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The role of Hepatitis B virus capsid protein in the host ubiquitin proteasome pathway

Role kapsidového proteinu virové hepatitidy B v hostitelském ubikvitin-proteazomovém systému

Diploma thesis

Supervisor: Mgr. Jan Weber, CSc.

Prague, 2018

Prohlášení:

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

V Praze, 25.04.2018

Podpis:

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Abstract

Hepatitis B virus (HBV) is a *Hepadnaviridae* virus infecting mammals. Its infection can result in an acute or chronic infection. Chronic infection can result in hepatocellular carcinoma and liver cirrhosis, potentially leading to death of the patient. HBV is a small 42 nm virus with a genome length of 3.2 kb encoding seven viral proteins. HBV Core protein (HBc) is a capsid forming protein which is pleiotropic in function. We have identified two ubiquitin ligases which could interact with this protein: F-box only protein 3 (FBXO3; E3 ubiquitin ligase) and Ubiquitin conjugating enzyme E2 O (UBE2O; E2/E3 ubiquitin ligase). By employing multiple methods we have confirmed these interactions. Co-immunoprecipitation and further western blot analysis unveiled multiple new insights into the ligases′ impact on HBc: FBXO3-mediated HBc polyubiquitination stimulation and UBE2O-mediated HBc monoubiquitination promotion. FBXO3´s and UBE2O´s role in HBV life cycle was investigated as well. By silencing the expression of FBXO3 and UBE2O respectively, we have observed changes in HBV replication levels: FBXO3 serves as an inhibitor of HBV replication, while UBE2O stimulates the course of HBV life cycle. Further investigation of these newly-discovered understandings may lead to a whole new HBV - host interplay perception.

Key words

Hepatitis B virus, Core protein, Ubiquitin, F-box only protein 3, FBXO3, Ubiquitin conjugating enzyme E2 O, UBE2O

Abstrakt

Vírus hepatitídy B (HBV) patrí do rodiny *Hepadnaviridae* infikujúcej cicavce. Infekcia HBV môže viesť k akútnej alebo chronickej hepatitíde typu B. Chronická hepatitída B môže vyústiť do hepatocelulárneho karcinómu a cirhózy pečene, ktoré čato nesú fatálne následky. HBV je malý vírus o 42 nm, ktorého genóm má dĺžku 3.2 kb. Kóduje sedem proteínov. HBV Core proteín (HBc) je proteínom zodpovedným za tvorbu kapsidy vírusu. Taktiež, však u ňho bolo odhalených mnoho ďalších funkcií. Podarilo sa nám identifikovať dve ubikvitín ligázy, ktoré by mohli interagovať s HBc: FBXO3 (E3 ubikvitín ligáza) a UBE2O (E2/E3 ubikvitín ligáza). Využitím viacerých metód sa nám tieto interakcie podarilo potvrdiť. Ko-imunoprecipitáciou, a následnou analýzou pomocou western blot metódy, sme odkryli nové náhľady na možnú funkciu týchto dvoch proteínov, čo sa ubikvitinácie HBc týka: FBXO3 zvyšuje hladinu polyubikvitinácie u HBc, zatiaľ čo UBE2O podporuje jeho monoubikvitináciu. Taktiež sme sa zaoberali celkovým dopadom FBXO3 a UBE2O ligáz na životný cyklus a replikáciu vírusu. Potlačením expresie oboch ubikvitín ligáz v HBV transfekovyných bunkách sme prišli k záveru, že FBXO3 inhibuje replikáciu vírusu, kým UBE2O ju naopak podporuje. Je potrebná podrobnejšia analýza novozískaných výsledkov, avšak ich potenciál na lepšie porozumenie vzájomného pôsobenia medzi HBV a hostiteľom je nepochybný.

Kľúčové slová

Vírus hepatitídy B, Core proteín, ubikvitiín, FBXO3, UBE2O

OBSAH

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Abbreviations

AA - acrylamide

aa - amino acid

AIRE - autoimmune regulator

ARD - arginine rich domain

BAP1 - ubiquitin carboxyl-terminal hydrolase BAP1

BCA - bicinchronic acid

BSL3 - bio-safety level 3

cccDNA - covalently closed circular deoxyribonucleic acid

CMV - cytomegalovirus

C_T - cycle threshold

CTD - C-terminal domain

ddH_2O - double-distilled water

DMEM - Dulbecco´s modified eagle´s medium

DNA - deoxyribonucleic acid

dsDNA - double-stranded deoxyribonucleic acid

E1 - ubiquitin activating enzyme

E2 - ubiquitin conjugating enzyme

E3 - ubiquitin ligase

ELISA - enzyme-linked immunosorbent assay

FBXL - F-box and leucine rich repeat protein

FBXO3 - F-box only protein 3

FBXW - F-box and WD repeat domain containing protein

GST - glutathione-S-transferase

HA - hemagglutinin

HBV - hepatitis B virus

HBc - hepatitis B virus Core protein

HBe - hepatitis B virus E antigen

HBs - hepatitis B virus Surface protein

HBx - hepatitis B virus X protein

HEK 293T - human embryonal kidney cells 293 expressing large SV40 T antigen

HIV - human immunodeficiency virus

hNTCP - human sodium taurocholate cotransporting polypeptide

HRP - horseradish peroxidase

Huh7 - human hepatocyte cells 7

IAV - influenza A virus

IFN - interferon

IgG - immunoglobulin G

IP - immunoprecipitation

LB - Luria-Bertani broth

Mo-MuLV - Moloney Murine Leukemia Virus

mRNA - messenger ribonucleic acid

MS - mass spectrometry

NES - nuclear export signal

NFκB - nuclear factor kappa-light-chain-enhancer of activated B cells

NIRF - Np95/ICBP90-like RING finger protein

NLS - nuclear localization signal

NS - nonstructural protein

NTD - N-terminal domain

ORF - open reading frame

PBS - phosphate buffer saline

PCR - polymerase chain reaction

pgRNA - pregenome ribonucleic acid

PLB - protein loading buffer

PMS - N-methyl dibenzopyrazine methyl sulfate

rcDNA - relaxed circular deoxyribonucleic acid

rcccDNA - recombinant covalently closed circular deoxyribonucleic acid

RNA - ribonucleic acid

rpm - revolutions per minute

RT - reverse transcription / reverse transcriptase

RT-qPCR - reverse transcription - quantitative polymerase chain reaction

RVFV - Rift valley fever virus

SCF - SKP1-Cullin1-Fbox

SDS - sodium dodecyl sulfate

SDS-PAGE - sodium dodecyl sulfate - poly acrylamide gel electrophoresis

siRNA - small interfering ribonucleic acid

SIV - simian immunodeficiency virus

SOC - Super Optimal broth with Catabolite repression

SUKH - Syd, US22, Knr4 Homology

SV40 - simian vacuolating virus 40

TAE - tris-acetate EDTA

Taq polymerase - DNA polymerase originally isolated from Thermus aquaticus

TBS - tris-buffered saline

TNFR - tumor necrosis factor receptor

TRAF - tumor necrosis factor receptor associated factor

TRIM22 - tripartite motif protein 22

Ub - ubiquitin

UBE2O - ubiquitin conjugating enzyme E2 O

WB - western blot

1 INTRODUCTION

Undoubtedly, we live in a great age. Humanity is thriving. The education level all over the world is peaking, healthcare has never been better and people from the first world countries are generally living in better conditions than ever before. Of course, there´s always many matters which are constantly putting us in the inevitable state of awareness and defense. One such concern has been present since the very early stages of evolution. A virus. Despite being one of the smallest living agents on Earth, it has always found its way through constantly changing evolution course. Even though the ancestral Hepatitis B virus (HBV) dates back to Mesozoic Era, it was not until the late 1960´s that the so-called "Australia antigen" has been identified as a part of the newly discovered HBV (Suh et al. 2013; Blumberg et al. 1968; Prince 1968). Ever since the virus was discovered by Dr. Blumberg in 1968, researchers all over the world realized, that an intensive effort for understanding the virus was needed. In 1982, a recombinant vaccine was prepared and has been available ever since. Even though it belongs to a mandatory set of vaccines in most of the developed countries, we are still far from winning this fight. HBV has over 257 million people chronically infected worldwide, with over 880 thousand fatalities per year (WHO 2017). Dr. Weber´s laboratory, where I was lucky enough to work on my master´s project, has been working on HBV research since 2014. Their recent publication was focused on HBV´s Core protein posttranslational modifications, where they identified arginine mono- and symmetric dimethylations as well as several putative ubiquitination sites (Lubyova et al. 2017). The complexity of the so-called "ubiquitin code" promises a lot of new information on how does the viral-host interplay evolve. We have identified several HBc specific interactors. I have focused on two ubiquitin ligases, namely the F-box only protein 3 ubiquitin ligase and the Ubiquitin conjugating enzyme E2 O. By analyzing their interactions via several different methods as well as their impact not only on HBV Core protein but also on the HBV as it is, I have tried to shed some more light onto the obscure viral endurance.

2 LITERATURE REVIEW

2.1 HEPATITIS B VIRUS

HBV is a partially-double-stranded DNA (dsDNA) virus from the Hepadnaviridae family. It contains 3.2 kb long DNA which encodes seven proteins. Mature particle also known as the Dane particle, is 42 nm in diameter. HBV forms multiple structures in the serum of an HBV infected person: the infective Dane particle and non-infective virus-like particles (filamentous or spherical particles) (Fig. 1). Only the mature Dane particle contains all three hepatitis B surface antigen forms (HBs) (Heermann et al. 1984). There are over 257 million people chronically infected worldwide. Virus infection can result in either acute or chronic hepatitis B. Patients who are HBs positive for more than 6 months are diagnosed with chronic hepatitis B. Chronic infection often leads to hepatocellular carcinoma and liver cirrhosis. These diagnosis result in over 880 000 deaths annually, which makes it a major global health problem. A person can get infected by HBV when exposed to infected blood or other body fluids (seminal and vaginal fluids, saliva), perinatal infection occurs as well. Highest risk of infection comes from unprotected sex and the reuse of needles (drug use and poor healthcare settings). Hepatitis B is the most spread disease in the third world countries, with low hygienic standards. Most affected are the countries in the south-east Asia and Africa. An effective vaccine has been developed in 1982, it is targeted against the HBsAg. It is a 3 or 4-dose vaccine, which ensures 95 % protection for over 20 years (WHO 2017). To this day we know 10 HBV genotypes (A-J) with different

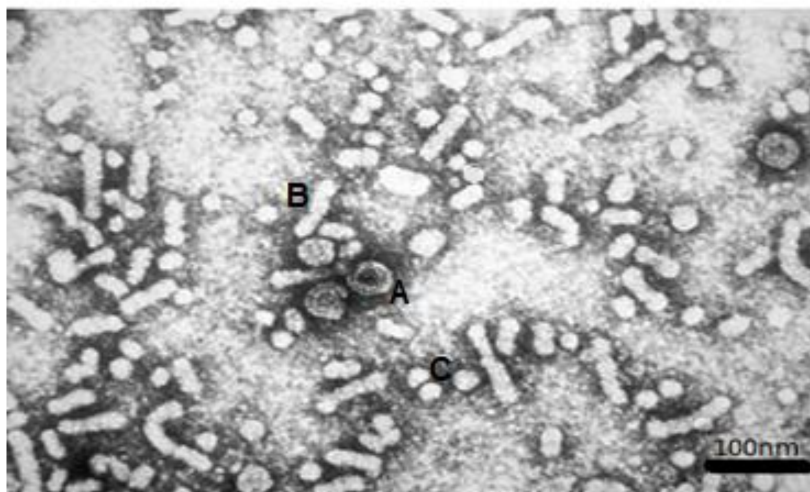

Fig. 1 HBV electron microscopy. A: Dane particle – mature HBV particle (42 nm in diameter). B: Virus-like filamentous particle (non infective). C: Virus-like spherical particle (non infective; 22nm in diameter). Adapted from CDC 2017.

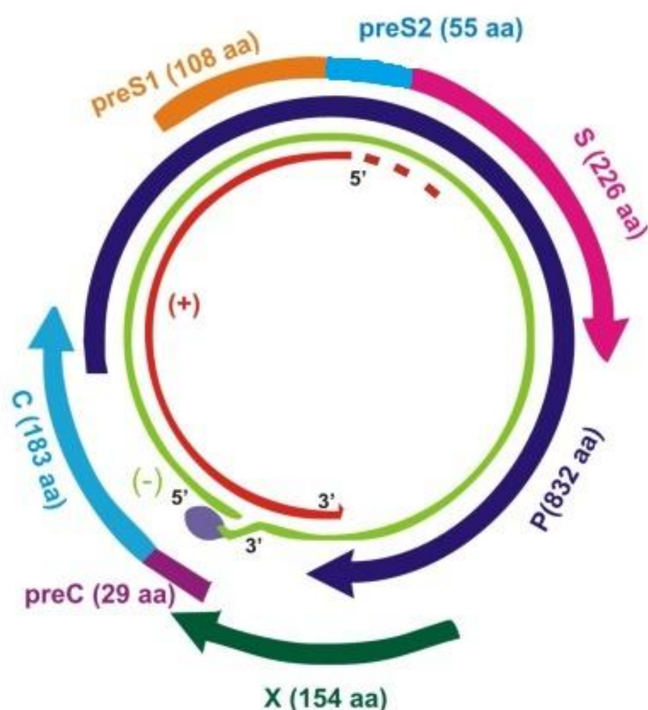

Fig.2 The HBV genome
HBV genome is 3.2 kb long with 4 ORFs: Core (C), Surface (S), Polymerase (P) and X. The genome encodes 7 viral proteins:P, HBc, HBe, Large HBs (PreS1), Medium HBs (PreS2), Small HBs (S) and X protein (HBx). Adapted from Jayalakshmi et al. 2013.

geographical distribution as well as different courses of infection (tendency towards chronicity). The genotypes are further classified into several subgenotypes (Sunbul 2014).

