Investigation of Interaction Between Hepatitis B Virus X Protein (HBx) and NF-κB Pathway in Carcinoma Cells

by

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Abstract

Hepatitis B virus (HBV) infection is a global health issue. With approximately 350-400 million chronic carriers worldwide, HBV causes an estimated 600,000 deaths annually, due in large part to the hepatocellular carcinoma (HCC). HBx, a promiscuous transactivator, is widely accepted as an important viral oncoprotein. The exact functions of HBx in tumorigenesis are poorly understood, but many studies have suggested an important role of NF-κB pathway. Currently available data on the relationship between HBx and NF-κB signal transduction are rather limited and simplistic. The molecular mechanisms underlying highly specific regulation NF-κB responses remain yet to be elucidated. Here, we provide evidence that activation of NF-κB by HBx depends on its association with a transcription factor, p65. HBx APAP mutant that could not activate NF-κB pathway also did not interact with p65. In the cytoplasm, HBx-p65 interaction may play an important role in IκBα phosphorylation and subsequent p65 nuclear localization. Based on real-time quantitative PCR (qPCR) and reverse transcription PCR (RT-PCR), HBx introduced into HeLa cells through ΔE3L vaccinia virus was able to modulate cytokine mRNA expression (e.g., IFNβ and TNFα) very different from APAP mutant and the control. As shown by chromatin immunoprecipitation (ChIP), regulation if IFNβ involved direct binding of HBx-p65 complex to the gene promoter in the nucleus. These findings support the importance of HBx-p65 interaction and suggest that it is potentially a promising target of novel therapeutics for HBV-associated liver diseases, including HCC.

Dedication

It is with my deepest gratitude and warmest affection that I dedicate this Master's thesis to my late supervisor Dr. Runtao He, who has been both a dear friend and a father to me. You will be remembered forever.

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List of Abbreviations

Akt	Protein kinase B
AP-1	Activator protein 1
ATP	Adenosine triphosphate
cccDNA	Covalently closed circular DNA
CDK2	Cyclin-dependent kinase 2
ChIP	Chromatin immunoprecipitation
CoIP	Co-immunoprecipitation
DMEM	Dulbecco's Modified Eagle Medium
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
ΔE3L	E3L deletion mutant of vaccinia virus
EMHT1	Euchromatic histone lysine N-methyl-transferase 1
ERα	Estrogen receptor α
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
Foxo3a	Forkhead box O3
GPT	Glutamic-pyruvate transaminase
GSK3B	Glycogen synthase kinase 3 beta
GST	Glutathione S-transferase
HAT	Histone
HBx	Hepatitis B virus X protein
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HDAC1	Histone deacetylase 1
Hpi	Hours post infection
Hpt	Hours post transfection
IFN	Interferon
IL	Interleukin
IPS-1	Interferon promoter stimulator 1
IRF3	Interferon regulatory factor 3
JAK	Janus kinase
Jnk	Jun amino-terminal kinases
MAPK	Mitogen-activated protein kinases
MEK	MAPK/ERK kinase
MSK	Mitogen and stress activated protein kinase
MTH assay	Mammlian two hybrid assay
mTOR	Mechanistic target of rapamycin protein
NEMO	NF-kappa-B essential modulator
NF-κB	Nuclear factor κB
NIK	NF-kappa-B-inducing kinase
p22-FLIP	NH2-terminal fragment of cellular FLICE-like inhibitory protein
PBS	Phosphate buffered saline

PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	Protein kinase C
PTM	Posttranslational modification
qPCR	Real-time quantitative PCR
rcDNA	Relaxed circular DNA
RPS3a	Ribosomal protein S3A
RT-PCR	Reverse transcription PCR
SRC	Steroid receptor coactivator
STAT	Signal transducer and activator of transcription
TAK1	Transforming growth factor beta-activated kinase 1
TFIIB	Transcription factor IIB
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TPL2	MAP3K8 gene in mice
TRAF2	TNF receptor-associated factor 2
TSC1	Tuberous sclerosis 1
XIAP	X-linked inhibitor of apoptosis protein

Chapter 1: Introduction

Hepatitis B virus (HBV) causes devastating liver diseases. Approximately 5% of the world population, 350-400 million people, are chronically infected with HBV (Ott, Stevens, Groeger, & Wiersma, 2012), and 15-40% are expected to develop hepatocellular carcinoma (HCC), cirrhosis, and liver failure (Lok, 2002). There is no cure, and once HBV develops persistence, the infection usually remains for a lifetime of the host. Annually, 600 000 deaths are attributed to HBV (Goldstein et al., 2005) due in large part to HCC (Szpakowski & Tucker, 2013). Despite the availability of an effective vaccine, the large number of currently existing HBV carriers and the high risk of viral transmissions in endemic regions collectively pose significant economic burdens worldwide. So far, the majority of HBV researchers have focused on virus-mediated carcinogenesis because it is arguably the most serious and costly end-stage manifestation of HBV infections; this thesis falls under the same category. Despite the extensive efforts, however, underlying molecular mechanisms for HBV-mediated carcinogenesis still remain a mystery. Part of the problem might be the extremely dynamic, complex interplay between the virus, the host, and the surroundings. Cancer, in and of itself, develops in multiple steps by involving various intrinsic and environmental factors. HBV directly and indirectly contribute to that process (Levrero & Belloni, 2010; Kremsdorf, Soussan, Paterlini-Breschot, & Brechot, 2006). Nonetheless, previous studies have collectively made significant contributions to further our knowledge in HBV pathogenesis. A growing evidence supports the importance of a few players in HBV-associated HCC: HBV X antigen (HBx) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) factors. Both have been independently linked to liver cancer (Kang et al., 2013), but not only that, they themselves are related to each other as HBx can activate the NF-κB signal transduction pathway (Su & Schneider, 1996). Their

interplay may very well be a critical component of HBV pathogenesis, thus HCC development. However, modulation of NF-κB pathway by HBx, especially the molecular aspects, is still poorly understood. To address this gap, this project was designed to investigates the relationship between HBx and NF-κB factors, with a particular focus on how their direct association with one another can affect NF-κB cell signaling pathway, which in turn may promote carcinogenesis. Beginning with a literature review on HBV pathogenesis and host factors involved, a rationale will be presented along with the hypothesis of the study, experimental approaches will be explained, results will be outlined, and interpretations, as well as limitations, will be discussed.

Chapter 2: Literature Review

To provide comprehensive background information necessary to understand the rationale and the findings of the research project, the literature review focuses on four topics: liver diseases caused by HBV (i.e., HCC and Hepatitis B), molecular virology of HBV, viral protein HBx, and the host cell NF-κB signaling pathway.

2.1. Hepatocellular Carcinoma (HCC)

Most cases of HCC are secondary to either cirrhosis or viral hepatitis (Aravalli, Steer, & Cressman, 2008). Alcoholism, diabetes, and other liver diseases are some of the important risk factors that may promote HCC development. The major cause, however, is chronic HBV infection that accounts for 50-80% of the total HCC cases and virtually all of childhood cases (Lin et al., 2013); HCC incidence and mortality closely follow HBV prevalence with high ecological correlation (El-Serag, 2012). The pathophysiology of HCC is not yet clear. As Aravalli, Steer, & Cressman (2008) reviewed, hepatocarcinogenesis is a complex process that involvew accumulation of genetic and epigenetic changes during initiation, promotion, and

progression of the disease These events in turn may affect cellular events, such as apoptosis and cell cycle, ultimately leading to the formation of cancerous cells. At any point in this process, multiple factors may contribute to the disease, having additive effects on clinical outcomes. For that reason, it is often difficult to determine the cause(s) of a HCC case, but correlation studies do provide strong evidence that certain common risk factors are more important than others - HBV is clearly one of the most, if not the most, important predisposing factors.

