ROMANIAN ACADEMY

School of Advanced Studies of the Romanian Academy

Institute of Biochemistry

PhD THESIS SUMMARY

Cellular factors involved in HBV infection

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The aim of the study

To date, approximately two billion of people have been infected with Hepatitis B Virus (HBV), and it is estimated that more than 350 million are chronic carriers of the virus. Chronic infection is the cause of severe liver pathologies, which evolve from inflammation and fibrosis to cirrhosis and hepatocellular cancer (HCC), which lead to the death of more than 1 million patients every year (WHO, 2017).

HBV infection can be prevented by vaccination and can be, to a certain extent, controlled by direct antiviral treatment, but it is not curable.

HBV infection stimulates the synthesis of fatty acids, and interferes with the metabolism of cholesterol and lipid droplets ("lipid droplets", LD). It has been demonstrated that HBV infection decreases the level of cholesterol and the amount of triglycerides in the cells (Suliman et. al, 2019). On the other hand, previous studies show that HBV replication leads to the expression of proteins that stimulate lipogenesis and cholesterologenesis (J Zhang et. al., 2021). Research carried out in our department has shown that the topology of the "large" envelope protein (L) of HBV depends on the intracellular cholesterol level, thus there is a direct link between the cholesterol content of the cell membranes and the efficiency of viral assembly (Dorobanţu C. et al, 2011).

The mechanisms by which HBV modulates lipid metabolism are not yet fully understood, although numerous studies in cell and animal models have addressed these interactions. The Sac1 protein, a type II phosphatidylinositol phosphatase, encoded by the SACM1L gene, catalyzes the dephosphorylation of PI4P to PI, with significant consequences on the cellular lipid metabolism and likely the HBV life cycle.

Despite the obvious existing connections, until the date of this study, how PI and the regulatory proteins of these lipids affect the life cycle of HBV has not been studied.

In this context, the aim of this work is to investigate the role of Sac1 in the HBV life cycle, using cellular models that allow detailed molecular analysis of viral particle replication, assembly and secretion.

Chapter I. Introduction

The mature HBV genome is represented by a circular relaxed DNA (rcDNA) molecule of approximately 3.2 kilobases (kb). HBV encodes seven viral proteins starting from four open reading frames: P encodes the viral polymerase (Pol), C encodes the capsid protein (HBc) and the HBe protein (an N-terminal extension of the HBc protein), X codes for the HBx protein (X) and S for the three surface (or envelope) proteins S (small), M (medium) and L (large). The envelope proteins are synthesized from three start codons S, M and L and have the sequence corresponding to the S protein in common, at the C-terminal end. The M protein additionally has the preS2 domain, at the N-terminal end, and the L protein, the preS1 and preS2 domains (Lambert and Prange, 2001; Tsukuda and Watashi, 2020). (Figure 1)

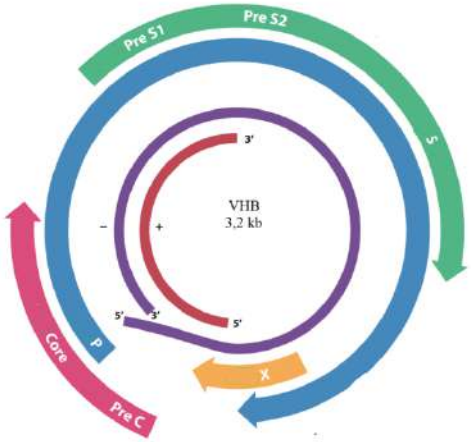

Figure 1. HBV encodes seven viral proteins starting from four different open reading frames (ORF): The first ORF encodes for the viral polymerase (Pol), the second encodes for the capsid protein (C) and the HBe protein (HBeAg), the third encodes for the HBx protein (X) and the fourth encodes three surface proteins that are synthesized from three start codons: S, M (preS2 and S domains) and L (preS1, preS2 and S domains). *Figure adapted from Graham Colm, 2020*

The HBV life cycle

The interaction of HBV with HSPGs (Heparan sulfate proteoglycans) especially with glypican 5, on the cell membrane of the hepatocyte, contributes to the localization of viral particles at the plasma membrane, facilitating their binding to the NTCP receptor and to the recently identified

co-receptor, the epidermal growth factor (EGFR), which mediates internalization of HBV through endocytosis (Tian J. et., al., 2021). (Figure 2)

This internalization complex is associated with E-cadherin (a calcium-dependent protein required for adhesion between cells). This association allows the re-localization of NTCP on the basolateral cell membrane, where the infection takes place (Hu, Q et. al., 2020).

The HBV-NTCP-EGFR complex is internalized in hepatic cells, mainly through clathrin-mediated endocytosis, although the participation of domains rich in caveolin, of the "lipid rafts" type, is not excluded (Macovei Alina et. al., 2010; Herrscher C. et. al., 2020). The interaction of HBV with clathrin is mediated by the preS1 domain of the L envelope protein, which also interacts with adapter protein 2 (AP-2) during internalization into hepatocytes. (Figure 2)