HBV encodes 4 open reading frames (ORFs) which yield the formation of seven proteins (fig.2). The biggest of the seven proteins is RNA dependent DNA polymerase (P), responsible for reverse transcription. Reverse transcription is a phenomenon where RNA is transcribed into DNA. Another ORF depicted encodes two forms of a core protein: Core (HBc), forming an icosahedral capsid, and pre-core which is further processed to a soluble HBe. Another gene encoding surface antigen yields a formation of three different proteins: large (preS1), medium (preS2) and small (S) HBs. These proteins are located in the viral envelope and are responsible for the first virion – host cell interaction during cell entry. Last protein encoded by HBV is the X protein (HBx). Role of this protein has not yet been fully elucidated. It has been shown that the protein is acting as a regulator on transcriptional level (Decorsière et al. 2016) as well as it interacts with the p53 tumor suppressor resulting in cell transformation (Wang et al. 1994).

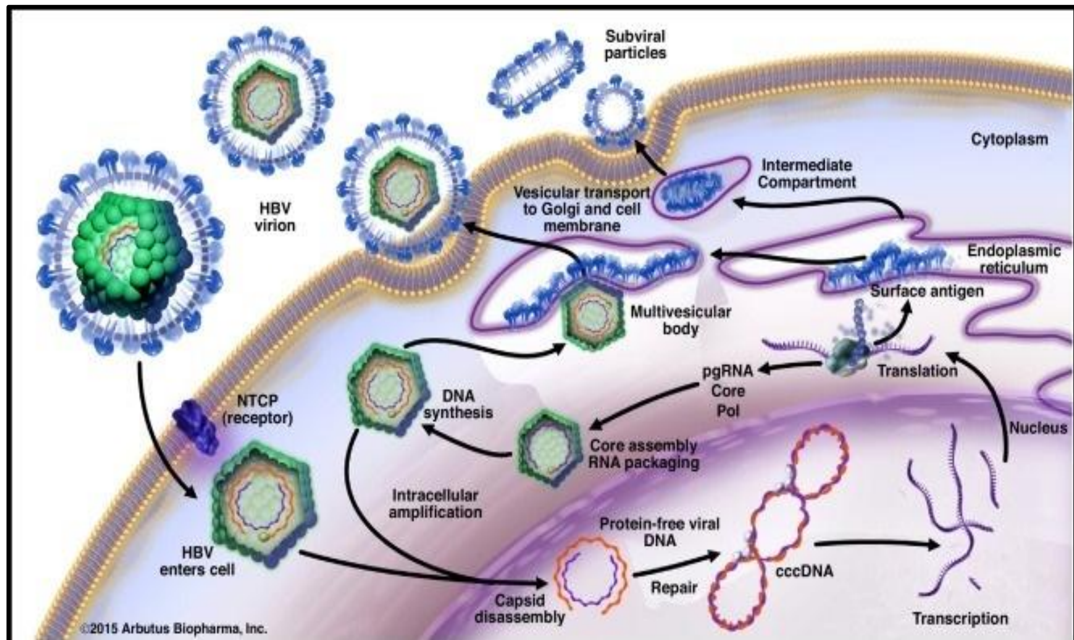

Fig.3 The HBV life cycle. HBV virion attaches itself to the NTCP receptor and entries the cell. Viral capsid is then directed towards the nucleus. Viral rcDNA enters the nucleus and is repaired using host proteins. Nascent viral cccDNA serves as a template for 7 viral mRNAs as well as for pgRNA. P protein attaches itself to pgRNA and starts the reverse transcription of pgRNA into DNA. At the same time capsid starts to form around the P-pgRNA complex. Nascent mature capsid can either bud out of the cell, enveloping itself with cellular membrane incorporated with the 3 viral surface proteins or enter the nucleus again and participate in the intracellular amplification. (Cole 2016)

HBV life cycle (fig.3) begins with the virion attaching to the human sodium taurocholate cotransporting polypeptide (hNTCP) specific for human hepatocytes (Yan et al. 2012; Huang et al. 2012). Once the virion attaches itself to the cell membrane, it releases the capsid into the cytosol. The capsid is further targeted towards the nucleus, where it disintegrates and viral relaxed circular DNA (rcDNA) is released into the nucleus. First, the HBV rcDNA is repaired using host proteins which leads to formation of a covalently closed circular DNA (cccDNA) (Königer et al. 2014). The nascent cccDNA is ready for transcription and further protein translation. Repaired cccDNA resides in the cell nucleus and acts as a viral minichromosome with the host nucleoproteins incorporated (histones H3, H2B, H4, H2A, H1) (Miller and Robinson 1984; Tuttleman et al. 1986). Two of the viral proteins act on the viral minichromosome as well: HBc and HBx. HBc bound to HBV cccDNA results in changes of nucleosomal spacing as well as in epigenetic regulation changes like lower methylation and deacetylation as well as higher CREB-binding protein bound to cccDNA (Bock et al. 2001; Y. Guo et al. 2011). HBV cccDNA residing in the nucleus is the cause for the chronic Hepatitis B development(Bock et

al. 2001; Belloni et al. 2009; Köck et al. 2010). The HBV cccDNA does not only yield mRNAs formation but the full genome transcript serves as a template for reverse transcriptase. This RNA transcript is called pregenome RNA (pgRNA) which is further reverse transcribed into rcDNA. Once the P protein attaches itself to nascent pgRNA, the capsid starts to form around the pgRNA - P protein complex, while the pgRNA is being reversely transcribed to its rcDNA form (Summers and Mason 1982). This unenveloped immature HBV capsid is then targeted towards the cytoplasmic membrane, where capsid envelopment occurs. The envelope consists of host cell cytoplasmic membrane integrated with all three HBs forms. Mature particle then buds out of the infected cell and is ready for further infection. However, this is not the only pathway for a newly formed capsid containing a nascent rcDNA. Portion of capsids is drifted back to the nucleus, where the DNA is again transcribed into mRNAs and pgRNAs. This phenomenon is called intracellular amplification.

2.2 HBV Core protein (HBc)

HBc protein is a small 21 kDa protein responsible for the assembly of the viral capsid. The 183 amino acid (aa) protein consists of two domains: N-terminal assembly domain (1-149 aa residues) and a C-terminal domain (CTD) which consists of four arginine rich domains (ARD I-IV; 149-183 aa residues) (fig. 4).

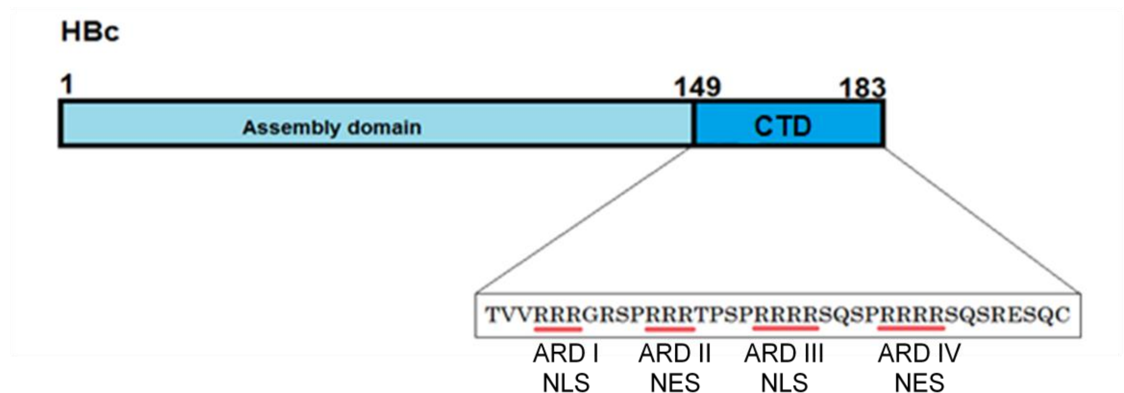

Fig. 4 HBc domains. The HBc protein contains two domains. The main assembly domain which is responsible for the correct capsid assembly and a positively charged C-terminal domain (CTD) containing four arginine rich domains (ARDs I-IV). ARD I and III are carrying NLS, while ARD II and IV contain NES.

The assembly domain of HBc is capable of capsid assembly *per se* (Zlotnick et al. 2002). At first, HBc proteins form dimers which regroup into trimers of dimers resulting in a T=3 (90 trimers of dimers) or a T=4 (120 trimers of dimers) capsid (Crowther et al. 1994; Stannard and Hodgkiss 1979). Besides serving as a major structural element for the virus, HBc carries multiple localization as well as signaling purposes. Once the capsid enters the hepatocyte, it is targeted towards the nucleus. HBc possesses a nuclear localization signal (NLS) as well as a nuclear export signal (NES). ARD I and III serve as NLS while the II and IV were identified as NES. Since the HBV capsid´s predominant subcellular localization is the cytosol, it can be concluded that the putative NES shows a certain dominance over the NLS. These two localization signals are not the only two mechanisms to direct the capsids position in the cell. Other cellular factors such as tip-associating protein (TAP), importin α and β have been shown to interact with HBc (Kang and Cullen 1999; Kann et al. 1999). HBc acts as an important epigenetic modulator of HBV cccDNA located in the host cell nucleus. By binding to the viral dsDNA it enhances viral mRNA transcription. The HBc-dsDNA nucleoprotein complex also correlates with lower histone deacetylase I activity on HBV cccDNA which would normally silence viral transcription. On the other hand, level of HBc bound to dsDNA corresponds with the level of the CREB binding protein responsible for transcription activation (Guo et al. 2011). While synthesizing rcDNA from the pgRNA performed by the P protein, the nucleic acids undergo multiple conformational changes. These have been proven to be directed by HBc´s nucleic chaperone activity as well as strand exchange activity which resides in the HBc CTD´s unfolded residues (Chu et al. 2014). Capsid envelopment and virion budding is also partly directed by HBc. As Basagoudanavar et al. in 2007 and Perlman et al. in 2005 have shown, the immature capsid (containing RNA) is formed by phosphorylated HBc residues. Accordingly to that, mature capsid containing rcDNA loses phosphorylation on its HBc residues and can proceed towards budding from the infected cell. Thus HBc serves as a control mechanism for immature capsids leaving the cell. These data have shown that HBc is

indeed, not only a structural element but serves as a crucial regulator of the viral life cycle.

2.3 UBIQUITIN MODIFICATION AND PROTEASOME PATHWAY

Ubiquitin-proteasome system of the cell is a complex network resulting in protein degradation. It is necessary for the right cell functionality as well as its right growth and defense system against antigens. Ubiquitin (Ub), a protein of 8.5 kDa plays a crucial role in this highly regulated pathway (fig. 5). It can be conjugated to protein´s lysine (isopeptide bond), cysteine

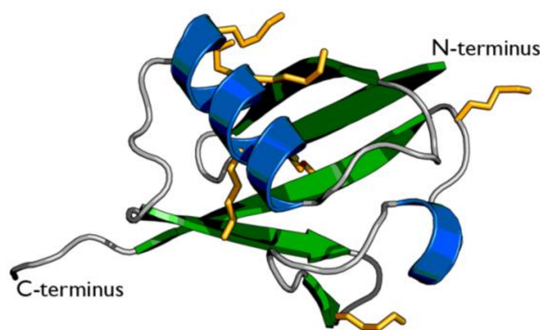

Fig. 5 Ubiquitin molecule. Ubiquitin, an 8.5kDa protein, consists of 76 amino acids represented in tertiary structure. C-terminal and N-terminal domains are shown. Adapted from (Ramage et al. 1994)

(thioester bond), serine and threonine (ester bond) residues (Pickart and Eddins 2004). Residues can be monoubiquitinated or polyubiquitinated - chain of ubiquitins attached to the residue. Each Ub contains seven lysine residues and an N-terminal methionine which can be further ubiquitinated (Fig. 6).A chain of ubiquitins attached to a protein via its Lys 48 residue is the most abbundant out of all Ub linkages (often more than 50 %). This modification results in a proteasome-mediated degradation of the protein. The second most common ubiquitin modfication is via its Lys 63 residue. This interaction leads to a non-degradative modification of the protein. Other Ub-protein modifications are rather atypical (Met1, Lys6, Lys11, Lys27, Lys29, Lys33) (Wagner et al. 2011; Kim et al. 2011; Kulathu and Komander 2012). Not only that Ub itself consists of eight other potential Ub sites yielding polyubiquitin

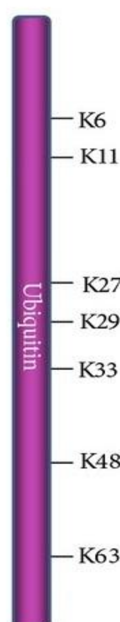

Fig. 6 Ubiquitin lysine residues
All seven lysine residues are represented: lysine 6, lysine 11, lysine 27, lysine 29, lysine 33, lysine 48 and lysine 63. Adapted from Lin and Man 2013

chain formation, the proteins can also be modified by other than Ub posttranslational modificators. The Small Ub-like Modificator family (SUMO family) is able to attach to (poly)Ub and further modify the protein′s function (Hendriks et al. 2015). New insights on the complex "Ubiquitin code" has led to a discovery of highly linkage-specific enzymes which recognize this Ub/Ub-like pattern. Although, degradation of the modified protein is one of the most recognized and connected to ubiquitination, it is by far not the only result of this posttranslational modification. The enormous number of different monoubiquitinations and polyubiquitinations ensures many possible outcomes for the protein; e.g. cellular localization, protein-protein interactions etc. (Glickman and Ciechanover 2002; Mukhopadhyay and Riezman 2007). There is no molecule with functional analogy to Ub in prokaryotes, however, some proteins share its fold and structure, which could represent possible Ub ancestors (Hochstrasser 2000). Ubiquitination is usually a three step process requiring three different enzymes (fig.7):

2.3.1 Ubiquitin-activating enzyme (E1)

E1 attaches ATP to Ub which results in the acyl-adenylation of the C-terminal domain of ubiquitin. The ATP-Ub intermediate is then transferred towards the active site Cysteine of the E1 which results in AMP release and thioester bond formation between E1 and Ub.

