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Studijní obor: Imunologie

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Exploring novel strategies targeting HBV

Nové přístupy cílené proti viru hepatitidy typu B

Diplomová práce

Vedoucí práce/Školitel:

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Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze Bc. Kristýna Šmilauerová

Ráda bych poděkovala především své školitelce Kláře Grantz Šaškové za inspiraci, povzbuzení a motivaci pro vědeckou práci. Dále bych ráda poděkovala celému týmu z laboratoře Jana Konvalinky, zejména Zuzaně Kružíkové, Janě Starkové a Irině Kontsevaye za rady ohledně diplomového projektu, Michalu Svobodovi a Monice Sivé za podporu při prvních krůčcích ve vědě. Velký dík patří i profesorům a garantům oboru Imunologie, kteří nejen mně výrazně pomohli na cestě za vědeckým povoláním předanými vědomostmi a zkušenostmi. Za podporu a trpělivost děkuji svým nejbližším přátelům a rodině, obzvlášť pak otci Pavlu Šmilauerovi za impuls věnovat se výzkumu.

Abstrakt

Přestože již existuje účinná a bezpečná vakcína proti viru hepatitidy B, nemocnost a úmrtnost na tuto nemoc jsou stále vysoké. Klíčem k vývoji spolehlivé léčby je detailní znalost životního cyklu viru a funkcí všech jeho složek. V předkládané práci jsme zkoumali interaktom kapsidového proteinu viru hepatitidy B. Použitím identifikační metody závislé na proximitní biotinylaci (BioID) spojené s hmotnostní spektrometrií jsme identifikovali seznam potenciálních proteinových kandidátů, kteří jsou buď významně nabohaceni (celkem 105 proteinů) nebo méně zastoupeni v buňce v přítomnosti kapsidového proteinu HBV (40 proteinů). Seznam také zahrnuje známé proteiny interagující s kapsidovým proteinem HBV: SRPK1 a SRPK2 a protein p53, o jehož expresi je známo, že je potlačen v důsledku interakce kapsidového proteinu HBV s transkripčním faktorem E2F1. Mnoho z nově identifikovaných možných proteinů interagujících s kapsidovým proteinem HBV se podílí na biologických procesech, u kterých je již známo nebo u nichž existuje podezření, že jsou ovlivněny HBV, jako jsou translační a transportní procesy nebo genová exprese a produkce makromolekul. Tato práce komplexně charakterizuje interaktom kapsidového proteinu HBV v živých buňkách a může tedy sloužit jako spolehlivý začátek pro hloubkovou analýzu interakce HBV-hostitel.

Klíčová slova: HBV, HBc, kapsidový protein, proximitní biotinylace

Abstract

An effective and safe vaccine against Hepatitis B virus already exists, yet morbidity and mortality of this illness are still high. The key to developing a reliable treatment is a deep knowledge of the virus' life cycle and functions of all its components. In the presented work we explored an interactome of the Core protein of the Hepatitis B virus. Using proximity-dependent biotin identification technique (BioID) coupled to mass spectrometry we have identified a list of potential candidates that are either significantly enriched (in total 105 proteins) or less abundant in the presence of the HBV Core protein in the cell (40 proteins). The list also includes known HBV Core interacting proteins SRPK1 and SRPK2, and p53 protein whose expression is known to be repressed due to the HBV Core interaction with the E2F1 transcription factor. Many of the newly identified possible HBV Core interacting proteins are involved in biological processes already known or are suspected to be influenced by the HBV such as translational and transporting processes or gene expression and macromolecule production. Overall, this work comprehensively characterizes the interaction landscape of the HBV Core protein in the live cells and might thus serve as a reliable start for in depth HBV-host interaction analysis.

Key words: HBV, HBc, Core protein, proximity biotinylation

List of abbreviations

HBV – Hepatitis B virus

HBc – HBV core protein

HBe – HVB precore protein

HBs – HBV surface protein

HBx – HBV X protein

HBcAg– HBV core antigen

HBeAg – HVB precore antigen

HBsAg – HBV surface antigen

HBxAg – HBV X antigen

pgRNA – pregenomic RNA

rcDNA – relaxed circular DNA

cccDNA – covalently closed circular DNA

NTCP – sodium taurocholate co-transporting polypeptide

HSPG – heparan sulphate proteoglycan

PBMCs – peripheral blood mononuclear cells

DMSO – dimethyl sulphoxide

PEG – polyethylene glycol

KCs – Kupffer cells

TLR – Toll-like receptor NFκB – nuclear factor kappa B

IFN – interferon

TGF – transforming growth factor

IRF – interferon regulatory factor

TNF – tumour necrosis factor

IL – interleukin

HIV – human immunodeficiency virus

PRRs – pattern recognition receptors

RIG-I – retinoic acid-inducible gene-I

DDX – DEAD-box helicase

APOBEC – apolipoprotein B mRNA-editing catalytic polypeptide

HCV – Hepatitis C virus

HDV – Hepatitis D virus

NLS – nuclear localisation signal

ARD – arginine rich domain

WB – western blot

SDS PAGE – sodium dodecyl-sulphate polyacrylamide gel electrophoresis

ER – endoplasmic reticulum

SRP – signal recognition partible

Contents

Introduction

Hepatitis B virus (HBV) is a small DNA virus (around 30-42 nm in diameter) from the *Hepadnaviridae* family. It was proven to cause the disease of hepatitis B by Baruch Blumberg, who was awarded the Nobel prize in Physiology or Medicine in 1976 (Blumberg 1977). Despite safe and functioning vaccine, about 250 million people worldwide currently suffer from a chronic hepatitis B infection and the HBV causes death of 880 thousand patients each year (WHO 2019). The virus in transferrable through blood and bodily fluids, making the mother-to-foetus transition the most common way of infection.

The virus is enveloped in a lipid bilayer originating from the host cell, which contains the viral surface proteins – HBs. Inside the lipid bilayer is a nucleocapsid consisting of the capsid protein – HBc or the HBV Core protein. The capsid carries the viral rcDNA (relaxed circular DNA) and viral RNA-dependent DNA polymerase. Two additional viral proteins – the precore protein (HBe) and the X protein (HBx) are usually not present in the capsid but have multiple important functions inside the host cell. The capsid can contain also some endogenous molecules, which might function either as enhancing or as restriction factors.

The life cycle of hepatitis B virus has been mostly elucidated, however various roles of its proteins have not been entirely discovered. A sufficient knowledge about mechanisms of the viral survival tactics is necessary for development of new treatment possibilities. This is important because current treatment causes only suppression of the infection but does not cure it completely. A better understanding of the viral life cycle and function of its components is crucial for future research and possibly even for HBV eradication.

It has been reported in several articles, how some of the viral proteins affect the immune system or exploit endogenous processes for their survival. However, the exact molecular mechanisms are often not described and many other roles and interactions of the viral proteins with endogenous molecules remain unknown.

In my diploma thesis I focused on exploring new roles of the HBV capsid or so-called “Core” protein by mapping its interactome. I showed that by using the proximity-dependent biotinylation technique (BioID) for the HBV Core interactome mapping in live cells, a list of significantly enriched or less abundant proteins in the cells expressing HBV Core can be produced. I analysed the network formed by the putative HBV Core interactants and speculated

about their possible roles in HBV infection. Finally, one of the hits was validated as a novel interacting partner of the HBV Core protein.

1 Literature Review

1.1 Hepatitis B virus

1.1.1 Life cycle of the virus

The HBV virion non-specifically binds the heparan sulphate proteoglycan (HSPG) on the surface of the hepatocyte (Schulze, Gripon et al. 2007) and then enters the target host cell by specific interaction with the sodium taurocholate co-transporting polypeptide (NTCP) that is expressed on the basolateral membrane of the hepatocyte (Yan, Zhong et al. 2012) (Figure 1). Recently, it has been found that HBV also requires the epidermal growth factor as a cofactor for the internalisation (Iwamoto, Saso et al. 2019).

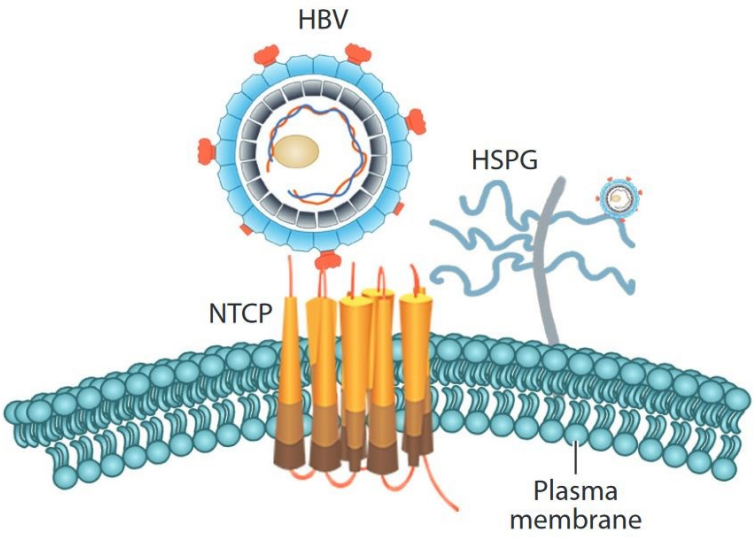


Figure 1: Entry of the virus is mediated by non-specific binding to the HSPG and by specific interaction with the NTCP receptor on the basolateral membrane of the host cell (Adapted from Li (2015)).

After binding to the NTCP receptor, the outer lipid envelope of the virus, containing the surface protein HBs, fuses with the hepatocyte membrane and the nucleocapsid, formed by the capsid protein HBc, enters the cell (Figure 2). The precise mechanism of the entry is still unknown, but it was shown that it might be mediated by the clathrin-dependent endocytic pathway (Huang, Chen et al. 2012). The uncoated nucleocapsid is subsequently transported to the nucleus where the HBV genome in the form of the rcDNA (relaxed circular DNA) is released.

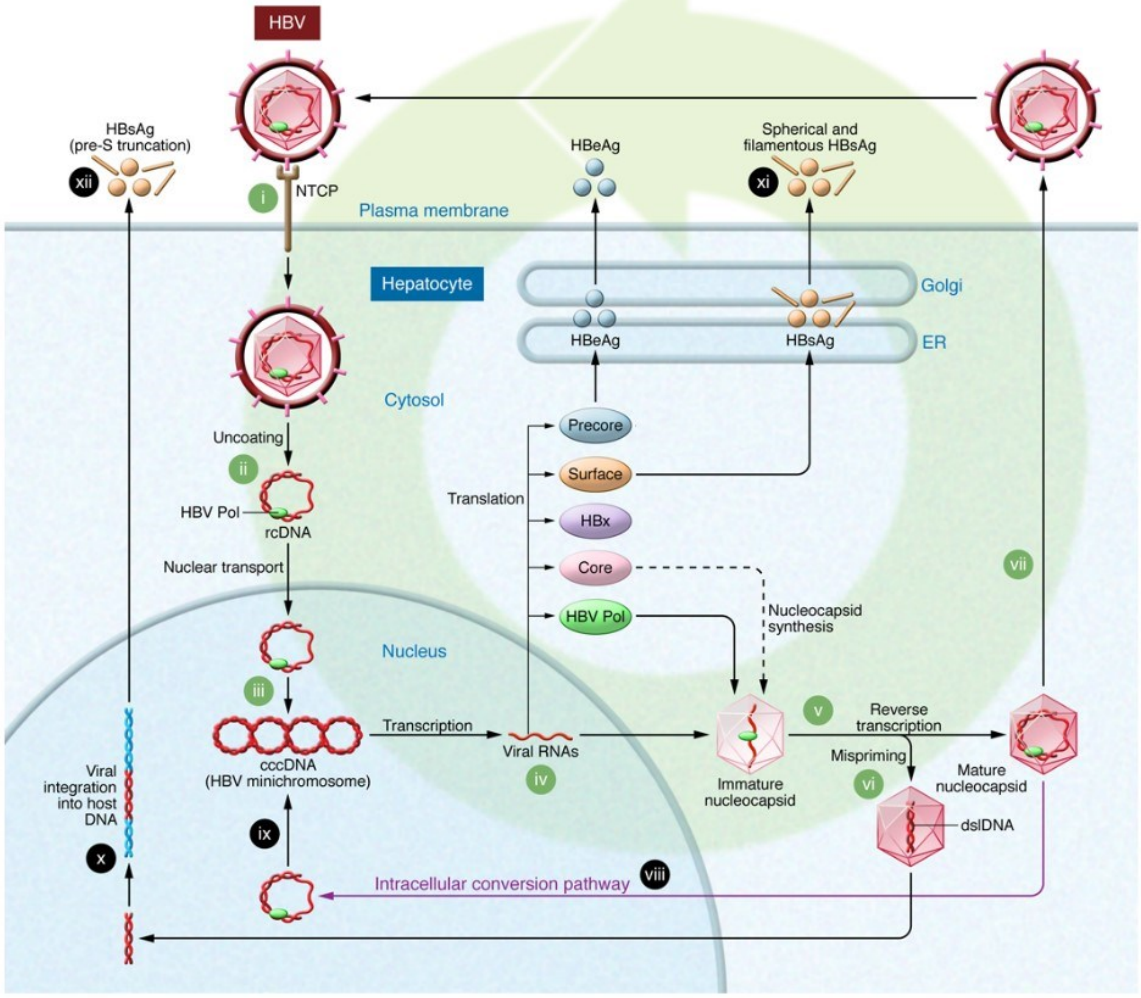


Figure 2: The life cycle of Hepatitis B virus. (I) HBV enters the cell via the NTCP receptor. (II) After uncoating, the nucleocapsid shuttles the rcDNA and the HBV polymerase into the host cell nucleus, (III) where it is transformed into the cccDNA. (IV) The minichromosome serves as a template for transcription of the viral mRNAs and the pgRNA, which serves as template for (V) reverse transcription. (VI) In rare cases the reverse transcriptase (RT) produces a linear dsDNA, which is then (X) integrated into the host genome. More usual is rather coating of the nucleocapsid in the host cell-derived membrane containing viral surface proteins and budding from the cell (VII) or (VIII & IX) reinfection of the nucleus thus replenishing the cccDNA reservoir. Besides being embedded in the lipid bilayer, surface proteins (HBsAg) are (XI) secreted from the cell in the form of subviral particles. (XII) This occurs also when the viral genome is integrated and theoretically serves as a suppressing mechanism of an adaptive immune system (Adapted from Revill and Locarnini (2016)).

The whole genome forms partially double stranded approximately 3.2 kbp long DNA with one complete (-) strand and one (+) strand with a 600–2100 bp gap (Summers, O'Connell et al. 1975). The complete (-) strand also bears the covalently attached HBV polymerase, which is upon translocation to the nucleus removed by the tyrosyl DNA phosphodiesterase (TDP2) (Koniger, Wingert et al. 2014). Furthermore, other host cellular factors, including endogenous

DNA polymerase κ and other still unknown DNA repair proteins, are recruited and the repair of the incomplete strand of the HBV genome proceeds (Kaplan, Greenman et al. 1973). The repaired form of the genome called cccDNA (covalently closed circular DNA) interacts with the viral proteins HBc and HBx (described further) and multiple host proteins including histones to create a nucleosome (Bock, Schranz et al. 1994, Bock, Schwinn et al. 2001, Belloni, Pollicino et al. 2009).

This episomal HBV minichromosome serves as a template for transcription of four viral mRNAs including the pre-genomic RNA (pgRNA). During virus maturation, the pgRNA serves as a template for reverse transcription into the rcDNA (Seeger, Zoulim et al. 2007, Seeger and Mason 2015). The viral DNA is transcribed into mRNAs by the endogenous RNA polymerase II, so the resulting transcripts include a 5'mG cap and a poly-A tail and are not recognised as extrinsic (Morikawa, Suda et al. 2016). It has also been shown that the hepatic nuclear hormone receptors are important for the transcription of the viral genome, but the exact mechanism of the transcription and its regulation still remains unknown (Tang and McLachlan 2001).

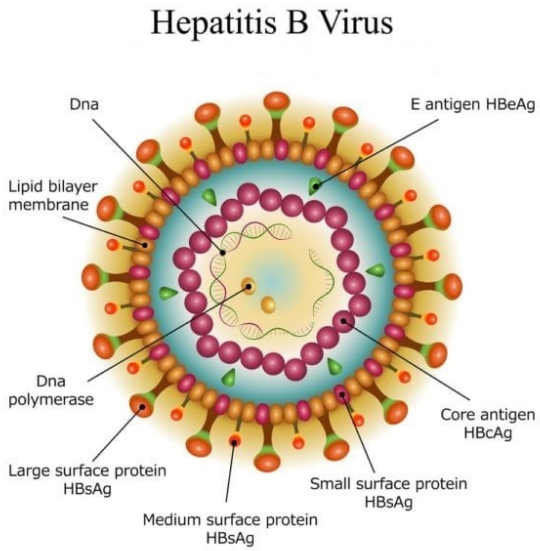


Figure 3: Structure of HBV virion: The HBV rcDNA and the reverse transcriptase are coated by nucleocapsid formed by the HBc protein (Core protein). The envelope is formed by the lipid bilayer from the host cell with the HBs proteins (Surface proteins; Large surface protein Pre-S1, Medium surface protein Pre-S2, Small surface protein S). The HBe protein (Precore protein) is a secretory protein which might be present in the virion and serves as an indicator of an active replication (Adapted from GoGraph.com-Moonnoon (2019)).

Viral mRNAs are translated into seven proteins: the small, the medium and the large surface proteins (Pre-S1, Pre-S2, S; HBs), the envelope/precore protein (HBe), the capsid protein ("Core protein", HBc), the RNA dependent DNA polymerase (P protein; RT) and the HBV X

protein (HBx), which is not part of the capsid. The P protein subsequently binds to the proximal 5′ end structure of the pgRNA called the ε-stem loop which initiates formation of the nucleocapsid consisting of 240 core protein subunits (Bartenschlager and Schaller 1992). Encapsidation, a process still not fully understood, triggers reverse transcription by the viral DNA polymerase. This whole process requires activity of the endogenous heat-shock proteins (Hu and Seeger 1996, Hu, Toft et al. 1997).

In the early stages of infection, the newly created capsid containing viral DNA is shuttled back to the host nucleus and disassembled to increase the number of cccDNA copies, ensuring viral persistence. Once there is enough of the surface protein, the nucleocapsid is enclosed into the host membrane forming a complete virion (Figure 3) and is secreted out of the cell (Summers, Smith et al. 1990). The mechanism of virion secretion is not entirely known but seems to be linked to the excretion pathway via Golgi apparatus and multi-vesicular body (Huovila, Eder et al. 1992, Watanabe, Sorensen et al. 2007). Besides the infectious mature virions, the smaller filamentous or globular bodies named sub-viral particles are created. They consist of the surface proteins (usually Pre-S2, S, less often Pre-S1) and are secreted in a similar manner to infectious virions but they are much more abundant. The high amount of theses immunogenic particles leads to exhaustion of immune cells, which are then unable to trigger an effective response (Dubois, Pourcel et al. 1980).

The HBV minichromosome has no replication origin and relies fully on the reinfection of the cell nucleus as described earlier. Even though the virus uses RNA intermediate and performs reverse transcription, incorporation of the viral DNA into the host chromosome is not its primal survival strategy, like in case of retroviruses. The HBV DNA integration is most likely due to an error of the stressed host cell. Integration into the host genome has been observed predominantly during chronic infection (Shafritz, Shouval et al. 1981, Murakami, Saigo et al. 2005) and, less often, during acute infection (Yoffe, Burns et al. 1990, Kimbi, Kramvis et al. 2005). However, it can later cause the remission of HBV infection in clinically healthy patients (free of serum HBsAg) (Wooddell, Yuen et al. 2017).

1.1.2 Infection

The virus is transmitted through a contact of an infected blood or body fluids with an open wound or genital and intestinal mucous membranes. This includes sexual contact (Fairley and Read 2012), injections (Hughes 2000), blood transfusions (Buddeberg, Schimmer et al. 2008)

or vertical transmission from mother to foetus. Infection occurs in most cases during birth (from mother to child) or during early childhood (Shapiro 1993).

Acute phase of HBV infection is in most cases asymptomatic. Some patients experience abdominal pain, jaundice, fatigue, nausea, and other symptoms typical for hepatic illness. For further development of the disease, a clearance of the viral particles from the body is crucial – if it is insufficient, the virus persists in the body and causes chronic infection (Chisari and Ferrari 1995). The risk of developing the chronic infection is the highest in infants and young children (25-90%) and low in adults (5-10%) (Chang 2007). In the long term, the chronic infection causes significant damage to the liver resulting in cirrhosis and eventually hepatocellular carcinoma (Yarrish, Werner et al. 1980, Michielsen and Ho 2011). The HBV infection can also result in the so-called occult phase, which seems asymptomatic, but in the long term still might lead to hepatocellular carcinoma. The replication of the HBV genome is stalled during occult phase, the serum HBsAg marker is undetectable but the cccDNA still remains in the host cell nucleus (Tabor, Hoofnagle et al. 1979, Makvandi 2016, Yip and Wong 2019).