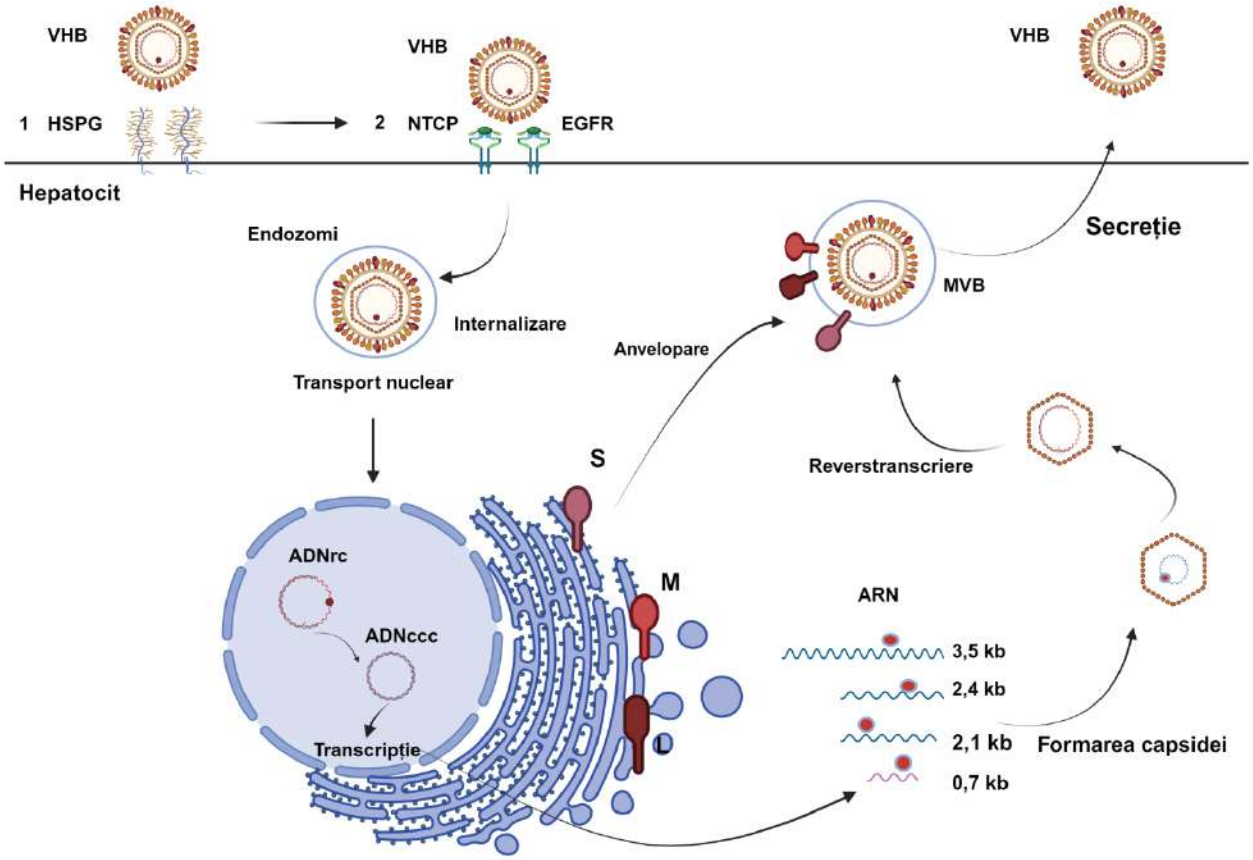

Figure 2. HBV life cycle. The first step in HBV infection is the interaction of the virions with the HSPG and the NTCP receptor on the cell membrane of the hepatocyte. The virus is internalized by endocytosis and the nucleocapsid is transported to the nucleus. In the nucleus, rcDNA is repaired and

cccDNA is formed, which is the template for the transcription of four different types of RNAs. pgRNA and the viral polymerase are included in the viral capsid, where reverse transcription and the formation of viral rcDNA takes place. Nucleocapsids are enveloped in the MVB where the surface proteins S, M and L, synthesized in the ER, are transported. The infectious viral particles are transported to the cell membrane via the secretory pathway mediated by MVB-ESCRT ("Endosomal Sorting Complex Required for Transport"). Following the fusion of the MVB with the plasma membrane, exosomes containing the viral particles are released. *The image was created by the author of this thesis with the BioRender.com program*

After internalization, the enveloped virus is carried via the endocytic pathway, being transported to early endosomes that mature into late endosomes and endolysosomes, where the release of the viral nucleocapsid into the cytoplasm takes place, mediated, most likely, by a membrane fusion process (Iwamoto M. et al., 2020).

After the release from the endosomal pathway, the viral nucleocapsid uses the microtubule network for the transport to the nucleus. In the nucleus, HBV rcDNA is converted to cccDNA.

cccDNA constitutes the template for the transcription of 5 types of RNA, a process mediated by RNA polymerase II, controlled by 4 promoters and two enhancers (Enhancer I and Enhancer II). (Prange R., 2012). The Precore/core promoter determines the synthesis of the 3.5 kb pregenomic RNA (pgRNA) which serves as a template for the translation of HBc proteins and viral polymerase but also of the pre-core RNA, which overlaps almost entirely with pgRNA and encodes for HBeAg. The promoters X, preS2 and preS1 determine the synthesis of subgenomic RNA of 0.7 kb, 2.1 kb and 2.4 kb, which encode for the proteins HBx, S, M and L. (Rall L. B. et. al., 1983; Prange R., 2012; Wand J. et al., 2020). (Figure 2). DNAccc transcription is regulated by numerous cellular factors with either ubiquitous expression, present in all cell types and involved in various processes, such as cell proliferation and differentiation or immune response, or strictly specific to hepatocytes (Turton K. L. et. al., 2020) .

The lipid metabolism in HBV infection

The mechanisms by which HBV infection affects lipid metabolism in hepatocytes have been analyzed in cell lines or transgenic mouse models. Thus, it has been demonstrated that HBV replication or assembly produce complex changes in the lipid metabolism of hepatocytes,

activating the expression of proteins involved in lipogenesis and cholesterologenesis, on the one hand, and decreasing fatty acid oxidation and bile acid synthesis, on the other hand (Yang F. et al., 2008; Zhang J. et al., 2021). Significant changes in phospholipid and sphingolipid metabolism were observed in hepatocytes or cell lines derived from liver tumors that were infected or transfected with HBV (Hyrina A et. al., 2022). Also, the concentration of sphingolipids in the serum of patients with hepatocellular carcinoma and especially those with acute liver failure caused by HBV infection is significantly higher than in healthy subjects (Schoeman J. C. et. al., 2016). (Figure 3)

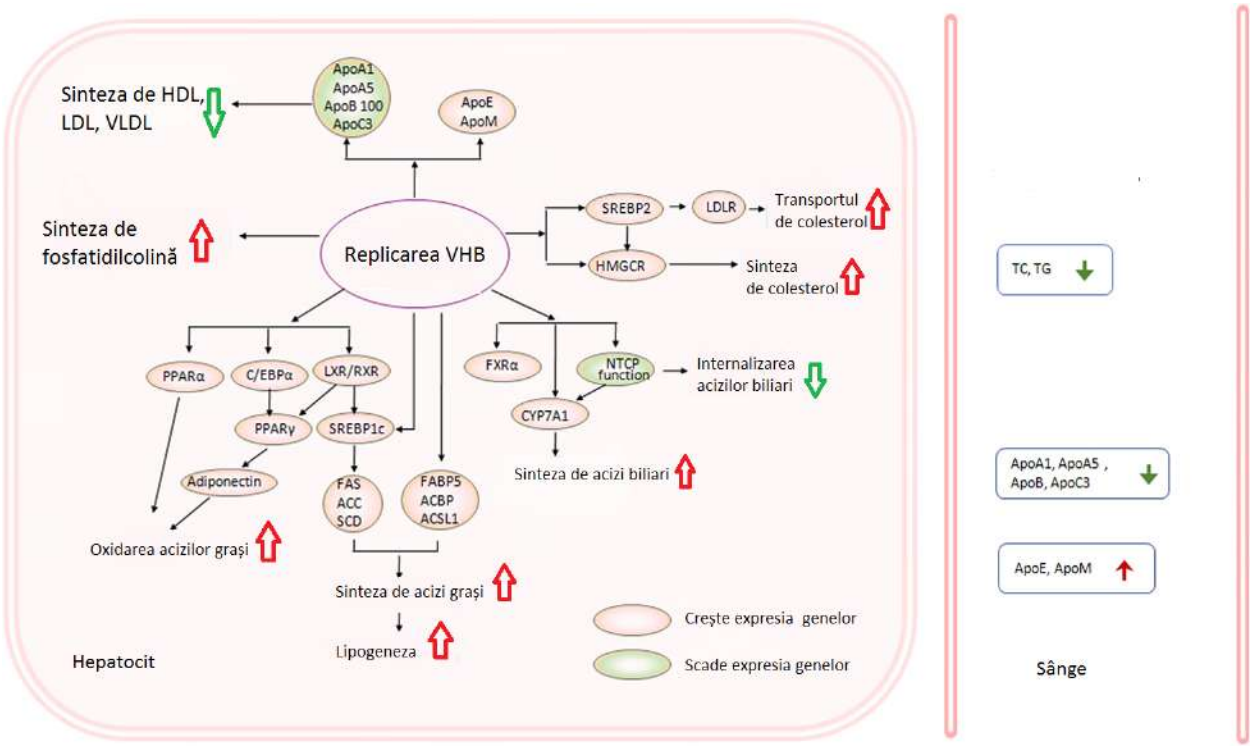

Figure 3. Hepatitis B virus produces major changes in the lipid metabolism of hepatocytes