2.3.2 Ubiquitin-conjugating enzyme (E2)

The Ub bound to E1´s cysteine is then targeted by E2. E2 is a second Ubiquitination mediator, which binds the Ubiquitin via its active site Cysteine. The E2-Ub complex is further processed by ubiquitin ligase - the last key player of ubiquitination.

2.3.3 Ubiquitin ligase (E3)

Ub ligase catalyzes the attachment of Ub to the substrate protein. E3 contains either a HECT domain (homologous to E6-AP C-terminal), a RING (really interesting new gene) or U-box domain (closely related to RING) (Metzger, Hristova, and Weissman 2012). It can provide the attachment from E2´s cysteine directly to a Lys/Cys/Ser/Thr of a substrate (RING, U-box), or it can transiently attach the Ub to its active site cysteine

and then catalyze the substrate ubiquitination itself (HECT). E3 is responsible for the substrate specificity.

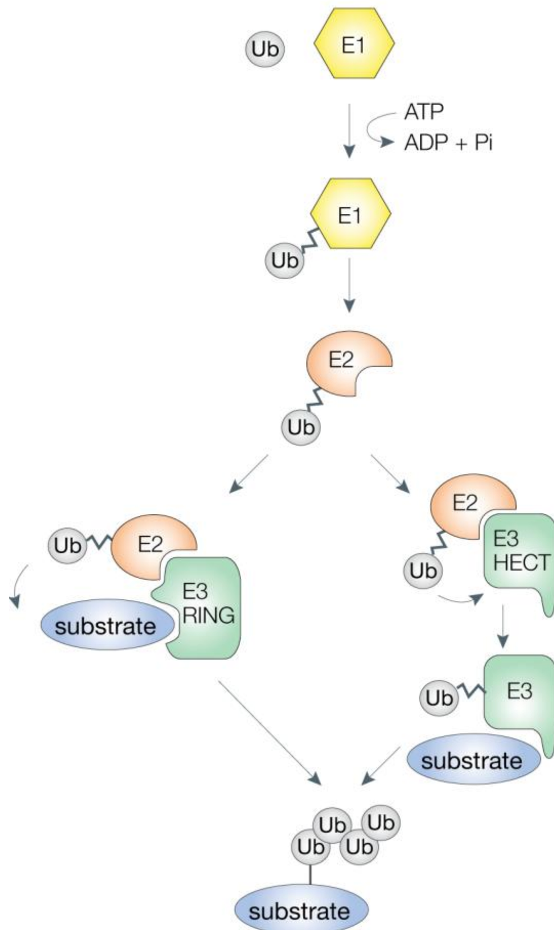

Fig. 7 Ubiquitination
The first step of ubiquitination is Ub activation. This reaction is catalyzed by ubiquitin-activating enzyme (E1). ATP is required for this step, where Ub is attached to E1´s active site cysteine residue. In the next step ubiquitin-conjugating enzyme attaches the Ub to its active site cysteine residue and interacts with ubiquitin ligase (E3). Depending on whether the E3 contains a RING/U-box or HECT domain the Ub is then attached to the substrate protein´s lysine/cysteine/serine/threonine residue directly (RING/U-box) or via transient E3 active site cysteine residue thioester bond. Substrate residue can be monoubiquitinated or polyubiquitinated (as shown in the figure). Adapted from Woelk et al. 2007.

2.4 Ubiquitin and HBV

Multiple projects have been focusing on Ub's role in HBV life cycle. HBc, being a crucial structural protein for HBV maturation, is downregulated by host's Np95/ICBP90-like RING finger protein (NIRF). NIRF is an E3 Ub ligase which is responsible for HBc polyubiquitination and further proteasome degradation (Qian et al. 2012, 2015). Another E3 ligase taking part in HBV life cycle regulation is Tripartite motif protein 22 (TRIM22) (Gao et al. 2009). TRIM22 is responsible for HBc promoter function inhibition. The E3 ligase can modulate protein levels via polyubiquitination or it can also act as a transcriptional repressor on a DNA level. HBV is by

far not the only virus inhibited by TRIM22 (Human Immunodeficiency Virus - 1 (HIV-1) - Barr et al. 2008, Influenza A - Di Pietro et al. 2013). Using mass-spectrometry (MS) analysis, my colleagues have shown multiple post-translational modifications of HBc. Such as phosphorylation, methylation and ubiquitination. Based on the MS, they indentified putative ubiquitination of K7, K96, S49, T53 and S157 residues. Analysis of K7R, K96R or double (K7/96R) HBc mutants confirmed that the K7 was the predominant site of HBc ubiquitination. Furthermore, both monoubiquitinated and polyubiquitinated HBc were identified. Combinations of different post-translational modifications lead to different outcomes for HBc (change of subcellular localization, different protein-protein interactions or proteasome-mediated degradation) which leads to different outcomes for the virus itself (Lubyova et al. 2017). HBc´s K96 monoubiquitination leads to an improved viral replication efficiency as well as the newly formed virion release (by changing the HBc´s subcellular localization) (Rost et al. 2006; Ponsel and Bruss 2003).

2.5 Other than HBV viruses and their Ub modifications

The HBV - ubiquitination connection is not the only case where Ub post-translational modification regulates viral life cycle. The TRIM superfamily is a group of E3 Ub ligases which take part in the complex innate immunity response. Their levels rise as a result of viral infection. TRIM22, in particular, has been connected to mediate viral proteins´ polyubiquitination and further degradation. This has been shown for Influenza A virus (IAV) nucleoprotein, Encephalomyocarditis virus 3C Protease (Eldin et al. 2009). TRIM19 has similar effects on HIV-1, Cytomegalovirus, Herpes simplex virus type 1, Ebola virus, Lassa virus and others (Nisole et al. 2005). TRIM28 restricts Murine leukemia virus (Wolf and Goff 2007). Another way of regulating viral life cycle is via monoubiquitination of Influenza A virus M2 protein by Itchy E3 Ub protein ligase which leads to a more efficient late endosome escape and virus release. IAV´s (Hubner and Peter 2012). As indicated above, IAV´s nucleoprotein is polyubiquitinated and degraded, however, this is not the only Ub-nucleoprotein interaction. Monoubiquitination of the nucleoprotein (K184) is crucial for the viral RNA

replication (Liao et al. 2010). This could be seen as a strong analogy between IAV´s nucleoprotein and HBV´s HBc monoubiquitination and polyubiquitination. New insights on this topic are needed to show whether this analogy is merely hypothetical or whether the mono/polyubiquitination system is indeed a conserved system for viral progression and at the same time eradication by polyubiquitination. Viral budding out of the cell has been connected to ubiquitination as well. Retroviral gag region has been shown to carry single ubiquitins, which are needed for the right budding of the virion out of the infected cell. This insight has been connected to HIV, Simian Immunodeficiency Virus (SIV) and Moloney Murine Leukemia Virus (Mo-MuLV) (Ott et al. 1998).

Two of the many proteins taking part in the Ub signaling pathway have been closely discussed in my diploma project. Those being F-box only protein 3 (FBXO3) and Ubiquitin conjugating E2 enzyme O (UBE2O).

2.6 FBXO3

FBXO3 protein, an E3 enzyme of 55 kDa, is a member of the SKP1-Cullin1-Fbox (SCF) E3 ligases superfamily regulating cell cycle progression as well as DNA repair and cell survival (Tyers and Willems 1999). The SCF complex consists of RING-box protein 1 and Cullin1 forming a scaffold for E2 binding and a SKP1 bound to F-box protein responsible for the substrate specific binding (Cenciarelli et al. 2017). There are three F-box groups known: FBXL (containing a leucine-rich c-terminal domain), FBXW (containing WD40 domain) and FBXO with other protein-protein interactions or no recognizable motifs (Jin et al. 2004). Protein of our interest, the FBXO3, belongs to the last group of F-box proteins. The FBXO3 consists of two putative protein-protein interaction motifs: a domain showing sequence similarity to SUKH (Syd, US22, Knr4 Homology) - poorly characterized so far, present in many immunity related proteins; an ApaG similar domain - mainly present in prokaryotes, only two eukaryotic proteins so far have been identified to contain the ApaG motif (FBXO3 and PDIP38) (Zhang et al. 2011; Krzysiak et al. 2016). However

this rare domain has been shown to be crucial for the right FBXO3 function in various protein interactions. FBXO3, being an E3 ligase, is often responsible for protein polyubiquitination, thus targeting the protein towards the proteasome. As an example of such activity, FBXO3 is included in the complex cytokine-driven inflammation control. It is responsible for FBXL2 polyubiquitination and further degradation. FBXL2, also an F-box protein, plays an important role in cytokine molecules expression and release. Tumor necrosis factor receptor (TNFR) is a membrane bound cytokine receptor which is responsible for inflammation, apoptosis and cell proliferation (Gravestein and Borst 1998). Signal from the TNFR is transduced to nucleus via TNFR-associated factors (TRAFs). There has been seven TRAF molecules identified until this day (TRAF1-7). FBXL2 is the regulator of these molecules, responsible for their polyubiquitination and targeting for degradation. FBXO3, to the contrary, sends the FBXL2 for degradation and thus promotes TRAF signaling. This leads to a higher cytokine expression and an inflammatory immune response (Inoue et al. 2000; Chen et al. 2013). An uncontrolled inflammation can result into pathologies like cappillary leakage, tissue edema, organ failure and even fatality (Nathan 2002; Aird 2003). FBXO3 has also been shown to be involved in autoimmune regulator (AIRE) ubiquitination and transcriptional activation. AIRE is a transcription factor expressed in thymic epithelial cells. It directs the expression of otherwise tissue-specific antigens, which serve as a bait for autoreactive T cells. Ubiquitination of AIRE by FBXO3 leads to its binding towards the positive transcription elongation factor b which results in AIRE expression. FBXO3 thus promotes the right functionality of the immune system (Shao et al. 2016; Zumer et al. 2013). Although FBXO3 is an undeniably important element in the complex cellular signaling network, it is also part of a non-cellular protein interaction: Rift valley fever virus (RVFV) and its nonstructural protein (NS) is a newly discovered FBXO3 interactor. RVFV, a serious pathogen of livestock and humans in Africa, is able to decrease levels of cell produced interferon type I (IFN-I), a crucial antiviral response to cellular infection. It is also inhibiting host transcription by destroying the general transcription factor TFIIH p62 subunit via the ubiquitin directed

proteasome pathway. The downregulation of both IFN-I and p62 is conducted by the FBXO3 E3 ligase. RVFV NS have been proved to drive the FBXO3 mediated TFIIH p62 degradation resulting in the downregulation of IFN-I transcription (Kainulainen et al. 2014). So far, there has been no FBXO3 HBV interaction described.

2.7 UBE2O

Another protein involved in the Ub signaling pathway is the Ubiquitin conjugating enzyme E2 O. This protein, of an unusually large size of 230 kDa, is one of the biggest E2 enzymes indentifies so far. Even though it is classified as an E2 enzyme, the UBE2O carries out E3 activity as well. As all other proteins acting in the Ub signaling pathway, UBE2O is also part of many sophisticated protein regulating networks. The widely investigated NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is one of the pathways regulated by UBE2O. The NF-κB pathway is an essential part of a cell response to cytokines, stress, bacterial or viral infection and many others (Gilmore 2006). TRAF6 is part of a signaling cascade leading to the activation of NF-κB transcription factor. TRAF6 autopolyubiquitination is necessary for the signal to proceed. UBE2O as an ihibitor of NF-κB expression, blocks the ubiquitination site (lysine 63) on the TRAF6 molecule. By the inhibition of this polyubiquitination it prevents further signal progression (Zhang et al. 2013). UBE2O is also a major cell proliferation regulator. It interacts with BAP1, a chromatin bound protein which acts as a tumor suppressor. By multi-monoubiquitinating BAP1´s NLS, UBE2O promotes BAP1´s cytoplasmic localization, thus promotes cell growth and proliferation eventually resulting in tumor development. However, BAP1 is able to deubiquitinate its NLS and thus reestablish its nuclear retention and chromatin interactions (Dey et al. 2012; Yu et al. 2010; Mashtalir et al. 2014) (fig. 8). Au contraire, UBE2O regulates c-Maf activity in an opposite manner. C-Maf is a transcription factor that is in need of high regulation in order to prevent cell transformation leading to myeloma. UBE2O is one of these regulators, as it acts on c-Maf as a Ub ligase. By polyubiquitinating c-Maf it directs the protein towards degradation, thus preventing tumor development (Eychène et al. 2008; Xu et al. 2017). Besides operating as a member in the cell growth and

proliferation regulation machinery, UBE2O works as a protein stoichiometry sustainer as well. Many proteins form sophisticated complexes, which are built out of numerous polypeptides translated separately. There is no controlling mechanism for setting the exact number of synthesized proteins. Thus there is a need for maintaining the protein stoichiometry at the posttranslational level. UBE2O targets the hydrophobic domains of "orphan proteins" which are normally hidden inside the multi-protein complex. Such proteins are then polyubiquitinated by UBE2O and destinated for degradation. In this matter UBE2O functions as a self-contained quality control factor which recognizes the excess polypeptides present in the cell (Yanagitani et al. 2017). In a similar manner - by UBE2O-mediated protein degradation, cell differentiation occurs - when from a highly broad and complex proteome of an undifferentiated cell becomes a simple cellular proteome. This phenomenon is significant in erythrocyte maturation. A mature erythrocyte´s proteome is 98 %globin. The Ub proteasome pathway is the leading machinery behind this transformation, UBE2O being the central element. It targets and multi-monoubiquitinates countless number of proteins which are further degraded (Nguyen et al. 2017). UBE2O´s broad specificity towards numerous substrates as well as its E2/E3 joined activity underlines the enzyme´s significance in cellular processes. Until this day, no UBE2O interplay with HBV has been identified.