The epidemiology of HCC mainly exhibits a bilateral distribution, resembling to some extent the East-West dichotomy. Its prevalence and incidence in so called, 'developed' countries are significantly lower (<5 per 100,000 in North America) compared to the 'developing' countries (>20 per 100,000 in China), due in large part to the availability and administration of HBV vaccines (Venook, Papandreou, Furuse, & de Guevara, 2010). In Western countries, HCC is generally seen as a rare cancer that follows other metastatic tumors and/or underlying liver conditions, such as cirrhosis. For individuals living in the West, certain risk factors related to nutrition and metabolism, such as alcoholism and type II diabetes, are considered more crucial than others. On the contrary, HCC is one of the most common cancers in East Asia, sub-Saharan Africa, and other regions, including China where chronic hepatitis B is found in 90% of the cases (Bisceglie, 2009). In these areas, fungal aflatoxin, a food contaminant and potent hepatotoxin, is likely an important causative agent, which may play a role in 4.6-28.2% of all global HCC cases (Yan Liu & Wu, 2010). In 2002, 82% of liver cancer cases occurred in developing countries, with 55% in China alone (Parkin, Bray, Ferlay, & Pisani, 2002). The HCC incidence is on the increase in North American and most of Europe, with the burden of this devastating cancer also expected to increase in coming years (Venook et al., 2010).

Worldwide, HCC is the third leading cause of cancer related mortality and the fifth most frequent tumor type (Ferlay et al., 2010), affecting twice as more males than females, typically between the ages of 30 to 50 (Beasley, 1982). HCC is a deadly disease that usually has poor prognosis characterized by high fatality and frequent treatment failures. Due to the very large regenerative capacity of the liver, HCC does not become clinically apparent in most patients until it is too late for curative interventions, such as surgical resection or liver transplantation (Feitelson et al., 2011). In a retrospective study of German HCC, overall median survival was determined to be 11 months after diagnosis (Greten et al., 2005), but it could be low as several months in underdeveloped countries. Given the severity of HCC, individuals at risk, especially those living in highly endemic regions, are in dire need of effective preventive measures and treatments. One promising area under extensive investigation is HBV. These studies have enormous potential in two aspects: 1) they may help to better understand the processes that lead to cancer, and 2) the findings may provide insights on how to target HBV infection in order to block the development and progression of HCC.

2.2. Hepatitis B

Hepatitis B is a liver disease caused by HBV. Based on the duration of seropositivity for the HBV surface antigen, the viral infection can be defined as acute (<6 months) or chronic (>6 months). The latter stage that lasts a lifetime is typically more damaging, although for the most part, it may lack any serious symptoms (Elgouhari, Abu-Rajab Tamimi, & Carey, 2008). This is usually the time period in which the cumulative molecular effects of HBV and its proteins (e.g., X protein) show clinical symptoms of advanced liver diseases. More than one-third of the world population (2 billion) have been infected with the virus, and about 20% of these individuals remain infected as chronic carriers (Elgouhari et al., 2008). Originally known as 'serum hepatitis'

in the late 1960's (Barker et al., 1970), a vast amount of information about HBV exists, but there is no cure and only a limited number of treatment options exists for chronic HBV infection.

2.2.1. Prevalence and Incidence. Consistent with the epidemiology of HCC, chronic HBV infection is highly prevalent (10-20% of population) in Asia, sub-Saharan Africa, and other parts of the developing world, but less so (0.1-2% of population) in North America, except some areas inhabited primarily by indigenous populations (Elgouhari et al., 2008). This rate also varies significantly based on gender, age, and ethnicity depending on the geographical location. In Canada, for instance, overall prevalence of hepatitis B infection is low (0.1-1%), but an assessment of distinct subsets of population reveals a striking 6.9% and 7.4% among the Inuit and immigrants respectively (J. Zhang, Zou, & Giulivi, 2001). As well, incidence rate of acute hepatitis B in Canadian males is twice as high as in females and peaks (6.1 per 100,000) at the age of 30-39 years (J. Zhang et al., 2001). Such a spike is often associated with much younger populations in countries with high HBV prevalence (e.g., China and Africa) due to the predominance of vertical transmission, which usually becomes chronic. To prevent HBV infections, vaccine, hepatitis B immune globulin, and/or post-exposure prophylaxis, in the case of perinatal exposure, have been utilized with great success, decreasing the prevalence of HBV infection in many countries (Hwang & Cheung, 2011). Indeed, a safe vaccine has existed for more than 25 years with scientific data demonstrating that it is 95% effective in preventing the development of chronic infection and its manifestations (Lavanchy, 2004). Cleary, HBV infection and its diseases are preventable. It is unfortunate that the overall number of individuals with chronic infection is continuing to increase, along with its economic burdens (Ott et al., 2012).

2.2.2. Transmission. As a sexually transmitted infection, HBV can spread through body fluid both vertically (perinatal) and horizontally, with the former being the most common route of transmission in a global setting, particularly in highly endemic areas (Kidd-Ljunggren, Holmberg, Bläckberg, & Lindqvist, 2006). In regions of low prevalence, most HBV infections occur through unprotected sexual intercourse and intravenous drug abuse that involves sharing of needles. Besides blood, saliva, nasopharyngeal fluid, breast milk, semen, urine, and cervical secretions can also harbor HBV (Kidd-Ljunggren et al., 2006). Some of these routes of transmission might be insignificant in reality as there are no documented cases. HBV is, however, is 50-100 times more infectious than human immunodeficiency virus and can survive outside the body for at least seven days (Ward & Averhoff, 2011), thus the risk of transmission cannot be underestimated. To make matters worse, many HBV carriers are asymptomatic. People at risk of HBV infection include hemodialysis patients, health workers who handle blood samples, infants born to HBV-infected mothers, and people with multiple sexual partners (Elgouhari et al., 2008). Approximately 45% of the global population live in areas of high chronic HBV prevalence (Mahoney, 1999). Due to the continuing surge of international travel, the risk of HBV acquisition is growing larger. Thus, the lack of appropriate protections against HBV, such as vaccination and other health interventions, can have devastating health outcomes, including HCC.

2.2.3. Pathogenesis. HBV primarily targets and replicates within liver cells, although viral constituents have been detected in extrahepatic sites, such as peripheral blood mononuclear cells (Coffin et al., 2011). HBV is generally considered non-cytopathic, meaning it does not directly cause cell damage. Rather, host immune responses against the viral antigens, namely core and surface protein (Nayersina et al., 1993), function as a double-edged sword, promoting

both viral clearance and hepatocellular damage. Cytotoxic T lymphocytes (CTLs) of the cell-mediated immunity contributes to most of the liver injury as they eliminate HBV infection by killing infected cell and producing antiviral cytokines Antigen-nonspecific inflammatory cells, such as neutrophils, can worsen immunopathology by direct killing of hepatocytes and recruitment of even more immune cells (Iannacone, Sitia, Ruggeri, & Guidotti, 2007). Acute hepatitis can manifest into jandice and rarely, fulminant hepatic failure that may cause death. As the infection progresses into a chronic stage, liver inflammation also persists, leading to cirrhosis and then HCC over a period of several years and decades (30-50 years) respectively (Shin et al., 2003). In order to minimize the liver damage, long-term treatment with administration of antiviral drugs (e.g., lamivudine) and/or immune system modulators (e.g., interferon alpha-2a) is often required (Aspinall, Hawkins, Fraser, Hutchinson, & Goldberg, 2011). These therapies, however, may have limitations associated with cost, side-effects, and more seriously, viral resistance.

2.2.4. Chronic Hepatitis B Virus Infection. **W**ithout any interventions, 95% of immunocompetent adults can resolve the acute HBV infection spontaneously, which is in sharp contrast to the 10% among infants (Elgouhari et al., 2008). Most infections acquired during birth and early children lead to asymptomatic, chronic HBV infection, which is defined as the existence of surface antigen, hence viral persistence, for more than six months. To clinically diagnose and identify the phase of HBV infection, several molecular markers found in blood are used. These include the viral antigens (i.e., surface and e antigens), antibody against core antigen, alanine aminotransferase (ALT), and HBV DNA (Elgouhari et al., 2008). Based on the patterns of these markers, chronic HBV infection can be further classified with one of four phases: immune tolerant, immune active, inactive, and clearance (McMahon, 2014).