During HBV infection, the synthesis of HDL, LDL and VLDL and the internalization of bile acids decrease. At the same time, the synthesis of phosphatidylcholine, the synthesis of fatty acids, cholesterol and the oxidation of fatty acids increase. These changes are determined by the decrease or increase in the activity of specific genes. *Figure adapted from Zhang J. et. al., 2021*

Phosphatidylinositol phosphates (PIP) are derivatives of PI that regulate numerous intracellular processes, signaling pathways, cytoskeleton organization, endo- and exocytosis, as well as autophagy. Mammalian cells produce 7 types of PIP: phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol 4-phosphate (PI4P), phosphatidylinositol 5-phosphate (PI5P), phosphatidylinositol 3,5-bisphosphate (PI3,5P2), phosphatidylinositol-4,5-bisphosphate (PI4,5P2), phosphatidylinositol 3,4 biphosphate (PI3,4P2), and phosphatidylinositol 3,4,5-triphosphate (PI3,4,5P3), each interacting with specific effectors (Fruman D. Aet. al. 1998).

Regulation of the level of each of these PI species is essential for vesicular transport, apoptosis, metabolism, reorganization of actin filaments, proliferation and cell signaling (Martin T. F. J. et. al., 1998).

PI4P is the precursor of phosphatidylinositol-4,5-bisphosphate (PI4,5P2) and of phosphatidylinositol 3,4,5-triphosphate (PI3,4,5P3) and is a molecule with an essential role in cell signaling (Fruman D.A et. al. 1998). Although PI4P is predominantly located in the trans-Golgi network (TGN), high levels of PI4P are also found at the plasma membrane, and a small amount of PI4P is located in the membranes of other intracellular organelles. Several studies have shown that Sac1 reduces the level of PI4P, creating a gradient in the secretory pathway that is used for the active transfer of other lipids between different cellular organelles, thus ensuring lipid homeostasis in the cell (Guo S. et. al. , 1999; Hughes W. E. et al., 2000; Xu J. and Huang X., 2020). In Sac1-deficient cells from yeast, insects, or mammals, PI4P accumulates not only in the ER and the Golgi Apparatus, but also in peripheral compartments, including secretory vesicles, the plasma membrane, and lysosomes (Faulhammer F. et. al., 2007).

Because viral proteins use the same transport mechanisms as the host cell proteins, it is expected that the depletion of Sac1 will affect the level of PI4P, with major consequences on the life cycle of HBV. Thus, this is the central hypothesis of this doctoral thesis, which will be verified via the experimental approach described in the following chapters.

CHAPTER II. Materials and methods

In this study I analyzed how modulation of the SACM1L gene expression in a transient or stable manner interferes with assembly and secretion of viral and subviral HBV particles, by using cell models such as Huh7, HEK293T, HepG2.2.15 and HepG2NTCP cells.

The depletion of the SACM1L gene was achieved with siRNAs, or by using the CRISPR/Cas9 genome editing technique. Depletion was confirmed in both cases by western blotting.

The amount of viral and subviral particles and HBsAg in the cell supernatant was then analyzed via ELISA and the amount of viral DNA and mRNA via real-time qPCR. Viral particles assembly was determined by ultracentrifugation in sucrose gradients. Through immunoprecipitation experiments, by using antibodies specific for the core protein and the envelope proteins, respectively, I quantified by qPCR the amount of intracellular viral nucleocapsids and secreted enveloped particles.

Finally, through immunofluorescence experiments I co-localized Sac1 and PI4P with viral proteins and cellular markers for compartments relevant for viral assembly, such as the ER and MVB.

Chapter III. Results and discussion

Transient depletion of the SACM1L gene in Huh7 cell line results in inhibition of HBV production

To analyze the role of the Sac1 protein in the production of HBV viral and subviral particles, Huh7 cells were transfected with the pTriExHBV1.1 plasmid for 3 days to allow efficient transfection, with fresh medium added each day, then treated with SACM1L siRNA or control siRNA for 24 or 48 hours. Cell lysates were then analyzed by western blot. As shown in Figure 4 A, the Sac1 protein was silenced in the Huh7 cell line by approximately 85% comparative to the control line at 24 hours and 70% at 48 hours after transfection. In this experiment, calnexin detection was used as a control for equivalent loading of the samples. The quantification of

subviral particles by ELISA (Fig. 4 B) and of viral DNA contained in nucleocapsids by qPCR (Fig. 4 C) in the cell supernatant, indicates a significant reduction, more evident at 24 hours. This is most likely due to more efficient silencing of Sac1 during this time frame. These initial results show that the Sac1 protein is involved in one of the stages of the HBV life cycle, and more experiments are needed to elucidate the mechanism of action.