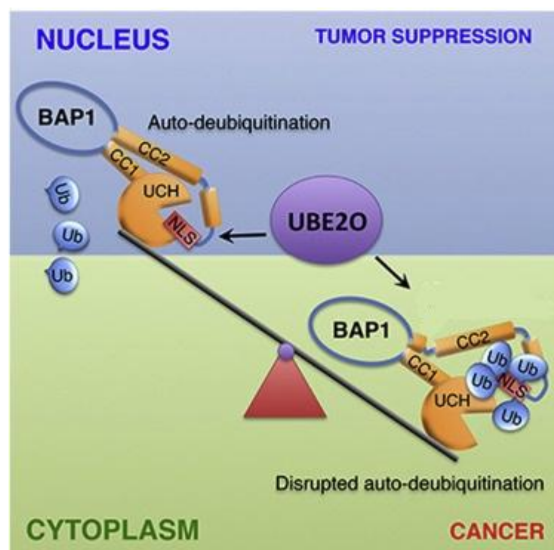

Fig. 8 UBE2O impact on BAP1 BAP1 is a vital tumor suppressor carrying a nuclear localization signal (NLS). This motif is multi-monoubiquitinated by UBE2O which leads to BAP1´s cytoplasmic sequestration preventing BAP1 to act on gene expression level. However, BAP1 is able to deubiquitinate its NLS, thus relocate itself back into the nucleus, where it acts as a functional tumor suppressor. Adapted from Mashtalir et al. 2014.

3 AIMS

HBV, remaining one of the world´s most life-threatening viruses, requires intensive studies by research groups all over the world. Its complex life cycle and simplicity at the same time are a perfect puzzle for the world of today.

We have focused on its HBc protein, which is known to interact with several host proteins. New insights onto these virus-host interactions could be a valuable fragment for a possible HBV cure.

Main aims of my master´s project were:

- to identify HBc interacting host proteins involved in ubiquitin proteasome pathway
- to confirm the HBc specific interactions with FBXO3 and UBE2O ligases
- to analyze the impact of FBXO3 and UBE2O on HBc
- to analyze the impact of FBXO3 and UBE2O on HBV replication

4 MATERIAL AND METHODS

4.1 MATERIAL

4.1.1 CELL LINES

HEK 293 - adherent cell line derived from human embryonal kidney cells

Huh 7 - adherent cell line derived from hepatocellular carcinoma, originally isolated from a 57 year old Japanese patient

HepG2 hNTCP - adherent cell line derived from hepatocellular carcinoma originally isolated from a 15 year old American overexpressing human NTCP receptor

4.1.2 BACTERIAL STRAINS

One Shot™ TOP10 Chemically Competent E. coli (Invitrogen)

BL21 Competent E. coli (New England BioLabs)

4.1.3 VECTORS

pcDNA 3.1 (-) (ThermoFisher)

pcDNA-3.2/capTEV-CT/V5-DEST (ThermoFisher)

pCMV-MYC (Clontech)

4.1.4 PLASMIDS

Core-HA - generated by PCR amplificationof HBc ORF (as a template we used plasmid pHY92CMV from Dr. Huiling Yang, Gilead Sciences. Inc., USA) followed by subcloning into pcDNA3.1

Core-FLAG - generated by PCR amplificationof HBc ORF (as a template we used again plasmid pHY92CMV) followed by subcloning into pcDNA3.1

Core-V5/AP - generated by PCR amplificationof HBc ORF (as a template we used again plasmid pHY92CMV) followed by subcloning into pcDNA3.1

GST-HBc - generated by PCR amplificationof HBc ORF (as a template we used again plasmid pHY92CMV) followed by subcloning into pcDNA3.1

FBXO3 - OriGene

UBE2O - OriGene

rcccDNA - HBV minichromosome DNA prepared by Guo et al. in 2016.

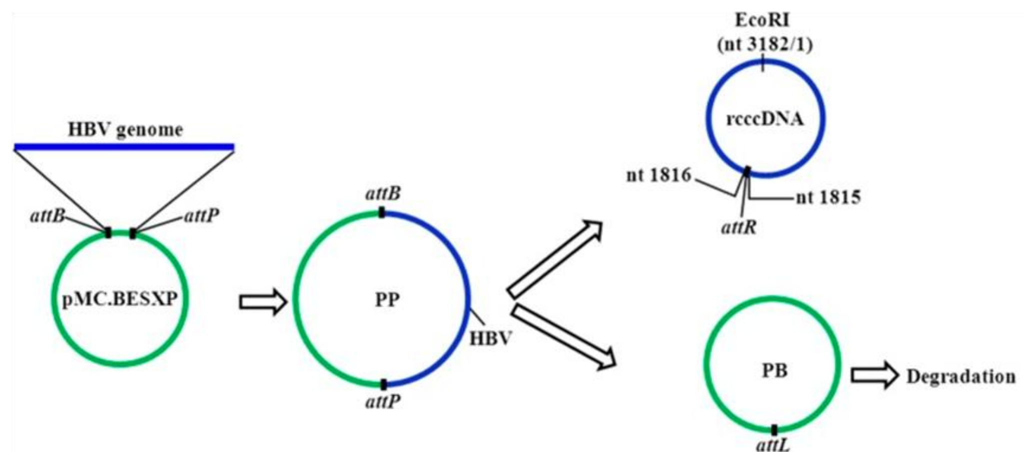

Fig.9. Scheme illustration of rcccDNA production by Dr. Guo. Synthesized linear HBV genome was subcloned into an empty minicircle producing pMC.BESXP plasmid (PP). PP was used for E. coli transformation (ZYCY10P3S2T strain). An overnight culture was treated with L-arabinose which induced the expression of E. coli genome coded ΦC31 DNA recombinase and I-SceI endonuclease. ΦC31 mediated the recombination between the attB and attP built-in in the PP, resulting in the minicircle with a attR recombination site, i.e., the rcccDNA. I-SceI endonuclease linearized the plasmid backbone (PB) which was further degraded by bacterial exonucleases. Adapted from Guo et al. 2016.

4.1.5 Antibodies

4.1.5.1 Primary antibodies

αHA - mouse monoclonal IgG against HA tag; dilution 1:4000 (Santa Cruz)

αFLAG - mouse monoclonal IgG against FLAG tag; dilution 1:4000 (Sigma-Aldrich)

αMYC - mouse monoclonal IgG against MYC tag; dilution 1:1000 (Sigma-Aldrich)

αHBc - rabbit polyclonal IgG against HBV Core protein; dilution 1:1000 (Dako)

αFBXO3 - rabbit polyclonal IgG against human FBXO3 protein; dilution 1:1000 (Sigma-Aldrich)

αFBXO3 - rabbit polyclonal IgG against human FBXO3 protein; dilution 1:1000 (OriGene)

αUBE2O - rabbit polyclonal IgG against human UBE2O protein; dilution 1:100-1:250 (ThermoFischer Scientific)

αUBE2O - rabbit polyclonal IgG against human UBE2O protein; dilution 1:1000 (OriGene)

αUbiquitin - mouse monoclonal IgG against Ubiquitin (P4D1); dilution 1:100-1:1000 (Santa Cruz Biotechnology)

αUbiquitin - mouse monoclonal IgG against Ubiquitin (P4D1); dilution 1:1000 (Cell Signaling Technology)

αUbiquitin - rabbit polyclonal IgG against Ubiquitin; dilution 1:1000 (Cell Signaling Technology)

αβActin - mouse monoclonal IgG against human β-actin; dilution 1:4000 (Abcam)

Neutravidin-HRP - specially prepared peroxidase-conjugated form of avidin biotin-binding protein (ThermoFisher)

4.1.5.2 Secondary antibodies

αRabbit - goat polyclonal anti-rabbit HRP ; dilution 1:10 000 (Sigma-Aldrich)

αMouse - goat polyclonal anti-mouse HRP ; dilution 1:10 000 (Sigma-Aldrich)

4.1.6 Enzymes

Trypsin (Sigma)

4.1.6.1 DNA polymerases

GB Elite PCR Master Mix - contains a hot-start DNA polymerase (GeneriBiotech)

SuperScript™ III Reverse Transcriptase (ThermoFisher)

4.1.7 Markers

4.1.7.1 DNA marker

MassRuler DNA Ladder Mix (ThermoFisher)

4.1.7.2 Protein marker

Precision Plus Protein™ WesternC™ (Bio-Rad)

4.1.8 Primers

FBXO3 and UBE2O primers were designed using the NCBI Primer designing tool. All primer were prepared in Generi Biotech.

FBXO3-B F - 5'-TACCAATGTCCAGACCAAATGGC-3'

R - 5'-GGGGAAGCCACCTGATACAA-3'

UBE2O-A F - 5'-CACATGCGATCCACCGACAG-3'

R - 5'-CAGCCAGCAGTCATAGGCAA-3'

βActin F - 5'-CTCTTCCAGCCTTCCTTCCT-3'

R - 5'-AGCACTGTGTTGGCGTACAG-3'

4.1.9 Frequently used solutions

Lysis buffer - 1 % Triton X-100, 150 mM NaCl, 20 mM Hepes pH7.8, 5 mM $MgCl_2$, 2 mM EDTA

Transfer buffer - 390mM Glycine, 480mM Tris-HCl, 0.37 % SDS

Wash buffer - 1xTBS, 0.05 % Tween20

TAE - 40 mM Tris, 20 mM Acetic acid, 1 mM EDTA

Tris-Glycine-SDS - 25 mM Tris, 192 mM glycine and 0.1 % SDS, pH 8.6

Protein loading buffer 2x - 100mM Tris HCl pH6.8, 3 % Mercaptoethanol, 4 % SDS, 0.2 % Bromphenol Blue, 20 % Glycerol

Protein loading buffer 6x - 300mM Tris HCl pH6.8, 9 % Mercaptoethanol, 12 % SDS, 0.6 % Bromphenol Blue, 60 % Glycerol

4.1.10 CULTURE MEDIA

4.1.10.1 BACTERIAL CULTURE MEDIA

LB medium - 1 % bacteriological Peptone(w/v); 0.5 % Yeast extract (w/v); 1 % NaCl (w/v)

SOC medium - 2 % bacteriological Peptone(w/v); 0.5 % Yeast extract (w/V); 10 mM NaCl; 2.5 mM KCl; 20 mM glucose; 10 mM $MgCl_2$;10 mM $MgSO_4$

4.1.10.2 CELL CULTURE MEDIA

DMEM serum complemented with antibiotics

DMEM serum free

4.1.11 CHEMICALS

96 % Ethanol

30 % Acrylamid

Agarose

Albumin from Bovine Serum

Amonium Persulfate

Ampicilin

Ethylendiamintetraacetate Disodium (EDTA)

Glycerol

Glycine

Hepes

Igepal CA-630

Magnesium Chloride

Methanol

MG132

N-methyl dibenzopyrazine methyl sulfate (PMS)

N, N, N′, N′ - Tetramethylethylendiamin

Potassium Chloride

Protease Inhibitor Cocktail Tablets EDTA-free

Sodium Chloride

Sodium Dodecyl Sulphate - SDS

Tris(hydroxymethyl)aminomethan - TRIS

Tris Buffer Saline (TBS)

Triton X-100

Tween 20

XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)

4.2 MACHINES AND EQUIPMENT

Trans-blot SD-semi- dry transfer cell (Bio-Rad)

Real-time PCR Realplex 4 (Eppendorf)

Biospectrometer (Eppendorf)

Multiporator (Eppendorf)

Innova 42 rockers (New Brunswick scientific)

Allegra X-15R centrifuge (Beckman Coulter)

Victor X3 Multimode plate reader (Perkin Elmer)

Optima Max XP centrifuge (Beckman Coulter)

Avanti J-301 centrifuge (Beckman Coulter)

Digital heatblock (VWR)

Analog vortex mixer (VWR)

Countess TM Automated cell counter (Invitrogen)

Nikon Eclipse T100 Microscope (Nikon)

Brady BMP 51 label maker (Brady)

Microplate shaker (VWR)

CO_2 incubator (Sanyo)

Nanodrop 2000 (Thermo Fischer Scientific)

4.3 METHODS

4.3.1 STERILIZATION

All plastic equipment used for bacterial or cell culture was sterilized by manufacturer. Surface of all equipment (pipettes, tips, flasks, tubes...) used in the Biohazard Box was sterilized using 70 % Isopropanol. Tips for automatic pipettes, bacterial spreaders and cryotubes used for bacteria were sterilized by flaming. Glassware was sterilized by high temperature.

4.3.2 WORK WITH RNA

4.3.2.1 RNA ISOLATION

RNA isolation from the cell lysate was conducted using the RNeasy kit (Qiagen) following the manufacturer´s instructions.

4.3.2.2 RNA CONCENTRATION DETERMINATION

Concentration of RNA in sample was determined with Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific) spectrophotometrically using absorbance at 260 nm wavelength.

4.3.2.3 DNASE TREATMENT

Samples were treated with DNase using the standard DNase I Amplification Grade (Thermo Fisher Scientific) following the manufacturer´s protocol.

4.3.2.4 REVERSE TRANSCRIPTION

Reverse transcription was conducted using the Superscript III First-Strand Synthesis SuperMix for qPCR kit (Thermo Fisher Scientific) 8 µl out of the 2 µg DNase treated RNA was mixed with 10 µl of 2xRT Enzyme Mix and 2 µl of RT Enzyme. After a 10 min incubation at 25°C, the sample was held at 50°C for 45 min. Reaction was terminated by a 5 min incubation at 85°C. 1 µl of RNase H was added to each sample. Followed by final incubation at 37°C for 20 min.

4.3.3 Work with DNA

4.3.3.1 Plasmid isolation from bacteria

Plasmids were isolated from cultures by using endo-free commercial kit (Qiagen Endofree Plasmid Maxi Kit) according to manufacturer´s instructions.

4.3.3.2 DNA concentration determination

Concentration of DNA in sample was determined with Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific) spectrophotometrically using absorbance at 260 nm wavelength.

4.3.3.3 DNA electrophoresis

DNA was separated in 1 % agarose gel. Agarose was dissolved completely in 1xTAE buffer by heat. This solution was then poured into the electrophoresis chamber with an electrophoresis comb. Once the gel solidified, it was transferred into the electrophoresis apparatus and covered with 1xTAE buffer. Then the sample mixed with a loading dye (Bromphenol blue -Thermo Scientific) was loaded into the wells of the gel. Marker was loaded as well (10 µl). The electrophoresis ran at 90 V-110 V for 45-70 min. The gel was then submerged in EtBr solution for 15 min. After this incubation time the gel was iluminated by UV and a picture was taken.