Although mainly hepatotropic, HBV can be found in other tissues (Yoffe, Burns et al. 1990), from which the most studied are peripheral blood mononuclear cells (PBMCs). The infected PBMCs play a significant role in mother-to-child transmission (Xu, Liu et al. 2015) most likely because they carry transcriptionally active HBV (Mei, Yatsuhashi et al. 2000) and can also cause re-infection in patients in clinical remission (absence of HBV surface antigen in serum) (Brind, Jiang et al. 1997) by releasing new virions into the bloodstream.

A lot of information about HBV infection was obtained from studies in animal models. Historically most used animal models were Peking ducks infected by duck hepatitis B virus (Omata, Uchiumi et al. 1983) and American woodchucks infected by woodchuck hepatitis virus (Roggendorf and Tolle 1995). Nowadays, human cell lines, transgenic and humanised mice are much more usual but research is also conducted on primates including chimpanzees (Wieland 2015) and macaques (Dupinay, Gheit et al. 2013) which are susceptible to human strain of HBV (Dandri, Volz et al. 2005). Human cell lines used for research are often treated with stress-inducing factors, such as dimethylsulphoxide (DMSO) or polyethylene glycol (PEG), to be more susceptible to infection, HBV replication and the whole viral life cycle. DMSO also inhibits cell division to reduce dilution of minichromosome pool in infected cells (Gripon, Diot et al. 1993, Zhou, Zhao et al. 2017).

1.2 HBV interaction with the host

1.2.1 HBV and innate immunity

1.2.1.1 Innate immune response to HBV

Acute HBV infection is often asymptomatic and resolves on its own without transmission into a chronic phase, most likely due to an innate immune response. It has been known that the noncytolytic clearance of HBV from the liver cells is possible, but the exact mechanism has not been entirely elucidated (Guidotti, Rochford et al. 1999). A protective type I IFN mediated response has been shown to occur in HepaRG cell line, after infection of a recombinant virus carrying HBV genome (Lucifora, Durantel et al. 2010). However, this model does not exactly mimic the natural state, in which the replication is much lower in the early stages of infection.

Kupffer cells (KCs), the liver residing macrophages, have been proposed to have a crucial role in the regulation of immune response and fibrosis of infected tissue during a chronic phase (Kolios, Valatas et al. 2006). It was suggested that the most efficient response is mediated by interleukin-6 (IL-6) production by activated non-parenchymal liver cells which inhibits destructive T-cell response, but also blocks HBV genome expression and thereby also its replication (Hosel, Quasdorff et al. 2009). However, when co-cultured with the HBV particles, rat-derived KCs were shown to produce the transforming growth factor beta (TGF-β), rather than any inflammatory cytokines, which indicates that HBV might be inducing their pro-fibrogeneic function (Li, Zheng et al. 2012). Similar results were obtained from mouse-derived parenchymal and non-parenchymal liver cells (including KCs), where HBV proteins suppressed mechanisms of Toll-like receptor (TLR) activation. Specifically, the HBs, the HBe or the complete virions were able to reduce the production of IFN-β and inhibit activation of the nuclear factor kappa B (NFκB) and the interferon regulatory factor 3 (IRF-3). The same study also reported that after the induction of HBV replication, the pre-infected murine cells had reduced expression of the tumour necrosis factor alpha (TNF-α), IL-6 and favoured production of TGF-β (Wu, Meng et al. 2009). The HBV core antigen (HBcAg) has been shown to activate THP-1 macrophage cell line via TLR2 to produce pro-inflammatory cytokines (Cooper, Tal et al. 2005). It was reported that activation of KC in the mouse HBV carrier model via TLR2 decreases effectivity of the $CD8^+$ cells leading to an exhaustion phenotype which promotes chronic persistence of the virus (Li, Sun et al. 2015) and that the murine KCs are the main cause of immune tolerance of the viral infection by secretion of IL-10 (Xu, Yin et al. 2014). Interestingly, the expression of TLR2 in HBeAg seropositive patients was reported to be

significantly decreased in KCs, hepatocytes and peripheral monocytes, (Visvanathan, Skinner et al. 2007) which, when combined with tolerogenic cytokine environment, reduces their ability to properly activate an adaptive part of immune reaction.

1.2.1.2 HBV strategies to suppress an innate immune response

HBV has a long quiescent phase after it infects a new patient that can be described as an early phase stealth virus (Wieland, Thimme et al. 2004). When the virus enters the organism, it is not directly recognised due to the outer envelope made from the membrane of the original host cell. Even when the virus enters the host cell and is uncoated, the innate immune response is poorly induced, compared to, for example, the human immunodeficiency virus (HIV) (Stacey, Norris et al. 2009). This is mainly due to the fact that the rcDNA is transformed into the cccDNA nucleosome by the host factors and subsequently transcribed by the endogenous RNA polymerase II. That makes both the viral DNA and RNA virtually invisible for the innate pattern recognition receptors (PRRs). The low level of the innate immune response is also likely caused by the virus suppressing or blocking the effector mechanisms (Figure 4).

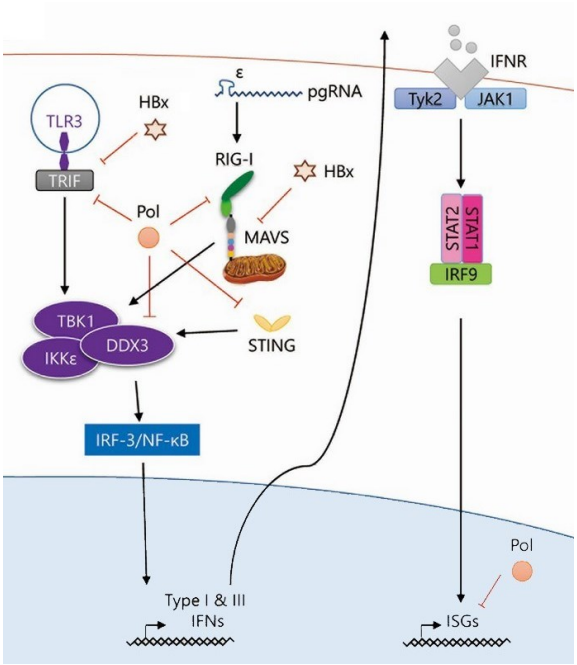


Figure 4: Viral products interfere with an innate pathogen sensing on many levels and still not all the inhibitory functions have been discovered yet (Adapted from Morikawa, Shimazaki et al. (2016).

It was shown that the HBx protein has a significant role in blocking the intracellular immune response. It binds the mitochondrial antiviral signalling protein (MAVS, also called virus-induced signalling adaptor (VISA), beta interferon promoter stimulator-1 (IPS-1) and Cardif)

and prevents its binding to other components of the activation cascade (Kumar, Jung et al. 2011). This includes the cytoplasmic RNA helicases RIG-I and the melanoma differentiation-associated gene 5 (MDA5), the main viral nucleic acid receptors (Wang, Li et al. 2010, Sato, Li et al. 2015). The HBx also causes degradation of MAVS by inducing its ubiquitination. Moreover, HBx downregulates the expression of MAVS and thus complexly blocks the MAVS-dependent retinoic acid-inducible gene-I (RIG-I)-mediated interferon beta (IFN-β) production (Wei, Ni et al. 2010). Furthermore, HBx downregulates TIR-domain-containing adaptor inducing interferon-beta (TRIF) which when uninhibited strongly hinders expression of the HBV genome (Hong, Zhou et al. 2015).

The HBV polymerase, besides reversely transcribing the viral pgRNA into the rcDNA, also has various effects on PRRs and their downstream elements. It was reported that HBV polymerase inhibits the response of the host cell to IFN-α and IFN-γ (Foster, Ackrill et al. 1991). Moreover, the HBV polymerase binds with its terminal protein domain to the promoter region of the alpha interferon (IFN-α)-inducible myeloid differential primary response protein (MyD88) gene. This region serves as a binding site for the signal transducer and activator of transcription 1 (STAT1) protein. Besides occupying the binding place, the HBV polymerase additionally prevents activation of STAT1 by IFN-α and its translocation to the cell nucleus (Wu, Xu et al. 2007, Chen, Wu et al. 2013). It was reported that HBV polymerase inhibits IFN-β expression by restricting interaction of the TANK-binding kinase 1 (TBK1), the IκBε kinase (IKKε) and the RNA helicase DDX3 (Wang and Ryu 2010, Yu, Chen et al. 2010). The HBV polymerase was also found to be blocking the cytosolic viral sensing by deubiquitinating the stimulator of interferon genes (STING). STING is upon activation polyubiquitinated via K63-ubiquitin chains which serves not as a degradation signal but regulates protein localisation and thereby enables transduction of an activation signal. Removal of the polyubiquitin chain inhibits activation of IRF3 and IFN-β induction (Liu, Li et al. 2015). This viral survival strategy was re-evaluated when it was determined that the hepatocytes do not express STING (Thomsen, Nandakumar et al. 2016). Another report showed that the overexpression of STING in the NKNT-3/NTCP cells (human immortalized hepatocytes exogenously expressing NTCP receptor) re-instates the resistance of the host cells to HBV infection, suggesting it as a possible future therapeutic option (Dansako, Imai et al. 2019).

1.2.2 HBV and the adaptive immune response

1.2.2.1 Adaptive immune response to HBV

During an acute phase, an effective adaptive T-cell response triggered by the expressing IFN-γ-induced genes can achieve the viral clearance. The $CD8^+$ T cell activity is crucial in this step and the virus will not be eradicated when the T cells are depleted. However, the T-cell activity also leads to pathogenesis depending on whether non-cytolytic (IFN-γ and TNF-α production) or cytolytic (apoptosis) functions of $CD8^+$ prevails (Thimme, Wieland et al. 2003, Wieland, Thimme et al. 2004). The $CD4^+$ T cells enable CD8+ T cell migration to the liver after primed with presented antigen in a suitable concentration. Too high (10 genome equivalents[1]) or too low (1 genome equivalent) dose causes slow immune response followed by increased immunopathology (Asabe, Wieland et al. 2009). Insufficient priming leads to T-cell deletion, anergy, exhaustion and tolerance of the antigens.

A damage of the liver during a chronic phase is not done by the virus itself but by a dysregulated immune reaction. A pathogenic effect is caused mainly by the $CD8^+$ cytotoxic lymphocytes (CTL), which can mediate desired virus clearance but also tissue destruction in a chronic phase (Maini, Boni et al. 2000). The $CD4^+$ cells producing IL-17 (T_H17) also play a significant role in inducing the pro-inflammatory environment during a chronic phase of infection and a liver damage (Zhang, Zhang et al. 2010).

1.2.2.2 HBV strategies to evade an adaptive immune response

The virus has developed several strategies to contradict the effects of the adaptive immune system, starting with inducing neonatal tolerance in foetuses by trans-placental migration of HBeAg, which has been documented on transgenic HBV mice (Milich, Jones et al. 1990). HBeAg induces tolerance in adult organism as well. Even though this has been shown again only in mice (Chen, Billaud et al. 2004), the data obtained from the patients correspond with the putative tolerogenic effect of the HBeAg. The patients infected by the mutant HBV strain, which was unable to produce HBeAg, showed more severe liver inflammation than patients infected by the wild-type HBV. The outcome of the liver-disease between both groups was not significantly different (Brunetto, Giarin et al. 1991).

[1] A genome equivalent is defined as amount of nucleic acid that is needed to be detectable in a purified sample to ensure presence of all genes of a given organism/virus.

The role of the dendritic cells in the whole immune process is up-to-date still unclear. Since HBV is an intracellular virus, the role of the B-cells is mainly producing the neutralisation antibodies, which ensures the clearance of the HBsAg-subviral particles from the blood and prevents the virus from entering the host cell. Positive immunisation results into the production of the antibodies against HBsAg in protective titre (Wismans, van Hattum et al. 1989, Shokrgozar and Shokri 2001).

1.2.3 HBV treatment – current and new approaches

1.2.3.1 HBV Vaccine

The anti-hepatitis B vaccine is in the most countries administered within 24 hours after birth with two or three follow-up booster shots. The vaccine or the single booster shot is also highly recommended to at-risk groups such as health-care workers, patients who need frequent blood transfusions or dialysis, injecting drug users, promiscuous people or male homosexuals. The vaccine is produced by the recombinant DNA methods, it is safe for use even during pregnancy and any side effects are very rare. The immunisation is ensured by the injection of HBs produced in yeast and leads to the production of the anti-HBs antibodies which provide protection against infection (WHO 2019).

Despite the global neonatal vaccine administration, there are still around 257 million people worldwide infected by the HBV. The most convenient way how to cure an HBV infected patient, would be the augmentation of effects of the endogenous HBV restriction factors.

1.2.3.2 Endogenous HBV restriction factors

Concerning the intrinsic immune response targeting HBV, the most discussed proteins are from the APOBEC family – apolipoprotein B mRNA-editing catalytic polypeptides. These proteins were initially identified during the HIV research. The *APOBEC3G* gene was the only one that could turn the state of the cell from “permissive to the virus” to “nonpermissive” during heterokaryon fusion experiments (Sheehy, Gaddis et al. 2002). During the HBV infection, APOBEC3G inhibits the viral replication by preventing capsid assembly (Turelli, Mangeat et al. 2004). Additionally, APOBEC3F was also reported to have equal role, however, neither of these two proteins initially seemed to have utilised their mRNA-editing function (Rosler, Kock et al. 2005). This conclusion was later shown to be a result of a faulty method protocol and the APOBEC3 proteins were shown to wield the DNA editing function. Upregulation of the

APOBEC3 proteins results in hypermutation of both DNA strands which leads to reduced replication of the HBV genome (Noguchi, Ishino et al. 2005, Suspene, Guetard et al. 2005).

It has been reported that several hepatocytic cell lines and the primal human hepatocytes upregulate production of the activation-induced cytidine deaminase (AID) when pre-treated by TNF-α and IL-1β. Unless the virus restricts the activation, these two pro-inflammatory cytokines trigger NFκB response pathway and increase the expression of AID. The enzyme restricts HBV infection as well as its replication by yet unrevealed mechanism (Watashi, Liang et al. 2013).

Proteins from the tripartite motif family (TRIM) are known for wide variety of cellular functions, including PRR signalling pathways. TRIM22 has been reported to have an anti-HBV function by suppressing activity of the HBc promoter by its RING (really interesting new gene) finger domain. Since the HBc is crucial for reverse transcription and TRIM22 blocks its function, it lowers the virus replication rate without direct interaction (Gao, Duan et al. 2009). Several other TRIM proteins were tested for their potential anti-HBV activity in human hepatoma cells. A number of them lowered the secretion of HBV proteins, however this could have been a false positive result due to the overexpression of TRIMs. When the group of the putative restrictive TRIM proteins was further tested only, TRIM41 was proven to inhibit both HBV enhancers I and II. The antiviral effect of TRIM41 seems to be dependent on its C-terminal domain but also on its E3 ubiquitin ligase activity[2] (Zhang, Guo et al. 2013). Another report showed that TRIM25 reduces HBV replication in cell lines, but HBV downregulates its expression in patients. TRIM25 is an IFN-stimulated gene (ISG) and requires both IFN I type and IL-27 for upregulation (Tan, Xiao et al. 2018).

Two unrelated studies have recently reported that SMC5/6 complex is targeted by HBx and consequently degraded. SMC5/6 is a protein complex containing Structural maintenance of chromosome protein heterodimer and proteins named “Non-SMC elements” and inhibits extrachromosomal expression of the HBV genome. To hinder this activity, HBx marks SMC5/6 complex for degradation by the proteasome by hijacking E3 ubiquitin ligase consisting of the

[2] Ubiquitinylation is a post-translation modification by adding ubiquitin protein to a lysine residue of a target protein. Depending on the specific type of the modification, ubiquitin tag serves many purposes – degradation, gene expression, cellular localisation, DNA repair, etc. There are three types of ubiquitin-associated enzymes: E1 ubiquitin-activating enzyme, E2 ubiquitin conjugating enzyme and E3 ubiquitin ligases, which determine the target protein for ubiquitinylation.

DNA-binding protein 1 (DDB1) and the Histone H3 methyltransferase CLR4 (Decorsiere, Mueller et al. 2016, Murphy, Xu et al. 2016).

The latest revealed endogenous restriction factor of the HBV is the Sterile alpha motif and HD domain-containing protein 1 (SAMHD1). The protein depletes the host cell supply of dNTPs, so the retroviruses are unable to perform reverse transcription. It has been shown that IFN-α induces expression of SAMHD1 in the liver cells and that the protein is able to restrict HBV replication even when catalytically inactive. However, HBV itself reduces expression of SAMHD1 (shown only in HepG2 cell line) (Chen, Zhu et al. 2014, Jeong, Park et al. 2016).

1.2.3.3 Currently used clinical approach

The treatment of the hepatitis B depends on the phase of infection. A patient who was exposed to the hepatitis B virus without being previously vaccinated receives an immunoglobulin injection which prevents the infection. Acute infection usually does not require any medication, only steady nutrition, higher fluid intake and rest. Severe cases might result in hospitalisation and administration of antiviral medication.

Once the patient is in a chronic phase of infection, the treatment aims to block the transmission to others and to minimalize damage of the liver. This often includes a combination of antiviral medication, which consists of nucleos(t)ide analogues to block reverse transcription and IFN-α, often PEGylated. The most widely used therapeutics are entecavir (Tang, Griffin et al. 2013), tenofovir (Kearney, Flaherty et al. 2004), adefovir (Dando and Plosker 2003) and lamivudine (Perry and Faulds 1997). All treatment has to be continually administered (often life-long) and has various side effects, including nausea, vomiting and other gastrointestinal complications, rash or change of mental state. Treatment with IFN-α is often accompanied by difficult breathing and fever-like symptoms (Sleijfer, Bannink et al. 2005). After progression of the disease and tissue destruction, patients often need liver transplant, either from a deceased donor or only partial liver transplantation from a living donor (MayoClinic 2019, WHO 2019).

1.2.3.4 New approaches towards HBV treatment

Current treatment of HBV hinders replication of the virus and restricts its spreading but does not remove the cccDNA from nuclei of the host cells. Clearing the minichromosome could be considered a complete cure, however, this task is rather complicated. According to Levrero, Testoni et al. (2016), current approaches to HBV cure can be divided into four categories based on their target. The first one is represented by the restoration of an innate and an adaptive

immune response of the host including the usage of the TLRs agonists, the checkpoint inhibitors and the re-activation of suppressed endogenous restriction factors and exhausted T cell population. The second approach represents the targeted sensitisation of the infected cells to immunological processes to achieve HBV elimination. The third approach aims to completely inhibit HBV replication, which includes blocking of entry of the virus into the host cell, inhibiting capsid assembly or disabling production of capsid protein by RNA interference. Last and lately widely researched option is a direct targeting of the cccDNA.

1.2.3.5 Treatment enhancing immune response

The innate immune system is primarily activated upon pathogen associated molecular patterns (PAMPs) recognition by PRRs, including TLRs. As an example, it has been shown that activation of TLR7 by engineered agonist GS-9620 induced IFN type I response, which suppressed HBV without decreasing load of the cccDNA in nuclei. The drug also increased antigen presentation, albeit only in animal models of chronic hepatitis B. GS-9620 passed phase Ib clinical trial as generally safe and its efficacy is to be tested (Gane, Lim et al. 2015, Niu, Li et al. 2018). TNF-α induced signalling leads under normal conditions to apoptotic death of infected cells. During chronic phase of hepatitis B infection, the signalling pathway is blocked by the cellular inhibitors of apoptosis proteins (cIAPs) (Ebert, Preston et al. 2015). The mechanism has not been completely elucidated but the depletion or inhibition of these endogenous proteins re-sensitizes infected hepatocytes to TNF-α effect (Ebert, Allison et al. 2015).

As mentioned before, RIG-I helicase functions as an RNA sensor and induces intracellular antiviral response. Although it has been tested only in chimeric mice with humanised hepatocyte, the research group isolated specific region of the HBV RNA which stimulated IFN type III response. The 5′ ε-stem loop of pgRNA was delivered in the form of vector to the cells in targeted liposomes and upon transcription induced the IFN type III production and suppressed viral replication (Sato, Li et al. 2015). IFN type III can suppress HBV infection in a similar manner as IFN type I but with lower toxicity (Robek, Boyd et al. 2005, Pagliaccetti, Chu et al. 2010). IFN type III have not been researched thoroughly in context of HBV infection, however IFN-λ1a has passed phase IIb clinical trial for use in HCV infected patients (Muir, Arora et al. 2014).