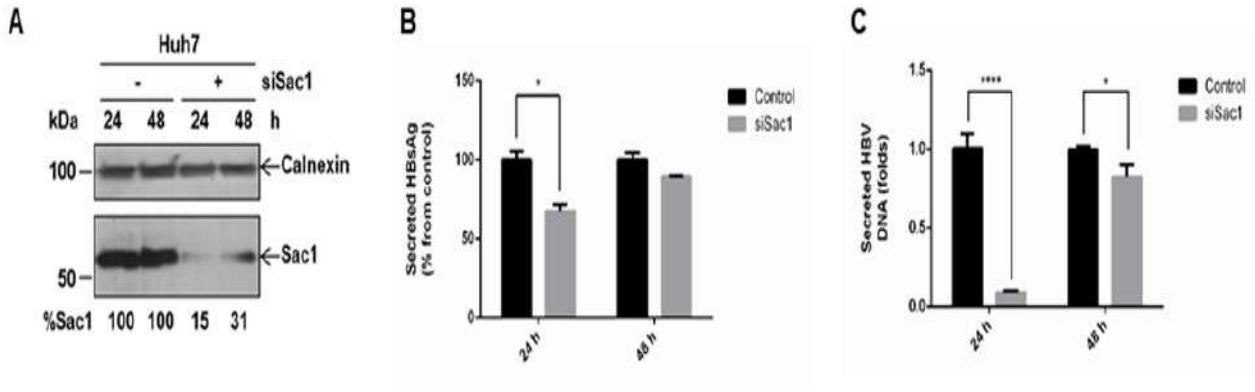

Figure 4. Effect of SACM1L silencing on SVP and HBV DNA production. (A) Huh7 cells were transfected with pTriEx HBV 1.1, then treated with 25nM Sac1 siRNA (+) or control siRNA (-) at 3 days post-transfection. At 24 or 48 hours after siRNA treatment, cells and supernatants were harvested. (A) Equal amounts of proteins were separated by SDS-PAGE and then detected by western blot with corresponding antibodies, using calnexin as sample loading control. The expression of Sac1 in cells treated with Sac1siRNA is compared to cells treated with control siRNA and expressed as a percentage. (B) HBsAg secretion was quantified by ELISA at 24 and 48 hours post-transfection with Sac1 siRNA and control siRNA. (C) Secreted viral particles were quantified by real-time PCR at 24 and 48 hours post-transfection with Sac1 siRNA and control siRNA. The value of $p<0.05$ (*) and $p<0.0001$ (** **). *Figure adapted from Popescu M. et al., 2022*

Transient depletion of the SACM1L gene in the HepG2.2.15 cell line

To confirm the results obtained in the Huh7 cell line and the correlation between the expression level of the Sac1 protein and the production of HBV viral and subviral particles, transient

depletion of the Sac1 protein was achieved in HepG2.2.15 cells. This cell line contains the HBV genome integrated into the nucleus of the host cell, thus enabling the analysis of the replication and secretion stages of the virus life-cycle. I chose this cell line for this experiment because transfection with pTriEx HBV 1.1 plasmid was no longer required, only transfection with siRNA to silence the SACM1L gene. Thus, we prevented the possibility that the cells would divide too much and the subsequent silencing with siRNA would not be effective. Sac1 expression was analyzed in HepG2.2.15 cells after siRNA treatment at 24 hours post-transfection, after electrophoretic separation of proteins from lysates, by western blot, with anti-Sac1 primary antibodies (Fig. 5 A). Western blot analysis of HBV envelope proteins (L, M, S) in the same cell lysates, using antibodies that bind to the common S domain, indicates similar expression in the control cell line and in the depleted HepG2.2.15 cells (Fig. 5A).

The results obtained show that envelope proteins S, M and L are detected at a higher level in cells in which Sac1 expression was silenced (Fig. 5 A), suggesting that the secretion of HBV envelope proteins may be disturbed in the absence of the Sac1 protein, resulting in intracellular accumulation.

Changes of viral protein levels in the cellular environment can be the consequence of several processes: a) disruption of cellular traffic b) inhibition of viral RNA transcription and implicitly of protein synthesis; therefore I decided to analyze the level of intracellular viral transcripts, in order to discriminate between the two main possibilities. Cellular RNA was purified in control and Sac1-depleted cells, and viral RNA was amplified by qPCR with specific primers. The obtained results show similar levels of intracellular viral RNA in both control and Sac1-depleted samples (Fig. 5 B). Together with the experimental data from Fig. 5 A showing that viral protein synthesis is not significantly altered between the depleted cells and control cells, these results demonstrate that RNA transcription and respectively translation of envelope proteins are not influenced by Sac1 protein silencing.

To determine whether the level of Sac1 observed by transient depletion (Fig 5 A) has an impact on the production of HBV viral and subviral particles (SVPs), samples from the supernatant of HepG2.215 cells were further analyzed. For virion analysis, viral DNA was purified from nucleocapsids, followed by qPCR quantification. SVPs were determined by ELISA that measures the level of HBsAg, the major constituent of SVPs. The data obtained showed an

inhibition of both SVP (Fig. 5 C) and viral particles (Fig. 5 D) produced in the silenced HepG2.2.15 cells, confirming the results obtained in the Huh7 cell line.

The ELISA technique quantifies the secretion of HBV subviral particles, an analysis by which the particles of interest interact with monoclonal antibodies that recognize the native conformation of the protein. Therefore, to check whether the decrease in the level of secretion of subviral and viral particles previously observed in the Huh7 cell line depleted of Sac1, was not caused by the change in the structure of HBsAg (in which case the antigen would no longer be recognized by the monoclonal antibodies on which we used), I first performed a pulse-chase experiment, by labelling proteins in their native state. HepG2.2.15 cells were treated with ^{35}S methionine/cysteine for 30 minutes to radiolabel proteins during their synthesis, then left for 3 hours in medium supplemented with 10 mM L-methionine, to quench the labeling by competition of excess unlabeled methionine with the radiolabeled one for protein incorporation (Fig. 5 E). The results indicated an important reduction of the S, M and L proteins in cell supernatants, confirming the inhibitory effect observed previously on SVP (Fig. 5C).

In addition to HBsAg that assembles in the SVP, HBV-infected cells also produce HBeAg, a derivative of the capsid (core) protein that, following N-terminal extension, can be translocated to the ER. HBeAg is processed by furin at the trans-Golgi level, which removes a sequence of 34 amino acids from the C-terminus, after which the mature antigen is secreted (Messageot F. et. al., 2003). Thus, the two HBV antigens are secreted following different processes. Quantification of HBeAg antigen in the supernatant of HepG2.2.15 cells treated with Sac1 siRNA and control siRNA, shows similar amounts of protein produced in Sac1 silenced cells and in the control cell line (Fig 5 F). Since the two viral antigens are exported from the cell through the common secretory pathway, this result suggests a specific effect of Sac1 depletion on HBsAg and not a general, non-specific action on cell trafficking.

