBV infection increased RAB5A expression obviously in the separated ER fractions of HepG2.2.15 cells (Figure S9C) and HBV-transfected Huh7 cells (Figure S9D). Additionally, endogenous Co-IP assay results showed that HBV infection increased the interaction between RAB5A and EEA1 as well as VPS34 (Figure 8F), indicating that HBV infection enhances dual activation of EE-LE system and autophagy by increasing the interaction between RAB5A and EEA1 as well as VPS34. VPS34 can catalyze phosphatidylinositol (PI) to generate phosphatidylinositol 3-phosphate (PI3P) in EE or ER to activate EE-LE vesicle pathway or initiate autophagy [21,41]. We observed that HBV infection substantially increased PI3P expression on EE and ER (Figure 8G,H), indicating that HBV infection caused VPS34 activation on EE and ER by increasing the interaction between RAB5A and VPS34. Collectively, our data support that HBV infection enhances RAB5A-mediated dual activation of EE-LE vesicle system and autophagy (Figure 8I).

Discussion

Accumulating evidence indicates that RAB5A plays crucial roles in mediating HBV endocytosis and viral replication [4,9]. Herein, we found that HBV infection increased RAB5A mRNA and protein expression obviously in HBV-expressing hepatoma cell lines as well as clinical liver tissue samples from chronic HBV-infected patients. Moreover, RAB5A silencing inhibited HBV replication and SVP expression significantly, whereas RAB5A overexpression increased them. Mechanistically, RAB5A increases HBV replication through enhancing EE-LE system activation by interacting with EEA1 and through enhancing autophagy induction by interacting with VPS34. Additionally, HBV infection enhances RAB5A-mediated EE-LE system activation and autophagy induction. In short, HBV exploits RAB5A-mediated dual activation of endosomal and autophagic vesicle systems for its own replication and persistence.

We have revealed that silencing RAB5A has a significant inhibitory effect on HBV replication and SVP expression in HBV-transfected and -infected