Exhausted populations of the CD8+ T cells, present in patients with chronic HBV infection, express high levels of inhibitory molecules on their surface. This includes a well-studied

programmed cell death protein 1 (PD-1), the cytotoxic T lymphocyte antigen 4 (CTLA-4) and the lymphocyte activation gene 3 (LAG-3). Checkpoint inhibitors such as pembrolizumab and nivolumab (both anti-PD-1) already passed through phase III clinical trials for oncological treatment. Nivolumab has recently entered the phase I trials for the use in patients with chronic HBV infection and hepatocellular carcinoma (Sangro, Crocenzi et al. 2013, El-Khoueiry, Sangro et al. 2017). Another approach to support the T cell-mediated response is a production of genetically modified T cells carrying a receptor specific for HBsAg. The engineered cells were tested in a single patient and proven to be viable and safe, though clinical efficacy was not determined (Qasim, Brunetto et al. 2015). Attempts to reverse immune tolerance of the T cells towards HBV by a therapeutic vaccine were so far unsuccessful (Lok, Pan et al. 2016), nevertheless, new vaccine candidates are being tested in mice (Martin, Dubois et al. 2015).

1.2.3.6 Targeting HBV life cycle steps

To prevent the first step of infection, there are several compounds which block entry of the virus into the cell. A synthetic N-acylated preS1 lipopeptide named Myrcludex B combined with PEGylated IFN-α has already passed through phase IIb of clinical trials. The drug is generally well tolerated by patients and seems to be blocking entry of both HBV and HDV (a subviral satellite of HBV). Myrcludex B is a competitive inhibitor of HBV entry. It mimics the binding of the HBV to the NTCP receptor as it is structurally similar to large surface protein preS1. Suggested treatment consists of the administration of the Myrcludex B and the reverse transcriptase inhibitors to prevent the virus from spreading while the immune system clears out infected cells (Urban, Bartenschlager et al. 2014).

The cccDNA molecule of the HBV is a key to the virus persistence in the host cells. After transformation of the rcDNA into the cccDNA, the minichromosome is exceptionally stable and it has a half-life of around 30 days (Chevaliez, Hezode et al. 2013). Targeting the host factors which enable the transformation is an intriguing option, but also hazardous, since their inhibition might disrupt normal functioning of the healthy cells. Current research often focuses on direct degradation of the cccDNA by various gene-editing tools, such as TALENs (Bloom, Ely et al. 2013) or recently discovered CRISPR/Cas9 nucleases (Ramanan, Shlomai et al. 2015, Sakuma, Masaki et al. 2016). This treatment would require a targeted delivery of the effector molecules to the hepatocytes and in case of CRISPR/Cas9 also a delivery of several guide RNAs to ensure specific and proper cleavage of the cccDNA.

The RNA interference approach aims to reduce the viral mRNA and pgRNA levels in infected cells by specific siRNAs. It aims solely on patients in chronic phase to reduce the viral load by targeting conserved sequences of the HBV transcripts. Since this approach requires to be site-specific, several strategies are focusing on engineering the siRNA to be hepatotropic, for example, by conjugating cholesterol to the siRNA (Wooddell, Rozema et al. 2013). ARC-520 liver-targeted therapeutic containing anti-HBV siRNAs passed phase II clinical trial and was shown to not only reduce the viral antigens but also the levels of the cccDNA (Yuen, Chan et al. 2015).

Another potentially effective strategy is targeting HBc (Core protein), since it plays many important roles in HBV life cycle (described further in chapter 1.2.4.1). Blocking its function might lead to complete inhibition of capsid assembly and reverse transcription, lower the HBV transcription rate and reverse inhibition of ISG. A capsid assembly modulator NVR 3-788 successfully passed clinical trial phase Ib, having very little adverse effects and significant efficacy at HBV DNA reduction (Yuen, Kim et al. 2015, Lam, Espiritu et al. 2019). Capsid inhibitor AB-423, sulfamoylbenzamide (SBA) from HBV capsid inhibitors class II inhibitors is currently in phase I clinical trials. After the treatment, infected cells secrete capsid without pgRNA or rcDNA reducing total replication rate to 50%. When tested in mice, the treatment has best efficacy when combined with RT inhibitors resulting in significant decrease of the HBV DNA serum levels (Mani, Cole et al. 2018). Hetero-aryl-dihydropyrimidine HAP12 and phenyl-propenamide AT130 compounds were tested in cell lines and almost completely abolished HBV replication, strongly disrupted transcription (shown on HBeAg production) and partially reduced cccDNA pool in the host cell (Belloni, Palumbo et al. 2015).

From the data already shown, it is clear that the Core protein is a suitable target for antiviral therapy. The HBV Core protein is involved in many mechanisms of HBV life cycle and survival strategies, many of them still unrevealed. The first step to discover new possible treatment targets is exploring Core interacting partners.

1.2.4 Known functions of HBV Core protein

1.2.4.1 Core protein function in HBV life cycle

As mentioned previously (in chapter 1.1.1), the main role of the Core protein is assisting during replication of the HBV genome. This includes encapsidation of the pgRNA and the HBV RNA-dependent DNA polymerase into a capsid to enable reverse transcription, followed by either packaging into the complete virion or shuttling of the rcDNA back to the host cell nucleus

(Summers, Smith et al. 1990, Bartenschlager and Schaller 1992). The Core protein is part of the minichromosome formed by the cccDNA and adjacent proteins. As mentioned further (in chapter 1.2.4.2), the Core protein increases transcription rate of the HBV genome by interacting with NFκB and by increasing its DNA affinity. The Core protein is mostly bound to CpG islands of the genome, epigenetically upregulating its transcription. Amount of the Core protein attached to CpG island 2 was shown to positively correlate with binding of the CREB binding protein and hypomethylation of the site (Guo, Li et al. 2011). Additionally, it was reported that depletion of the Core protein leads to lower replication rate of the HBV genome (Kohno, Tsuge et al. 2014). The C-terminal ARD of the Core protein is necessary for binding of the pgRNA (Nassal 1992) and the phosphorylation state is also crucial during encapsidation (Zhao, Hu et al. 2018). The Core protein is not just an inert capsid component, it actively contributes to the reverse transcription by its nucleic acid-chaperone activity (Chu, Liou et al. 2014) and supports synthesis of second DNA strand (Tan, Pionek et al. 2015).

Based on these findings, there has been an effort to develop drug targeting specific Core protein functions, mainly capsid assembly. In theory, two possible approaches can disrupt capsid formation – either conformational change of a single Core protein subunit, which would prevent assembly, or inhibition of inter-subunit interaction. Conformation of the Core protein can be *in vivo* influenced by phosphorylation, as described in chapter 1.2.4.2. Therefore, the ability of the Core protein to form capsid can be blocked by regulation of affiliated kinases and phosphatases. For disruption of the capsid assembly on the inter-subunit level, number of effective inhibitors were discovered such as a small molecule fluorescent probe (Zlotnick, Ceres et al. 2002), non-nucleos(t)ide HBV replication inhibitors like heteroaryldihydropyrimidine (Stray and Zlotnick 2006) or phenylpropenamide derivatives (Feld, Colledge et al. 2007) and sulfamoylbenzamide (SBA) derivatives, that were previously shown to inhibit the cccDNA formation (Cai, Mills et al. 2012, Campagna, Liu et al. 2013). On the other hand, zinc ions were shown to cause a conformational change that facilitated capsid formation (Stray, Ceres et al. 2004).

To sum up, the HBV Core protein plays several crucial roles in the viral life cycle. Understanding its importance relies on knowledge about its behaviour in host cell and mainly on mapping of its endogenous interacting partners.

1.2.4.2 Endogenous interacting partners of HBV Core protein

The HBV Core interactome is mostly unknown with several exceptions, which are summarised in the following chapter. In theory, HBV Core protein has multiple functions during the virus

life cycle (as described in chapter 1.2.4.1) but also in blocking the immune response or subjugating mechanisms of the host cells for the virus survival and spreading.

The Core protein increases expression of the HBV by binding to NF-κB (p50/p65 heterodimer) to form a complex which then interacts with DNA sequence upstream of the pregenomic promoter (Kwon and Rho 2002).

On the other hand, the Core protein was reported to be the target of the E3 ubiquitin ligase Np95/ICBP90-like RING finger protein (NIRF), which leads to destruction of the Core protein and subsequently reduction of HBV replication (Qian, Jin et al. 2012, Qian, Hu et al. 2015). Another adverse interaction was reported between the Core protein bound to the cccDNA and APOBEC cytidine deaminases (which causes hypermutations of the HBV DNA as described earlier). The Core protein, when part of the minichromosome structure, interacts with several members of APOBEC family and mediates their binding to the cccDNA prior to deamination (Lucifora, Xia et al. 2014).

One of the proteins functioning as a restriction factor by interacting with the Core protein is the interferon-inducible cytoplasmic dynamin-like GTPase MxA. By binding to the Core protein, MxA inhibits the formation of the capsid necessary for reverse transcription and thus restricts the increase of the HBV DNA in the host cell (Li, Zhang et al. 2012). Expression of the MxA was previously reported to be reduced in HBV infected cells and it was also shown that the Core protein interacts with MxA promoter to prevent its antiviral effect (Fernández, Quiroga et al. 2003).

Once the reverse transcription is complete and the Core protein subunits of the capsid are phosphorylated, their C termini translocate to the exterior of the capsid and reveal a nuclear localisation signal (NLS) (Rabe, Vlachou et al. 2003). The NLS is recognised by importins (karyopherins) α/β which bind to the capsid and target it to the cell nucleus via a nuclear pore. The cargo is arrested in the nuclear basket, specifically by interaction of the Core protein with the nucleoporin 153 (Nup153). If the capsid is mature (meaning the pgRNA is completely reversely transcribed to rcDNA), the subunits disassociate and release the genome into the host cell nucleus, while immature capsids are arrested (Schmitz, Schwarz et al. 2010). Interestingly, the Core protein monomer was reported to physically interact with Tip-associated protein/nuclear export factor-1 (TAP/NFX1) which associates with the Core protein arginine rich domain (ARD). When in the form of capsid, the ARD is hidden inside, however, once the mature capsid arrives in the nucleus and disassembles, as previously described, the nuclear

export signal is recognised by TAP/NFX1 and the Core protein is recycled back to the cytoplasm (Li, Huang et al. 2010).

Phosphorylation of the HBV Core protein influences its conformation and therefore its function. It was first shown in ducks that phosphorylation of the Core protein induces conformational change of its C terminus (Yu and Summers 1994). Followingly, another protein kinase, distinct from all known kinases, was described and termed the Core-associated kinase (CAK) (Kau and Ting 1998). At the same time, glyceraldehyde-3-phosphate dehydrogenase (GAPD) was shown to phosphorylate the Core protein (Duclos-Vallee, Capel et al. 1998). Later, it was discovered that phosphorylation of the Core protein is necessary for encapsidation of the pgRNA (Gazina, Fielding et al. 2000). Two kinases specifically interacting with the HBV Core protein were identified by purification followed by mass spectrometry as SR protein-specific kinase 1 (SRPK1) and SR protein-specific kinase 2 (SRPK2). Among other protein kinases, the Protein kinase C, which was previously thought to phosphorylate Core protein (Kann and Gerlich 1994), was tested during these experiments and the claim was disproven (Daub, Blencke et al. 2002). SRPK kinases were further shown to reduce the efficiency of the pgRNA packaging and thus repress the HBV replication, even when catalytically inactive (Zheng, Fu et al. 2005). Next identified Core-phosphorylating kinases were the α-type CK2-activated protein kinases A (PKA), PKAIα and PKAIIα, which can be inhibited by drug Suramin (Enomoto, Sawano et al. 2006, Okabe, Enomoto et al. 2006). Since phosphorylation enhances the Core protein function, then the removal of phosphate should in theory hinder it. This hypothesis was tested with the protein-tyrosine phosphatase PTPN3, which suppresses the HBV genome expression. The phosphatase binds the C-terminal region of the Core protein, however, the downregulating effect on the viral expression is not dependent on the interaction (Hsu, Lin et al. 2007). Phosphorylation of the Core protein by the cyclin-dependent kinase 2 (Cdk2) was shown several years ago, but its inhibition had no effect on HBV genome replication so its importance was questioned (Ludgate, Ning et al. 2012). Recently, Polo-like-kinase 1 (PLK1) was shown to phosphorylate the Core protein and required phosphorylation priming by Cdk2. This means that the first step of the phosphorylation performed by the Cdk2 enables phosphorylation by the PLK1. Moreover, the inhibition of PLK1 led to the reduction of the HBV genome replication and it was suggested that the inhibition of both Cdk2 and PLK1 might hinder virus biosynthesis (Diab, Foca et al. 2017). Since inhibitors for both kinases have already been researched for cancer treatment, their application for HBV infection should not be complicated (Asghar, Witkiewicz et al. 2015, Gjertsen and Schoffski 2015).

The Core protein was reported to bind to Filamin B (actin-binding protein, ABP-276/278). Filamin anchors actin cytoskeleton to plasma membrane and can function as a scaffold for IFN signalling. The proteins bind each other by their C-termini and their interaction is necessary for HBV replication (Huang, Chen et al. 2000, Li, Sun et al. 2018).

Nucleophosmin (NPM1 or B23) is a nucleolar protein that plays a role in many processes including cell growth, cell cycle, proliferation and ARF-p53 tumour suppression pathway regulation, DNA repair, genomic stability and functions as a histone chaperone (Okuwaki 2008). B23 was shown to interact with the HBV Core protein (Lee, Shim et al. 2009) and to facilitate capsid assembly and stability in HepG2.2.15 cells. The Core protein utilizes chaperone function of B23, B23-promoted capsids are stable even in higher temperatures (Jeong, Cho et al. 2014). The Heat shock protein 90 (Hsp90), another protein with known chaperone activity, was reported to interact with the Core protein in a similar manner. Capsids facilitated by Hsp90 were also more stable in various temperatures and when treated with detergents. HBV replication rate was reduced upon inhibition of the B23 and Hsp90 in HepG2.2.15 cell line, underlining their contribution to the viral life cycle (Shim, Quan et al. 2011, Jeong, Cho et al. 2014). On the other hand, two members of the Hsp40 family, Hdj1 and hTid1, have been shown to accelerate degradation of the Core and HBx proteins and when ectopically expressed, suppressed HBV replication (Sohn, Kim et al. 2006).

The Core protein quite possibly plays a role in egress of new viral particles from the cell. It has been shown that the Core protein interacts with the γ2-adaptin and the endosomal E3 ubiquitin ligase Nedd4. Both of these endogenous host factors and the ubiquitinase activity of the Nedd4 are necessary for budding of new virions from the cell. The Core protein, pre-S1 γ2-adaptin and Nedd4 were reported to be colocalised in late endosome which further suggests that HBV might be exploiting endosomal pathway for its production (Rost, Mann et al. 2006).

A wide experiment to discover new Core protein interactants resulted in several putative hits. The study was conducted in yeast using plasmids expressing the Core protein and the lymphocyte DNA library and focused primarily on proteins produced by yet unknown genes. It was shown that the Core protein might interact with endogenous proteins as follows: Hypermethylated in cancer 2 (HIC2), Eukaryotic translation elongation factor 2 (EEF2), acetylCoA synthetase 3, DNA polymerase γ, chemokine (C-C motif) receptor 5 (CCR5), mitochondrial ribosomal protein L41 (MRPL41), programmed cell death 2 (PDCD2), putative translation initiation factor (SUI1), Kyot binding protein and HBeAg-binding protein 3

(HBEBP3) (Lin, Cheng et al. 2006). However, none of these putative interactants have been validated.

Moreover, the Core protein interacts with E2F1 transcription factor and reduces its DNA binding ability. Inhibition of the E2F1 represses expression of the p53, an important anti-oncogene, which might contribute to the development of hepatocellular carcinoma (Kwon and Rho 2003). The Core protein also inhibits TRAIL-induced activation of apoptosis. It was discovered that the Core protein represses promotor activity of *DR5* gene, which leads to lower production of Death receptor 5 protein. The impact of the Core protein on DR5 expression was initially tested in cell lines and further in mouse model. Finally, expression of DR5 was determined to be significantly reduced in patients with chronic hepatitis B (Du, Liang et al. 2008). Inhibition of apoptosis might also support cancer development.

The list of published HBV Core protein interactants is rather short, despite its obviously crucial role in viral survival. To further explore biology of HBV and possibly find new therapeutic targets, it is necessary to identify endogenous interacting partners of Core protein. For example, HBV uses a pgRNA intermediate during its replication, which can be targeted by various endogenous RNA sensing receptors, including RNA helicases. Since Core protein encapsidates pgRNA and assists during reverse transcription it might also come in contact with these enzymes, which have been shown to play various roles in viral infections.

1.2.5 DDX3 and role of RNA helicases in viral infections

DEAD-box proteins (DDX) belong to the family of the ATP-dependent RNA helicases present in almost all prokaryotes and eukaryotes. They partake in metabolic processes such as mRNA processing (mainly splicing), transcription, RNA turnover and translation initiation. They have similar structure to the viral helicases which suggests that their function might be similar as well. They possess an enzymatic activity which enables them to unwind RNA duplexes and disrupt the protein-RNA complexes after activation by ATP hydrolysis. Each DDX helicase has unique function based on its interacting partners (Rocak and Linder 2004).

DDX3 helicase (also termed DDX3X) was shown to play a role in cell growth (Lai, Chang et al. 2010), which implicates its function in cancer development. For example, its expression is increased in breast cancer cells during hypoxia (Botlagunta, Krishnamachary et al. 2011). DDX3 also influences metastatic properties of melanoma cells by controlling mRNA

translation of the Melanocyte inducing transcription factor oncogene (Phung, Ciesla et al. 2019).

DDX3 helicase has 279 putative interactors based on BioGrid database (Chatr-Aryamontri, Oughtred et al. 2017) including viral proteins. Additionally, the role of DDX3 helicase was described in several viral infections, including the hepatitis C virus (HCV) and the Human immunodeficiency virus (HIV). It was reported that knockdown of DDX3 in cell cultures causes suppression of the HCV RNA replication and release of the viral protein in the supernatant (Ariumi, Kuroki et al. 2007). In case of HIV infection, DDX3 was shown to enable export of unspliced HIV mRNA from the host cell nucleus (Yedavalli, Neuveut et al. 2004).

First connection between DDX3 and HBV infection was studied based on its cell cycle-control function. It was suggested and eventually confirmed that DDX3 is deregulated in HBV and HCV infection which leads to the development of hepatocellular carcinoma. During this study it was also determined, that expression of DDX3 is downregulated during HBV but not HCV infection (Chang, Chi et al. 2006). This mechanism has not yet been elucidated, but it was shown that HBV has a reason to suppress DDX3 expression. As mentioned previously, DDX3 is important in TBK1/IKKε-mediated IRF signalling (Schroder, Baran et al. 2008), which is disrupted by the HBV polymerase (chapter 1.2.1.2). Another study has reported that DDX3 is present in HBV capsids and prevents reverse transcription, thus functioning as a restriction factor (Wang, Kim et al. 2009). DDX3 was also reported to reduce transcription of HBV genome and its knockdown leads to increased production of the viral RNAs (Ko, Lee et al. 2014).

Further exploration of DDX3 interactome and its role during viral infection might lead to better understanding of mutual host-virus impact and future treatment possibilities.

2 Aims of the thesis

The aims of the thesis were to explore the interactome of the Hepatitis B virus Core protein by the proximity-dependent biotin identification (BioID) using mass spectrometry and validation of acquired hit/s by the pull-down protein-protein interaction assay.