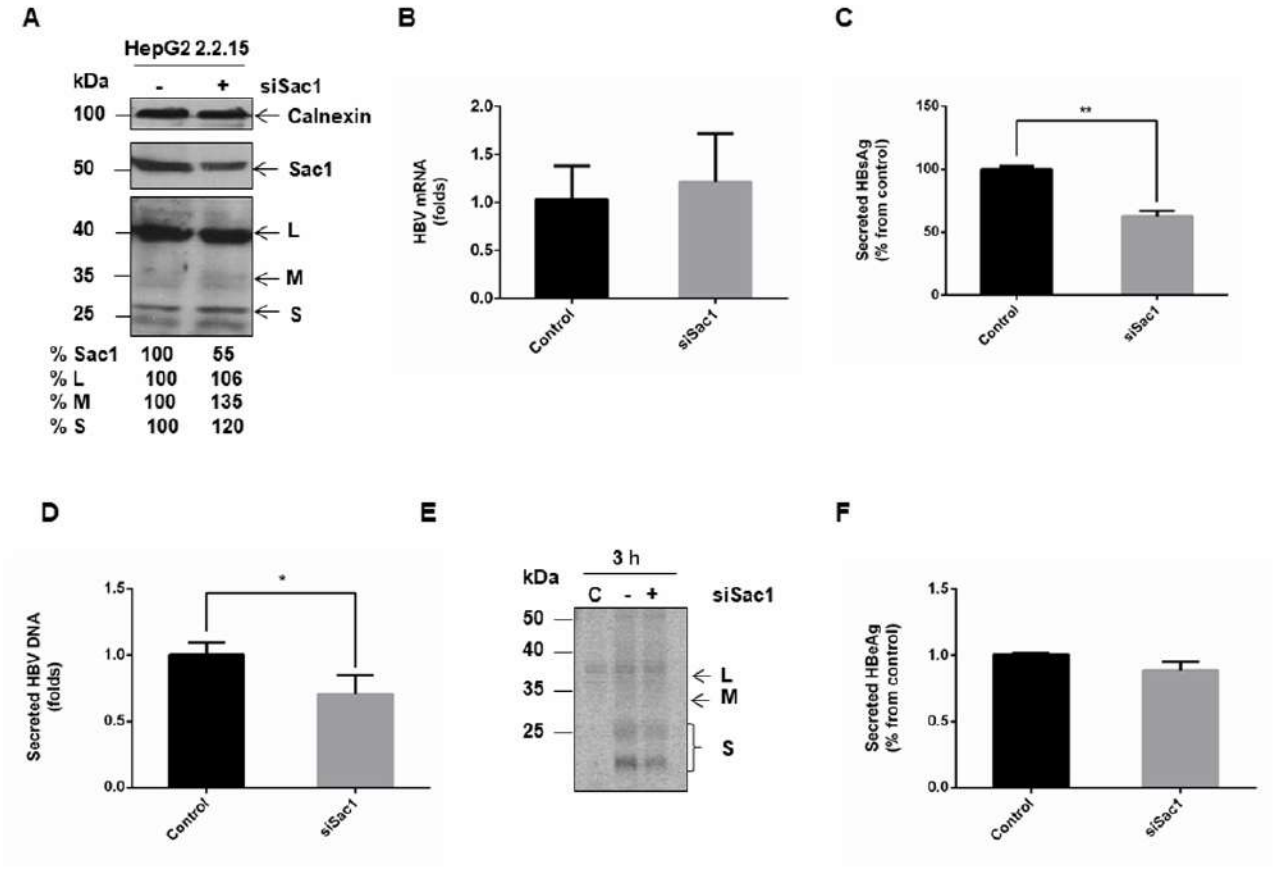

Figure 5. Effect of SACM1L gene depletion in HepG2.2.15 cells. HepG2.2.15 cells were treated with 25nM Sac1 siRNA or control siRNA for 24 hours. At 24 hours after siRNA treatment, cells and supernatants were harvested. (A). Equal amounts of protein were subjected to western blot analysis, using calnexin as a sample loading control. (B) Quantification of intracellular viral mRNA was performed via qPCR, 24 hours after transfection. (C) Secretion of subviral particles was analyzed by quantification of HBsAg via ELISA, 24 hours after transfection (D). Secreted viral particles were quantified by real-time qPCR at 24h post-transfection with SACM1L siRNA and control siRNA. (E) Cells were radiolabeled with 35S methionine/cysteine, and supernatants were immunoprecipitated with a mixture of anti-S and anti-preS1 antibodies. (F) The amount of secreted HBeAg was quantified 24 hours post-transfection with SACM1L siRNA (or control siRNA). The amount of viral RNA in Sac1 depleted cells and control cells was analyzed by qPCR at 24 hours post-transfection. p-value<0.01 (**) (C) and p<0,05 (*) (D). *Figure adapted from Popescu M. et al., 2022*

Secretion of SVP and viral DNA from Huh7 cells that have the SACM1L gene stably depleted by CRISPR/Cas9 gene editing

Previous experiments using siRNA for transient silencing of the SACM1L gene indicate a short period of time in which the reduction of Sac1 expression is significant (24 or 48 hours). This short period is not enough to be able to experimentally analyze the "recovery" of the pre-silencing phenotype by overexpression of Sac1 in cells depleted of endogenous Sac1 and to investigate in detail the mechanism of action that determines the effects observed in the secretion of viral and subviral particles. For this reason, I used a Huh7 cell line with the SACM1L gene depleted with the CRISPR/Cas9 genome editing technique, obtained in our department by my colleague, David Patriche, in another project. The method of obtaining, sequencing and characterization of these lines are the subject of another doctoral thesis.

Three clonal lines, denominated Sac1(-) c1-3 were initially analyzed by qPCR (Fig. 6 A) and western blot (Fig. 6 B) to determine Sac1 expression and gene inactivation efficiency at the RNA and protein level, respectively. One of the clonal lines named Sac1(-)c1, which has a similar growth rate to the parental line Huh7, was selected from the 3 initial clones for further experiments and to investigate the mechanism of action of Sac1 depletion on HBV.

As expected, reduced expression of Sac1 was observed by western blot in all three initially selected clones relative to the control (Fig. 6B). To determine whether the stable depletion of the SACM1L gene produces the same observed effects on HBV and subviral particle production as transient depletion, clonal Sac1(-)c1-c3 cells were transfected with the pTriExHBV1.1 plasmid for 24 h, and the supernatants were analyzed via ELISA 5 days post-transfection, to allow the accumulation of viral and subviral particles. The results show a significant reduction in the amount of subviral particles (SVP) in the supernatants of clonal cells obtained by the CRISPR/Cas9 genome editing technique, as determined by HBsAg quantification (Fig. 6 C).

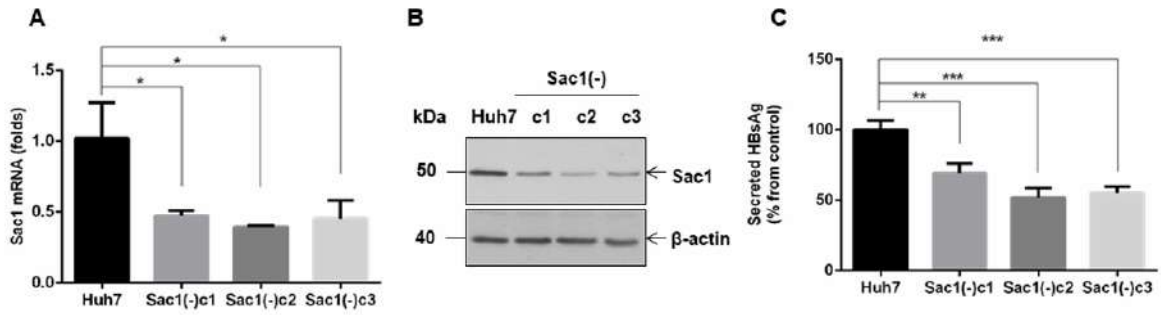

Figure 6. The effect of stable depletion of Sac1 by CRISPR/Cas9 genomic editing was investigated in Huh7 cells. (A) Quantification of Sac1 mRNA by qPCR from Huh7 Sac1(-) c1-c3 clones was performed by qPCR. (B) The amount of Sac1 protein was quantified by western blot, using actin detection as a control for equal sample loading. (C) Huh7 cells clones Sac1(-) c1-c3 were transfected with the pTriexHBV1.1 plasmid for 24 h and then maintained in culture for an additional 5 days. HBsAg secretion was quantified by ELISA 5 days post -transfection. p<0,05 (*) (A), p-value<0,01 (**) and p-value<0.001 (***) (C). *Figure adapted from Popescu M. et al, 2022*