4.3.3.4 PCR

Primers for qPCR were designed using the NCBI Primer designing tool.

qPCR program:

Step	Cycles
Initial Denaturation 95°C 5 minutes	
Denaturation 95°C 15 seconds Annealing + Elongation 60°C 1 minute	40x
95°C 15 seconds 60°C 15 seconds 20 minute heating up to 95°C 95°C 15 seconds	Melting curve
Cooling at 4°C	

4.3.3.5 SEQUENCING

Sequencing was performed by GATC Biotech company (Germany). Samples for sequencing were prepared in a 10 µl volume: 1.5 µl of DNA, 2.5 µl of primers with 6 µl of ddH_2O. Sanger sequencing method was employed for all sequencings.

4.3.4 WORK WITH BACTERIA

4.3.4.1 CULTIVATION FOR PLASMID PRODUCTION

For the production of all plasmids were used single colonies of bacterial culture spread on nutrient agar plate with appropriate antibiotics. Single colony was inoculated into 5ml of LB media with appropriate antibiotics and incubated for 2 hours on shaker at 200 rpm, 37°C. Then 0.2 ml of this bacterial culture was inoculated into 100 ml of LB media and incubated with appropriate antibiotics (200 rpm., 37°C) overnight.

4.3.4.2 Bacteria transformation by electrophoresis

For one electroporation, 50 μl of competent bacteria were mixed with 1 μl of plasmid. Mixture was transferred to electroporation cuvette (Bio-Rad) with interelectrode distance 2mm. The electroporation pulse had capacitance 25 μF, voltage 2.5 kV, resistance 200 Ω and last for 4.5 - 5 miliseconds. After the pulse was applied, 1 ml of SOC media was added to cuvette and suspension was transferred to Erlenmeyer flask. Suspension was incubated at 37°C with shaking (200 rpm) for 1 hour. After incubation, variety of volumes (1 μl, 10 μl, 100 μl and the rest of culture) of bacteria were spread on nutrient agar plates and incubated overnight at 37°C.

4.3.4.3 Minipreparation of plasmids

Single colonies from nutrient agar plate were inoculated in 0.75 ml of LB with appropriate antibiotics in culture tubes and incubated overnight with shaking (200 rpm) at 37°C. Plasmids from bacteria were isolated according to the chapter 5.3.3.1.

4.3.4.4 Bacterial conserves

1.5 ml of freshly grown culture was added to 0.6 ml of sterile 50 % glycerol. The bacteria was then frozen on dry ice and stored at -80°C.

4.3.5 Work with tissue culture

All work with tissue culture was done with mammalian cells (Huh7, HEK 293T). Cells were cultivated at 37°C in 5 % CO_2 atmosphere. Maintenance passaging was done with serum supplemented DMEM medium with antibiotics.

4.3.5.1 Passaging of cell culture

Medium was removed from confluent cells and cells were washed two times with PBS (13 ml for a T75 flask). Tissue culture was then trypsinized (2 ml for a T75 flask). Tissue culture was then incubated at 37°C in 5 % CO_2 atmosphere for about 5-7 min until the cells detached from the surface. Serum supplemented DMEM with antibiotics was then added to the trypsinized culture (8 ml for a T75 flask). Tissue culture was then centrifuged at 1200 rpm for 5 min, supernatant was removed and cells

were resuspended in serum supplemented DMEM with antibiotics and passaged onto new flasks.

4.3.5.2 Transfection of HEK 293 cells

HEK 293T cells were transfected using Lipofectamine 2000 reagent, following the manufacturer´s protocol.

4.3.5.3 Transfection of Huh 7 cells

Huh 7 cells were transfected using GenJet reagent, following the manufacturer´s protocol.

4.3.5.4 siRNA transfection

Huh7 cells were using RNAiMax Lipofectamine reagent, following the manufacturer′ protocol.

4.3.5.5 Medium harvest for ELISA

Medium was harvested followed by centrifugation at 10 000 rpm for 20 min. Supernatant was then transferred into new microcentrifuge tubes.

4.3.5.6 MG132 treatment

Cells were incubated at 37°C in 5 % CO_2 atmosphere in a serum supplemented DMEM with antibiotics with 50 μM MG132 (Sigma-Aldrich) for 5 hours.

4.3.5.7 XTT cell viability assay

Cells were grown on a 12 well plate. Medium was either harvested or disposed. 250 μl of fresh phenol red free DMEM Complete was pipetted onto each well. For each well, 250 μl of XTT-PMS (50:1 ratio) solution was needed. XTT PMS mix was prepared just before the actual application on cells, with a light turned off in the biohazard box. After a 4 hour incubation at 37°C in 5 % CO_2 atmosphere, the XTT cell viability was checked on the Perkin Elmer station at 450 nm wavelength.

4.3.5.8 ELISA

On day six post HBV rcccDNA transfection, medium was harvested and centrifuged at 10 000 rpm for 20 min. Supernatant was then transferred into new microcentrifuge tubes and the cell debris was disposed. Two ELISA kits were employed: ELISA HBeAg kit (Bioneovan) detecting HBe

and ELISA HBsAg kit (Bioneovan) detecting HBs. Work was conducted following the manufacturer´s protocol for each ELISA kit. Both ELISA kits´ results were obtained via Perkin Elmer measurement at 420 nm wavelength.

4.3.6 WORK WITH PROTEINS

4.3.6.1 PROTEIN HARVEST

Prior to protein harvest, cells were washed with 12 ml of pre-chilled PBS (1 ml for each well of a 12 well plate). Protein was harvested using Lysis buffer (1 % Triton X-100, 150 mM NaCl, 20 mM HEPES pH7.8, 2 mM EDTA, 5 mM $MgCl_2$) - 800 µl for a T75 flask, 200 µl for one well of a 12 well plate. After a short incubation time with the lysis buffer (1-2 min at RT), cells were scraped using a sterile scraper into a sterile eppendorf tube. Cell lysate was then spinned down at 15000 rpm for 30 min at 4°C. Supernatant was transferred into a new eppendorf tube and held at -80°C.

4.3.6.2 PROTEIN CONCENTRATION DETERMINATION

Protein concentration was measured using Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific) following the standard kit protocol. Protein concentration was then determined at 560 nm wavelength.

4.3.6.3 CO-IMMUNOPRECIPITATION

Pierce™ antiHA Magnetic beads (Thermo Fisher Scientific) which were used for our experiments were first equilibrated by extensive wash (3 times 1 ml of Lysis buffer). Beads were then added to cell lysate and left for incubation at 4°C overnight. On the next day, the unbound lysate was washed out (4 times 1 ml of Lysis buffer) followed by one wash with 1 ml of PBS. Beads were then spinned down for 5 min at 10 000 rpm and residual PBS was removed.

4.3.6.4 GST PULL-DOWN

GST-HBc fusion protein or GST alone (0.5 µg) bound to glutathione-Sepharose beads (GE Healthcare Life Sciences) were incubated with 500 µg of the cell lysate isolated from FBXO3- UBE2O-transfected HEK 293T cells at 4°C for 3 h. After five 10-min washes with lysis buffer

supplemented with 1% Igepal CA-630, the proteins that were bound to the beads were analyzed by Western blotting with antiFLAG specific antibodies.

4.3.6.5 Mass spectrometry (MS)

4.3.6.5.1 On-beads digestion

Immunoprecipitated proteins on Glutathione Sepharose 4B beads were washed 3x with 1ml of 50 mM ammonium hydrocarbonate. Proteins in the sample were reduced by dithiothreitol (65°C, 30 min) and alkylated by Iodoacetamide (RT for 30 min in dark). Solvent was removed, 100 ul of 50 mM ammonium hydrocarbonate including 0,1 μg of chymotrypsin were added and proteins were digested at 37°C for 10 hours. Resulting peptides were separated from beads by magnet, dried in the SpeedVac (Labconco) and dissolved in 15 μl of 0.1 % formic acid.

4.3.6.5.2 Liquid chromatography MS/MS

All samples were analyzed on UltiMate 3000 RSLCnano system (Dionex – Thermo Scientific) coupled to a TripleTOF 5600 mass spectrometer with a NanoSpray III source (Sciex). The instrument was operated with Analyst TF 1.7 (Sciex). The peptides were trapped and desalted with 2 % acetonitrile in 0.1 % formic acid at flow rate of 5 μl/min on Acclaim PepMap100 column (5 μm, 2 cm×100 μm ID, Thermo Scientific). Eluted peptides were separated using Acclaim PepMap100 analytical column (3 μm, 25 cm×75 μm ID, Thermo Scientific). The 70 min elution gradient at constant flow of 300 nl/min was set to 5 % of phase B (0.1 % formic acid in 99.9 % acetonitrile, phase A 0.1 % formic acid) for first 5 min, then with gradient elution by increasing content of acetonitrile. TOF MS mass range was set to 350–1500 m/z, in MS/MS mode the instrument acquired fragmentation spectra within m/z range 100-2000.

4.3.6.5.3 Data processing

Protein Pilot 4.5 (Sciex) was used for protein identification from raw (*.wiff) spectra using database consisting of HBV proteins and their mutant variants, human proteins and common contaminants (Uniprot). The search was set to choose iodoacetamide as alkylation substance, trypsin as digestion agent and TripleTOF 5600 as instrument. All samples were

evaluated by Paragon algorithm in the regime „Thorough“. Set of biological modification as defined by the vendor with different probabilities of potential modifications was employed.

4.3.6.6 Western blot

4.3.6.6.1 Sample preparation

Protein loading buffer was added to a sample (6xPLB:protein - 1:5). Sample was then incubated at 100°C for 5min followed by a 2min incubation on ice.

4.3.6.6.2 SDS-PAGE

The apparatus for gel preparation was set up following the manufacturer's instructions. Tightness of the two glasses was checked with water. The running gel was prepared following a standard protocol. Space between the two glasses was filled with the newly prepared gel (reaching to about 1.5 cm under the upper edge of the glasses). Gel was overlaid with water (ddH_2O) and left for 30 min on the bench. The right polymerization of the gel was checked with the remaining volume of the gel in the tube. Water was removed using vacuum and upper gel was stacked on top of the running gel, comb was inserted, and gel was left for polymerization. Gel was then set in the electrophoresis apparatus and immersed in SDS-PAGE running buffer (25 mM Tris, 192 mM Glycine, 0.1 % SDS). Wells were washed with the SDS-PAGE running buffer. Protein sample was then loaded (2-20 µl). Marker was loaded as well (1.5 µl). Electrophoresis was then started - 60 V for the first 20 min of the run, 90 V for the rest of the run (until the bromphenol blue reached the bottom of the gel).

4.3.6.6.3 Western blot

Once proteins were separated on the gel, they were transferred onto a PVDF membrane using semi-dry western blot. The membranes were immersed in methanol and incubated in Transfer Buffer (390 mM Glycine, 480 mM Tris-HCl, 0.37 % SDS) for 15min together with the gel. Then the two were transferred into the Trans-Blot Turbo Transfer System (Bio-Rad). Blotting sandwich was assembled: filter paper, membrane, gel, filter paper. Transfer was performed at 25 V, 2.5 A for 10 min.

4.3.6.6.4 DETECTION

Successful protein transfer was checked with the marker visible on the membrane. PVDF membrane with transferred proteins was then blocked by incubating it in 5 % milk (dry milk completely dissolved in Wash buffer: 1xTris-buffered Saline - TBS, 0,1 % Tween20) for one hour on a rocker at room temperature. Then the membrane was left for an overnight incubation at 4°C on a rocker in 6ml of 5 %milk with a properly diluted primary antibody. Next, membrane was washed with Wash buffer 3 times, each time for 15 min on a rocker at room temperature. Then, it was incubated in 6ml of 5 % milk with properly diluted secondary antibody and Horse-Radish Peroxidase (HRP; 1:10 000) for one hour on a rocker at room temperature. Then the membrane was washed again with Wash buffer, in the same manner as above. Proteins were detected based on the used antibodies. Detection was performed using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and FujiFilm LAS-3000 Imager.

4.3.7 WORK IN BSL3

All work with tissue culture was performed in a special Bio-safety level 3 laboratory. This required extreme caution and sterile environment. A person performing any kind of experiments in this laboratory was required to be wearing an overall, head mask, air filtration unit, two pairs of gloves and proper laboratory shoes with girdles. Every flask/dish/plate needed to be checked via microscope before opening in biohazard box. All disposed material needed to be autoclaved before leaving the facility.

4.3.8 DATA ANALYSIS

4.3.8.1 GRAPHPAD PRISM SOFTWARE

Data for FBXO3 and UBE2O silencing experiments have been analyzed using the GraphPad Prism 7 Software for Windows. To compare difference between control and each data set, t-test was performed.

4.3.8.2 Image Quant TL 8.1 Software

The Image Quant TL 8.1 Software (General Electric Healthcare Life Sciences) was used for image quantifications (Western blot images). All quantification results have been normalized to the overall expression of the quantified protein.

5 RESULTS

5.1 HBC PROTEOMIC ANALYSIS

The fact that HBc is a crucial protein for a successful HBV replication is undeniable. Its employment in multiple viral processes makes it an intriguing subject for closer interrogation. That is why, we have decided to analyze the interactome of the protein. Cell lysates from HepG2 hNTCP cells were incubated with HBc - Glutathione-S-transferase (GST) or GST recombinant proteins bound to Glutathione Sepharose 4B beads. After excessive washes the HBc interacting proteins were analyzed by MS. Among potential HBc interactors we have decided to focus on two proteins: FBXO3 and UBE2O. FBXO3 (E3 ligase) and UBE2O (E2/E3 ligase). In a publication from my laboratory, Lubyova et al. identified several HBc posttranslational modifications (PTMs): R150 and R156 methylations by protein arginine methyltransferase 5 and possibly 7 (PRMT 5, PRMT7); K7 ubiquitination by a yet to be identified Ub ligase as well as other potential Ub acceptor sites (K96, Serine and Threonine residues) which are in need of further investigation (Lubyova et al. 2017). These findings served as a backbone for our subsequent work on HBc ubiquitination.