1. Mapping of new HBV Core interactants by the proximity-dependent biotinylation in live cells coupled to mass spectrometry
    a. Validation of expression of the DNA constructs used for BioID method
    b. Proximity-dependent biotinylation in live cells
    c. Affinity purification of the biotinylated proteins
    d. Validation of proximity-dependent biotinylation by western blot analysis
    e. Analysis of biotinylated proteins by mass spectrometry
    (M. Hubálek, A. Křenková)
    f. Network modelling of the newly identified interactome

2. Validation of the interaction between a chosen protein and the HBV Core protein
    a. Co-transfection of the HBV Core protein and a chosen protein hit
    b. Pull-down of the HA-tagged HBV Core protein
    c. Pull-down of the FLAG-tagged target protein
    d. Western blot analysis of the pull-down assay

3 Materials and methods

3.1 Materials

3.1.1 Chemicals and solutions

- acetonitrile (Sigma-Aldrich, St. Louis, USA)
- acrylamide (Sigma-Aldrich, St. Louis, USA)
- Albumin standard (2 mg/ml) (Thermo Fisher Scientific, Waltham, USA)
- All Blue pre-stained protein standard (Bio-Rad, Hercules, USA)
- ammonium persulfate (Serva, Heidelberg, Germany)
- Ampicillin (Sigma-Aldrich, St. Louis, USA)
- Benzonase nuclease (Sigma-Aldrich, St. Louis, USA)
- Blocker casein in PBS (Thermo Fisher Scientific, Waltham, USA)
- bromphenol blue (Sigma-Aldrich, St. Louis, USA)
- cOmplete^{TM} Mini, EDTA-free, Protease Inhibitor Cocktail (Roche, Basel, Switzerland)
- biotin (Sigma-Aldrich, St. Louis, USA)
- DMEM High Glucose w/o L-Glutamine (Biosera, Nuaille, France)
- foetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, USA)
- formic acid (Sigma-Aldrich, St. Louis, USA)
- glycerol (PENTA, Chrudim, Czechia)
- glycine (Duchefa, Haarlem, Netherlands)
- Igepal CA-630 (Sigma-Aldrich, St. Louis, USA)
- iodoacetamide (Sigma-Aldrich, St. Louis, USA)
- isopropanol (PENTA, Chrudim, Czechia)
- Kanamycin (Sigma-Aldrich, St. Louis, USA)
- LB agar (Sigma-Aldrich, St. Louis, USA)
- LB medium (Sigma-Aldrich, St. Louis, USA)
- L-glutamine (Sigma-Aldrich, St. Louis, USA)
- 2-mercaptoethanol (Sigma-Aldrich, St. Louis, USA)
- methanol (PENTA, Chrudim, Czechia)
- N,N'-methylene-bis(acrylamide) (Thermo Fisher Scientific, Waltham, USA)
- opti-MEM medium (Thermo Fisher Scientific, Waltham, USA)
- Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, USA)

- polyethyleneimine (Sigma-Aldrich, St. Louis, USA)
- potassium chloride (Lach-Ner, Neratovice, Czechia)
- potassium phosphate monobasic (Lach-Ner, Neratovice, Czechia)
- sodium chloride (Lachema, Brno, Czechia)
- sodium dodecyl sulphate (Sigma-Aldrich, St. Louis, USA)
- sodium phosphate dibasic (Lach-Ner, Neratovice, Czechia)
- TCEP (tris(2-carboxyethyl)phosphine)
- TEMED (tetramethylethylendiamine) (Fluka, Buchs, Switzerland)
- Tris (tris(hydroxymethyl)aminomethane) (PENTA, Chrudim, Czechia)
- Tris-HCl (tris(hydroxymethyl)amino methane hydrochloride) (Thermo Fisher Scientific, Waltham, USA)
- Trypsin (Sigma-Aldrich, St. Louis, USA)
- Trypsin/EDTA solution (Sigma-Aldrich, St. Louis, USA)
- Tween 20 (Thermo Fisher Scientific, Waltham, USA)

3.1.2 Antibodies

- Anti-Hepatitis B Virus Core Antigen rabbit-produced (BioGenex, California, USA, Cat. No. PU082-UP)
- Secondary anti-rabbit goat-produced IRDye® 800CW (LI-COR Biosciences, Lincoln, USA, Cat. No 926-32211)
- Secondary anti-rabbit goat-produced Alexa Fluor® 680 (Thermo Fisher Scientific, Waltham, USA, Cat. No. A27042)
- Secondary anti-mouse goat-produced IRDye® 680 RD (LI-COR Biosciences, Lincoln, USA, Cat. No 926-68070)
- IRDye® 800CW Streptavidin (LI-COR Biosciences, Lincoln, USA, Cat. No. 926-32230)
- Anti-HA-Tag (C29F4) rabbit-produced (Cell Signalling, Cat. No. 3724S)
- Anti-FLAG® M2-Peroxidase (HRP) mouse-produced (Sigma-Aldrich, St. Louis, USA, Cat. No. A8592)
- Anti-β-actin mouse-produced (Exbio, Prague, Czechia, Cat. No. 1A-644-T025)

3.1.3 Cell cultures

E. coli Top10 cells (Invitrogen, Carlsbad, USA)

HEK293 cells (original strain ATTC, Manassas, USA)

HEK293T–provided by Jana Starková (IOCB, Prague)

Huh7 provided by Dr. Jan Weber (IOCB, Prague) (originally from Okayama University, isolated cells from differentiated hepatoma, age 57, male)

3.1.4 Commercial kits

QIAGEN Plasmid Maxi Kit (QIAGEN, Hilden, Germany, Cat. No. 12163)

- Online protocol: (https://www.qiagen.com/us/resources/resourcedetail?id=46205595-0440-459e-9d93-50eb02e5707e&lang=en)

ZYPPY™ plasmid miniprep kit (Zymo research, Irvine, USA, Cat. No. D4020)

- Online protocol: (https://dwo0hlbtc3ypb.cloudfront.net/amasty/amfile/attach/_D4019_D4020_D4036_D4037_Zyppy_Plasmid_Miniprep_Kit_ver.1.2.7.pdf)

3.1.5 Vectors

Table 1: Vectors used in the study.

Expressed construct	Backbone	Tag	Source
Core-GS-BioID2-HA	pcDNA3.1	HA	Dr. Šašková lab (FOS, CU)
Myc-BioID2-GS-Core	pcDNA3.1	Myc	Dr. Šašková lab (FOS, CU)
Core-13×GS-BioID2-HA	pcDNA3.1	HA	Dr. Šašková lab (FOS, CU)
Core-HA	pcDNA3.1	HA	Dr. Weber lab (IOCB Prague)
Myc-BioID2	pcDNA31.	Myc	Addgene no. 74223*
DDX3-Myc-Flag	pCMV6-Entry	Myc, Flag	Origene (Cat.No.RC204171)
DDX1-Myc-Flag	pCMV6-Entry	Myc, Flag	Origene (Cat. No. TP308769)
-	pcDNA3.1	-	Dr. Weber lab (IOCB Prague)

*myc-BioID2-MCS was a gift from Kyle Roux (Addgene plasmid # 74223) (Kim, Jensen et al. 2016)

3.1.6 Consumables

Nitrocellulose membrane (Bio-Rad, Hercules, USA)

Streptavidin agarose (Thermo Fisher Scientific, Waltham, USA, Cat. No. 20353)

Anti-HA magnetic beads (Thermo Fisher Scientific, Waltham, USA, Cat. No. 88837)

Anti-FLAG M2 magnetic beads (Sigma-Aldrich, St. Louis, USA, Cat. No. M8823)

96-well transparent plate, F-bottom (P-lab, Prague, Czechia)

Cell culture dishes and plates (Biotech, Prague, Czechia)

Millex GP syringe-driven filter unit 0.22 μm (Millipore, Massachusetts, USA)

Microcon filters YM-10 (Sigma-Aldrich, St. Louis, USA)

C18 SPE column (Thermo Fisher Scientific, Waltham, USA)

Acclaim PepMap100 column (Thermo Fisher Scientific, Waltham, USA)

3.1.7 Instruments and devices

3.1.7.1 Instruments

centrifuges:

- Beckman Allegra X-15R (Beckman Coulter, Brea, USA)
- Centrifuge 5415R (Eppendorf, Hamburg, Germany)
- Fresco Heraeus 21, IEC CL10 (Thermo Fisher Scientific, Waltham, USA)

electrophoresis:

- Electrophoresis power supply EPS 301 (GE Healthcare, Chicago, USA)
- Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad, Hercules, USA)
- Mini Trans-Blot® Cell (Bio-Rad, Hercules, USA)

imaging systems:

- Odyssey® CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, USA)
- Monochrome scientific grade camera Quantum ST4 (Vilber Lourmat, Collegién, France)

incubators:

- CO2 incubator MCO-19AIC, (Sanyo, Osaka, Japan)

- Innova 4300 (New Brunswick Scientific, New Jersey, USA)
- Thermocell Mixing Block MB102 (BIOER Technology, Hangzhou, China)
- Peltier-cooled incubator IPP400 (Memmert, Schwabach, Germany)

laminar flow box:

- Laminar flow box (Clean Air®, Telstar)

water bath:

- TW8 Water Bath (JULABO, Seelbach, Germany)

shakers:

- KS 260 basic shaker (IKA, Staufen, Germany)
- WiseMix rotator Wisd RT-10 (Verkon, Prague, Czechia)
- SkyLine Digital Rocking Shaker DRS-12 (ELMI, Riga, Latvia)

water filtering unit:

- Milli-Q® Integral Water Purification System for Ultrapure WaterbyMerck (Millipore, Massachusetts, USA)

magnetic stand:

- 16-Tube SureBeads™ Magnetic Rack (Bio-Rad, Hercules, USA)

laboratory scales:

- EK-400H (A&D Company, Tokyo, Japan)
- PLS 4000-2 (KERN & Sohn GmbH, Postfach, Germany)
- XA 116/X (Radwag, Sumperk, Czechia)

microscopes:

- Stemi 305 EDU Microscope (Zeiss, Oberkochen, Germany)
- Axio Imager Z2 (Zeiss, Oberkochen, Germany)
- AxioScan Z1 (Zeiss, Oberkochen, Germany)

pH-meter:

- pH 50 (XS instruments, Carpi, Italy)

sonicator:

- Sonication bath S 30 Elmasonic (Elma, Singen, Germany)

spectrophotometers:

- NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, USA)
- Infinite® microplate reader M1000 PRO (Tecan, Männedorf, Switzerland)

Mass spectrometer:

- UltiMate 3000 RSLCnano system (Dionex) coupled to an Orbitrap Fusion Lumos Tribrid with an EASY-Spray source (Thermo Fisher Scientific, Waltham, USA), operated with Xcalibur (Thermo Fisher Scientific, Waltham, USA)

3.1.7.2 Software

Microsoft Office (Microsoft Corporation, Redmond, USA)

Image Studio Lite Software (LI-COR Biosciences, Lincoln, USA)

Gimp 2.10.4 (The GIMP Development Team)

Inkscape (The Inkscape Development Team)

Andromeda search engine in MaxQuant (Cox, Neuhauser et al. 2011)

Perseus (version 1.6.2.3 (Tyanova, Temu et al. 2016))

3.2 Methods

3.2.1 Transformation of bacteria for DNA amplification

The host strain *E. coli* Top10 competent cells (Invitrogen, Carlsbad, USA) was used for transformation and amplification of all plasmids listed in chapter 3.1.5. The transformation was carried out according to previously published protocol (Sambrook et al, 1989). Briefly, 2 µg of DNA (c=4,375 µg/µl) was added to 30 µl of freshly unfrozen competent bacterial cells and left to incubate for 30 minutes on ice. Heat shock was performed at 42°C for 90 seconds, followed by cooling of the bacterial suspension down on ice for 5 minutes. Subsequently, the bacteria were incubated with 500 µl of LB media (without antibiotic) at 37°C for 1 hour, then spread over the agar plates containing relevant antibiotic (100 µg/ml ampicillin or 50 µg/ml kanamycin) and incubated at 37°C overnight.

After overnight incubation, freshly grown colonies were individually picked and inoculated into 500 ml (for maxipreparations) or 10 ml (for minipreparations) of sterile LB medium supplemented with antibiotic (100 mg/ml ampicillin or 40 mg/ml kanamycin) for DNA amplification. Bacteria were grown in a rotatory incubator Innova 4300 (New Brunswick Scientific) at 37°C and 220 rpm overnight. The following day, the culture was centrifuged at 4000g, 4°C for 10 minutes and cell pellets were further processed with QIAGEN Plasmid Maxi Kit (QIAGEN) or ZYPPY™ plasmid miniprep kit (Zymo research) according to manufacturer recommendation (chapter 3.1.4). Isolated DNA was eluted from columns with 40 µl (for minipreparations) or from precipitated DNA pellet by 500 µl (for maxipreparations) of sterile water and its concentration and purity was measured using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Plasmids were sequenced by GATC Biotech (Konstanz, Germany).

3.2.2 Cell line transfection

- DMEM complete medium (DMEM High glucose with 10% foetal bovine serum)
- PBS buffer (pH 7.4 – 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, 137 mM sodium chloride 2.7 mM potassium chloride)
- Opti-MEM medium
- Trypsin/EDTA solution
- Polyethyleneimine (PEI)

All experiments with cell lines were carried out in laminar flow box (Clean Air®, Telstar). The HEK293T cell line was provided by Jana Starková (IOCB Prague) and the Huh7 cell line was originally provided by Jan Weber PhD. (IOCB Prague). Both cell lines were kept in a CO_2 incubator (5% CO_2, Sanyo) at 37°C in DMEM complete medium (containing L-glutamine and 10% FBS) freshly prepared by Jana Starková (IOCB Prague). The cells were seeded on a 100 mm dish, in the density of 2.6×10^6 cells per dish. The following day, the transfection was carried out using a mixture of 20 µg of plasmid DNA and 80 µl of PEI dissolved in 750 µl Opti-mem medium per one dish. The mixture was incubated 20 minutes at room temperature before the cell transfection. The cells were incubated for 24 hours in a CO_2 incubator (5% CO_2, Sanyo) at 37°C. After the incubation, the medium from each dish was removed and the dish was carefully washed with PBS buffer. Subsequently, 1.8 ml of Trypsin/EDTA solution was added to each dish and the cells were incubated in the Trypsin/EDTA solution until the cell detachment was observable. The cells were then washed from the surface of the dish by repeatedly pipetting the Trypsin/EDTA solution containing detached cells and then the cells were transferred into a sterile, previously weighted Eppendorf tube. The cells were centrifuged at 2000g, 25°C for 3 minutes, the supernatant was carefully removed, and the cells were resuspended in the sterile PBS buffer and centrifuged again (2000g, 25°C, 3 min.). After supernatant removal, the tube contacting the cell mass was weighted.

3.2.3 In cell proximity biotinylation

- DMEM complete medium (DMEM High glucose with 10% foetal bovine serum)
- PBS buffer (pH 7.4 – 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, 137 mM sodium chloride 2.7 mM potassium chloride)
- Opti-MEM medium
- Trypsin/EDTA solution
- Biotin (1mM; 12.2 mg biotin dissolved in 50 ml DMEM High Glucose filtered by 0.22-µm syringe-driven filter unit)
- PEI

In cell proximity biotinylation was performed in HEK293T cell line transfected with plasmids encoding HBV Core protein with BioID2 proximity biotin ligase and respective controls, as described in chapters 3.2.2 and 4.1.3. After cell transfection, 50 µM (final concentration) of biotin was added to the media to induce biotinylation. The cells were then incubated for 24 hours in a CO_2 incubator (5% CO_2, Sanyo) at 37°C. After the incubation, the medium from

each dish was removed and the dish was carefully washed with cold PBS buffer. Subsequently, 1.8 ml of Trypsin/EDTA solution was added to each dish and the cells were incubated in the Trypsin/EDTA solution until the cell detachment was observable. The cells were then washed from the surface of the dish by repeatedly pipetting the Trypsin/EDTA solution containing detached cells and then the cells were transferred into a sterile, previously weighted Eppendorf tube. The cells were centrifuged at 2000g, 25°C for 3 minutes, the supernatant was carefully removed, and the cells were resuspended in the sterile PBS buffer and centrifuged again (2000g, 25°C, 3 min.). After supernatant removal, the tube contacting the cell mass was weighted.

3.2.4 Cell pellet lysis

- RIPA buffer (pH 7.4, 50 mM HEPES, 150 mM NaCl, 0.4% Igepal dissolved in autoclaved milliQ water from Milli-Q® Integral Water Purification System supplemented by cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail tablet

Pre-chilled RIPA buffer was supplemented by cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail tablet (Roche). After the tablet dissolved, the lysis buffer was added to the harvested cell pellets in a ratio 4:1 (the buffer volume in µl to the mass of the cells in µg). The cells were suspended in the lysis buffer by pipetting and incubated on ice for 30 minutes. The suspended pellet was sonicated in the water bath sonicator in three cycles (30 s of sonication followed by 30 s of incubation on ice). After the sonication, 1 µl of Benzonase nuclease was added to each sample and the lysate was incubated on ice for 30 minutes. The lysates were then centrifuged at 16000g, 4°C for 20 minutes. The resulting supernatant with cellular proteins was carefully removed without disturbing the remaining cell debris and used for further analysis. From each supernatant, the sample was taken for Bradford protein concentration analysis (chapter 3.2.5), SDS-PAGE analysis (chapter 3.2.7) and affinity purification coupled with MS/MS analysis (chapters 3.2.6 and 3.2.9) in case of previous proximity biotinylation. The samples were processed immediately or frozen in -80°C if needed.

3.2.5 Bradford protein assay

From each cell supernatant, usually 1/100 was separated into a clean Eppendorf tube (1 µl from each sample was diluted 100× into 99 µl of sterile MilliQ water). To acquire the most accurate results, the measured samples were diluted so that the absorbance of each reached approximately the middle of the calibration curve. The concentration of diluted samples was measured using Bradford Protein Assay Dye (Bio-Rad), according to original publication

(Bradford 1976). Diluted Bovine serum albumin standard samples ranging from 0 µg/ml to 200 µg/ml were used to create a calibration curve. 20 µl of each sample (in triplicates) and diluted standards (in duplicates) were incubated with 180 µl of diluted Bradford protein assay reagent on transparent 96-well flat bottom plate (P-lab) and left at room temperature for 5 minutes. The absorbance was measured at 595 nm on microplate reader (Infinite® microplate reader M1000 PRO, Tecan (Männedorf, Switzerland)). The concentration of the samples was calculated based on known concentration and measured absorbance of calibration line using Microsoft Excel software.

3.2.6 Affinity purification

- RIPA buffer (pH 7.4, 50 mM HEPES, 150 mM NaCl, 0.4% Igepal dissolved in autoclaved milliQ water from Milli-Q® Integral Water Purification System supplemented by cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail tablet
- SDS-PAGE sample buffer (360 mM Tris, pH 6.8, 30% glycerol, 10% SDS, 4% 2-mercaptoethanol, 0.01% bromophenol blue)

The biotinylated proteins from the cells were purified using agarose Streptavidin coated beads (Thermo Fisher Scientific, Waltham, USA). First, 80 µl of the original Streptavidin beads solution were washed three times for 5 minutes in 1 ml of the RIPA buffer simply by centrifugation (1000g, 4°C for 2 minutes). Next, 500 ug of total protein worth of supernatants were incubated with the equilibrated beads for one hour at room temperature on a rotary shaker. Then, the beads were spin down at 1000g, 4°C for 2 minutes and the flow-through fraction was collected for later SDS-Page/western blot analysis (chapter 3.2.8). The beads were washed five times for 5 minutes with 1 ml of the RIPA buffer to remove any non-specificities and the washed samples were again collected for later SDS-Page/western blot analysis. After removing the final wash, the beads were boiled in 140 µl of reducing SDS-sample buffer at 98˚C for 10 minutes. Next, 100 µl of the boiled sample were submitted for mass spectrometry analysis. The remaining amount was used for SDS-PAGE and western blot analysis.

3.2.7 SDS-PAGE (Sodium dodecyl-sulphate polyacrylamide gel electrophoresis)

- SDS-PAGE sample buffer (360 mM Tris, pH 6.8, 30% glycerol, 10% SDS, 4% 2-mercaptoethanol, 0.01% bromophenol blue)

- 5% stacking gel (250 mM Tris-HCl pH 6.8, 5% (v/v) acrylamide solution (acrylamide with N,N'-bisacrylamide in a ratio 35.7:1), 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate (APS), 0.02% (v/v) TEMED)
- 10% resolving gel (375 mM Tris-HCl pH 8.8, 10% (v/v) acrylamide solution (acrylamide with N,N'-bisacrylamide in the ratio 35.7:1), 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate (APS), 0.01% (v/v) TEMED)
- SDS-PAGE running buffer (25 mM Tris pH 8.8, 250 mM glycine, 0.1% SDS)

The samples acquired during DNA construct validation and proximity biotinylation were analysed by SDS-PAGE followed by western blot (chapter 3.2.8).