As the initial phase of chronic infection, 'immune tolerant phase' is characterized by normal liver function, high HBV DNA, and high e antigen, but no anti-e antibody, suggesting an early, active viral replication (Elgouhari et al., 2008). A spontaneous clearance of HBV e antigen may occur in up to 15% of infected individuals, but the (~85%), proceed to develop e antigen-positive chronic hepatitis (Elgouhari et al., 2008). During 'immune active phase,' the immune system attempts to clear HBV infection. Indicated by high level of HBV DNA (McMahon, 2014), the fragile attempts at viral clearance is accompanied by concomitant hepatocellular injury that accumulates and eventually leads to cirrhosis, as well as HCC (Elgouhari et al., 2008). On the other hand, chronic HBV infection may enter the 'inactive phase' if the immune responses succeed in containing and reducing the viral infection, as represented by the e antigen seroconversion (i.e., detectable anti-e antibody) and low viral DNA level (McMahon, 2014). Some of the individuals at this stage may experience spontaneous loss of serum surface antigen (HBsAg) that represents the 'clearance phase,' which is an ideal outcome for the host because it usually indicates that the chronic HBV infection has been cured (Kobayashi et al., 2007). Usually correlated with HBV DNA level, the risk of HCC is the highest for individuals in immune tolerant and active phases, but there is still some risk with inactive phase as well (McMahon, 2014). Transitions into each phase is neither permanent nor linear, and chronically infected individuals may experience recurrent viremia and one or more seroconversions of e antigen (Elgouhari et al., 2008). There seems to an equilibrium that is reach between the host immunity and HBV; presence and absence of certain factors, such as HBV X protein (HBx), may potentially disrupt this balance, allowing the infection/disease to progress to or regress from specific stages. Accurate, precise diagnosis of chronic HBV infection may improve health outcomes by allowing health professionals to prescribe management and

treatment regime most appropriate for each patient. This attests to the important fact that molecular studies of infectious agent, such as HBV, essentially form the foundation for any clinical applications.

2. 3. Biology of Hepatitis B Virus

In order to win the battle against HBV, understanding its molecular virology is essential. One of the challenges in HBV research is the lack of cell culture system that accommodates natural viral infection, which makes it difficult to study certain aspects of HBV biology, including viral entry. Based on the available data, however, the extent to which the virus can harm and affect the host through its limited number of proteins is remarkable. In this section, some of the potential molecular mechanisms underlying HBV-mediated carcinogenesis will be outlined.

2.3.1. Structure and Genome. HBV, a member of the Hepadnaviridae family, is the smallest DNA virus that consists of 3.2 kb partially double-stranded relaxed, circular DNA (rcDNA) genome with one end linked to DNA polymerase (Block, Guo, & Guo, 2007). This viral rcDNA is enclosed within a nucleocapsid (core antigen) that is surrounded by a lipid bilayer envelope studded with viral glycoproteins (large, medium, and small surface antigens). HBV produces multiple proteins from four overlapping open reading frames. P gene encodes the viral RNA-dependent DNA polymerase, C gene produces core and e antigen via alternative splicing, S gene yields three surface antigens of different sizes, and lastly, X gene is for a non-structural regulatory protein called X antigen (HBx). These viral proteins may have various important functions during specific stages of HBV replication and pathogenesis. To date, eight genotypes (A-H) of HBV have been identified, but their clinical significance remains somewhat obscure,

partly due to the lack of data. Previously, genotype C has been found to be an independent risk factor for HCC (Elgouhari et al., 2008).

2.3.2. HBV Life Cycle. As one of the few non-retroviral viruses that use reverse transcription during replication, HBV has a complex life cycle (Block et al., 2007) that can be divided into six steps: Attachment, penetration, uncoating, replication, assembly, and release. To briefly elaborate, HBV surface antigens bind to hepatocyte surface receptors, identified as NTCP sodium/bile acid transporter (Yan et al., 2014). The virus enters the cell by endocytosis, during which virus and host cell membranes fuse, releasing the nucleocapsid into the cytoplasm. Then core protein dissociates from rcDNA, which is transported into the cell nucleus and repaired into a plasmid-like covalently closed circular DNA (cccDNA) by host enzymes (Beck & Nassal, 2007a). This highly stable viral cccDNA serves as a template for pre-genomic RNA (pgRNA), which is packaged into the virions, and subgenomic RNA that encodes viral proteins. HBV DNA replication is unique as it is initiated by the viral DNA polymerase that binds to pgRNA to prime DNA synthesis and recruit core proteins (Block et al., 2007). Immature nucleocapsid then proceeds to the endoplasmic reticulum, where it matures and acquires the envelope required to become a functional virion (Beck & Nassal, 2007b). These virus progenies exit the hepatocytes via a secretory pathway. This complicated lifecycle and its underlying mechanisms ensure optimal HBV replication, as well as survival within a hostile cellular environment.

2.3.3. Immune Evasion and Viral Persistence. Recent developments on HBV infection has shown that the virus is not just a stealth pathogen, but also that it actively employs strategies to escape the innate immune system (Busca & Kumar, 2014). Formation of cccDNA during viral replication plays a key role in HBV persistence by residing indefinitely in a small

number of hepatocytes and acting as a reservoir for further infection (Levrero et al., 2009). Detection of cccDNA in serum of inactive carriers is indicative of HBV reactivation. In its life cycle, HBV is able to maintain the cccDNA pool through an intracellular recycling of immature nucleocapsids that contains viral DNA (Beck & Nassal, 2007b). Moreover, viral proteins may play important roles in avoiding the innate immune responses by inhibiting two interconnected processes: Type I Interferon (i.e., IFNβ) production and interferon regulatory factor 3 (IRF3) activation (Busca & Kumar, 2014). While HBV polymerase may directly inhibit the activity of IRF3, HBx seems to bind to and inactivate IFN promoter-stimulator 1 (IPS1/VISA/MAVS), abolishing its ability to trigger gene expression (Kumar et al., 2011). By crippling the antiviral defence, HBV persists in hepatocytes, establishes chronic infection, and causes liver diseases.

2.3.4. Carcinogenesis. Exact mechanisms by which HBV infection causes HCC remains yet to be elucidated, but many potential contributing factors have been proposed. Progression of HCC may be the direct effect of the virus itself or the indirect effect through the processes of inflammation, regeneration, and fibrosis (Bisceglie, 2009). The latter is perhaps the more popular view, which argues that the chronic liver damage due to the immune responses triggers chronic inflammation, oxidative DNA damage, continuous death, and subsequent cell proliferation, increasing the likelihood of cell transformation and potentiating the action of exogenous carcinogenic factors (Levrero & Belloni, 2010). There is, however, evidence that support the direct effects of HBV on HCC. Previous studies have reported positive correlation between elevated serum level of HBV DNA, which indicates active HBV replication, and higher risk of HCC, even in the absence of advanced liver diseases (Yang et al., 2002). HBV may directly contribute to HCC by at least four different mechanisms: genomic instability, insertional mutagenesis, telomerase reactivation, and long-term expression of viral proteins.

The majority of HCC cells display a high incidence of chromosome instability as measured by the fractional allelic loss, which is an independent prognostic marker (Levrero & Belloni, 2010). HBV may contribute to genomic instability by general integration of viral DNA into the host genome, which is observed in about 80% of HCC cases, and by HBx that affects mitotic checkpoints (Forgues et al., 2003). Particular viral DNA integrations, such as those inserted within or near oncogenes, may provide a growth advantage to a clonal cell population, allowing these mutations to accumulate (Levrero & Belloni, 2010). Moreover, telomerase can be up-regulated during HBV infection by HBx, surface antigen, and viral DNA integration into telomerase reverse transcriptase (TERT) gene (Ozturk, Arslan-Ergul, Bagislar, Senturk, & Yuzugullu, 2009). By constantly replenishing telomeres, telomerase reactivation may allow infected hepatocytes to bypass senescence, an irreversible state of cell cycle arrest. Last, but not least, HBV within chronically infected hepatocytes produces surface protein and HBx, which have been independently linked to tumorigenesis in a transgenic mouse models (Terradillos et al., 1997). As evident in this section, the predominant mechanisms of HBV carcinogenesis seems to be based on the ability of viral proteins, in particular HBx, to modulate cell proliferation and sensitize liver cells to mutagens (Levrero & Belloni, 2010). Once introduced into the cellular environment, HBV proteins may change the dynamics of how the host factors behave and thus interfere with normal cellular processes. In order to understand the precise mechanisms of HBV-mediated tumorigenesis, further analysis of individual viral and host factors are critical. Henceforth, the main topics of this paper - HBx and NF-κB pathway - will be discussed.