5.2 HBC-FBXO3 AND HBC-UBE2O INTERACTION DETECTION

In this chapter we have focused on confirming the obtained MS results (see chapter 6.1). In order to do so, we have performed several co-immunoprecipitations and GST pull-down assays. We have tried using several cell lines (HepG2, Huh7 and HEK 293T cells). HEK 293T turned out to be the most efficient considering protein overexpression, which has been crucial for most of conducted experiments.

5.2.1 CO-IMMUNOPRECIPITATION

Human embryonic kidney cells (HEK 293T) were transfected with FBXO3, UBE2O and HBc (Core-HA) plasmids. pcDNA 3.1 was used as a control plasmid. Core-HA plasmid was carrying a human hemagglutinin (HA) tag (amino acid sequence: YPYDVPDYA) (Lubyova et al. 2017). Expression plasmids of FBXO3 and UBE2O were purchased from OriGene and

contained two tags: Myc (EQKLISEEDL) and FLAG (DYKDDDDK). Cells were transfected using Lipofectamine 2000 reagent. In order to have the same HBc levels in all transfections, HBc was transfected separately, prior to the transfection of FBXO3 and UBE2O. This transfection resulted in six combinations of over-expressing cell cultures:

- pcDNA3.1 pcDNA3.1
- pcDNA3.1 FBXO3
- pcDNA3.1 UBE2O
- Core-HA pcDNA3.1
- Core-HA FBXO3
- Core-HA UBE2O

Forty-eight hours post transfection cells were lysed and harvested. 20 µg of protein lysate were denaturated and resolved on SDS-PAGE to check for levels of protein expression. In order to find out whether the proteins of our interest interact, we have performed co-immunoprecipitation using antiHA beads. 500 µg of total protein from each sample were incubated overnight with 25 µl of antiHA magnetic beads. On the next day the beads were washed, denaturated and analyzed by SDS-PAGE (fig.10). The membrane was then incubated with antiFLAG antibody. UBE2O was detected on the membrane, confirming our hypothesis that HBc and UBE2O interact. However, there was no sign of FBXO3 band on the membrane, thus HBc-FBXO3 interaction has not been confirmed. Probably due to low FBXO3 expression levels - FBXO3 might have been lost during the wash of the beads. This experiment has been repeated five times with small changes (protein load, number of washes, antibodies etc.). Since co-immunoprecipitation did not work for FBXO3-HBc interaction confirmation - probably due to low FBXO3 expression in cells, a slightly different experimental approach was conducted. HEK 293T cells were transfected with HBc carrying a V5/AP tag (which enables biotinylation during expression in cells) in combinations with pcDNA3.1, FBXO3 or UBE2O. 500 µg of protein was then immunoprecipitated with antiFLAG magnetic beads. Immunoprecipitate containing biotinylated HBc was visualized on WB via antiNeutravidin HRP antibody. Results indicate

that HBc specifically interacts with both FBXO3 and UBE2O E3 ligases (fig.11).

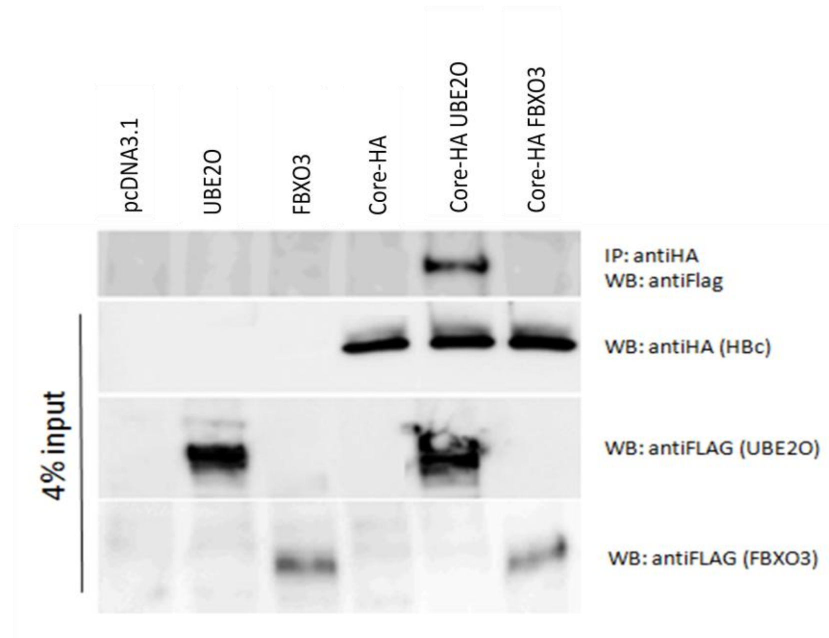

Fig.10 UBE2O interacts with HBc. Co-immunoprecipitation of HBc in transfected HEK 293T cells. HEK 293T cells were transfected with HA-tagged HBc in combination with Flag-tagged FBXO3 or UBE2O expression plasmids. Forty-eight hours after transfection, the cells were harvested and protein lysates were prepared. Protein lysates (500 μg) were immunoprecipitated (IP) with anti-HA antibodies (HBc), and the immunoprecipitated complexes were analyzed by Western blot (WB) with antiFLAG antibodies. The relative levels of FBXO3, UBE2O and HBc in 20 μg of protein lysates are shown for comparison (4 % input).

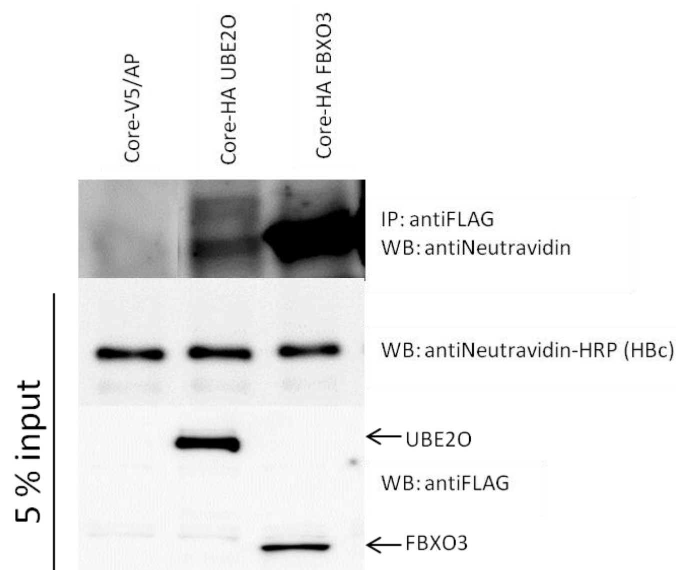

Fig.11 HBc interaction with FBXO3 and UBE2O. Co-immunoprecipitation of biotinylated HBc in transfected HEK 293T cells. HEK 293T cells were transfected with V5-tagged HBc conjugated with AP in combination with Flag-tagged FBXO3 or UBE2O expression plasmids. Forty-eight hours after transfection, the cells were harvested and protein lysates were prepared. Protein lysates (500 μg) were immunoprecipitated (IP) with anti-FLAG antibodies, and the immunoprecipitated complexes were analyzed by WB with

antiNeutravidin-HRP (HBc). The relative levels of FBXO3, UBE2O (antiFLAG) and HBc (antiNeutravidin-HRP) in 25 µg of protein lysates are shown for comparison (5 % input).

5.2.2 GST PULL-DOWN

The interaction between HBc and FBXO3 and UBE2O was further confirmed by GST pull-down assay. HEK 293T cells were transfected with three plasmids: pcDNA3.1 as a control, FLAG-tagged FBXO3 and UBE2O. Cell lysates were incubated with GST or GST HBc recombinant protein prepared in bacteria. After extensive washes, the HBc-bound proteins were eluted, resolved on SDS-PAGE and detected with antiFLAG antibodies. Results indicate that both FBXO3 and UBE2O specifically interact with HBc (fig.12a-c).

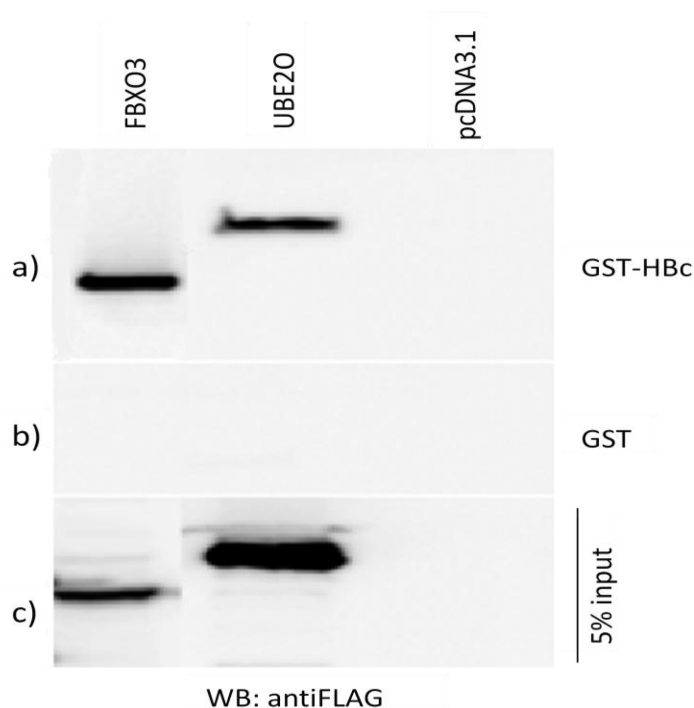

Fig.12 UBE2O and FBXO3 interact with GST conjugated HBc. Flag-tagged FBXO3 and UBE2O were transfected into HEK 293T cells and 500 µg of total protein were incubated with a) HBc fused to GST or b) GST alone immobilized on glutathione-Sepharose beads. The bound proteins were eluted and resolved on 10 % SDS-PAGE followed by WB with anti-Flag antibodies. c) Five percent of total protein input is shown.

5.3 HBC UBIQUITINATION

After having confirmed, that the proteins of our interest specifically interact, we have moved on to the PTM itself. By WB analysis we observed HBc ubiquitination levels – by comparing the control (HBc) to HBc in combination with either FBXO3 or UBE2O. For this experiment, we have

used once again the HEK 293T cell line. This cell line has been reliable in growth and transfection outcomes were undeniably higher than any other tested cell line. Two sets of experiments were conducted. One, where endogenous Ub was detected and one with overexpressed Ub.

5.3.1 HBc UBIQUITINATION LEVELS WITH ENDOGENOUS UBIQUITIN

HBc, FBXO3 and UBE2O transfected HEK 293T cells were treated with MG132 proteasome inhibitor (50 µM concentration; 5 hours). Cell lysate was harvested using Lysis buffer. 1500 µg of overall protein from samples: pcDNA3.1, Core-HA, Core-HA FBXO3 and Core-HA UBE2O have been incubated overnight with 50 µl of antiHA beads each. On the next day, the beads were washed and denaturated. In order to detect ubiquitination of the samples we have performed SDS-PAGE followed by Western blot with antiUbiquitin antibodies. This experiment has been repeated three times. Representative results are summarized in fig.13a-c. Bands representing HBc monoubiquitination were quantified and normalized to overall HBc expression (input) using the Image Quant TL software. Results seem to indicate that FBXO3 predominantly promotes HBc polyubiquitination, while UBE2O may promote HBc´s monoubiquitination. This result is further supported by the image quantification, where the relative level of HBc monoubiquitination in UBE2O co-transfected sample is approximately 1.6-fold higher compared to control (pcDNA3.1 transfected) cells. The overexpression of FBXO3 also led to moderate (1.3-fold) increase of HBc monoubiquitination. It is known that FBXO3 Ub ligase is a compelling mediator of polyubiquitination in human cells. While UBE2O´s role is often connected to monoubiquitination of target proteins. Our results tend to agree with these findings but further investigation by my colleagues is necessary.

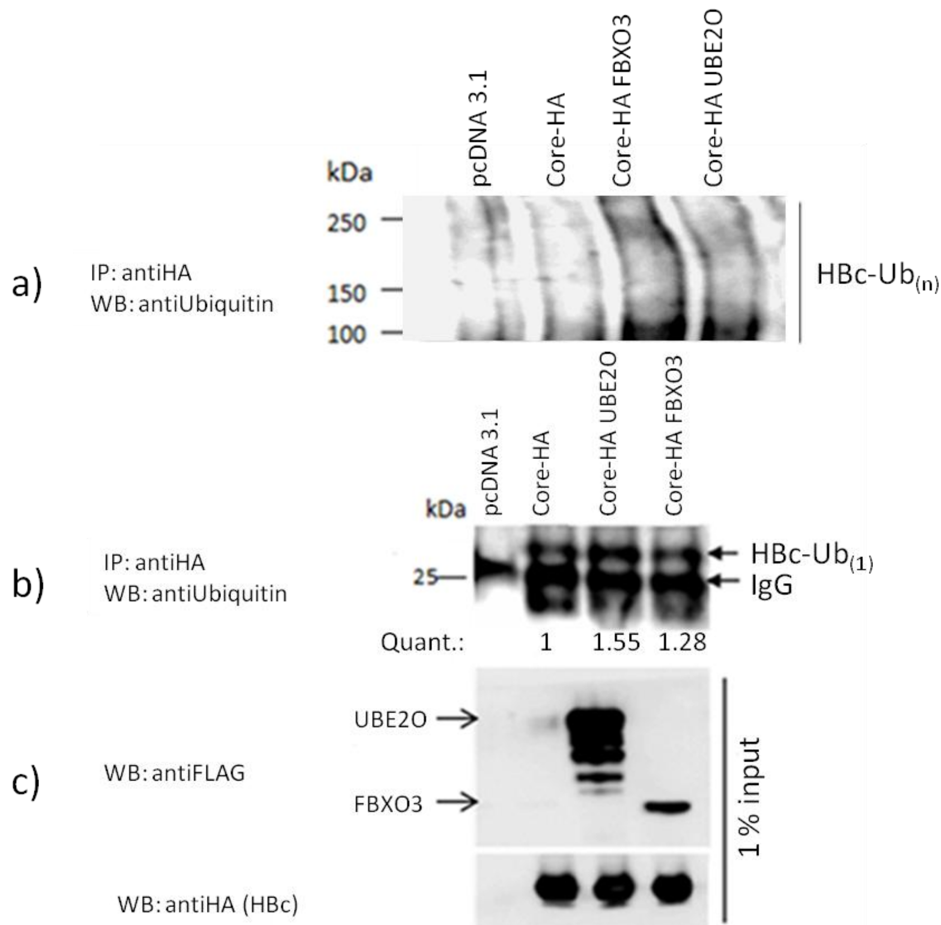

Fig.13 HBc ubiquitination with endogenous Ub. Co-immunoprecipitation of HBc in transfected MG132 treated HEK 293T cells. HEK 293T cells were transfected with Flag-tagged FBXO3 and UBE2O expression plasmids as well as with HA-tagged HBc. Forty-eight hours after transfection, the cells were harvested and protein lysates were prepared. Protein lysates (1500 µg) were immunoprecipitated with anti-HA antibodies (HBc), and the immunoprecipitated complexes were analyzed by WB. a) WB membrane was incubated with antiUbiquitin antibodies. HBc-$Ub_{(n)}$ represents the polyubiquitination levels of HBc (smears). b) WB representing the monoubiquitinated HBc (HBc-$Ub_{(1)}$) band. Band quantification was normalized to the overall HBc expression (input) The bands were quantified using Image Quant TL. c)The relative levels of FBXO3, UBE2O and HBc in 15 µg of protein lysates are shown for comparison (1 % input).