The samples were dissolved in reducing SDS sample buffer to reach uniform concentration in 5:1 ratio, vortexed and boiled for 10 minutes. All samples were boiled for 10 minutes at 98 ˚C. A 10% polyacrylamide gel with 10 or 15 wells was prepared according to standard protocol using vertical electrophoresis apparatus (Bio-Rad). If not stated otherwise in the Results section, 20 µg of total protein from each sample was loaded on the gel. In case of samples created by boiling purification beads, the concentration could not be determined and a uniform volume of 20 µl was loaded on the gel. The electrophoresis was run at 120 V for usually 1 hour and 25 minutes in SDS-PAGE running buffer or until the All Blue pre-stained protein standard (Bio-Rad) reached the bottom of the gel.

3.2.8 Western blot analysis

- WB running buffer (12.5 mM Tris-glycine pH 8.3, 10% (v/v) methanol)
- Blocking buffer (PBS with 0.5% Casein blocker)
- PBS-T buffer (PBS, 0.1% Tween 20)
- PBS buffer (pH 7.4 – 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, 137 mM sodium chloride 2.7 mM potassium chloride)

The samples separated with SDS-PAGE were transferred to nitrocellulose membrane (0.45 µm, Bio-Rad) by wet protein transfer using Mini Trans-Blot® Cell (Bio-Rad) according to the manufacturer's protocol. The membrane was blocked with 0.5% Casein in PBS-T for one hour at room temperature. Primary antibodies (Streptavidin conjugated with green fluorescent dye, rabbit anti-Core) were added to the blocking solution in concentration 1:10000 and incubated over night at 4 ˚C. Following day, the membrane was washed three times with PBS-T to remove residual primary antibodies. Secondary anti-rabbit antibody (conjugated with red fluorescent

dye) was added in concentration 1:10000 to 5 ml of PBS-T and incubated with the membrane for 1 hour at room temperature. The membrane was washed three times in PBS-T buffer and dried before caption. The resulting image was captured on Li-Cor machine (Odyssey® CLx Infrared Imaging System, LI-COR Biosciences (Lincoln, USA) at 680 and 800 nm.

3.2.9 Mass spectrometry measurement and data analysis

The mass spectrometry measurement and data analysis were performed by the IOCB Prague Mass Spec facility, namely Dr. Martin Hubálek and Dr. Alena Křenková.

The samples were reduced with TCEP (37 °C, 30 minutes), alkylated with iodoacetamide (RT for 30 minutes in dark) and digested by trypsin on Microcon filters YM-10. The resulting peptides were desalted on a C18 SPE column (PepClean, Thermo Fisher Scientific) and dried in the SpeedVac. Peptides dissolved in 0.1 % formic acid were analysed on UltiMate 3000 RSLCnano system (Dionex) coupled to an Orbitrap Fusion Lumos Tribrid mass spectrometer with an EASY-Spray source (Thermo Fisher Scientific). The instrument was operated with Xcalibur (Thermo Fisher scientific). After the injection, the samples were trapped and desalted with 2 % acetonitrile in 0.1 % formic acid at flow rate of 30 µl/min on Acclaim PepMap100 column (5 µm, 5 mm×300 µm ID, Thermo Fisher Scientific). Eluted peptides were separated using Acclaim PepMap100 analytical column (3 µm, 15 cm×75 µm ID, Thermo Fisher Scientific). The 65 minute elution gradient at constant flow of 400 nl/min was set to 5 % of phase B (0.1 % formic acid in 80 % acetonitrile, phase A 0.1 % formic acid) for first 1 minute, then with gradient elution from 5 % to 35 % B over 52 minutes, from 35 % to 99 % B over 5 minutes, stayed at 90 % B for 10 minutes and descended to 5 % B and remained there for 7 minutes. Orbitrap was set as mass analyser for MS acquisition with resolution of 120000 selecting peptides in range 350-2000 m/z with charge state from 2+ to 6+, excluding the masses for 10s over threshold level 1.5 e4. MS2 was recorded on orbitrap in the same range with resolution 30000 using HCD activation type with isolation window 1.5 Da and set collision energy 30%. MaxQuant (Cox and Mann 2008) was used for protein identification and quantification from raw spectra using *Homo sapiens* database. The search was set by choosing carbamidomethyl as static modification. Trypsin was set as digestion agent, with two allowed miscleavages. All experiments were evaluated by Andromeda search algorithm in MaxQuant (Cox, Neuhauser et al. 2011), allowing 20 ppm peptide tolerance in the first search and 4.5 ppm peptide tolerance in the main search. Unique and razor peptides were selected for quantification, minimal label ratio count was set as 2 and re-quantification was enabled. Missing peptide

intensities were artificially imputed into the data as the normal distribution of background signal and relative quantities of all proteins were estimated, comparing protein intensities in sample from cells transfected with Construct 1 DNA to negative control, using Perseus (version 1.6.2.3) (Tyanova, Temu et al. 2016). Statistical analysis was also performed in Perseus, resulting in list of significantly enriched or downregulated proteins (with p value <0.05), compared to negative control.

3.2.10 Data analysis in Enrichr and Panther software on-line tools

The acquired lists of significantly enriched or downregulated proteins was analysed by Enrichr and Panther software on-line tools (https://amp.pharm.mssm.edu/Enrichr/, http://www.pantherdb.org/) (Chen, Tan et al. 2013, Kuleshov, Jones et al. 2016, Mi, Muruganujan et al. 2019) during June 2019. The resulting data was visualized in Microsoft Excel.

3.2.11 Co-transfection experiments

The plasmids encoding DDX3 protein (DDX3-Myc-Flag) and the HBV Core protein (Core-HA in pcDNA3.1) were co-transfected into the 100 mm dish, in the density of 2.6×10^6 cells per dish, of HEK293T cells as described previously in chapter 3.2.2. (10 µg of each respective plasmid in total of 20 µg was used for each co-transfection). Plasmids encoding DDX1 protein (DDX1-Myc-Flag) and HBV Core protein (Core-HA) were co-transfected as negative control for specific protein-protein interaction. Plasmid pcDNA 3.1 was used as a negative control. The HEK293T cells were handled as described in chapter 3.2.4. The cells were trypsinised 24 hours after the co-transfection.

3.2.12 Protein-protein interaction affinity pull-down assay

- RIPA buffer (pH 7.4, 50 mM HEPES, 150 mM NaCl, 0.4% Igepal dissolved in autoclaved milliQ water from Milli-Q® Integral Water Purification System supplemented by cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail tablet
- SDS-PAGE sample buffer (360 mM Tris, pH 6.8, 30% glycerol, 10% SDS, 4% 2-mercaptoethanol, 0.01% bromophenol blue)

The co-transfected cells were harvested and lysed as described previously in chapter 3.2.4. and the concentration of the resulting supernatant was measured according to chapter 3.2.5. From each sample, 300 µg of total protein were incubated on pre-equilibrated anti-HA coated magnetic beads and similarly, 300 µg were incubated on pre-equilibrated anti-FLAG coated

magnetic beads for one hour at 4 ˚C. The equilibration consisted of three washes of 20 µl of the original bead suspension by 1 ml of the RIPA buffer. Collection of the beads was done by using magnetic rack (BioRad). After incubation, the beads were washed three times with 1 ml of RIPA lysis buffer and after removal of the final wash boiled in 40 µl of reducing SDS sample buffer. The samples were separated by SDS-PAGE and blotted to nitrocellulose membrane as described previously in chapter 3.2.7 and 3.2.8. The membranes were blocked for 1 hour at room temperature and stained with anti-HA (rabbit) primary antibody and anti-FLAG HRP-conjugated antibody over night at 4 ˚C. Secondary anti-rabbit antibody with conjugated red fluorescent dye was added the following day after washing of the membrane and incubated for 1 hour at room temperature. Resulting pictures were captured on Li-Cor machine at 680 nm followed by incubation of membranes in 5 ml Luminata Forte substrate to visualise HRP-conjugated antibody and captured on chemiluminiscence camera (Monochrome scientific grade camera Quantum ST4, Vilber Lourmat (Collegién, France).

4 Results

4.1 Mapping of possible HBV Core interacting proteins in live cells

To identify novel HBV Core protein interactions in living cells, we used proximity-dependent biotin identification (BioID) (Roux, Kim et al. 2013). The BioID technique uses promiscuous biotin ligase (named BioID2), which is in our case fused to HBV Core protein and, when expressed in the cell, biotinylates proximal endogenous proteins. Therefore, it enables identification of weak or transient protein-protein interactions that are typically hard to identify by yeast two-hybrid or affinity purification. The newly identified HBV Core – protein interactions were further analysed bioinformatically in the cellular context. Based on the analysis, one of the putative newly identified HBV Core interacting proteins was validated on the molecular level.

4.1.1 HBV Core - BioID2 fusion constructs used for BioID experiments

The DNA constructs encoding three different fusion proteins of HBV Core and biotin ligase were previously cloned by Dr. Irina Kontsevaya (Dr. Grantz Šašková group). The DNA construct encoding HBV Core with C-terminal HA-tag was obtained from the laboratory of Dr. Weber (IOCB Prague) and the DNA plasmids encoding DDX3 protein and DDX1 with C-terminal Myc- and Flag-tag were purchased commercially (OriGene). All protein variants used in the study are summed in Table 1 (chapter 3.1.5) and schematically depicted in Figure 5. The "Core-GS-BioID2-HA" and "Myc-BioID2-GS-Core" DNA constructs encode protein variants where HBV Core protein is connected to the biotin ligase with either HA- or Myc-tag by a short "glycine-serine" linker either on C- or N-terminus of the Core encoding sequence. The "Core-13xGS-BioID2-HA" encodes the protein variant in which a 13-times repetitive "glycine-serine" amino acid linker is used to theoretically reach a wider interactome space. The constructs expressing only Myc-BioID2 proximity biotin ligase and HA-Core where used as controls.

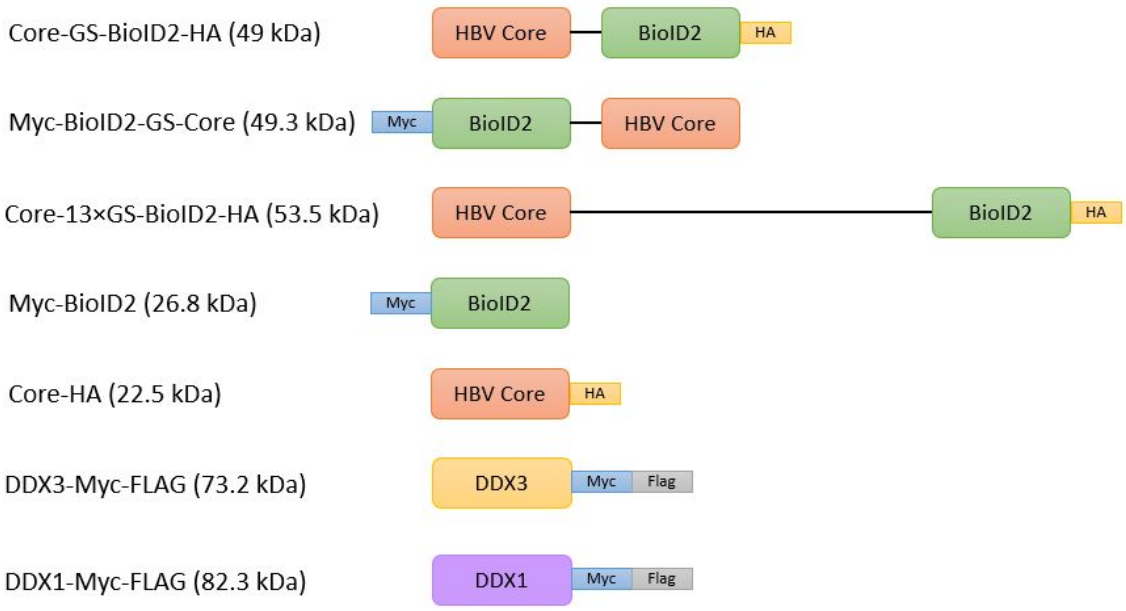


Figure 5: Schematic representation of all protein variants used in the study.

4.1.2 Validation of expression of the HBV Core - BioID2 fusion protein variants

Transient expression of the Core-GS-BioID2-HA, Myc-BioID2-GS-Core and Core-13xGS-BioID2-HA protein variants was tested in Huh7 cells as described in chapters 3.2.1, 3.2.2 and 3.2.4. The lysates were analysed by western blotting as described in chapters 3.2.7 and 3.2.8 (Figure 6). The blot was visualized by anti-Core antibody and subsequently by the green fluorescent dye-conjugated secondary anti-rabbit antibody. The anti-β-actin antibody was used as a loading control, visualised by red fluorescent dye-conjugated secondary anti-mouse antibody. The Myc-BioID2-GS-Core protein variant was not expressed. The Core-GS-BioID2-HA and the Core-13xGS-BioID2-HA protein variants were equally expressed. No degradation of the two expressed protein variants was observed. For the initial in-cell biotinylation experiments described in the study, the Core-GS-BioID2-HA variant with short "glycine-serine" linker was used. The amino acid sequence with individual "protein domains" is depicted in Figure 7. The variant encoding Core-13xGS-BioID2-HA protein was kept for the following future experiments.

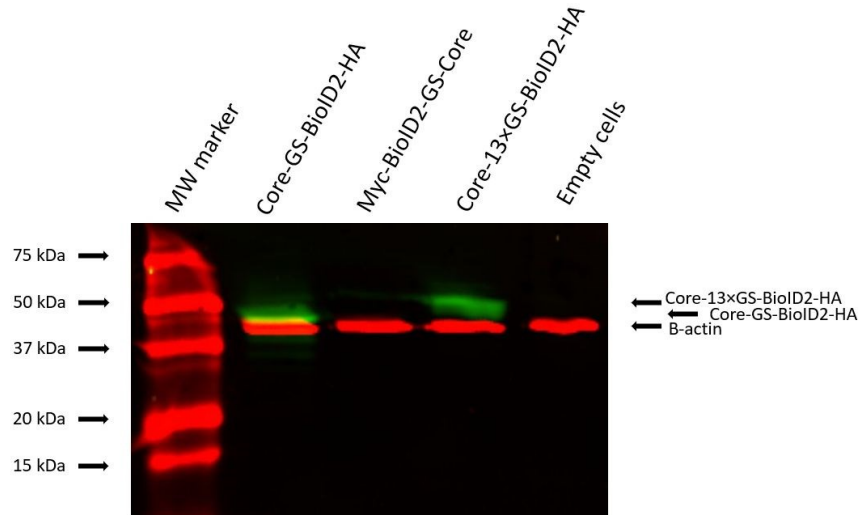


Figure 6: Expression validation of the Core-GS-BioID2-HA, Myc-BioID2-GS-Core and Core-13xGS-BioID2-HA protein variants in Huh7 cells compared to non-transfected cells. The Huh7 cells were transiently transfected with the respective DNA constructs as described in chapter 3.2.2. Two days after transfection, the cells were harvested, and the lysates were separated on 12% AA gel and subsequently transferred to a nitrocellulose membrane for western blotting. The proteins were visualized by anti-Core antibody (green). β-actin was used as a loading control (red). All blue marker was used as a MW standard.

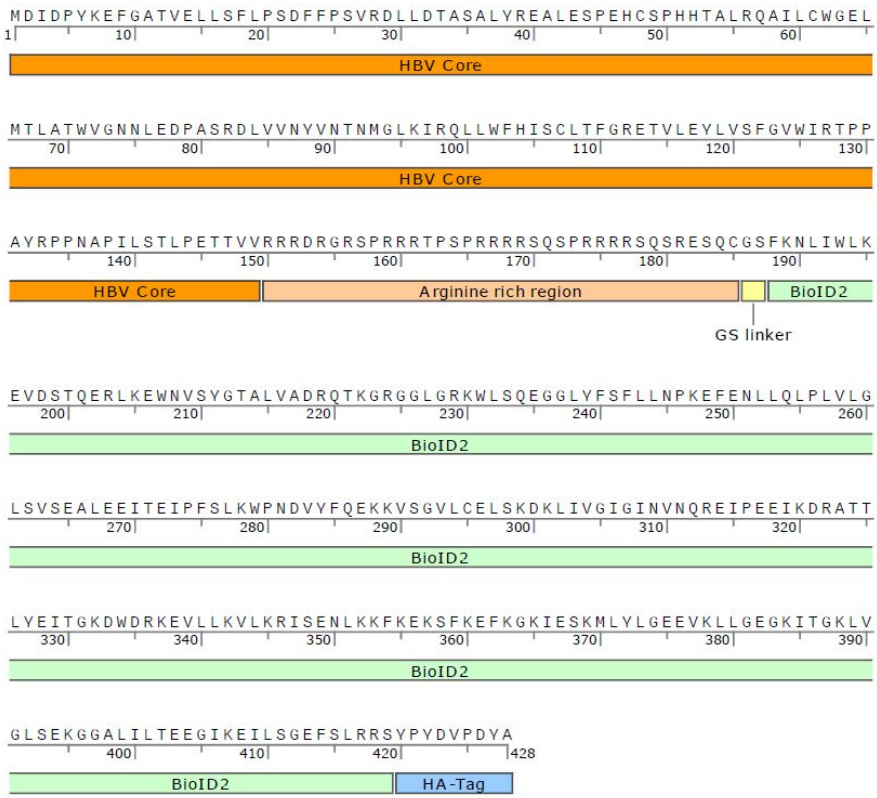


Figure 7: Amino-acid sequence of the Core-GS-BioID2-HA protein variant used for the proximity biotinylation in live cells. The HBV Core (orange) is linked to biotin ligase (BioID2, green) via a short “glycine-serine” linker

(yellow) and N-terminally HA-tagged (blue). The Arginine rich region of the HBV Core protein is depicted in light orange.

4.1.3 Proximity-dependent biotin identification of the HBV Core - host interacting proteins

The workflow of the proximity-dependent biotin identification experiment used to identify novel HBV Core interacting proteins is depicted in Figure 8. Briefly, the HEK293T cells were transiently transfected with the Core-GS-BioID2-HA DNA construct and respective controls. Next, biotin was added to the media to induce biotinylation. The cells were then incubated for 24 hours, then harvested and lysed. The biotinylated proteins were subsequently purified on the Streptavidin coated agarose beads. The whole procedure was analysed by western blot before the samples were subjected to mass spectrometry analysis. To obtain significant results for statistical analysis, the whole procedure was repeated in 6 biological replicates. Finally, the potential target list was analysed, and one target protein was validated.

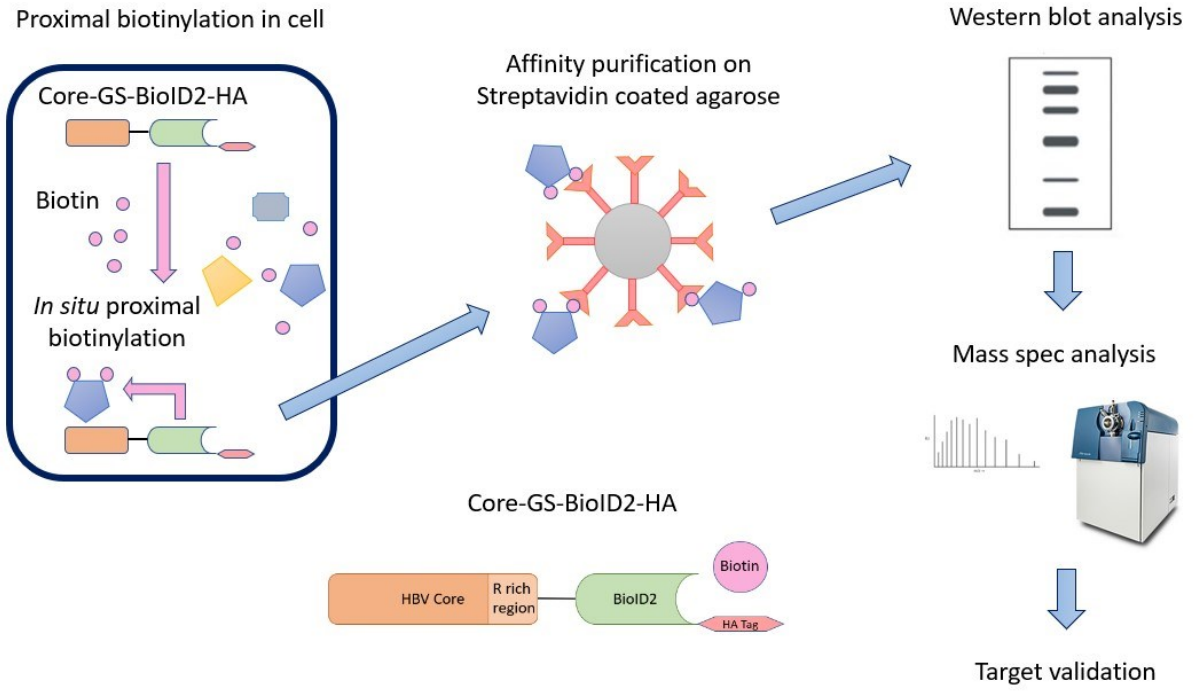


Figure 8: Schematic representation of the complete workflow of the proximity-dependent biotin identification of the HBV Core - host interacting proteins.