2.4. HBV X Protein (HBx)

HBx encoded by HBV is a multi-functional regulatory protein essential for gene expressions and virus replication (Bouchard & Schneider, 2004); it is highly conserved across all genotypes. This 17-kDa polypeptide consists of 154 amino acids, with those from 52 to 148 being essential for the protein's function (Bouchard & Schneider, 2004). HBx is both versatile and promiscuous in that it not only activates transcription, but also modulates many different signal transduction pathways, including NF-κB pathway. This viral protein does not bind to DNA directly, but it does interact with numerous host factors. A single study identified 127 HBx-interacting proteins in HepG2 using GST pull-down assay coupled with mass spectrometry (T. Zhang et al., 2013). Protein-protein interaction may very well be the cornerstone of HBx function. Due to the complex nature of HBx function, studies have often reported conflicting results, but the overarching theme - HBx is oncogenic - has remained constant throughout. As a product of one of the viral genes most frequently integrated into host genome (Paterlini, Poussin, Kew, Franco, & Brechot, 1995), HBx is expressed in roughly 70% of chronically infected patients with HCC and is mostly found in the peritumor liver samples, suggesting its early role in carcinogenesis (Feitelson et al., 2011). At low expression level, HBx localizes primarily in the nucleus where it has a significantly longer half-life compared to those that accumulate in the cytoplasm at elevated levels (Cha, Ryu, Jung, Chang, & Ryu, 2009). HBx has been detected in the mitochondria as well (S. K. Li, Ho, Tsui, Fung, & Waye, 2008). Given the multiple functions of HBx in different cellular compartments, its dynamic distribution during HBV pathogenesis may have implications for the development of HCC. Indeed, tumorigenesis is likely an outcome of consorted efforts by multiple cellular processes, but the impacts of each

HBx function are still ambiguous. This section will focus on HBx functions that may directly contribute to HCC.

2.4.1. Transcription Regulation in the Nucleus. Many studies have demonstrated the ability of HBx to transactivate and transrepress many viral and cellular genes (Herceg & Paliwal, 2009). Although cytoplasmic HBx can control gene expression by activating signal transductions, HBx in the nucleus can also regulate transcription by four interrelated mechanisms: direct interaction with nuclear transcription machinery, association with transcription factors, recruitment of cofactors, and regulation of epigenetic modifications.

It was previously reported that HBx interacts with several components of the basal transcriptional apparatus, including TFIIB and TFIIH (Cheong, Yi, Lin, & Murakami, 1995), collectively known as general transcription factors. Recruitment and assembly of the transcription machinery can be greatly expedited by another class of transcription factors called activator proteins that bind to enhancer regions on DNA. HBx can bind to and modulate the functions of some transcription activators, most notably Myc, cAMP response element-binding protein (CREB/ATF), and NF-κB factors (Y. Wei, Neuveut, Tiollais, & Buendia, 2010), all of which have been linked to various cancers (You, Madrid, Saims, Sedivy, & Wang, 2002). Further optimization of gene expression is achieved through coactivators and corepressors that cooperate with transcription factors to increase or decrease the rate of transcription. Like HBx, these factors cannot bind to DNA and thus must rely on protein-protein interactions. As one of the most well known group of coregulators, steroid receptor coactivators (SRC) have intrinsic histone acetyltransferase (HAT) that acetylates histones to make DNA more accessible for transcription. A member of SRC, Amplified in breast cancer 1 (AIB-1/SRC-3), which has been associated with breast cancer, can physically bind to HBx (Hong et al., 2012). Moreover,

studies have shown that HBx can interact with epigenetic modifiers, such as histone deacetylase 1(HDAC1) that may suppress transcriptional activity of ERα, as observed in HCC patients (Han et al., 2006). In addition to acetylation, HBx can regulate DNA methylation and other types of histone modifications (Tian, Yang, Song, Wu, & Ni, 2013). In the context of HCC, these direct regulations of transcription in the nucleus is critical because the genes involved may encode for tumor suppressors, oncogenes, and proteins involved in cell life cycle. To build on this point, some specific examples of NF-κB target genes regulated by HBx will be presented later. Since nuclear HBx heavily relies on the availability of certain host factors, which in many cases depend on their nuclear import and/or activation status, the ability of HBx to activate signaling cascades from other cellular compartments might be equally important.

2.4.2. Calcium Signaling in Mitochondria. HBx localized to mitochondria may have pivotal roles during HBV pathogenesis because it can regulate cytosolic calcium levels, to which many of the protein's activities have been attributed (Clippinger & Bouchard, 2008). In rat primary hepatocytes, HBx was shown to regulate the mitochondrial membrane potential by modulating the mitochondrial permeability transition pore that spans through the outer membrane (Clippinger & Bouchard, 2008); this regulation coincided with elevation of calcium level in the cytoplasm. As a ubiquitous secondary messenger, calcium activates and/or modulates many cancer-inducing signaling transduction pathways, including, but not limited to Src kinase, MAPK (Ras-Raf-MEK-ERK), p53, PI3K/Akt, and NF-κB pathways (Gearhart & Bouchard, 2011; Katz, Ayala, Santillán, & Boland, 2011; Truant, Antunovic, Greenblatt, Prives, & Cromlish, 1995). In effect, HBx may alter the function of mitochondria to regulate various cytoplasmic cellular processes that may contribute to malignant transformation of hepatocytes.

2.4.3. Signal Transduction in Cytoplasm. HBx activates many cytoplasmic signal transduction pathways responsible for cell proliferation, survival, migration, and angiogenesis (Bouchard & Schneider, 2004). Src tyrosine kinase may play an important role in this process as an upstream antecedent, from which particular signals can diverge to a number of different pathways. As a non-receptor kinase that transfers a phosphate group from ATP to a protein, Src serves as a molecular switch in many cellular processes. HBx can activate Src not through physical interactions, but by releasing mitochondrial calcium (Bouchard, Wang, & Schneider, 2006). Studies have also shown that HBx may bypass this step to activate the immediate downstream protein, a small GTPase known as Ras, in the absence of calcium (Benn & Schneider, 1994). Both Src and Ras are examples of most common oncogenes found in human cancers; mutations that lead to permanent activation of Ras are found in 20% of all human tumors and up to 90% in certain types, including pancreatic cancer (Downward, 2003). The molecular mechanisms underlying HBx carcinogenesis may involve the following three interrelated signal pathways that are controlled by Src and/or Ras: cell cycle, PI3K/Akt, and NF-κB pathways.