5.3.2 HBc ubiquitination levels with exogenous ubiquitin

To further evaluate the role of FBXO3 and UBE2O in HBc ubiquitination, we have performed a set of experiments where exogenous ubiquitin was expressed in HEK 293T cells. For this experiment, we have prepared a new plasmid carrying ubiquitin DNA with a Myc tag on the protein´s C end. Prior to cell harvest, HEK 293T underwent MG132 treatment (50 µM; 5 h). 1500 µg of total protein was immunoprecipitated with anti HA antibodies

(50 µl). Cell lysate immunoprecipitate was then resolved on 10 % SDS-PAGE (fig.14a-c). Same experiment has been repeated two times. Obtained results indicate that FBXO3 protein supports HBc polyubiquitination: smear (representing HBc polyubiquitination) in HBc transfected cells which were co-transfected by FBXO3 was visibly stronger compared to cells co-transfected with UBE2O. On the other hand, the levels of HBc monoubiquitination (represented by HBc-$Ub_{(1)}$ band in fig. 14b) appeared to be higher in UBE2O-overexpressing cells compared to control (pcDNA 3.1) or FBXO3-transfected cells. Monoubiquitinated HBc representing bands (HBc-$Ub_{(1)}$) were quantified using the Image Quant TL software and normalized to the overall HBc expression (input). In UBE2O-overexpressing cells, the level of HBc monoubiquitination was increased by 1.4-fold compared to mock (pcDNA3.1) transfected cells. The slight increase (1.2-fold) in HBc monoubiquitination was also observed in cells transfected with FBXO3. This data further confirmed the UBE2O′s putative role in HBc monoubiquitination.

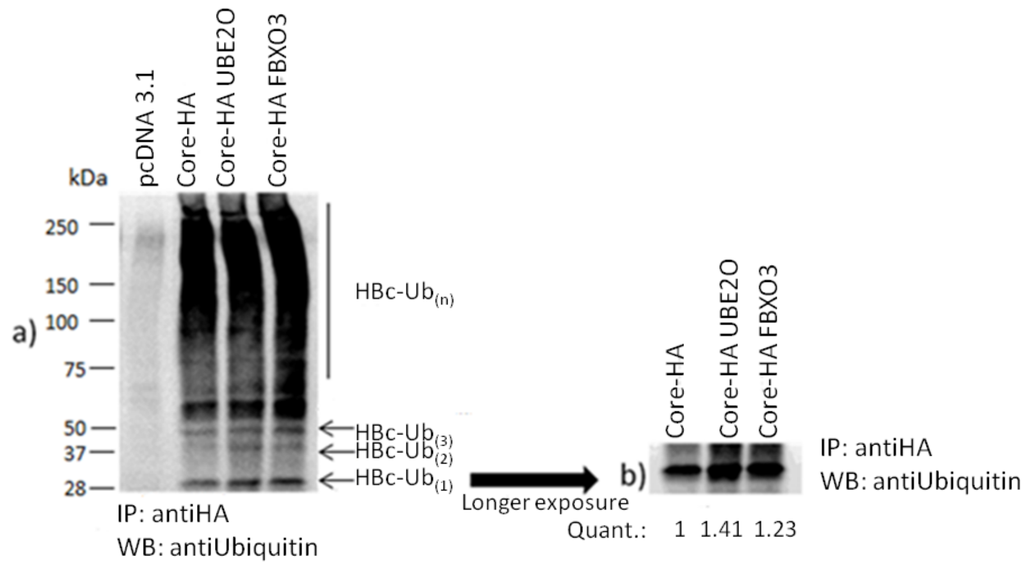

Fig.14 HBc ubiquitination with overexpressed Ub. Co-immunoprecipitation of HBc in transfected MG132 treated HEK 293T cells. Cells were transfected with Flag-tagged FBXO3 and UBE2O expression plasmids as well as with HA-tagged HBc and Myc-tagged Ub. Forty-eight hours after transfection, the cells were harvested and protein lysates were prepared. Protein lysates (1000 µg) were immunoprecipitated with anti-HA antibodies (HBc), and the immunoprecipitated complexes were analyzed by WB. a) Membrane incubated with antiUbiquitin antibody represents levels of HBc ubiquitination. Mono-, di- and tri- ubiquitinated HBc (HBc-$_{Ub(1/2/3)}$) bands are indicated. b) Lower part of the antiUbiquitin membrane representing HBc-Ub$_{(1)}$ is shown after longer exposure. Band quantification was normalized to the overall HBc expression (input). Bands were quantified using the Image Quant TL software. c) The relative levels of FBXO3, UBE2O, Ub, HBc and βActin in 20 µg of protein lysates are shown for comparison (2 % input).

5.4 FBXO3 AND UBE2O LIGASES´ EFFECT ON HBV REPLICATION

So far, we have focused on FBXO3´s and UBE2O´s effect on a protein level (HBc ubiquitination). In pursuance of a more complex picture, we have moved on to study HBV replication's dependence on the two Ub ligases. By modulating the expression of endogenous FBXO3 and UBE2O, we were able to investigate changes of HBV replication levels. For this, we have used ELISA kits measuring the levels of HBeAg (HBV e antigen) or HBsAg (HBV surface antigen) proteins in the harvested cell media of HBV recombinant cccDNA-transfected (rcccDNA) Huh7 cells. As a mean of down-regulating FBXO3 and UBE2O levels in cells, small interfering RNA (siRNA) interference assay was employed. These experiments were supported by a control siRNA (CTRLsi). Two sets of siRNAs specific for interfering with FBXO3 mRNA and three sets of siRNAs were used for UBE2O´s mRNA. In order to provide a reliable outcome from this experiment, siRNAs´ knock-down efficiency was determined via reverse transcription quantitative PCR (RT-qPCR) (fig.15a-c). Huh7 cells were transfected with six sets of siRNAs (control siRNA, two sets of FBXO3 siRNAs and three sets for UBE2O siRNAs). Cells were harvested on day 6 after the transfection and the total RNA was isolated. Prior to reverse transcription, each sample was treated with DNase. Obtained cDNAs were then amplified and quantified via qPCR. Both siRNAs for FBXO3 silencing (FBXO3si-1 and FBXO3si-2) have proven to be working sufficiently. With FBXO3si-2 yielding a slightly stronger gene expression silencing. UBE2O siRNAs worked as well. UBE2Osi-3 siRNA had a slightly stronger silencing effect than UBE2Osi-1 and UBE2Osi-2. PCR products (5 μl) were analyzed on gel electrophoresis (1.8 % agarose gel; 110 V; 70 min). The electrophoresis outcome shows a single specific PCR product for each RT-qPCR run. The agarose gel representing FBXO3 silenced cDNA amplicons does not indicate any changes in cDNA levels for the FBXO3 siRNA transfected samples. This may be due to the saturation of the sample after forty cycles of qPCR. Whilst UBE2O representing agarose gel indicates a significant decrease of UBE2O cDNA in the UBE2O siRNA transfected samples. This phenomenon is probably significant due to the higher UBE2O siRNAs efficiency.

After checking the silencing efficiency of each siRNA we have moved on to observing the changes in HBV replication levels with knocked-down FBXO3/UBE2O. Huh7 cells were transfected by siRNAs one day prior to HBV rcccDNA transfection. The rcccDNA minichromosome was prepared by Guo et al. in 2016 (fig.9). RNAiMax Lipofectamine reagent was used for siRNA transfection, Genjet reagent was used for rcccDNA transfection. Six days post transfection, cell media was harvested and analyzed by ELISA (fig.16a-b). Cell viability in each well (each siRNA have been introduced into three wells) were determined by XTT cell viability assay.

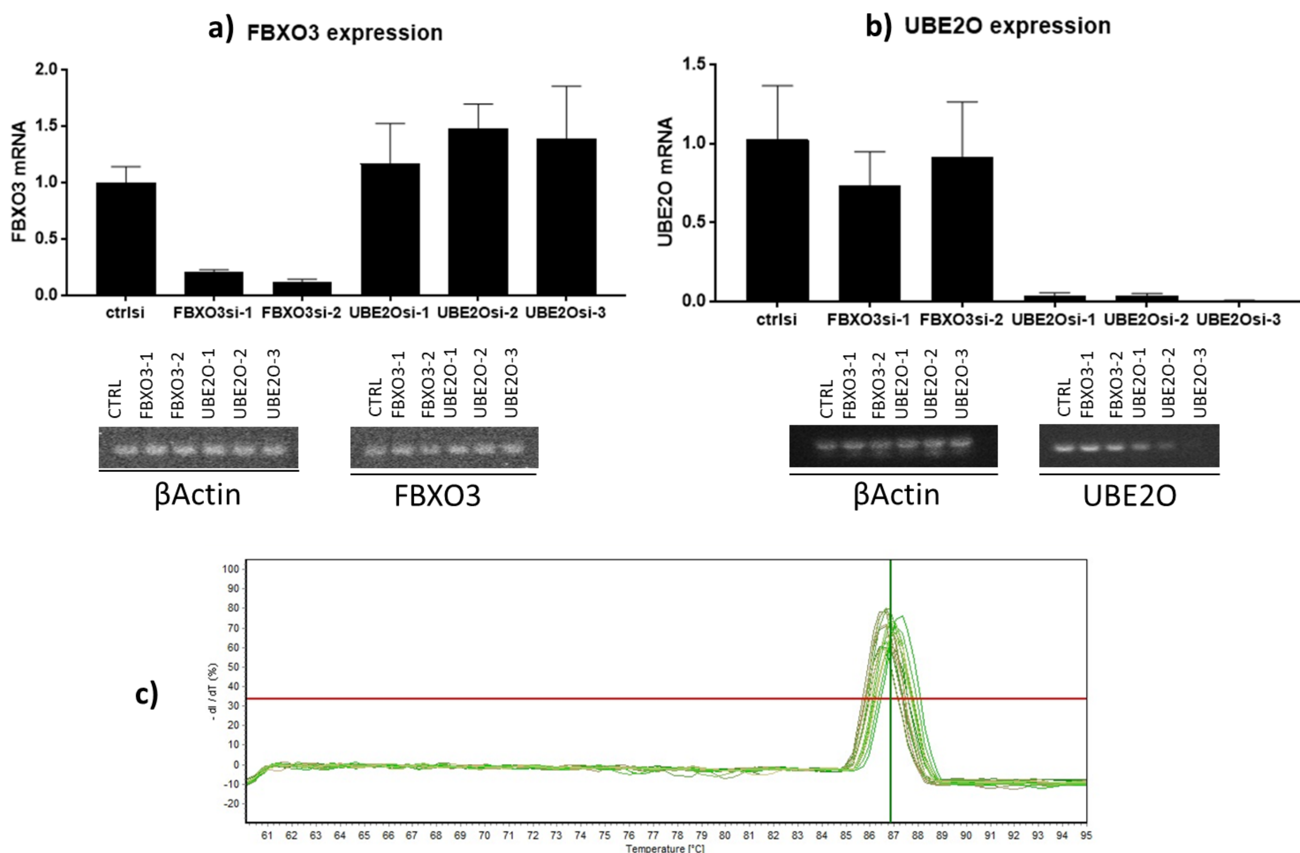

Fig.15 siRNA gene expression silencing efficiency. Huh7 cells were transfected with six sets of siRNAs: control siRNA (CTRLsi); FBXO3si-1 and FBXO3si-2 specific for FBXO3 mRNA; UBE2Osi-1, UBE2Osi-2 and UBE2Osi-3 specific for UBE2O mRNA. On the sixth day post transfection, cells were lysed and harvested. RNA was isolated and RNA concentrations were measured. Samples were then treated with DNase and 2 μg of RNA from each sample was reversely transcribed. cDNA was then amplified using qPCR with specific primers for FBXO3 and UBE2O DNA. βActin DNA was amplified as well and used for normalization. Results were then quantified and summarized into graphs: a) representing FBXO3 mRNA levels and b) representing UBE2O mRNA levels. c) Melting curve for βActin mRNA (housekeeping gene) indicates a reliable specific qPCR run.