Each out of the six biological experiments was done as follows: the HEK293T cells were seeded on four 100 mm dishes and transfected according to Table 2.

Table 2: HEK293T cell transfection for proximity-dependent biotin identification experiment

DNA plasmid	Description
none	Non-transfected HEK293Tcells, negative control (NC)
Core-HA	HEK293T cells transfected with Core-HA plasmid, positive control for WB analysis (CC)
Myc-BioID2	HEK293T cells transfected with plasmid expressing only BioID2 proximity biotin ligase, negative control for MS analysis
Core-GS-BioID2-HA	HEK293T cells transfected with Core-GS-BioID2-HA

The cells were harvested 24 hours after transfection, lysed, the supernatant was separated from the cell debris and its concentration was measured. 500 µg of total protein were incubated on Streptavidin coated agarose beads, washed and boiled in 140 µl of the reducing SDS sample buffer. From each sample, 100 µl were submitted for mass spectrometry analysis and 40 µl were kept for western blot analysis. The representative of the six resulting pictures of the western blot analyses of all six biological replicates is shown in Figure 9. It is possible to discern between endogenously biotinylated proteins (three green bands in the negative control and the Core-HA control) from the BioID2 mediated biotinylation (Myc-BioID2 control and Core-GS-BioID2-HA). The Core-GS-BioID2-HA protein variant is visible as a yellow band, which is created by combination of the red signal (presence of Core protein) and the green signal (biotinylated Core protein). The positive control Core-HA is clearly detected in the CC-lysate fraction and the same amount in the CC-Flow-through fraction. None is detected in the CC-purified fraction as the purification was performed on Streptavidin beads. As clearly shown, the Core-GS-BioID2-HA fusion protein biotinylates with similar effectivity as the biotin ligase alone, but different variety of endogenous proteins.

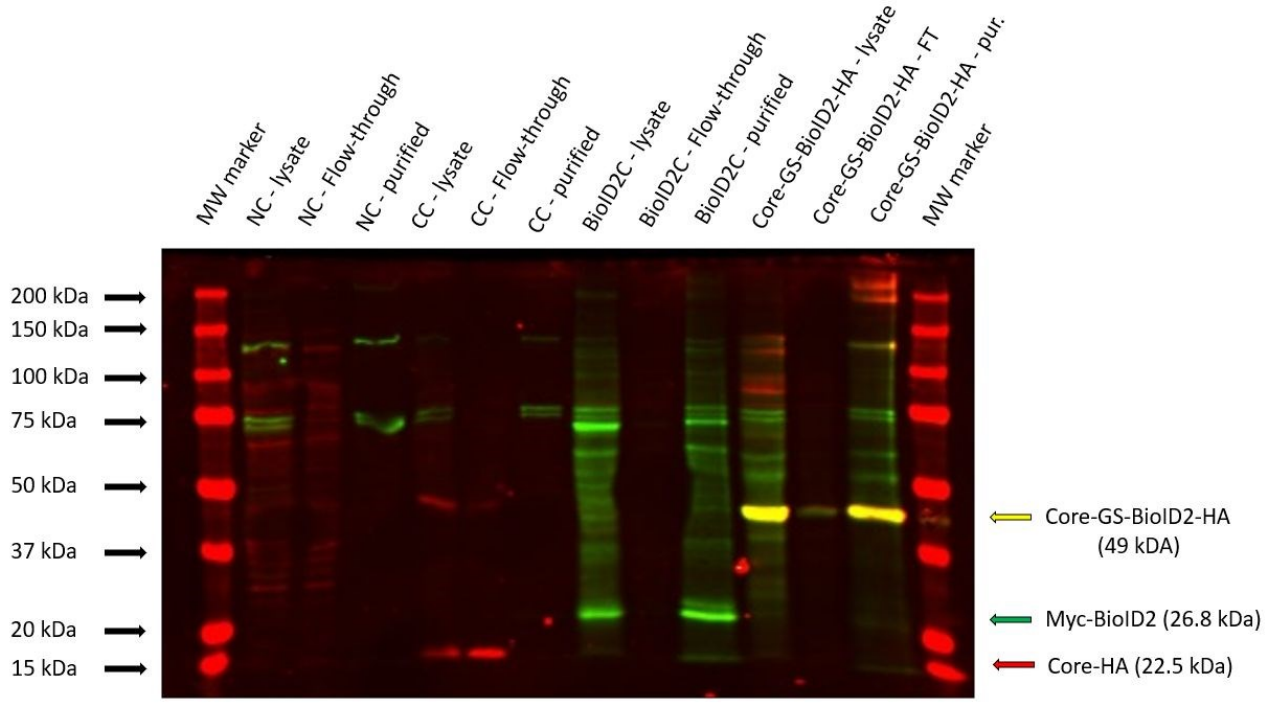


Figure 9: Western blot analysis of the proximity-dependent biotin identification experiment with the Core-GS-BioID2-HA protein variant in HEK293T cells. 500 µg of the lysates (according to Table 2) were incubated on Streptavidin agarose beads. After incubation, the flow-through fractions (FT) were collected and the beads were boiled in reducing SDS sample buffer (sample - purified). 20 µg of FT and lysate samples and 20 ul of samples after affinity purification were separated in 10% AA gel. The membrane was visualized with anti-Strep (green) and anti-Core antibody (red). The image was acquired on Odyssey® CLx Infrared Imaging System, LI-COR Biosciences (Lincoln, USA). n=6

4.1.4 Mass spectrometry analysis and statistical analysis

The mass spectrometry measurements were performed in the Mass Spec Core facility of the IOCB Prague under the supervision of Dr. Martin Hubálek. Statistical analysis was done by Dr. Alena Křenková from the same facility. The statistical analysis performed as described in chapter 3.2.9. resulted in a list of proteins that were significantly enriched or downregulated in the samples produced from cells transfected with the Core-GS-BioID2-HA DNA plasmid, compared to non-transfected cells or the cells transfected with the Myc-BioID2 plasmid. Only the results comparing the Core-GS-BioID2-HA transfected cells and the non-transfected cells are shown and discussed in the study. The total list of 105 significantly enriched and 40 significantly downregulated proteins was identified. The results are visualised as a Volcano plot (Figure 10), which shows the difference of the spectra intensity (X - axis) and the significance of the difference (Y - axis) in the scope of six biological replicates.

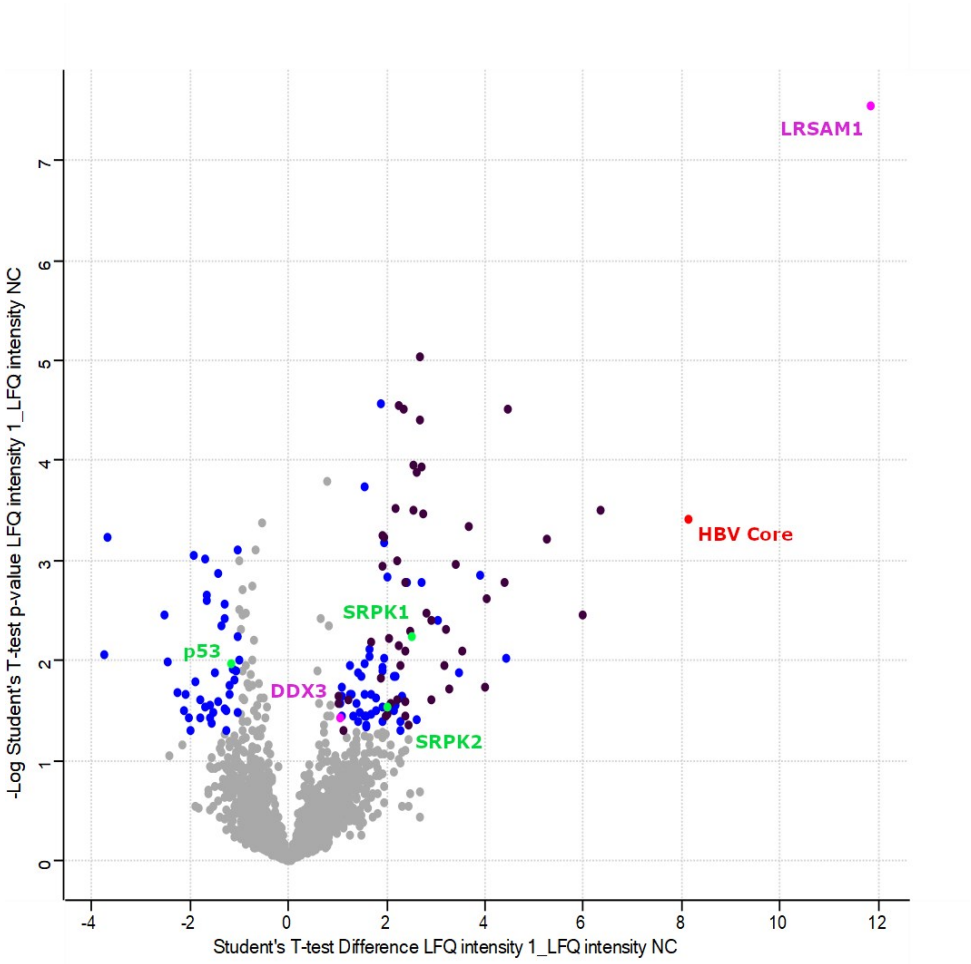


Figure 10: A volcano plot showing statistically significant proteins identified by the BioID technique with significantly increased or decreased intensity of the spectra of the proteins present in samples produced by the Core-GS-BioID2-HA compared to the intensity of spectra in the respective negative control samples. All statistically significant proteins in blue, ribosomal proteins in black, previously confirmed HBV Core interactants in green, HBV core in red and two proteins further analysed or commented in the study in violet.

4.1.5 Network modelling and clustering analysis

To visualize the whole HBV Core protein interactome network, the protein list was analysed by Enrichr (Chen, Tan et al. 2013, Kuleshov, Jones et al. 2016) and PANTHER (Mi, Muruganujan et al. 2019) on-line software tools. Initially we were interested if our newly identified HBV Core interactome network shares proteins already identified as interactors of other viruses infecting humans. The significantly enriched proteins were thus compared to all known human proteins previously identified as interacting with any virus using Enrichr on-line tool (Chen, Tan et al. 2013, Kuleshov, Jones et al. 2016). The list of the newly identified HBV Core putatively interacting proteins shared with other viruses is shown in Table 3.

Table 3: List of putative newly identified HBV Core interacting proteins shared with previously identified interactors of other human viruses

	VIRUS	PROTEINS (GENE NAME)
1	Epstein-Barr virus (strain GD1)	*SRSF1, NCL, CAPZA1, H1FX, RSL1D1, RPS2, RPS3A, RPS6, RPS8, RPS9, RPS13, RPS17, RPS23, RPL7, RPL7A, RPL8, RPL9, RPL13, RPL18, RPL31*
2	Human papillomavirus type 16	*RPS25, UBE2I, ATP6VOA1, SRPK1*
3	Human immunodeficiency virus 1	*ANXA2, NCL*
4	Bovine papillomavirus type 1	*UBE2I*
5	Human herpesvirus 1 (strain 17)	*RBBP4*
6	Epstein-Barr virus (strain B95-8)	*SRSF1*
7	Human herpesvirus 1	*SRPK1*
8	Hepatitis C virus subtype 1b	*DDX3*

Next, the list of the newly identified putative HBV Core interacting proteins (enriched) and also the list of the proteins that were less present ("downregulated") in the samples after the expression of the HBV Core protein compared to negative control, were both analysed in terms of the biological processes individual proteins are involved in (Figure 11). The assignment into specific categories was done by Enrichr on-line that employs The Gene Ontology (GO) database (Ashburner, Ball et al. 2000, The Gene Ontology 2019). Relation between individual categories was curated manually based on AmiGO web searching tool (Carbon, Ireland et al. 2009).

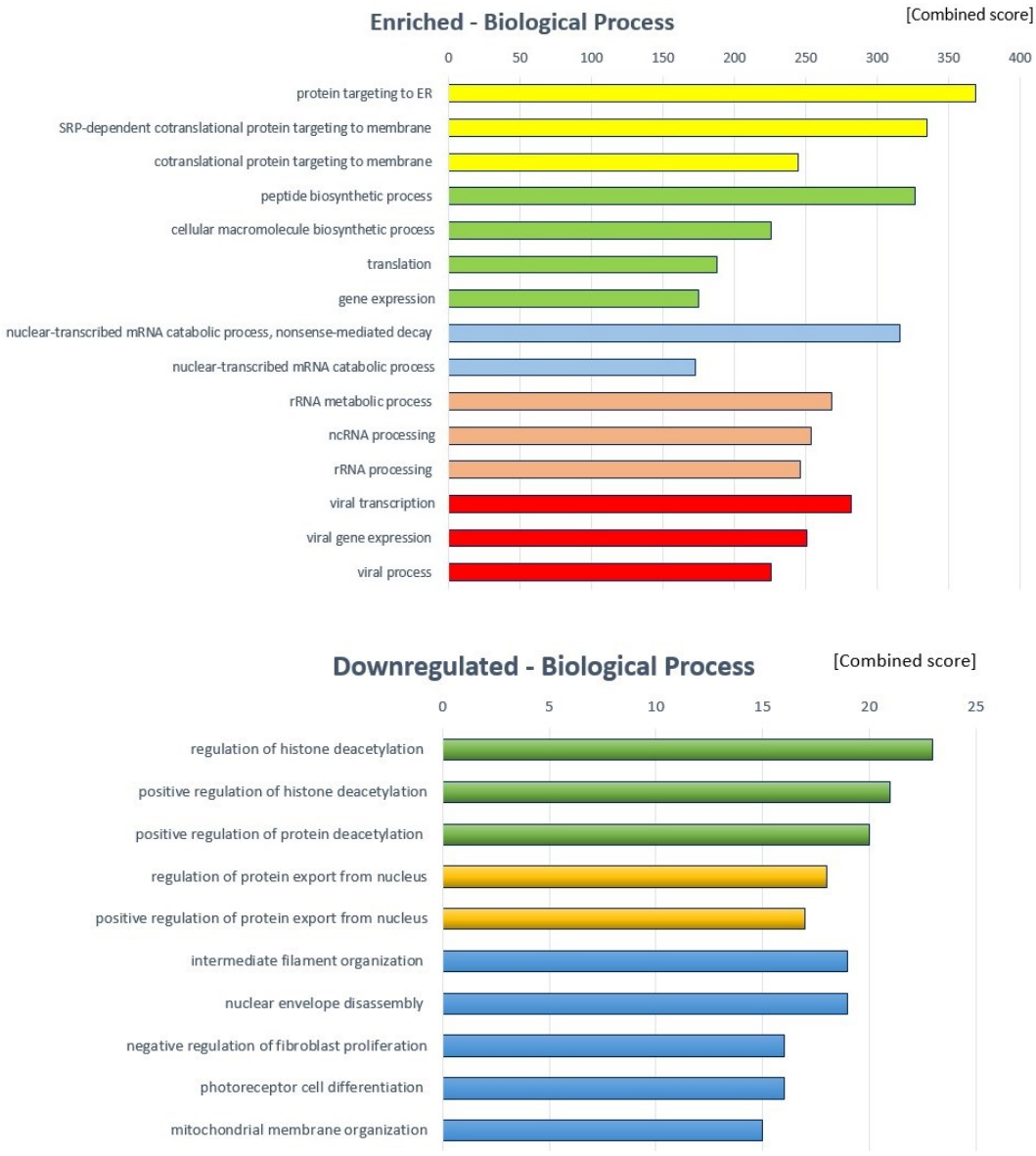


Figure 11: The significantly enriched proteins identified by the BioID experiments are employed in biological processes which can be divided among 5 main groups: co-translational transport (yellow), gene expression (green), catabolic processes (blue), RNA processing (orange) and viral processes (red). The proteins that are present in significantly lower amount in the samples expressing HBV Core are mainly involved in processes linked to nuclear homeostasis – deacetylation (green), protein export from nucleus (yellow) and other, nonrelated processes (blue). The data are shown as a combined score, computed by taking the log of the p-value from the Fisher exact test and multiplying that by the z-score of the deviation from the expected rank.

Most of the putative HBV Core newly identified interactors are involved in the co-translational processes, such as proteins targeting to Endoplasmic Reticulum (ER) (e.g. LRSAM1, SRP54, SRP72) or proteins involved in gene expression (U2AF, SRSF1, DHX30, etc. including previously identified SRPK1 and SRPK2) (Daub, Blencke et al. 2002). The newly identified HBV Core interactors involved in viral processes are mostly ribosomal proteins (e.g. RPL4,

RPS3) but also VCP or LRSAM1. The proteins that are present in significantly lower amount in the samples expressing HBV Core are mainly involved in the processes linked to nuclear homeostasis, including previously identified p53 (Kwon and Rho 2003).

Next, we employed similar approach to analyse the molecular functions of the newly identified HBV Core target proteins (see Figure 12). The majority of the significantly enriched proteins potentially interacting with HBV Core belongs to the RNA binding proteins. Some HBV Core interactors are involved in ubiquitin-proteasome pathway (i.e. RPL5, RPL11) and some have RNA helicase activity (DDX18, DHX30, DDX3).

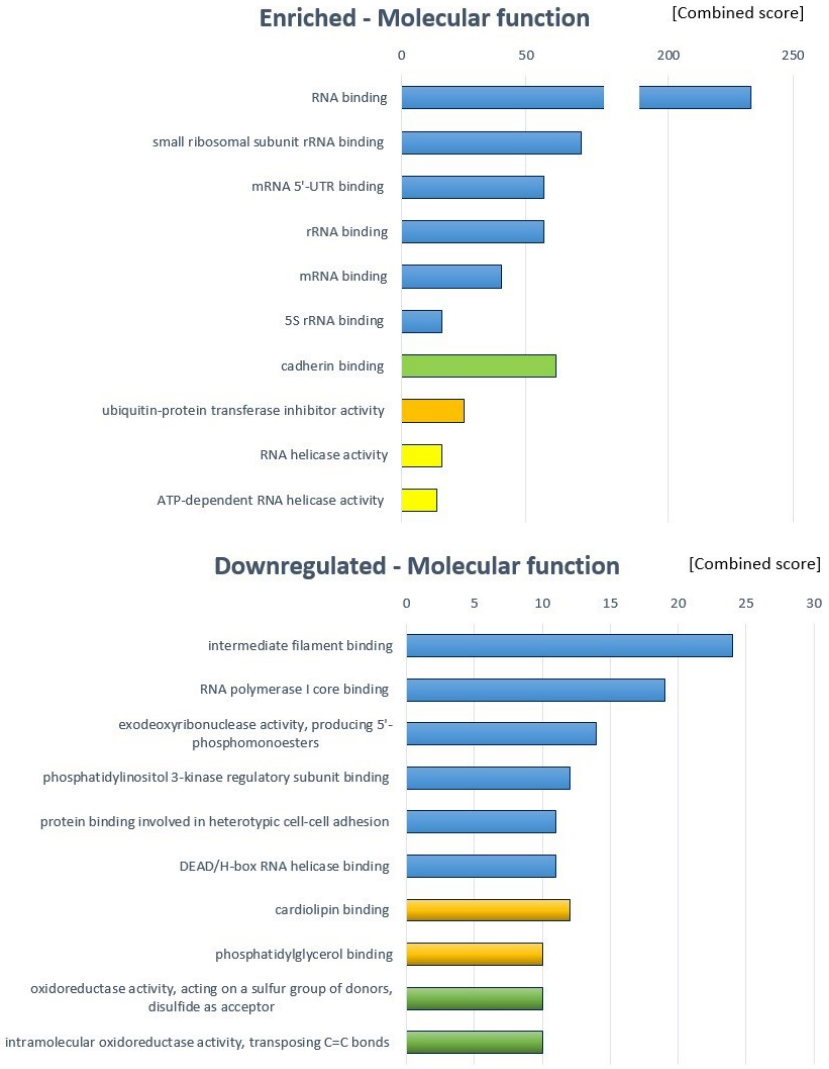
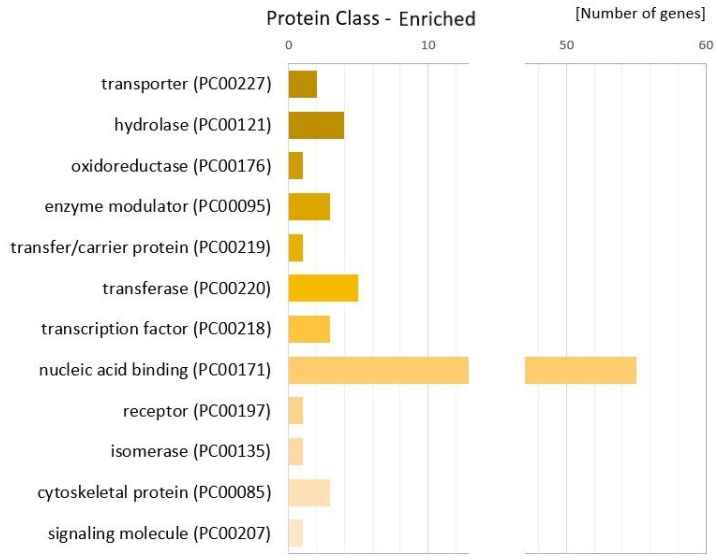
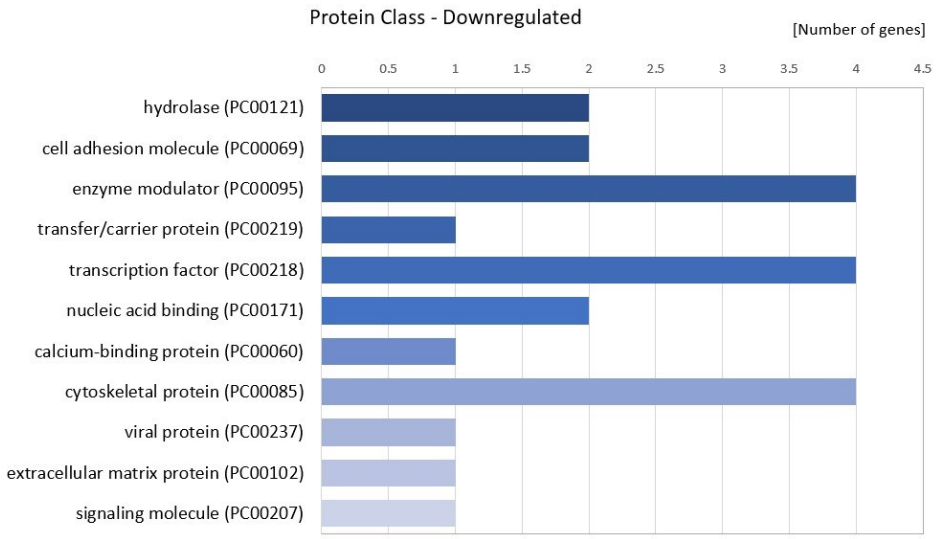


Figure 12: The proteins that are significantly enriched after HBV Core expression compared to negative control mostly belong to the category of RNA binding proteins (blue), proteins with helicase activity (yellow) and others. The downregulated proteins have various unrelated molecular functions (blue), some have oxidoreductase activity (green), some are involved in phospholipid binding (yellow). The data are shown as a combined score, computed

by taking the log of the p-value from the Fisher exact test and multiplying that by the z-score of the deviation from the expected rank.