2.4.3.1. Cell cycle and p53 pathway. Cell cycle refers to the series of events that lead to cell division and proliferation. Divided into three different stages (interphase, mitotic phase, and cytokinesis), cell cycle is monitored and regulated by various check points that allow the process to continue only under appropriate conditions (e.g., absence of DNA damage). For instance, within the interphase is a G_1 checkpoint that controls the rate of cell cycle, making the decision on whether the cell will divide or enter a resting stage, quiescence. As such, impaired regulations of this essential process due to mutations and exogenous factors may lead to uncontrolled cell growth and transformation, inherent in tumorigenesis. Due to the increasing

evidence that linked HBx to HCC, the effects of this viral protein on cell proliferation have been studied extensively in various models under different conditions, including immortalized cell lines and primary hepatocytes (Madden & Slagle, 2001). The results are conflicting. There are evidence to suggest that HBx can limit cellular proliferation (Qiao et al., 2001), induce cell cycle with subsequent 'stall' during S phase (Gearhart & Bouchard, 2011), or increase proliferation by removing barriers to cell cycle entry (Benn & Schneider, 1995) in cell cultures. Similarly, *in vivo* studies using HBx transgenic mice have shown mixed results, suggesting that HBx may both promote and inhibit cell proliferation. Transgenic mice with high susceptibility to HCC had a HBx-associated increase in hepatocellular proliferation (Madden & Slagle, 2001), while those with liver damage had impaired liver regeneration due to HBx (Quétier et al., 2013). Given the dual function of HBx, perhaps it is not surprising that HBx can regulate multiple host factors involved in cell cycle. It was reported that HBx can increase the activity of cyclin dependent kinase 2 (CDK2), activate p21 promoter, and regulate the levels of several cell cycle regulatory proteins, such as p16, p27, and cyclin A (Qiao et al, 2001). The discrepancies from previous studies might be attributable to the difference in experimental methods, including cell type and concentration of HBx. It is entirely possible that HBx may have completely opposite functions on cell proliferation depending on the molecular context that consist of various factors, such as cytokines and p53, at different levels. Such paradoxical function of HBx is not without a logical explanation as their intricate balance may synergistically contribute to HCC. During the course of natural chronic HBV infection, HBx may block the cell cycle to impede hepatocyte recovery, while promoting cell proliferation by allowing the cells to circumvent cell cycle arrest and even senescence.

Human interleukin-6 (IL-6) and tumor suppressor p53, both important regulators of cell cycle, may mediate the function of HBx on cell proliferation. As a cytokine important in inflammation, IL-6 plays an important role in immune responses and modulates cell signal transduction, such as JAK/STAT pathway, that may contribute to oncogenesis. Moreover, IL-6, along with NF-κB factors, is one of the most important initiating factors in the liver regenerative response (Fausto, Campbell, & Riehle, 2006). A recent study of HBx transgenic mice that underwent partial hepatectomy revealed that HBx delayed hepatocyte proliferation, hence liver regeneration, by up-regulating IL-6 expression (Quétier et al., 2013). The relationship between HBx and p53 has been well documented. In response to various stimuli, including DNA damage and oncogene activation, p53 modulates gene transcription to activate DNA repair, apoptosis, and cell cycle arrest (May & May, 1999). As a tumor suppressor, p53 prevents tumorigenesis by conserving genomic stability and inhibiting angiogenesis; its impaired function due to mutation (e.g., TP53 gene) and viral proteins (e.g., E6 produced by human papilloma virus) has been linked to cancers of the breast, colon, lung, and cervix (Thomas, Pim, & Banks, 1999; Wang et al., 2011). HBx can bind to p53, inhibit its DNA binding, and suppress p53 transactivation activity (Wang et al., 1994). Interestingly, studies have shown that expression of p21, a cyclin-dependent kinase inhibitor, is activated in the presence of functional p53, but is suppressed in the absence of p53 (Ahn et al., 2002). To complicate things even further, p21 also has dual functions. Although generally considered an inducer of cell cycle arrest, p21 has been known to promote cell proliferation, particularly when expressed in the cytoplasm (Yano et al., 2013). Regulation of cell cycle by HBx is evidently complex and perplexing, but its connection to HCC seems indisputable. To better understand the effect of HBx on cell differentiation, it is important to look at other relevant signal pathways, such as PI3K/Akt and NF-κB pathways.

2.4.3.2. PI3K/Akt pathway. Activated by Src kinase, phosphotidylinositol 3-kinase (PI3K)/Akt (protein kinase B) pathway sends a survival signal that abrogates apoptosis, a programmed cell death (Cooray, 2004). Akt, which is downstream of PI3K, targets and enhances the activity of proteins, such as Raf, GSK3B, and NF-κB factors, that are involved in cell growth, glucose metabolism, cell cycle, and protein translation (Khattar, Mukherji, & Kumar, 2012). Some of the biological consequences of abnormal PI3K/Akt pathway are important contributors to carcinogenesis, and the up-regulation of the pathway has been linked to poor prognosis in many human cancers (Khattar et al., 2012). In addition, the pleiotropic nature of this pathway may have significant impacts on HBV replication and pathogenesis (Cooray, 2004). HBx can interact with both PI3K and Akt to induce their activation (Khattar et al., 2012). During chronic HBV infection, PI3K/Akt pathway may prevent apoptosis to promote hepatocyte survival, thereby ensuring cellular transformation (Cooray, 2004). A previous study has illustrated that Akt phosphorylates HBx serine residue at position 31 through direct interaction, augmenting its oncogenic potential (Khattar et al., 2012). In turn, HBx may activate PI3K/Akt pathway via cytokines, such as epidermal growth factor and interleukin-6 (Khattar et al., 2012), effectively forming a positive feedback loop. This cooperation between the two proteins might be crucial for the development of HBV-mediated HCC. Moreover, the amplification of HBx and Akt activations may affect other signaling pathways since the targets of Akt are often important players of other signal cascades, including MAPK, mTOR, and NF-κB pathways (Khattar et al., 2012). Interestingly, it was reported that Akt promotes oncogenesis in a NF-κB-dependent manner and that oncogenic PI3K mutation led to a NF-κB-dependent cytokine expression profile that play a critical role in promoting a microenvironment amenable to tumor progression (Hutti et al., 2012).

2.5. NF-κB Signal Transduction Pathway

As one of the most downstream pathways, many signal cascades, including PI3K/Akt and MAPK, may converge into NF-κB signaling (Figure 1). More than 25 years of extensive research revealed that NF-κB is expressed in almost all cell types and tissues; specific NF-κB binding sites are present in the promoters/enhancers of a large number of genes (Oeckinghaus & Ghosh, 2009). Involved in cellular responses to stimuli, such as genotoxic stress (e.g., UV radiation), cytokines (e.g., TNFα and IL-1), and infections (e.g., viral dsRNA and bacterial lipopolysaccharide), NF-κB pathway plays a critical role in host immune responses and cell proliferation (Gilmore, 2006). Indeed, many viruses, including HIV, have evolved to subvert NF-κB pathway in order to avoid host immunity and/or to promote viral replication (Hiscott, Kwon, & Génin, 2001). Moreover, constitutive activation of this pathway has been implicated in many human cancers, including HCC (Seki & Brenner, 2007). Considering HBV pathogenesis, it is perhaps not coincidental that HBx targets NF-κB pathway. Through such regulation, HBx may suppress the antiviral defence mechanisms to promote viral persistence, while inducing cell cycle and inflammatory cytokine expression, which may contribute to tumorigenesis. HBx has multiple ways to modulate the NF-κB signalling in both nucleus and cytoplasm. In order to understand the molecular context in which HBx functions, NF-κB signal pathway will be described in detail.

2.5.1. Role in Viral Infection and HCC. NF-κB transcription factor has been termed the central mediator of the immune system (Hiscott et al., 2001) as it can control the expression of well over 100 target genes, majority of which participate in immune responses. Activation of NF-κB is a rapid, immediate early event that occurs within minutes after exposure to a relevant inducer and does not require de novo protein synthesis (Hiscott et al., 2001). Type I

interferon, including IFNα and IFNβ, is a critical component of innate viral immunity and is under the control of the NF-κB factors, especially p65 (Wang et al., 2010).

Persistent, constitutive activation of NF-κB has been linked to various cancers, including colon, breast, and liver (Prasad, Ravindran, & Aggarwal, 2010). In addition to those involved in the immune responses, target genes of NF-κB pathway also include growth factors and cytokines that have profound impacts on cell cycle and apoptosis (Hiscott et al., 2001). Yet some other targets, such as proto-oncogenes, have been linked to invasion, angiogenesis, and metastasis of cancer (Prasad et al., 2010). NF-κB likely contributes to HCC development and progression through the collective effects of multiple mechanisms, such as cytotoxicity of inflammation induced by NF-κB controlled cytokines (e.g., TNF, IL-6 and IL-19), cell transformation through heightened proliferation, and oncogene expression (Prasad et al., 2010). These processes may occur at different clinical stages of liver damage, in which chronic inflammation of the liver leads to fibrosis, followed by cirrhosis and finally, HCC (Sun & Karin, 2008).