Recovery of Sac1 expression and cellular phenotype by exogenous production of Sac1-GFP in cells stably depleted of Sac1

To unequivocally demonstrate the relationship between the production of HBV viral and subviral particles and the level of Sac1 protein in the cell, a "phenotype rescue" experiment was performed in Sac1-depleted cells. Thus, increasing amounts of Sac1-GFP plasmid were added to the Sac1(-) c1 clonal line, before and after transfection with the pTriExHBV1.1 plasmid that ensures viral replication. This approach was aimed to ensure sufficient amount of Sac1 during HBV replication, which was analyzed at day 8 post-transfection.

Exogenous expression of Sac1-GFP confirmed by western blot, shows an electrophoretic mobility corresponding to a higher molecular mass than the exogenous protein, as expected, due to the fusion with GFP (Fig. 7 A). Subviral particles and viral and were analyzed from the supernatant of transfected cells by ELISA (Fig. 7 B) and qPCR (Fig. 7 C). Experimental data show that both the level of SVP (Fig. 7 B) and virions (Fig. 7 C) secreted from Sac1(-)c1 cells in the absence of Sac1-GFP overexpression is significantly reduced, compared to the parental line

Huh7. It is important to mention that these results confirm the inhibitory effect also obtained in the experiments with transiently depleted Sac1 in Huh7, HepG2.2.15 and HepG2NTCP cell lines.

Analysis of the secretion of subviral and viral particles in the Sac1(-)c1 clonal line in the presence of Sac1-GFP, showed a normalization of their production (Fig. 7 B and 7 C), likely induced by Sac1. Moreover, this recovery effect is more accentuated at higher concentrations of the Sac1-GFP plasmid. The relatively high concentrations of Sac1-GFP plasmid required to compensate for the effect of endogenous Sac1 depletion may be explained by the lower number of cells simultaneously expressing Sac1-GFP and the HBV genome post-transfection compared to those in which only one of the plasmids was successfully delivered.

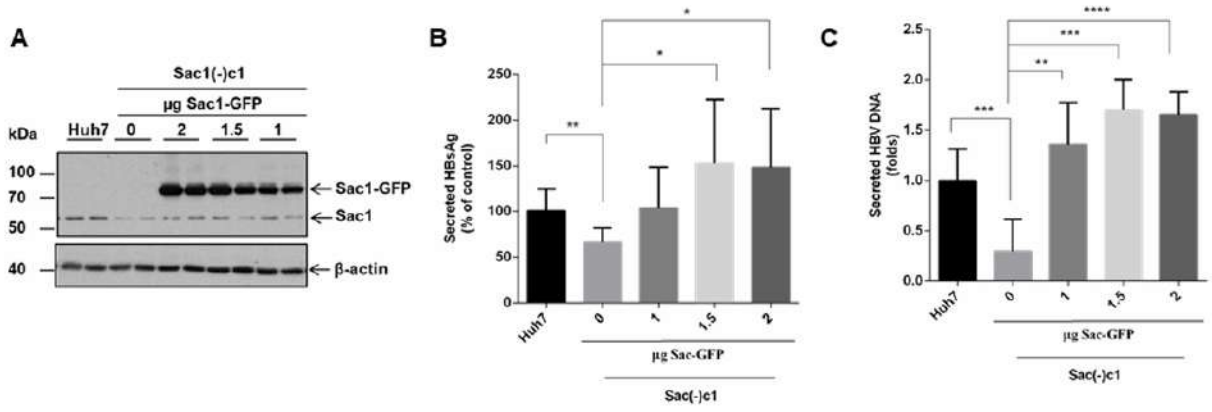

Figure 7. Recovery of Sac1 expression and cell phenotype by exogenous production of Sac1-GFP in cells stably depleted of Sac1. Control Huh7 cells and the Sac1(-)c1 cell line were transfected with 2 µg of pLNCX-GFP plasmid, as a control, or increasing amounts of pLNCX-GFP-Sac1 plasmid for 24 hours. One day post-transfection the cells were transfected with 0.3 µg of pTriEx-HBV 1.1 for 5 days, then they were transfected with 2 µg of plasmid pLNCX-GFP, or with increasing amounts of plasmid pLNCX-GFP-Sac1, as shown in the figure. Cells and medium were harvested 3 days after the last transfection. (A) Cell lysates were analyzed by western blot for analysis of endogenous and exogenous (GFP-fused) Sac1 protein expression. Actin detection was used as a sample loading control. (B) HBsAg secretion was analyzed in cell supernatants via ELISA, and expressed as a percentage of the Huh7 control line. (C) Viral nucleocapsids were purified from the cell supernatant and the amount of viral DNA was quantified by qPCR. The value of $p<0.05$ (*), 0.01(**), 0.001 (***) and 0, 0001 (****).*Figure adapted from Popescu M. et al, 2022*

Analysis of viral particle envelope in Huh7 cells with stably depleted SACM1L gene

The lower number of HBV particles secreted from clonal cell lines that have transiently or stably depleted SACM1L gene may show reduced production, or impaired transport to the extracellular space. To discriminate between these possibilities, an analysis of the intracellular level of particles was necessary. Also, the secreted viral particles can be enveloped or represented by nucleocapsids without an envelope. The qPCR quantification method, because it strictly analyzes the encapsulated viral genome, regardless of whether it is enveloped or not, does not provide information about the completion of the morphogenesis process with the uptake of the coating proteins. This differentiation can be done by immunoprecipitation of cell lysates and supernatants with antibodies that specifically recognize different forms of virus particles (with, or without envelope), followed by quantification of the viral genome precipitated in the antibody-virus complexes, by qPCR. Control Huh7 and Sac1(-)c1 cells were first transfected with the HBV genome to initiate viral replication. Both lysates and supernatants were then immunoprecipitated, either with anti-core antibodies (which will react with non-enveloped particles, as this protects the capsid and implicitly the access of antibodies), or with anti-pre-S1 which will react exclusively with the L protein present in the viral envelope, thus excluding non-enveloped nucleocapsids. After immunoprecipitation, the resulting complexes were adsorbed on a Sepharose matrix conjugated with Protein G and then eluted. Viral nucleocapsids were purified from the complexes, and viral DNA was isolated and quantified by qPCR. A modest but significant reduction in the number of nucleocapsids and enveloped virions was observed in the supernatant of Sac1(-)c1 cells compared with control Huh7 cells (Fig. 8 A), suggesting that the inhibitory effect of Sac1 silencing on HBV secretion is predominantly in terms of enveloped particles.