Finally, we categorized the newly identified HBV Core target proteins by protein class using PANTHER (Mi, Muruganujan et al. 2019) on-line software tool (Figure 13). The majority of the proteins belongs to the family of proteins binding nucleic acids. Second most common protein class are transferases, followed by hydrolases, transcription factors and cytoskeletal proteins. We were also interested in those protein classes that are less abundant in the samples where HBV Core was expressed. Three major classes are enzyme modulators, transcription factors and cytoskeletal proteins. Altogether, when analysing both "enriched" and "downregulated" datasets, six major protein classes are influenced by HBV Core expression with the majority being proteins binding nucleic acids, followed by hydrolases, enzyme modulators, transferases, transcription factors and cytoskeletal proteins.





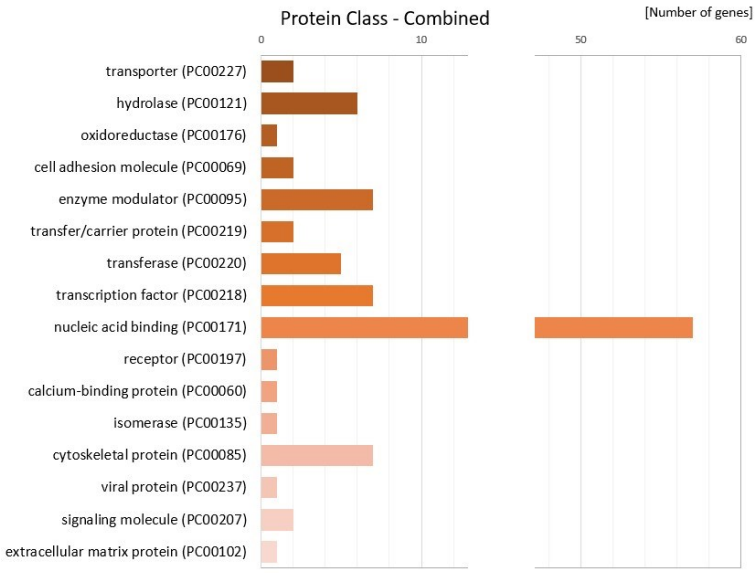


Figure 13: Categorization of the identified HBV Core interactors by protein class. Analysis was done using web-server PANTHER classification system. Graphs were created in Microsoft Excel.

4.1.6 Clustergrams

To inspect how the newly identified HBV Core target proteins cluster together in the categories of Biological processes (Figure 14) and Molecular function (Figure 15), we used Enrichr on-line tool (Chen, Tan et al. 2013, Kuleshov, Jones et al. 2016) to produce clustergrams from the list of "enriched" proteins. Five proteins including RAB3GAP2, RAB3GAP1, LRSAM1, Annexin A2 and VCP are involved in the establishment of protein localization in the ER, vacuole and autophagosome assembly. The second cluster form CTTN, ATP6V0A1, GOLGAG4, IFT22, SNX9 and SRP72 proteins in a more general process "protein transport". Several clusters are formed by SRKP1 and SRKP2 serine/arginine-rich protein-specific kinases that were previously identified as HBV Core interacting partners that play various roles in the cell such as those in splicing, mRNA maturation, chromatin reorganisation and cell cycle progression. They cluster with their substrates SRSF1 and SRSF6 and further with proteins U2AF, PUF60, DDX3X, PAT1, SUPT5H and AHNAK. The last cluster worth attention is formed by TFB1M, FTSJ3, NOP2, TRMT61A and NOP10 proteins.

The largest cluster formed when analysing Molecular function of the enriched proteins from our dataset are RNA binding proteins. Second cluster is formed by proteins binding cadherin, where belongs H1x, AHNAK, HDLBP, PUF60, Annexin A2, MAPRE1, DDX3X, CTTN, CAPZA1 and SNX9. DDX3X is further clustering with RNA helicases DHX30, DDX18 and DDX21. The last cluster is formed by SRPK1, SRPK2, SRP54, GTPBP4 and TSR1.

It would be interesting to further determine the molecular details of HBV Core interaction with individual proteins and to inspect the interplay, if any, between HBV Core and individual proteins forming the clusters.

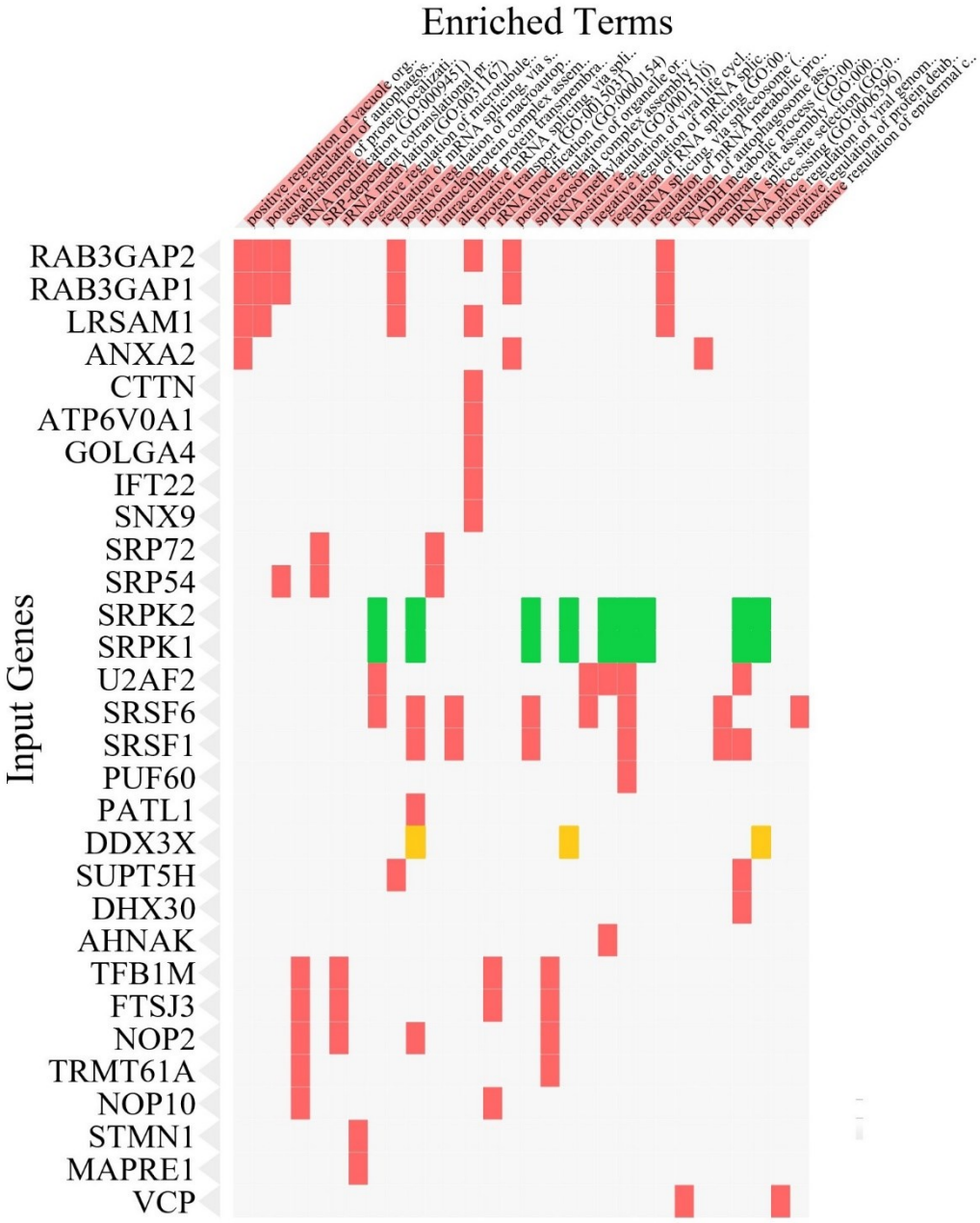


Figure 14: Clustergram analysis of the Biological processes where the enriched proteins identified in the samples expressing HBV Core are involved in. SRPK1, SRPK2 (previously identified HBV Core interactors) shown in green, target protein DDX3 in yellow.

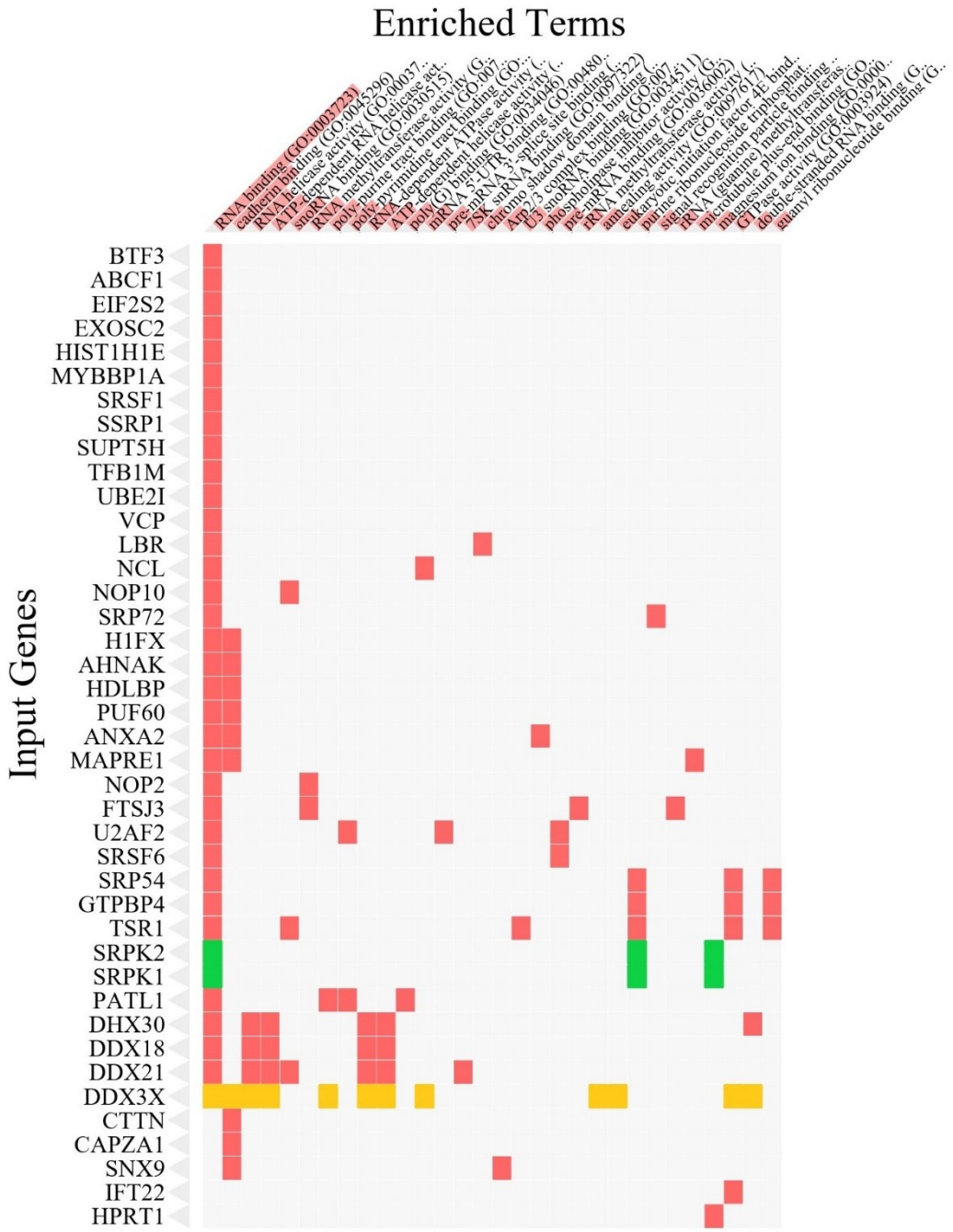


Figure 15: Clustergram analysis of the Molecular function where the enriched proteins identified in the samples expressing HBV Core are involved in. SRPK1, SRPK2 (previously identified HBV Core interactors) shown in green, target protein DDX3 in yellow.

4.2 Validation of HBV Core – DDX3 interaction

4.2.1 Protein – protein interaction pull-down experiment

The RNA helicase DDX3 was chosen to be validated as the HBV Core interacting protein partner mainly due to its known role in HBC viral infection. This was further supported by our protein network analysis. To be able to affinity-purify DDX3 and HBV Core protein from the cell lysate, both proteins were C-terminally tagged (Figure 5), the HBV Core by the HA-tag and the DDX3 protein by the FLAG-tag. C-terminally FLAG-tagged DDX1, related RNA helicase that plays role in HBC viral infection, but that was not present among the significantly enriched proteins, was chosen as a negative control. HEK293T cells were seeded and transfected with the respective plasmids as described previously, under conditions described in Table 4.

Table 4: Design of the protein-protein interaction pull-down assay

Condition	First plasmid	Second plasmid
Negative control	pcDNA 3.1	pcDNA 3.1
Negative Core protein control	pcDNA 3.1	Core-HA
Negative control DDX1	pcDNA 3.1	DDX1-FLAG
DDX1 + Core protein	Core-HA	DDX1-FLAG
DDX3 negative control	pcDNA 3.1	DDX3-FLAG
DDX3 + Core protein	Core-HA	DDX3-FLAG

The cells were processed as previously described and 300 µg of the total protein was incubated on both anti-HA and anti-FLAG beads to pull out tagged proteins and potentially also its interacting partner. The beads were washed sufficiently after incubation and boiled in 40 µl of the reducing SDS-sample buffer. Resulting samples were separated by SDS-PAGE, transferred to nitrocellulose membrane and stained with anti-FLAG HRP conjugated antibody and anti-HA primary antibody which was detected by anti-rabbit red fluorescent dye-conjugated secondary antibody. The proteins were visualized by the Odyssey® CLx Infrared Imaging System, LI-COR Biosciences (Lincoln, USA) or incubated with the Luminata Forte substrate and captured on Monochrome scientific grade camera Quantum ST4, Vilber Lourmat (Collegién, France). As shown in Figure 16, the Core-HA protein was pulled down via DDX3-FLAG protein incubated with the anti-FLAG beads, but not via DDX1-FLAG. Furthermore, the DDX3-FLAG

protein was pulled down via Core-HA incubated with the anti-HA beads. In agreement with the previous pull-down using anti-FLAG beads, the DDX1-FLAG protein as not pulled down by Core-HA incubated with the anti-HA beads. No unspecific binding of tested proteins to the beads was observed. The pull-down experiment was performed in three biological replicates and only representative blots are shown.

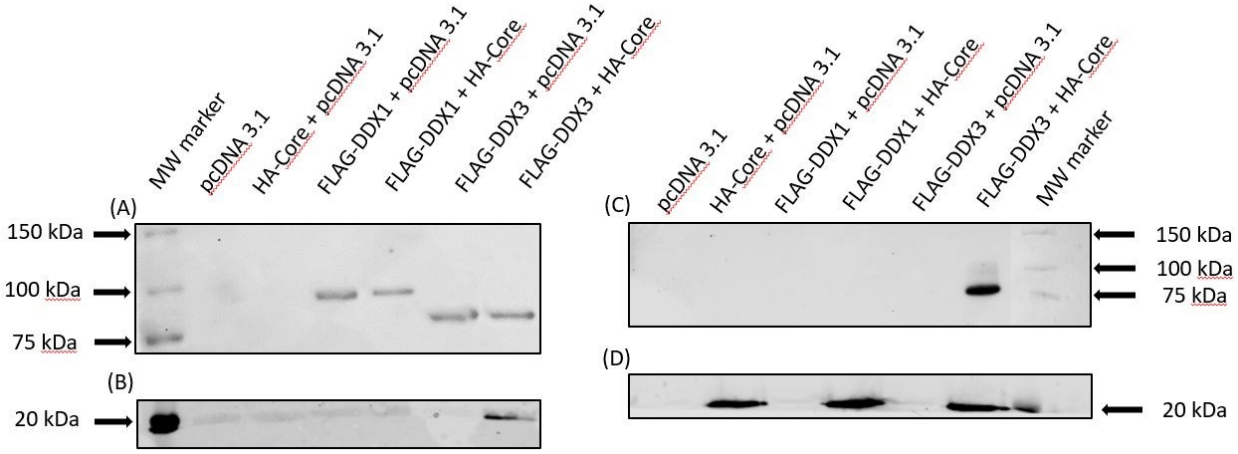


Figure 16: Protein-protein interaction pull-down assay. The protein samples from the anti-FLAG (A, B) and anti-HA (C, D) affinity purification were separated in 10% AA gel, followed by wet transfer to the nitrocellulose membrane. The membranes were stained by anti-FLAG (A, C) and anti-HA (B, D) antibodies.

5 Discussion

Despite a safe and effective vaccine, hepatitis B virus still infects more than 250 million people worldwide, leading to death of around 800 thousand of them annually. Hepatitis B virus could be in general described as “stealth” virus, since the acute phase and the early phase of chronic infection often go unnoticed, leading to the development of hepatocellular carcinoma. Current treatment inhibits replication of the virus but does not cure the illness entirely, resulting in mandatory life-long treatment accompanied by side effects. The key to viral persistence is cccDNA molecule residing in the host cell nucleus, hidden from the immune system. Other components of HBV also partake in its survival within the cell, either by hijacking processes of the host cell for the virus’ benefit or by inhibiting immune response. To be able to target these processes specifically, we must first discover what endogenous molecules are hijacked by the viral products, how do the viral components influence their function and with what outcome. By improving the current knowledge of the mechanisms of intracellular HBV infection, it could be possible to eradicate the illness entirely.