2.5.2. Mechanisms of NF-κB Signal Cascade. Mechanistic aspects of NF-κB signal transduction has been thoroughly reviewed elsewhere (Oeckinghaus & Ghosh, 2009). Involving a cascade of protein interactions, this signalling consists of two similar, and yet distinct pathways - canonical and non-canonical - that involve different patterns of subunit activations and downstream genetic responses. This study focuses on the more common, classical pathway because it controls the nuclear localization of a prototypical NF-κB dimer, p65(RelA)-p50 (NF-κB1), the most abundant NF-κB factor in cells (Oeckinghaus & Ghosh, 2009). Major players, starting from upstream, include: IKK (IκB kinase) complex, IκBs (Inhibitors of nuclear factor κB), and NF-κB transcription factors. Each of these portions comprise of multiple members that have distinct roles in regulatory mechanisms.

Classic, canonical pathway, involves activation of IKK complex with subsequent phosphorylation-induced proteolysis of IκBα inhibitors and consequent nuclear translocation of the p65-containing transcription factor (Oeckinghaus & Ghosh, 2009). Under normal conditions, NF-κB dimer is sequestered and inactivated in the cytoplasm by IκBα that binds to p65. Upon activation, however, IKKβ subunit of the complex phosphorylates the NH2 domain of the IκBα to allow its binding to E3 ubiquitin ligase, initiating IκBα degradation by calpain protease (Sun & Karin, 2008). Liberated from the inhibitory protein, the dimer translocates into the nucleus where it activates the target genes by recruiting coactivators to the promoters. Non-canonical pathway follows essentially the same scheme of activation (i.e., liberation of transcriptionally active NF-κB dimer through processing of IκB), but involves different proteins and subunits that regulate unique classes of genes downstream. In the alternate pathway, NF-κB inducing kinase (NIK) phosphorylates IKKα subunit that initiates the degradation of a different IκB protein, p52/NF-κB2 precursor (Oeckinghaus & Ghosh, 2009). Activated RelB-p52 dimer targets specific κB *cis* elements, controlling a distinct set of genes (Oeckinghaus & Ghosh, 2009). There are many types of NF-κB dimers, but their physiological roles remains yet to be fully understood. Given the broad, important functions of NF-κB pathway, it is perhaps not surprising that its activity is tightly regulated at multiple levels.

2.5.3. NF-κB Pathway Regulation and Crosstalk. The regulation of NF-κB pathway mainly involves post-translational modification of cytoplasmic IKK and IκB proteins, as well as NF-κB transcription factors in the nucleus (Oeckinghaus & Ghosh, 2009). In addition, NF-κB-dependent gene expression is closely coordinated with other signaling pathways that intervene at the regulatory points. This section will outline some of the basic principles, key regulatory steps, and cross-regulation associated with NF-κB pathway.

2.5.3.1. IKK Complex. As a gate keeper of NF-κB signaling, IKK complex consists of three components: regulatory subunit IKKγ (NEMO) and two catalytically active kinases, IKKα and IKKβ. These kinases contain a carboxy terminal NEMO-binding domain (NBD), as well as a leucine zipper that allows dimerization. Activation of IKK involves the phosphorylation of key serine residues in T loops of IKKα and/or IKKβ, mediated by NEMO that either recruits IKK kinases (IKK-K) or facilitates oligomerization-induced autophosphorylation (Oeckinghaus, Hayden, & Ghosh, 2011). This specific stage represents a bottleneck in NF-κB pathway as many other signal cascades converge into and diverge from IKK, forming an intricate network of crosstalk. This also depicts the ability of IKK to function independently, outside of NF-κB pathway. Many upstream IKK-K (e.g., MAPKs, NIK, TAK1, MEKK1, and PKC) can phosphorylate T-loops or NBD of IKK to regulate its activity (Oeckinghaus et al., 2011). For instance, Akt pathway may control IKKβ indirectly through its downstream effector mTOR, a ser/thr kinase (Dan et al., 2008). In the absence of p53, the catalytic activity of IKKβ is enhanced due to its constitutive modification with O-linked N-acetylglucosamine (O-GlcNAc) monosaccharides that interferes with negative regulatory phosphorylation (Kawauchi, Araki, Tobiume, & Tanaka, 2009). IKKβ may, in turn, affect the function of NF-κB factors. However, IKKβ can also independently modulate apoptosis, mTOR, and MAPK pathways by modifying their respective regulatory proteins, Foxo3a, TSC1, and TPL2/COT (Oeckinghaus et al., 2011), all of which are associated with tumorigenesis.

2.5.3.2. IκB Proteins. NF-κB dimers are kept inactive in the cytosol by the IκB proteins that include three prototypical IκB proteins (i.e., IκBα, IκBβ, and IκBε), two Rel precursors (i.e., p100 and p105 that are cleaved to form NF-κB transcription factors), and two atypical members (i.e., Bcl-3 and IκBζ in the nucleus). Individual proteins are thought to preferentially associate

with a particular subset of NF-κB dimers, with IκBα having a higher affinity for p65-p50 complex than for other dimers (Oeckinghaus & Ghosh, 2009). In the course of canonical signaling to NF-κB, IKKβ subunit targets and phosphorylates the prototypical IκB proteins, of which IκBα is the most studied member. As previously mentioned, a steady-state localization of NF-κB dimers in a resting cell appears exclusively cytosolic, but such 'sequestering' of NF-κB complexes are more complex in reality. Crystallographic structural analysis of IκBα-p65-p50 complex revealed that IκBα masks only the nuclear localization signal (NLS) of p65, but not that of p50 (Jacobs & Harrison, 1998). As such, exposed NLS together with the nuclear export sequence (NES) of IκBα causes constant shuttling of IκB-p65-p50 between the nucleus and cytoplasm. Active NF-κB induces the expression of IκBα, which then removes NF-κB complex from the DNA in the nuclues and translocates it into the cytosol (Oeckinghaus & Ghosh, 2009). This important negative regulatory feedback loop plays a critical role in terminating the NF-κB responses. Given the important functions of IκB proteins, its posttranslational modification (PTM), hence its activation or degradation, is one of the hallmarks of NF-κB pathway. Interestingly, a study has shown that Jak2 of the JAK/STAT pathway phosphorylates IκBα, suggesting that IκB proteins may contribute to the NF-κB crosstalk (Digicaylioglu & Lipton, 2001).

2.5.3.3. NF-κB Transcription Factor Family. **The** NF-κB protein family in mammals consist of five transcription factors divided into two classes. Members of one class, p65 (RelA), c-Rel, and RelB, have a transcription activation domain that does not exist in the members of the second class, p50 (NF-κB1) and p52 (NF-κB2). Although the physiological existence and relevance remain elusive, these individual NF-κB proteins can form up to 15 different homo- and heterodimeric complexes that are transcriptionally functional (Oeckinghaus

& Ghosh, 2009). Each NF-κB dimers may have different cell-specific distributions, as well as unique functions. In mature B lymphocytes, the cRel-p50 complex, rather than the prototypical dimer, is the primary component of constitutively active NF-κB (Miyamoto, Schmitt, & Verma, 1994). Furthermore, p50-p50 homodimer has been found to repress κB-dependent transcription through posttranslational modifications (PTMs) by recruiting a HDAC1 (Elsharkawy et al., 2010) and EMHT1 (Ea, Hao, Yeo, & Baltimore, 2012). Individual dimers may target overlapping and yet distinct sets of genes as they have preferential affinities for particular DNA sites (Oeckinghaus & Ghosh, 2009). The sequence-specific cis elements alone, however, cannot explain the specificity. Regulation of gene transcription occurs in the context of nucleosomal chromatin, through a concerted action of the transcription factor, coactivators, and corepressors. As such, the complex structures of target promoters in conjunction with the ability of NF-κB dimers to promote protein-protein interactions at the site are likely to be essential (Oeckinghaus & Ghosh, 2009). Based on its functions, NF-κB factors are important targets of regulation.