Analysis of intracellular HBV particles showed a significant increase in non-enveloped particles in Sac1(-)c1 cells (Fig. 8 B), indicating that loss of Sac1 expression does not affect viral DNA synthesis (replication) and capsid assembly, while the envelope of the viral particles is strongly disturbed, resulting in their accumulation at the intracellular level (Fig. 8 C). The normalization of enveloped particles to the total amount of nucleocapsid particles, both in the intracellular (Fig. 8 B) and extracellular environment (Fig. 8 D), as well as the lack of accumulation of enveloped particles in Sac1-depleted cells (Fig. 8 B) support this hypothesis.

These results demonstrate that the effect of the Sac1 protein on viral particle secretion is due to a significant disruption of the envelope process. This may be the consequence of impaired transport of envelope proteins to multivesicular bodies (MVBs), where envelope proteins are taken up by nucleocapsids, or of defective assembly at the MVB level.

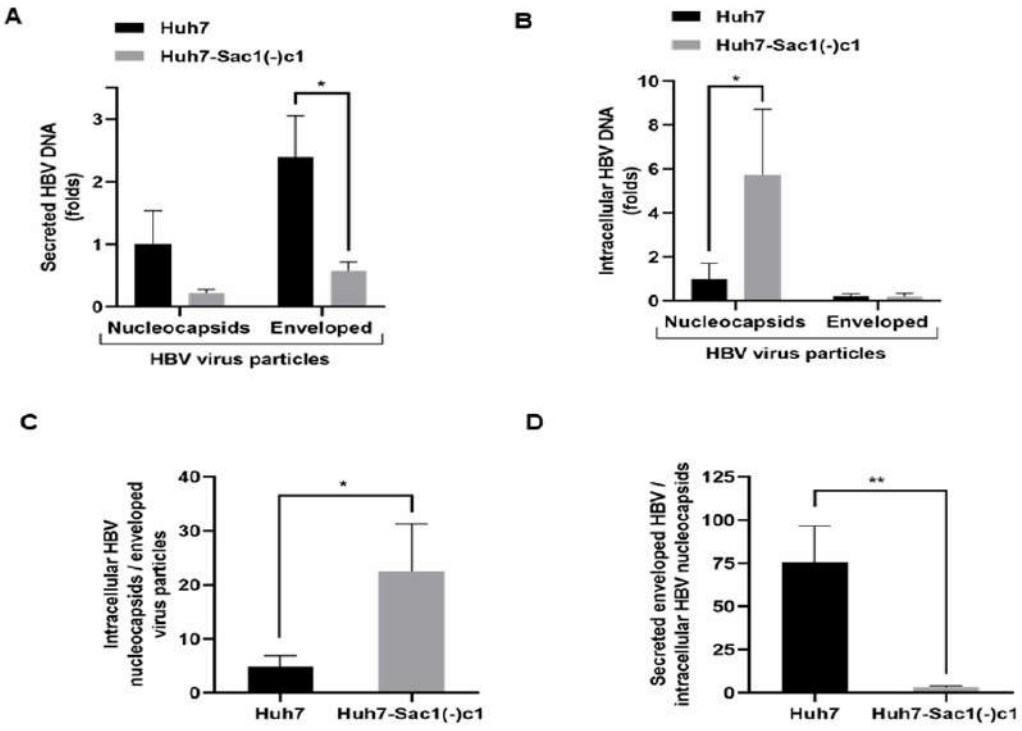

Figure 8. Analysis of viral particle envelope in Huh7 cells that have stably depleted SACM1L gene. (A-D) Huh7 and Sac1(-)c1 cells were transfected with pGEM-4Z-HBV 1.3 WT or P-null plasmid (control). Supernatants were collected 3 or 6 days post-transfection, and cells were harvested 6 days post-transfection. Cell medium (A) and cell lysates (B) were subjected to immunoprecipitation, using anti-core or anti-preS1 antibodies. Viral DNA was purified and quantified by qPCR. The results obtained for the Sac1(-) c1 cell line are compared to the Huh7 parental cell line. The ratio of nucleocapsids to enveloped HBV particles was investigated in cells (C) and supernatants (D). The value of $p<0.005$ (*) and $p<0.01$ (**). *Figure adapted from Popescu M. et al, 2022*

Transport of the viral envelope proteins in Huh7 cells that have the SACM1L gene stably depleted

Sac1 depletion causes a significant increase of PI4P in yeast, insect or mammalian cells followed by a disorganized redistribution of phospholipid in the Golgi Apparatus, ER, secretory vesicles, late endosomes and cell membrane (Liu Y. et. al., 2008). To determine the consequences of Sac1 depletion on intracellular PI4P localization, I performed immunofluorescence microscopy experiments, using normal and sac1-depleted Huh7 cells (the Sac1(-)c1 cell line). PI4P was observed to be distributed mainly in the secretory pathway in Huh7 cells, as revealed by the punctate fluorescence around the cell nucleus (Fig. 9, A and B, upper panels). PI4P does not significantly co-localize with GRASP65, a myristoylated membrane protein rich in cis-Golgi cisternae (Fig. 9 A) and partially overlaps the HBsAg (Fig. 9 B), which is predominantly localized in the ER and ERGIC. Notably, in Sac1(-)c1 cells, increased amounts and predominant intracellular distribution of PI4P in vesicular structures were observed, confirming the effects of Sac1 inactivation or depletion (Fig. 9 A and B, lower panels). Because secretion of the HBV envelope proteins was significantly reduced in Sac1(-)c1 cells, we further investigated the transport of the envelope proteins from the secretory pathway to MVBs, the organelles involved in the assembly and secretion of mature virions, by co-localization of the HBsAg with CD63, a protein found in MVB membranes (Poles et al. , 2009) (Fig. 9 C)

Importantly, a fraction of the total amount of HBsAg was found in the CD63-positive compartments in Huh7 cells (Fig. 9 C, upper panel), demonstrating the transport of the envelope proteins to the cellular sites used by the virus for nucleocapsid envelopment. This fraction appears to decrease in Sac1-depleted cells (Fig. 9 C, lower panel). To confirm this observation, a quantitative analysis of the HBsAg/CD63 co-localization was performd by determining the Mander's M1 co-localization coefficient. This analysis indicated a significant reduction in colocalization of the two proteins in Sac1(-)c1, cells compared to Huh7 control (Fig. 9 C, right). Together, these results suggest that transport of the HBV envelope proteins from the Golgi Apparatus to the MVB is impaired in Sac1-depleted cells.