To address this issue, we decided to explore the interactome of HBV Core protein. To achieve this, we chose to use BioID method, which is performed in living cells and thus provides more relevant and reliable data than other commonly used approaches. Three different plasmids were designed: The Core protein C-terminally linked to BioID2 enzyme with short or long linker and the Core protein N-terminally linked to BioID2. N-terminally linked Core protein to BioID2 was presumed not to be functional, based on accumulating evidence from the collaborating lab (unpublished data), yet we decided to see whether the fusion protein could be useful. The long linker was designed to ensure the flexibility in case the short linker would disturb functionality of the two fused proteins. The N-terminally fused construct was indeed non-functional, most likely due to disrupting correct fold of the Core monomers and preventing assembly of subunits into polymers. The N-terminus is partially hidden in the secondary structure of the protein, whereas the C-terminus is more flexible and accessible, thus less susceptible to conformational disruptions (Figure 17). Both C-terminally linked fusion proteins were expressed so we chose to work further with the Core-GS-BioID2-HA first. The one with longer linker was saved for future research, though it might produce less specific data due to wider radius of biotinylation.

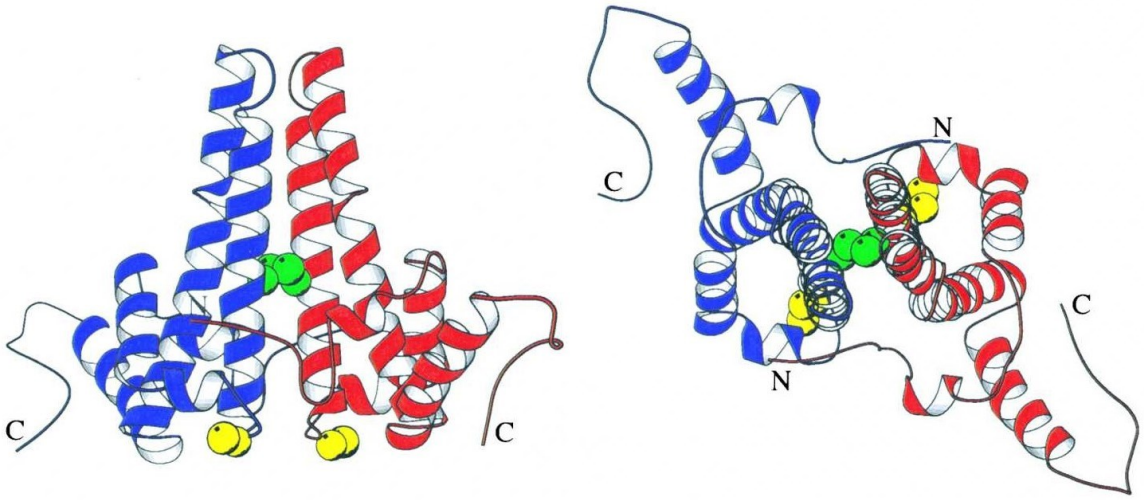


Figure 17: Structure of two interacting Core protein subunits (red and blue). The cysteine residues forming disulphide bonds are shown in green, whereas cysteine residues not forming disulphide bonds are shown in yellow. The N-termini are partially hidden in the interacting plane, contrary to the C-termini which are exposed for facilitated interaction. Adapted from Wynne, Crowther et al. (1999).

The biotinylation experiment showed, that the fusion construct is able to biotinylate endogenous proteins with similar effectivity as the BioID2 enzyme alone, when analysed on western blot. Even on the blot (Figure 9, chapter 4.1.3) it is visible, that the expressed constructs biotinylates slightly different range of proteins than BioID2 itself, though the final assessment was left for the mass spectrometry analysis. In the context of my diploma thesis, only the non-transfected cells and the Core-GS-BioID2-HA-transfected cells were compared.

The mass spectrometry produced several interesting datasets. Firstly, we looked at the outcome of binary subtraction, simply to see, what proteins were present only in the samples from the construct-transfected cells, when compared to empty cells. However, this method produced false negative results for proteins, that were present in both samples, yet in different concentrations. The employed biological system provides very little possibilities to normalise the samples. Cell lines often behave differently even when treated exactly the same, the transfection effectivity might vary etc. Instead of using the isotopic labelling method (such as SILAC), we decided to compare spectra intensity of single identified peptides and intensity of the whole sample. Thereby we could obtain an information of how big the fraction of a specific protein present in the whole purified sample was. This approach provided us with the list of proteins that were significantly enriched or even significantly downregulated, or less abundant in the presence of the Core protein. The presence of three positive controls (SRPK1, SPRK2, p53) and the HBV Core itself ensured us that the obtained results presented a reliable data. The

volcano plot (Figure 10) provided us with the information about the distribution of the enriched and downregulated proteins in our samples, compared to negative control. To be able to include even the proteins present only in the samples from the construct-transfected cells, they were assigned low, non-null value. Interestingly, we could determine that ribosomal proteins were present only in the enriched fraction of significantly altered proteins (shown in black), indicating that the Core protein does somehow interfere with protein biosynthesis.

The results from the mass spectrometry allowed us to evaluate how effective or accurate our experimental design was. Affiliated lab of Dr. Jan Weber (IOCB Prague) performed the mass spectrometry analysis of the affinity purified HBV Core interactants with endogenous proteins in the form of cell lysate. Their mass spectrometry results were partially distinct from ours, but also contained positive controls (previously published validated HBV Core interactants) and several proteins identified by our method. When compared together, the BioID method produced a smaller list of putative Core protein interactants than affinity purification. This might have several reasons. Firstly, the BioID method is performed in living cells so the "bait" protein, in our case the Core protein, is present only in cell compartments as in natural state and does not come into contact with other components present in the lysate. Secondly, single Core protein subunits quite likely spontaneously form capsids or dimers at least, since this mechanism has been shown even in the case of the recombinantly produced Core protein monomers (Zlotnick, Johnson et al. 1999). Formation of capsid might both enable or restrict some interactions. Lastly, the BioID2 enzyme labels endogenous proteins in their native state and they are later purified from the lysate by Streptavidin beads. The biotin-Streptavidin interaction is very stable and can be subjugated to more extensive and aggressive washes than protein-protein interactions, resulting in less non-specificities. However, both methods had 18 common hits, among them FTSJ3, DDX3, DHX30 etc. (Figure 18). Hereby we determined that our approach is both specific and sensitive and the results can be further processed.

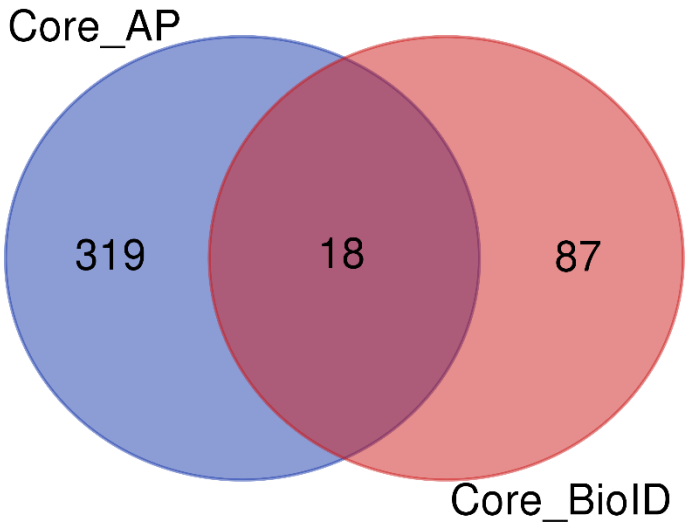


Figure 18: Comparison of the putative Core protein interactants produced by mass spectrometry analysis of the samples acquired by affinity purification method (AP, blue) or by BioID method (BioID, red) and their intersection (violet).

Both obtained lists of the putative Core-influenced proteins from the BioID method were analysed individually and combined as well, to see, what is the connection between identified proteins. Firstly, we searched for known interaction of viral proteins with proteins from our lists. We found that mainly ribosomal proteins and chromatin-associated proteins, but also modifying enzymes (kinases, ubiquitinases), are significant for viral infections.

Further, we investigated what biological processes do the putative interactants (i.e. enriched proteins) take part in. The results were not too surprising, since the virus needs to use the host cell translation apparatus and ER transport for its replication. It was determined that most abundant are proteins that are required for co-translational transport and SRP-dependent targeting to ER. Majority of the produced HBV proteins (HBs, HBe) are transported to the ER during translation, some of them to be incorporated in the membrane (HBs, all sizes). From this it seems that the Core protein might have a regulatory or assisting role in hijacking the translational and transporting apparatus. Second most abundant group of the putative Core protein interactants takes part in general gene expression and macromolecule production. Together with the third group of proteins which take part in mRNA processing, it is evident, that the Core protein might supervise or be present at every step of HBV expression and replication. In case of downregulated proteins, it is not entirely clear whether they directly interact with the Core protein, since their concentration is lower for several reasons. The Core protein might interact with them directly and cause their degradation in similar manner as HBx downregulates SMC5/6 (Decorsiere, Mueller et al. 2016, Murphy, Xu et al. 2016). Other

possibility is, that Core protein interacts with their promoter and hinders their expression like in case of p53 (Kwon and Rho 2003). Downregulated proteins from our list can be divided into two systematic groups and several single categories of biological processes. One group partakes in regulation of (de)acetylation. The other group concerns regulation of protein export from the cell nucleus. This might be quite likely linked to the Core protein role in keeping cccDNA chromosome stable, transcriptionally active and safe in the host cell nucleus, together with all necessary cccDNA minichromosome associated proteins.

The analysis of lists of enriched or downregulated proteins in terms of molecular functions also had an intriguing outcome. Overwhelming majority of enriched proteins takes part in some sort of RNA binding and several of them have RNA helicase activity. Theoretically, the Core protein might partake in exploitation of translational apparatus including the viral mRNA export and preferential processing. Specifically, the Core protein could function as an adaptor protein and target ribosomal subunits to bind the viral mRNA with higher occurrence than endogenous transcripts. RNA helicase activity is needed in various cellular processes, including signalling pathways or viral RNA sensing, which could be both inhibited by the interaction with the HBV Core protein. Some of the enriched proteins from out list play a role in ubiquitin-proteasome pathway, which further suggests, that the Core protein might hijack this system for degradation of restriction factors or other undesired molecules. Downregulated proteins mostly fall into categories that are often important in signalling pathways, including binding to phospholipids or cytoskeleton, suggesting role of the Core protein as signal transduction inhibitor. Some of the downregulated proteins are connected to oxidoreductase activity, which might in native state disrupt or hinder capsid assembly by oxidizing cysteine residues. Formed disulphide bonds (shown in Figure 17) are necessary for formation of secondary structure of HBV Core protein.

Protein classes, into which can be the proteins from our lists categorised, agree with the results of analyses of molecular functions and biological processes. Interactors of nucleic acids are by far the most abundant category, followed by transcription factors, cytoskeletal proteins, enzyme modulators, transferases and hydrolases. This means that RNA binding is the strongest common attribute for the obtained dataset, which further supports the importance of the Core protein for HBV replication and survival. By the interaction with transcription factors and cytoskeletal proteins, the Core protein could be able to regulate expression of both endogenous and viral proteins as well as compartmentalisation and possibly even signalling pathway scaffold. By affecting enzyme activity, the Core protein could influence degradation by hydrolases and chromatin remodelling. Downregulated proteins are predominantly in three protein classes also

present among the enriched hits – cytoskeletal proteins, transcription factors and enzyme modulators. This further suggests that the Core protein might disrupt signalling cascades and cause degradation or lower expression of the host restriction factors. When combined, both groups of significantly differently present proteins confirm that the most prominently by the Core influenced proteins are those binding nucleic acids. Whether the Core protein interacts with or downregulates these endogenous proteins, it is most likely to protect the cccDNA, facilitate translation of the viral mRNAs and enable reverse transcription of the rcDNA. The Core protein therefore seems to be truly "the core" of HBV infection.

To explore relation between enriched proteins from our data more closely, Clustergrams, another software tool provided by Enrichr, were employed. This feature depicts, which proteins fall into the same category, providing better perspective of the whole dataset. In case of the biological processes, protein clusters, closer described in chapter 4.1.6, can be divided into two groups – mRNA processing and protein trafficking. The second group comprises of proteins that regulate protein transport, autophagosome and vacuole organisation. The first, slightly larger group containing also previously published Core interactants SRPK1 and SRPK2, takes part in mRNA splicing, viral life cycle control and mRNA modification. It might be possible, that the Core protein interacts only with one of the proteins but since they could form a complex or cooperate in one pathway, they come in contact with the Core protein as well. Or alternatively, the Core protein might target these specific proteins because they are crucial for the native process. From these results it seems even more likely that the Core protein somehow influences budding of new virions from the host cell (LRSAM1, VCP) and processing of the viral mRNA, mainly during splicing (PAT1, AHNAK) and methylation, which might make the viral transcripts appear as endogenous (FTSJ3). When looking at molecular functions, the largest cluster is, as expected, RNA binding. The second biggest cluster are proteins connected to cadherin binding. This might seem unrelated to the rest of the functions; however, it is quite likely partially involved in transformation of the host cell in later stage of infection. Loss of E-cadherin and its substitution by N-cadherin has been shown to be causally related to formation of carcinomas (Cavallaro, Schaffhauser et al. 2002). Targeting this group of putative interactors might prevent transformation of infected cells and thus reduce mortality from HBV infection. The last two clusters are formed by RNA helicases, which might function as either restriction factors or facilitate the viral replication, as already mentioned SRPK1/2 and their substrates.

In theory, it is possible to assign enriched proteins from the statistical analysis of the mass spectrometry results to single steps of the viral life cycle (Figure 19). Their putative role/s in

HBV infection can be suggested based on known function of the protein, and our bioinformatical analyses. Next aim of our research is also to validate putative Core protein interactants in HBV infected cells.

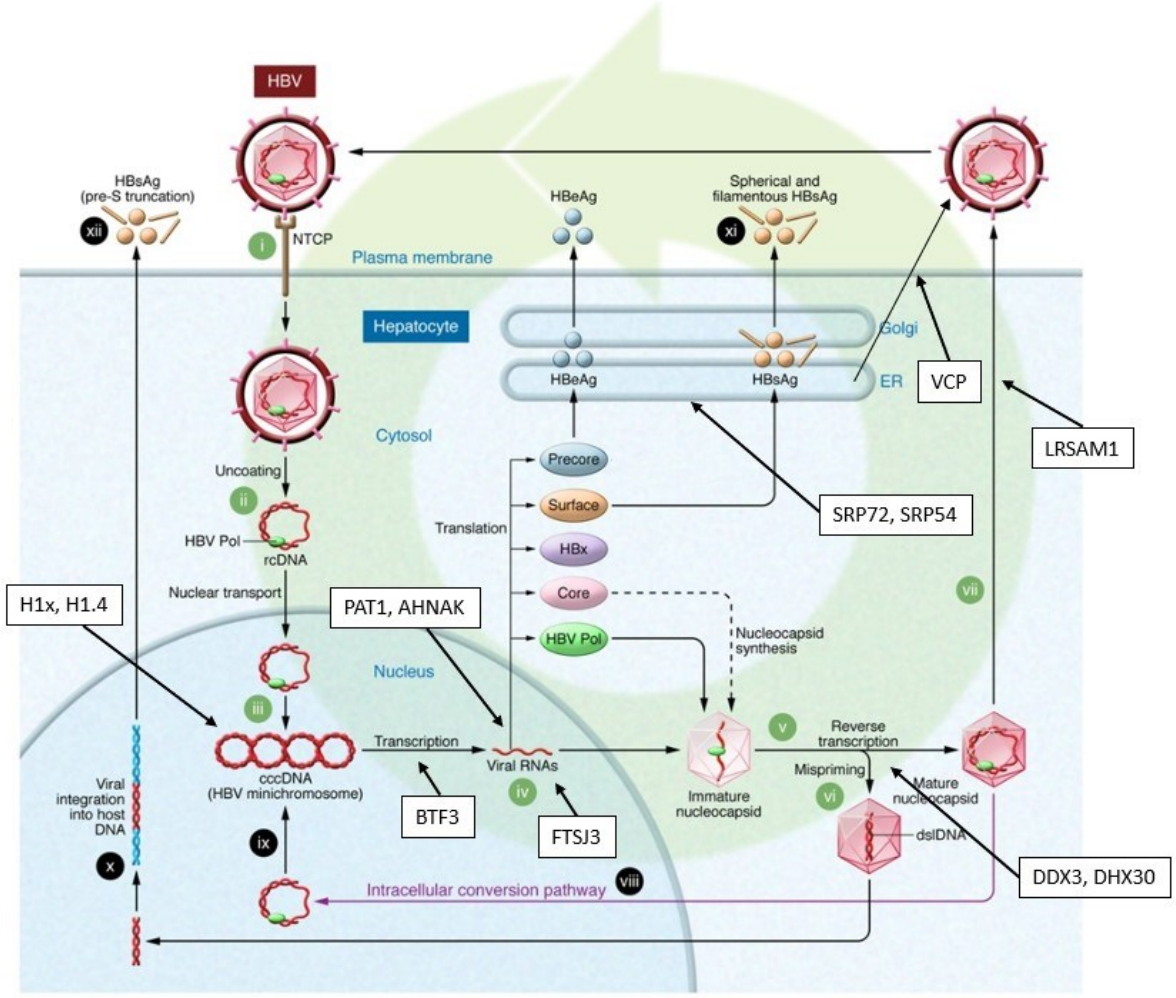


Figure 19: Novel putative HBV Core interacting partners and their suggested role in the HBV life cycle. Putative HBV Core protein interactants from our results might assist or restrict specific steps of the HBV life cycle such as cccDNA stability (histones H1x, H1.4), gene expression (transcription factor BTF3), viral RNA methylation (FTSJ3) or splicing (PAT1, AHNAK proteins), SRP-dependent transport to ER (SRP54, SRP72), restriction of reverse transcription (DDX3, DHX30) and virion budding (LRSAM1, VCP) (Adapted from Revill and Locarnini (2016)).

Besides already published interacting partners (SRPK1, SRPK2), DDX3 RNA helicase is the only protein from our list which has been studied in context of HBV infection. It has been shown that DDX3 functions as a restriction factor for HBV and that its knockdown increases HBV replication rate (Ko, Lee et al. 2014). The bioinformatical analysis has shown that DDX3 is part of the most abundant group of RNA-binding proteins, possesses RNA-helicase activity and participates in signalling pathways leading to cellular immune response and clusters with

multiple other putative interactants. We chose DDX1 as a negative control for validation of specific protein-protein interaction, since it is closely related to DDX3 but was not significantly enriched in our hexaplicate. Role of the RNA helicases in viral infections has been reported multiple times, specifically both DDX1 and DDX3 play an important part in HIV infection (Yasuda-Inoue, Kuroki et al. 2013). The pull-down experiment in triplicate has shown, that the Core protein binds DDX3, but not DDX1 or negative control (empty beads). It is quite possible that the C-terminal arginine rich domain is crucial for this interaction and experiments with truncated version of the Core protein have been carried out, however so far without conclusive results. The exact mechanism of the interaction is yet unknown and will be a subject of our future research.

Several other significantly enriched proteins from obtained dataset have been shown to interact with the viral proteins (as shown in Table 3 chapter 4.1.5). However, some proteins play a role in viral infection without directly interacting with its products. E3 ubiquitinase LRSAM1 (also termed Tsg101-associated ligase) monoubiquitinates Tsg101 enzyme and thereby regulates sorting of exo- and endocytic vesicles including budding of new HIV virions (Amit, Yakir et al. 2004). Its significantly increased presence in all of the six replicates makes it next candidate for protein-protein validation which will be a subject of our future research.

6 Conclusions

1. Mapping of new HBV Core interactants by the proximity-dependent biotinylation in live cells coupled to mass spectrometry
    a. Expression of the DNA constructs used for BioID method was validated by western blot
    b. Proximity-dependent biotinylation was performed in live cells transfected by Core-GS-BioID2-HA construct
    c. Biotinylated protein separated by affinity purification on Streptavidin beads
    d. Effective biotinylation was validated on western blot
    e. Biotinylated purified protein samples were submitted for mass spectrometry and statistically analysed (M. Hubálek, A. Křenková)
    f. Newly identified putative interactors were linked via network model

2. Validation of the interaction between a chosen protein and the HBV Core protein
    a. HBV Core protein was co-transfected with DDX3 helicase
    b. HA-tagged HBV Core was purified by pull-down
    c. FLAG-tagged DDX3 was purified by pull down
    d. Results of pull-down experiments were analysed on western blot, showing, that DDX3 and HBV Core protein interact

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