NF-κB dimers can mediate the cross talk between NF-κB and other signal pathways in three aspects: PTMs of NF-κB subunits in the nucleus by various signals, interaction with heterologous transcription factors activated by other signal cascades, and involvement of target genes in other pathways. Numerous studies have illustrated that the p65 subunit is subjected to a wide range of PTMs, including phosphorylation, ubiquitination, acetylation, methylation, and O-GlcNAcylation (Campbell & Perkins, 2004). These protein modifications affect the transcriptional activity of NF-κB dimers, in particular by influencing cofactor recruitment and DNA binding (Oeckinghaus & Ghosh, 2009). NF-κB PTMs are important for both transcription fine-tuning and termination. Phosphorylation of p65 amino acid Ser276 by IKKβ or MSK proteins of the MAPK pathway and Ser536 by Akt pathway were shown to be important for p65-

mediated transcription activation (Campbell & Perkins, 2004). In contrast, acetylation of p65 Lys122 had a negative impact on its DNA-binding affinity, effectively attenuating the transcriptional activity (Kiernan et al., 2003). In addition, the activity of NF-κB dimers can be influenced by heterologous transcription factors that occupy adjacent sites on DNA or directly bind to them (Oeckinghaus & Ghosh, 2009). Notable examples are AP-1 and STAT3 of the Jnk and JAK/STAT pathway respectively. Both factors were shown to interact with p65, activating or repressing transcription depending on the cellular context and target gene examined (Oeckinghaus et al., 2011). Lastly, included among the vast target genes of NF-κB dimers are E3 ubiquitin ligase MDM2 that induces proteasomal degradation of p53 (Tergaonkar, Pando, Vafa, Wahl, & Verma, 2002), as well as inhibitors of Jnk signaling, A20 and XIAP (Papa, Zazzeroni, Pham, Bubici, & Franzoso, 2004). NF-κB signaling not only integrates, but also regulates heterologous pathways.

The network of cell signaling that revolves around NF-κB pathway is complex, broad, and dynamic. The regulatory nodes, IKK, IκB, and NF-κB transcription factors, serve as the points of intersection between different pathways and are thus appealing targets of oncogenic viruses. Based on current literature, HBx can interact with all three major regulatory proteins of NF-κB signal cascade, and relevant data will be presented in the next section. Moreover, studies have illustrated the direct and/or indirect effects of HBx on many signal cascades (e.g., mTOR, MAPK, apoptosis, p53, Jnk, and JAK/STAT pathways) that can communicate with NF-κB pathway (Feitelson et al., 2011). As an immediate early response, NF-κB signaling may play a central role as a conduit that mediates HBx functions to heterologous pathways. Given the multiple, dynamic links between HBx and various signal pathways, the range of NF-κB signaling's impacts on tumorigenesis may account for many, if not the majority, functions of

HBx previously identified. As such, the interaction between HBx and NF-κB is a crucial component of understanding HBV-mediated HCC. Examples of gene regulation by HBx that depend on NF-κB will be presented in the sections to follow.

2.6. Interaction between HBx and NF-κB Pathway

2.6.1. Points of Interactions. Regulation of NF-κB by HBx occurs at multiple levels and compartments within hepatocytes. HBx may up-regulate NF-κB activity using several different routes. Most upstream effects observed to date are at the level of TNF receptor associated factor 2 (TRAF2) and transforming growth factor β activated kinase 1 (TAK1), which have been shown to be important for HBx-mediated IKK phosphorylation (Zhou et al., 2010). Interestingly, HBx can directly interact with IKK complex. A study has demonstrated that HBx forms a ternary complex with anti-apoptotic protein p22-FLIP and IKKγ (NEMO), leading to NF-κB activation (Lim et al., 2013). HBx has been reported to interact also with two cytoplasmic IκB proteins, IκBα and p105, promoting their proteasomal degradation with subsequent liberation of NF-κB factors (Su & Schneider, 1996). Furthermore, direct binding of HBx to p65 creates a positive feedback loops that augments NF-κB activity (Shukla et al., 2011). In response to tumor necrosis factor (TNF), phosphorylated p65 that had a higher affinity to HBx was able to increase the half-life of HBx more significantly, compared to the non-phosphorylated p65 (Shukla et al., 2011). Moreover, nuclear HBx-p65 complex was shown to directly bind to the DNA promoter of certain genes, regulating its expression (Bui-Nguyen, Pakala, & Sirigiri, 2010). The effect of HBx-p65 transcription may also depend on coregulators and chaperones, including ribosomal protein S3 (RPS3a) that enhances NF-κB signaling by interacting with HBx (Lim et al., 2011). Interestingly, RPS3a is also a subunit of p65-containing enhancesome within uninfected cells, mediating selective gene regulation of NF-κB dimers (Wan et al., 2007).

Relatively high number of mechanisms with which HBx activates NF-κB pathway support the importance of this signaling in HBV pathogenesis.

2.6.2. Contribution to HCC. In the context of HBV pathogenesis, NF-κB may play a dual role: viral innate immune response and carcinogenesis. HBx can promote viral persistence by subverting NF-κB-mediated immune response, after which it may promote the carcinogenic effects of the pathway. As previously mentioned, HBx can directly bind to and suppress the function of IFNβ promoter stimulator (IPS-1/VISA/MAVS), which induces Type 1 interferon production via NF-κB pathway (Wei et al., 2010). Such inhibition of host immune response may lead to chronic HBV infection, paving the way for development and progression of liver diseases, including HCC. Mechanisms underlying tumorigenesis induced by HBx-activated NF-κB signaling can be indirect and/or direct. To elaborate, sheer activation of NF-κB through disturbance in its regulation may cause inflammation that lead to carcinogenic transformation, while specific modulation of NF-κB may affect the expression of tumor suppressors and oncoproteins. Through NF-κB pathway, HBx was shown to up-regulates proinflammatory cytokines, including TNF, IL-1β, IL-6, and leukocyte chemoattractant IP-10 (Hutti et al., 2012; Shukla et al., 2011; Zhou et al., 2010), while down-regulating anti-inflammatory cytokines, IL-4 and IL-13 (Lou, Hou, & Liang, 2013). Some of the NF-κB target genes may play a more direct role in carcinogenesis. A study has illustrated that HBx-p65 complex activates the transcription of MTA1, a master chromatin modifier and an oncoprotein commonly over-expressed in HCC (Bui-Nguyen et al., 2010). HBx may also stimulate cell-cycle progression by promoting the expression of cyclin D1 in an NF-κB-dependent manner (Park, Chung, Kang, Kim, & Jung, 2006). In the presence of HBx, the effects of NF-κB signaling on heterologous pathways that

contribute to HCC remains yet to be elucidated. Nevertheless, it seems clear that NF-κB pathway is an essential component of HBx functions that contribute to HCC.

Chapter 3: Gap in Literature and Rationale

HBV causes 600,000 deaths worldwide annually, largely due to HCC. Currently, there is neither a cure nor effective treatments. Vast amount of data suggest that two components - HBx and NF-κB pathway - are critical for HCC development. With regards to their relationships and cooperation, however, much remain in the dark. Current literature does explain how NF-κB signalling can be constitutively activated by HBx, but not the specificity in NF-κB responses, leaving some important questions unanswered. How does HBx target particular genes? How does HBx activate or repress those genes? Scholars have pointed out that different NF-κB homo- and heterodimers may exhibit an astounding degree of precision. Similarly, the oncogenic functions of HBx might be determined at the transcription level as it interacts with NF-κB factors and other coregulators. In particular, interaction between HBx and p65 (RelA) has been previously reported (Shukla et al., 2011), but its importance and functions remain poorly understood. These gaps warrant further investigation into the temporal and spatial modulation of NF-κB pathway by HBx. Such efforts will not only further our knowledge in molecular HBV pathogenesis, but also NF-κB-associated carcinogenesis. The findings have enormous potential to benefit the lives of chronic HBV carriers by providing insights into strategies and targets that can be exploited for novel therapeutics. Inhibiting the effects of HBx on NF-κB pathway may block or even reverse liver disease progression in order to prevent HBV-associated HCC.