HBsAg and HBeAg levels in the media were then normalized to XTT assay results for each well. t-tests were conducted by comparing each transfection to CTRLsi. FBXO3si-2, UBE2Osi-1 and UBE2Osi-3 were statistically significant. Results from the HBeAg ELISA indicate higher HBeAg levels in FBXO3-1 and FBXO3-2 siRNA transfected cells. On the other hand, the UBE2O-1, UBE2O-2 and UBE2O-3 siRNA transfected cells show lower HBeAg levels. These results indicate, that FBXO3 Ub ligase is employed in HBV replication inhibition. While UBE2O could work as a stimulator of the viral replication. HBsAg ELISA results do not show such significant differences in the HBsAg levels. This might be due to constant high expression of HBs proteins. HBeAg levels are generally accepted as an indicator of HBV replication level, while HBsAg levels are not usually used for such purpose. However, the results from HBsAg ELISA are not in disagreement with HBeAg ELISA results since the tendencies of the graph are preserved.

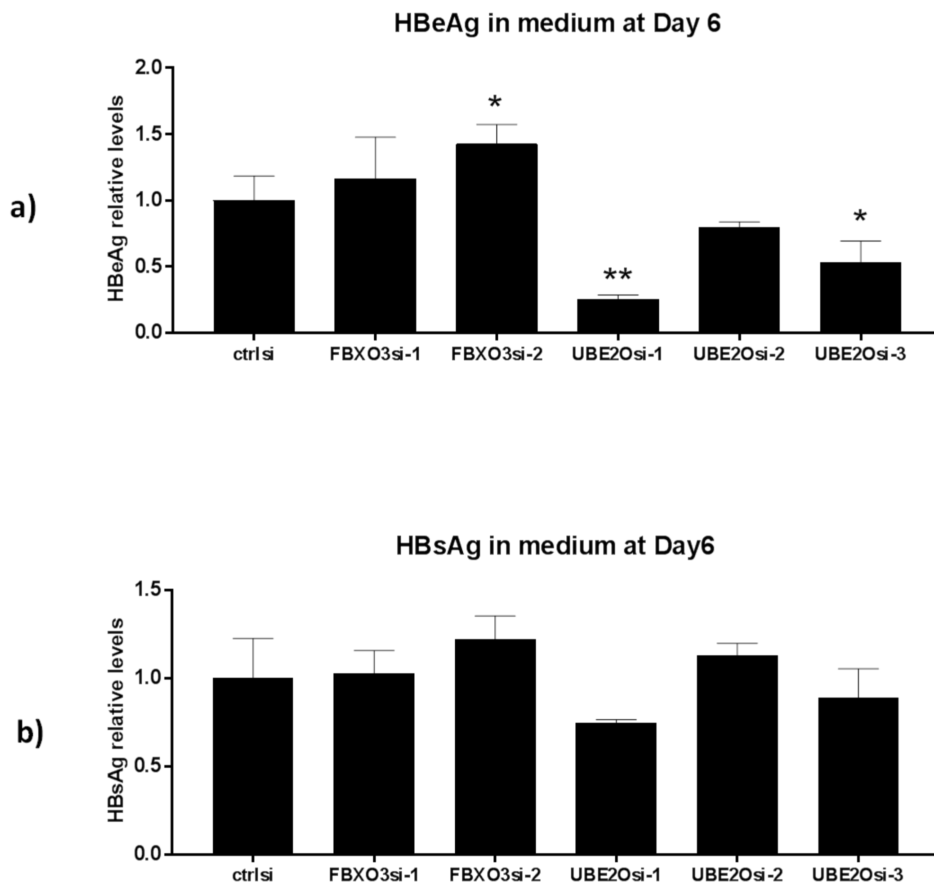

Fig.16 FBXO3 and UBE2O silencing in rcccDNA transfected Huh7 cells. On day zero, cells were transfected with six siRNAs (CTRLsi; FBXO3si-1,2; UBE2Osi-1,2,3). On day

one, the cells were transfected with HBV rcccDNA. On day six medium was harvested and cells underwent the XTT viability assay. Both HBeAg and HBsAg levels were measured via ELISA. Results from ELISA were normalized to XTT viability assay. Error bars represent standard deviations calculated from the results of three independent transfection experiments. t-test has been conducted by comparison of each result to CTRLsi transfected cells result. Asterisks indicate statistically significant differences (* $P<0.05$; ** $P<0.01$). a) HBeAg levels in the harvested medium. b) HBsAg levels in the harvested medium.

6 DISCUSSION

Despite an available vaccine which works as an effective prevention against the virus, Hepatitis B is still considered a major global health problem affecting more than 257 million people worldwide with over 880 thousands of casualties per year (WHO 2017). These numbers are a powerful boost for the science of today to continue in HBV research in hope for an efficient cure, which would save hundreds of thousands of lives. Plenty of research groups all over the world are tirelessly trying to solve this complex puzzle. Our group has focused on HBV topic since 2014. By concentrating on HBV proteomics, my colleagues have unveiled multiple elements which brought more light into the complex virus – host cell interactions riddle. HBc, a capsid forming protein, is a pleiotropic protein employed in multiple steps of HBV life cycle. It affects intracellular localization of the capsid (Haryanto et al. 2012; Kann et al. 1999; Li et al. 2010), viral transcription and further gene expression (Y. Guo et al. 2011), HBV reverse transcription (Lewellyn and Loeb 2011) as well as the capsid envelopment (Basagoudanavar, Perlman, and Hu 2007). A summarizing publication on HBc′s functions in viral life cycle was published by Zlotnick et al. in 2015. These findings together form a promising potential for HBc, as an inevitable piece in HBV replication, to act as a likely antiviral target. A starting point for my master′s project, focused on HBc ubiquitination, was work guided by Jan Weber and conducted by Barbora Lubyova and her colleagues which has been published in 2017 (Lubyova et al. 2017). Their effort was to identify several post-translational modifications of HBc as well as the enzymes responsible. One such finding concerned the putative K7, K96, S49, T53 and S157 HBc ubiquitination. We started by analyzing the protein′s interactors through GST pull-down of HBc and further MS analysis. Among multiple identified HBc interactors, two Ub ligases caught our attention. Our results indicated that FBXO3 (E3 ligase) and UBE2O (E2/E3 ligase) are interacting with HBc and thus, could be responsible for its ubiquitination. For further support and confirmation of our preliminary results we proceeded to test the interactions via co-immunoprecipitation, GST pull-down and WB. We have tried both co-immunoprecipitiation approaches: antiHA magnetic beads (with HBc

carrying an HA tag) and antiFLAG magnetic beads (with FBXO3 and UBE2O carrying antiFLAG tag). HBc-UBE2O interaction has been confirmed. However, the HBc-FBXO3 interaction could not be detected, which might be the result of generally lower FBXO3 expression in transfected cells. Thus, we have moved on to a slightly different approach, where we have used biotinylated HBc which yielded a far stronger pull-down assay product. Both FBXO3 and UBE2O interactions with HBc were detected. Following GST pull-down assay supported these results as well. Multiple publications on FBXO3 indicate that the ligase is predominantly responsible for polyubiquitination of cellular proteins which, in many cases, leads to proteasome-mediated degradation. Not only that FBXO3 is known to interplay with the immune system, it is also known to play a role in viral infection (Chen et al. 2013; Shao et al. 2016; Žumer et al. 2013). RVFV infection leads to suppressed antiviral cellular response (suppression of IFN-I) as well as to reduced host gene expression (degradation of p62). Both of these cellular modulations are mediated via FBXO3, a human protein which serves as a tool for successful RVFV spread (Kainulainen et al. 2014). All these findings together paint an interesting and rich pattern on how the FBXO3 could possibly influence HBV infection. The large UBE2O protein, which at the same time, catalyzes Ub conjugation and Ub ligation, has been shown to be an important modulator of cancer development in cells. UBE2O-mediated monoubiquitination of human BAP1 protein leads to its predominantly cytoplasmic localization which impedes with its transcription silencing activity, thus promoting cell transformation (Dey et al. 2012; Yu et al. 2010; Mashtalir et al. 2014). On the other hand, by polyubiquitinating human c-Maf transcription factor, UBE2O acts as an inhibitor of cell transformation (Eychène et al. 2008; Xu et al. 2017). Besides being an important cancer development regulator, UBE2O acts as a crucial player in development and protein cell homeostasis (Nguyen et al. 2017; Yanagitani, Juszkiewicz, and Hegde 2017). UBE2O′s relative promiscuity in human cells brings us to believe that it could play an interesting role in the HBV-host cell interplay. Since the specific interaction of our proteins of interest has been securely confirmed we have decided to focus more on ubiquitination of HBc. The

protein consists of several potential Ub acceptor sites which enable the protein ubiquitination. In an effort of understanding the effect of FBXO3 and UBE2O on HBc, we have performed a set of experiments where we analyzed ubiquitination via WB. HBc and FBXO3 co-transfected samples (both with or without overexpressed Ub) appeared to have a stronger polyubiquitination smear when analyzed on WB with an antiUbiquitin antibody. While the HBc UBE2O co-transfected cells (both with or without transfected Ub) bands representing the putative monoubiquitinated HBc were fairly stronger than the rest of the bands (HBc or HBc-FBXO3). Results from immunoprecipitated HEK 293T transfection cell lysate seems to indicate that FBXO3 stimulates HBc polyubiquitination, while UBE2O enhances HBc monoubiquitination. These results are in correlation with the publications which show strong tendency of FBXO3 towards polyubiquitination and UBE2O towards monoubiquitination. Of course, these conclusions are preliminary and in need of a further investigation by my colleagues. However, experiments have been repeated multiple times and the result has always stayed the same. As many publications have already shown, monoubiquitination often leads to changes in the modified protein′s function. One of such modifications is the cellular localization of the target protein. By altering HBc localization, the virus replication could be directed. When bound to cccDNA, HBc stimulates gene expression of its own. Thus, by UBE2O-mediated ubiquitination, HBc nuclear localization may be stimulated which would result in higher HBV transcription and subsequent protein translation. Nascent HBV capsids are not always targeted towards the endoplasmic reticulum where the envelopment occurs. The other option is so-called intracellular amplification, where the capsid heads back to the nucleus and newly formed rcDNA is repaired to cccDNA which can be further transcribed into viral mRNAs. This phenomenon could be also directed by HBc PTMs, potentially by UBE2O-mediated monoubiquitination itself. For further analysis of these interpretations, a closer look at the HBc and HBc-$Ub_{(1)}$ cellular localization would be in place. Thus, I suggest that the nuclear fraction specific as well as cytoplasm specific pull-down assay would be a suitable follow-up to my findings. Another approach allowing observation of HBc cellular

localization would be confocal microscopy of the HBV infected cells. By all means, both of these hypothesis would eventually lead to higher HBV titer. Thus, we may suggest that UBE2O is somehow employed in the viral replication stimulation. This intriguing question will be discussed later on. On the other hand, we have shown that FBXO3 Ub ligase yields higher levels of polyubiquitinated HBc. As the general consensus is that in most cases polyubiquitination results in proteasome-mediated degradation of the protein, we suppose that this case would not be any different. Thus, the FBXO3 Ub ligase may lead to HBc degradation resulting in the overall HBc downregulation, which would further suggest, that FBXO3 serves as a tool for HBV replication suppression. To further test the role of the two Ub ligases in HBV life cycle, we have proceeded to observe their impact on viral replication levels in cells. By knocking-down the expression of either FBXO3 or UBE2O in HBV rcccDNA transfected Huh7 cells we have observed the changes in HBV replication levels. HBV relative levels in FBXO3 knocked-down cells were higher than the control. On the other hand UBE2O silenced cells showed markedly lower HBV relative levels. Experimental results suggest following hypothesis: human FBXO3 protein inhibits HBV replication, while UBE2O promotes the viral replication. This experiment was conducted two times with similar results. Hence, our above-explicated working hypothesis on UBE2O-mediated monoubiquitination and FBXO3-mediated polyubiquitination outcomes are in a good correlation with these results. We believe that this new insight on HBV - host relation brings a lot of promising ways for manipulating the course of HBV infection. Since we have now identified two new potential HBV replication modulators, there is a possibility of HBV elimination. By better understanding this interplay, we will have more and more possibilities for a potential HBV cure. Of course, these results require further investigation. Ubiquitin ligases overexpression in HBV rcccDNA transfected cells would be a suitable mean for hypothesis interrogation. The next step would be HBV infection of HepG2 hNTCP cells and further observation of FBXO3 and UBE2O ligases influence on viral life cycle. A closer look on the HBc-FBXO3 and HBc-UBE2O interactions would be in place - knowing the exact domains and residues of both interacting sites is

undoubtedly needed for further research. The FBXO3 mediated viral inhibition could serve as a potential inspiration for a new antiviral substance. Certainly, these are all strong and confident statements, which are based on preliminary results. However, I believe that it could serve as a fresh motivation for further research. Any new insight on viral life cycle brings us closer to its understanding and thus opens the door to new ways of fighting against these deadly yet fascinating *beasts*.

7 CONCLUSIONS

I believe that my work in Dr Weber′s lab has led to several interesting and promising outcomes. As there was a time limitation, many results are in need of further analysis. However, I believe that my findings are complementing each other, which makes it a promising untouched pathway towards a new constructive and complex HBV understanding. We have identified two new HBc interactors: FBXO3 (E3 Ub ligase) and UBE2O (E2/E3 Ub ligase). Our results suggest the predominant function of FBXO3 in HBc polyubiquitination, while UBE2O seems to promote HBc monoubiquitination of HBc. FBXO3 seems to inhibit HBV replication, while UBE2O may stimulate the levels of HBV replication. Since polyubiquitination is closely connected to proteasome-mediated degradation of the modified protein, FBXO3 could act as an HBV replication inhibitor via HBc degradation. On the other hand, monoubiquitination can lead to multiple different changes in the protein′s function, cellular localization, protein-protein interaction etc. By promoting HBc monoubiquitination, UBE2O can play a crucial role in modulating the pleiotropic HBc′s functions and thus promoting the replication of the virus.

Following lines summarize my results into several main points:

- FBXO3 ubiquitin ligase interacts with HBc
- UBE2O ubiquitin ligase interacts with HBc
- FBXO3 ubiquitin ligase promotes HBc polyubiquitination
- UBE2O ubiquitin ligase promotes HBc monoubiquitination
- FBXO3 ubiquitin ligase inhibits HBV replication
- UBE2O ubiquitin ligase promotes HBV replication

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