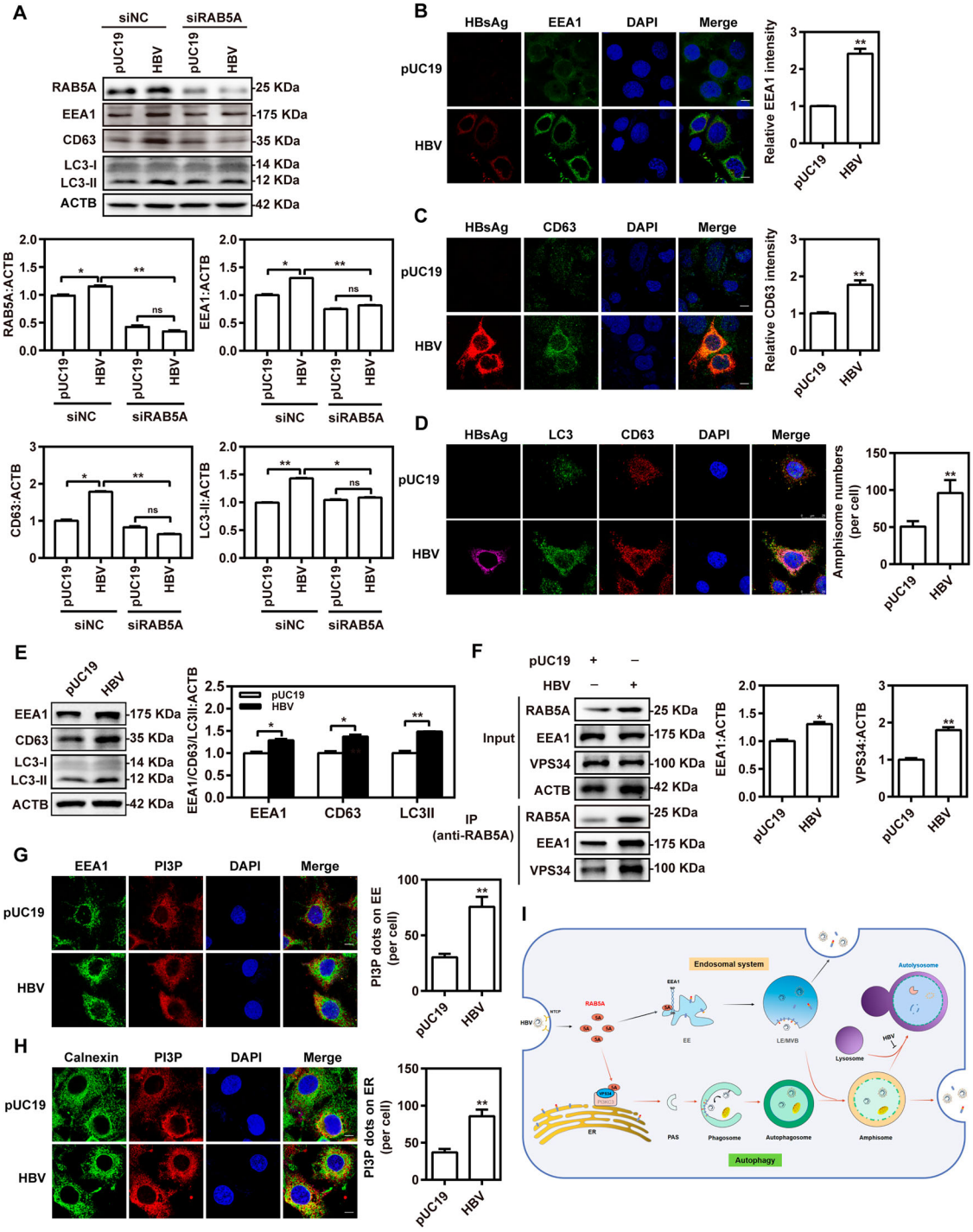

Figure 8. HBV infection enhances RAB5A-mediated dual activation of EE-LE system and autophagy. (A) Huh7 cells were co-transfected with pHBV1.3 or pUC19 and 40 nM siRNAs against RAB5A (siRAB5A) or siRNA negative control (siNC) for 72 h. The levels of EEA1, CD63 and LC3 were measured by western blotting analysis. (B–D) Huh7 cells were transiently transfected with pHBV1.3 plasmid or control vector pUC19 for 48 h. The dots of EEA1, CD63, and LC3 inside the cells were imaged by confocal microscopy. Scale bar, 10 μm. (E) The levels of EEA1, CD63, and LC3 were measured by western blotting analysis. (F) Endogenous co-immunoprecipitation of RAB5A and EEA1 or VPS34 were performed in Huh7 cells using anti-RAB5A antibody and immunoblotted with indicated antibodies. (G-H) PI3P dots on EE and ER were imaged under confocal microscopy. Scale bar, 10 μm. *$P < 0.05$, **$P < 0.01$; ns, not significant. (I) Schematic diagram of HBV utilizing RAB5A-mediated dual activation of endosomal and autophagic vesicle pathways for viral replication.

hepatoma cells. Inoue *et al.* evaluated the roles of three isoforms of RAB5 (including RAB5A, RAB5B, and RAB5C) in HBV replication and secretion process, observing that RAB5B silencing decreased the levels of secreted HBsAg and HBeAg [42]. They studied the effect of RAB5B on the intracellular and secreted HBV DNA expression in RAB5B-depleted cells and demonstrated that RAB5B is a key regulator of HBV production by regulating LHBs transport between the ER and LE. Our findings are also consistent with the effect of RAB5A on HBV replication, which preliminarily showed that RAB5A, as one of CCDC88A/

GIV downstream effectors, enhanced HBV production in HepG2.2.15 cells and HBV-transfected Huh7 cells [4], supporting that EE–LE vesicle system activation promotes HBV replication. However, the study did not test the promotion effect of RAB5A on HBV production in HBV-infected cell systems and the mechanisms of action. Our present study further revealed that RAB5A enhances EE biogenesis by recruiting EEA1 and thereby promotes the transition from EE to LE in HBV-infected cell models. Unfortunately, it remains unclear how EE–LE vesicle system activation results in increased HBV replication. We assumed that endosomal components might provide a physical scaffold for capsid/nucleocapsids (NC) assembly assemble or by sequestering related restriction factors of NC/virion degradation [11]. In addition to viral DNA replication, RAB5A also participates in increasing HBV infection. Macovei *et al.* reported that HBV infection strongly depends on RAB5A expression on the EEs of differentiated HepaRG cells [43], indicating that the transport through the network of endosomal vesicles is a crucial step for HBV uncoating and NC release, to establish viral infection. Therefore, RAB5A, may participate in modulation of multiple critical steps in the HBV life cycle.