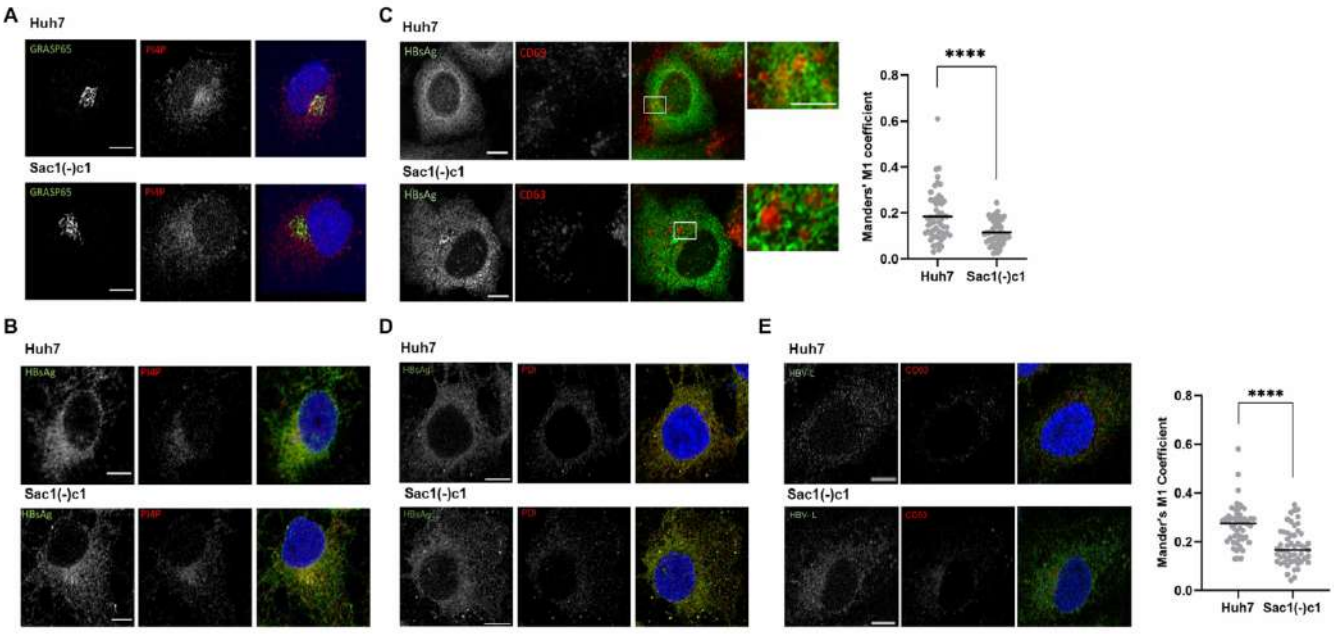

Figure 9. Immunofluorescence microscopy in Huh7 cells with stably depleted SACM1L gene. (A-C) Huh7 and Sac1(-)c1 cells were seeded on microscope slides. Cells were transfected with pTriExHBV1.1 for 48 hours, then fixed, and treated with specific primary antibodies, followed by incubation with Alexa Fluor 488 (green) or Alexa Fluor 594 (red) conjugated secondary antibodies. Nuclei were labeled with DAPI (blue). (A and B) Co-localization of PI4P with either GRASP65 (A) or the HBsAg (B) in Huh7 cells (upper panels) and Sac1(-)c1 cells (lower panels). (C) Co-localization of the HBsAg with CD63 in Huh7 (upper panel) and Sac1(-)c1 cells (lower panel) and analysis of the Mander's M1 co-localization coefficient (right). The images were obtained with the 40x objective of the Zeiss Axio Imager.M2 microscope. *Figure adapted from Popescu M.A et. al., 2022*

Conclusions

Previous published results by other groups have indicated a crucial role for PIP4 in the replication and morphogenesis of RNA viruses, that rely on formation of intracellular membranous webs. PI4P lipids may contribute to recruitment of viral/host proteins at the membranous web for efficient viral RNA synthesis, by providing efficient binding sites. The study performed in this PhD thesis demonstrates for the first time that, by regulating the intracellular PI4P levels, the Sac1 protein is a host factor involved in the morphogenesis of HBV, which is a DNA virus. The results obtained in this work suggest that Sac1 and PI4P are cellular factors involved in the assembly and transport of the HBsAg and viral particles and highlight the possibility of developing new therapeutic strategies targeting this lipid phosphatase. The proposed mechanism of action for the contribution of these factors in the HBV life-cycle is schematically illustrated in Fig. 10.

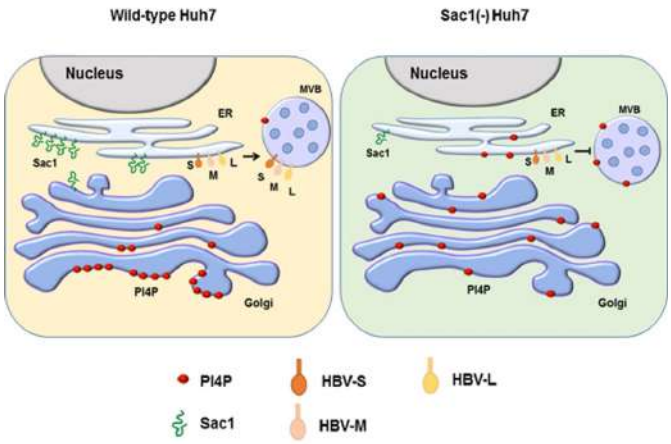

Figure 10. Proposed mechanism of action of the Sac1 protein in the HBV life cycle. The intracellular distribution of Sac1 and PI4P are closely related and mutually excluded. Sac1 located in the ER allows efficient transport of PI4P between different cellular compartments, especially at the Golgi Apparatus. In Sac1 depleted cells, the intracellular amount of PI4P increases and is redistributed non-specifically at the level of cell organelles, including the MVB where the HBV envelopment occurs. The transport of HBV envelope proteins to the MVB is impaired in these cells, which results in the intracellular accumulation of immature, non-enveloped nucleocapsids. *Figure adapted from Popescu M.A et. al., 2022.*

List of published articles within the doctoral thesis

Publications in ISI indexed journals

1. Sac1 phosphatidylinositol 4-phosphate phosphatase is a novel host cell factor regulating hepatitis B virus particles assembly and release

Popescu MA, Patriche D, Dobrica MO, Pantazica AM, FlintoacaAlexandru PR, Rouillé Y, Popescu CI, Branza-Nichita N. FI= 5,622

FEBS J. 2022 Jul 11. doi: 10.1111/febs.16575.

2. Class II phosphatidylinositol 3-kinase 2ß is a novel target for the potential development of antiviral drugs against the Hepatitis B virus

David-Sebastian Patriche, Mirela-AndreeaPopescu, Costin-IoanPopescu, NoricaNichita

PHARMACY JOURNAL, 2022 FI= 1.6

National conference papers

1. The role of phosphatidylinositol (Ptdins) phosphatase Sac1 in the HBV life cycle

Popescu MirelaAndreea, Patriche David-Sebastian, Popescu CostinIoan, NichitaNorica

RSBBM conference, Iaşi, 2019

2. Class II phosphatidylinositol 3-kinases regulate HBV life cycle in hepatoma cell lines

David-Sebastian Patriche, Mirela-AndreeaPopescu, Costin-IoanPopescu, NoricaNichita

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