Our findings provide evidence of an undefined molecular mechanism of RAB5A promotion of HBV replication through dual activation of endosomal and autophagic vesicle systems. We have elucidated that RAB5A combines with EEA1 to activate the endosomal vesicle system to promote HBV replication and SVP expression. Sequential transition between EEs and LEs is mediated by RAB5A and RAB7, and RAB5A is responsible for early endosome maturation [28,29]. Fauzyah *et al.* and Macovei *et al.* have demonstrated that RAB5A and RAB7 are involved in trafficking of HBV viral particles from EE to LE [9,43]. They observed that silencing of RAB5A or RAB7 blocked EE-LE maturation and inhibited early HBV infection markedly, and a considerable number of virions accumulated in the LEs. Similarly, knockdown of vital effectors, dynamin 2 and clathrin heavy chain for vesicle endocytosis, led to significant inhibition of HBV replication and progeny secretion [4]. We have obtained similar results that RAB5A activates the EE-LE transition by interacting with EEA1 as well as enhancing HBV replication and SVP production. Therefore, the RAB5A-mediated EE-LE vesicle pathway is closely related to promotion of HBV replication.

We have also elucidated that RAB5A promotes HBV replication by interacting with VPS34 to induce autophagy initiation. Accumulating evidence supports that autophagy plays a critical role in promoting HBV assembly and envelopment [11]. RAB5A participates in autophagy formation by changing MTOR kinase activity or forming PI3KC3 complex by interacting with VPS34 and BECN1 [36,37,39], indicating that RAB5A may modulate HBV replication by regulating the activity of PI3KC3 complex. Upon PI3KC3 activation, PI3P is generated in certain ER domains to form omegasomes, thus driving autophagy initiation [44]. Sir *et al.* have previously reported that HBx is able to bind to PI3KC3 and enhance its activation to initiate autophagy in Huh7.5 cells [45]. Unfortunately, they have not elucidated further how HBV affects PI3KC3 recruitment and activation for autophagy induction. To date, the definite mechanisms of HBV infection-induced autophagy initiation have not been elucidated. Our findings elaborate that RAB5A interacts with VPS34 as well as inhibiting MTORC1 activity to induce autophagy, leading to promote HBV DNA replication and SVP expression. We have further verified that a complete autophagic process is induced by RAB5A activation to form autophagosomes. Collectively, RAB5A-medaied autophagy induction as well as EE-LE vesicle pathway activation facilitates efficient HBV replication.

According to the results of the present study, HBV infection leads to the elevation of RAB5A mRNA and protein expression in clinical human liver tissues and *in vitro*. We attempted to identify which HBV protein is responsible for RAB5A elevation by overexpression of HBV encoding proteins, including HBc, HBx, SHBs, MHBs, LHBs, HBe and Pol. Notably, none of the HBV proteins increases RAB5A protein expression, suggesting that only one viral protein cannot elevate RAB5A expression. Furthermore, we observed that the intracellular HBV virions contribute to the elevation of RAB5A expression. Our previous studies have also identified that HBV infection decreases RAB7 expression and RAB7 interferes with autophagosome-lysosome fusion, which leads to the blockage of autophagic degradation and greatly promotes viral replication [46]. Endosomal RAB5A plays an essential role in the early-to-late endosome transition and the initiation of autophagy formation [28,37]. Previous studies have revealed that HBV infection causes massive autophagosome accumulation, which is beneficial for DNA replication [11,12,17,47]. Herein, we reveal that HBV infection markedly increases RAB5A expression and promotes the translocation of RAB5A from EEs to ERs. Therefore, HBV infection induced ER localization of RAB5A to activate VPS34 and initiate autophagic flux, which in turn enhances viral replication. In turn, the activated endosome system and autophagy were beneficial for viral replication and gene expression.

In summary, the present study has revealed that HBV infection elevates RAB5A expression in hepatocytes obviously and leads to dual activation of the EE-LE and autophagy vesicle pathways. We have elucidated that the activated EE-LE vesicle system and autophagy are conducive for HBV DNA replication

and SVP expression. Therefore, the authors speculate that RAB5 is one of major factors facilitating viral persistence in HBV infection, and RAB5A is a potential target for treatment of chronic HBV infection.

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Disclosure statement

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