Chapter 4: Hypothesis and Objective

HBx regulates NF-κB pathway in multiple ways. It is hypothesized that HBx modulates NF-κB activity by interacting with p65 (RelA) in both cytoplasm and nucleus, leading to a unique profile of gene expression. The objectives of this study are as follows: 1) to characterize the activation of NF-κB pathway by HBx, 2) to correlate HBx functions with its ability to interact with p65, and lastly, 3) to assess the biological significance of HBx-p65 complex in human carcinoma cell lines.

Chapter 5: Materials and Methods

5.1. Cell Culture

Huh7, HepG2, HeLa, and BHK21 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep). These cells were incubated at 37℃ in 5% CO_2.

5.2. Antibodies and Reagents

Antibodies for HBx and NF-κB p65 were purchased from Abcam. Anti-Flag M2 and α-actinin antibodies were from Sigma and Santa Cruz Biotechnology respectively. Antibodies for Phosphorylated IκBα (Ser32) and IκBα were from Cell Signaling Technologies. NF-κB1 p105/p50 antibody was purchased from New England Biolabs (NEB). Interleukin-1 (IL-1) purchased from Sigma was diluted to 100 ng/ml stocks and stored at -20℃ until use. To construct recombinant vaccinia viruses, selection media (2% FBS, 3% Xanithin, 0.125% hypoxanithin, and 0.25% mycophenolic acid in DMEM) was used during serial passage of the viruses. Non-denaturing lysis buffer (0.1% digitonin, 137 nM NaCl, 10% glycerol, 20 mM Tris-HCl pH 8, and 2 mM EDTA) was used throughout immunoprecipitations. To use with western blot, sodium dodecyl sulfate (SDS) sample buffer was prepared at 6X concentration (10% SDS, 30% glycerol, 0.5 M Tris/SDS pH 6.8, 6 mg Bromophenol Blue, 0.8 M β-mercaptoethanol).

5.3. Cloning of DNA Plasmids

HBx gene from HBV clone pEco63 (Genbank accession No. AY108092) was amplified by PCR and inserted into pVP16 (Clontech), pAmCyan-C1 (Clontech), p3xFlag, (Sigma-Aldrich) and pCMV-Myc (Clontech). Specific primers listed in Table 1 were used to construct

truncation (N1-N3) and substitution (APAP) mutants via sequential deletion and site-directed mutagenesis respectively. Utilizing agarose gel electrophoresis (1% agarose, TBE buffer, 120V), initial PCR amplicons and an empty vector were gel extracted (Qiagen), followed by double-digestion with appropriate high fidelity restriction enzymes (NEB) for 1 hour at 37℃. After the isolation of digested amplicons and vectors, the products were ligated overnight with T4 DNA Ligase (NEB) at 16℃. One Shot Top10 chemically competent *E. coli* cells (Life Technologies) were transformed with the ligated vectors and were spread on LB agar plates containing 100 µg/mL ampicillin or kanamycin. Colonies were PCR screened using GoTaq Green Master Mix (Promega) with vector and insert primers. Positive clones were grown overnight at 37℃ in LB broth supplemented with 1% ampicillin or kanamycin, while being shaken at 280 RPM. Plasmids were purified using PureYield Plasmid Maxiprep System (Promega) or QIAprep Miniprep Kit (Qiagen). To verify successful cloning, plasmids were sequenced at the NML Genomics Core. The p65 and p50 gene templates were purchased from Origene. PCR-amplified p65 or p50 was cloned in frame into pM (Clontech), pAsRed2-C1 (Clontech), or p3xFlag (Sigma-Aldrich). Truncation mutants (p65 C1-C2; p50 C1) and/or substitution (p65 DED3A) mutants were generated as described above. All constructs were sequenced using specific primers (Table 1) by NML Genomics Core.

Table 1. Primers used in this study.

Name of Primer	Sequence (5' -> 3')
HBx-Eco-S	GTACGAATTCATGGCTGCTAGGCTGTACTGCCAACT
HBx-N1-Xho-S	GTACCTCGAGTTCTTTGTTTACGTCCG
HBx-N2- Xho-S	GTACCTCGAGTTGCGGGGCCGCTTGGGA
HBx-N3- Xho-S	GTACCTCGAGTTCCAGCCGACCACGGGG
HBx-Hind-aS	CTACAAGCTTTTAGGCAGAGGTGAAAAAGTTG
HBx-APAP-S	[Phos]GCACCAGCACCATCTGCCGTTCCAGCCGACCAC
HBx-APAP-aS	[Phos]CGAGAGAGTCCCAAGCGG
HBx-ScrnAPAP-S	CTCTCTCGGCACCAGCACCA
p50-Eco-S	GTACGAATTCATGGCAGAAGATGATCCATATTTG
p50-Hind-aS	GTACAAGCTTCTAGGTTCCATGCTTCATCCCAG
p50-C1-Hind-aS	GTACAAGCTTCTAGAAACTATCCGAAAAATTGGGCA
p65-Bam-S	GTACGGATCCGTATGGACGAACTGTTCCCCCTC
P65-Xba-aS	GTACTCTAGATTAGGAGCTGATCTGACTCAGCAGGG
p65-C1-Xba-aS	GTACTCTAGATTAGAGGCCATTGGGGAGCCC
p65-C2-Xba-aS	GTACTCTAGATTAGCGAGTTATAGCCTCAGGGTA
p65-DED3A-S	[Phos]GCGGCAGCTTTCTCCTCCATTGCGGACA
p65-DED3A-aS	TCCTGAAAGGAGGCCATTG
p65-ScrnDED3A-aS	GGAGGAGAAAGCTGCCGC

5.4. Construction of Recombinant ΔE3L Vaccinia Vectors

HBx and APAP templates were cloned into the recombination Vaccinia vector pJS5 that encode green fluorescent protein (GFP) and xanthine-guanine phosphoribosyltransferase (GPT). While constructing a recombinant virus, GFP and GPT serve as selection markers for recombinat virus identification and selection respectively. Inserts and pJS5 vectors were double-digested with 'high-fidelity' restriction enzymes EcoRI and PstI (NEB) for 1 hour at 37℃. Digested DNA fragments were purified by gel extraction (Qiagen), followed by ligation and transformation as described above. Colonies were grown overnight at 37℃ in LB broth supplemented with 1% ampicillin while being shaken at 280 RPM. Plasmids were purified using QIAprep Miniprep Kit (Qiagen) and sent for sequencing at the NML Genomics Core. After confirming the correct sequences of inserts, HBx and APAP, the rest of the protocols were followed as described below.

5.4.1. Infection and Transfection for Recombination. Approximately 80% confluency BHK21 cells in six-well plates were infected with ΔE3L vaccinia virus that express red fluorescent protein (RFP) at a MOI of 0.2 with 500 µl media. Cells were incubated for 1 h at 37℃ and 5% CO_2 with gentle swirl every 15 minutes. After adding 1 ml of media to each well, transfection of recombination plasmids were performed using Attractene in order to insert HBx, GFP, and GPT genes into the TK locus of the parental ΔE3L vaccinia virus. 2 µg of plasmids, pJS5 HBx and pJS5 APAP, were each diluted in RNase free water to a final volume of 20 µl and were sterilized for 15 minutes at 85℃. At 24 hpt, cells were observed under fluorescent microscope to confirm efficient infection and transfection based on the expression of GFP and RFP. Viruses were grown for 96 h at 37℃.

5.4.2. Passaging and Virus Purification. The first stock of recombinant viruses were collected by 3 cycles of freeze (-80℃) and thaw in room temperature, followed by centrifugation for 5 minutes at 2000 RPM. A monolayer of confluent BHK21 cells in each well of 12-well plates was covered by 500 µl of selection media consisting of mycophenolic acid (MPA) that inhibits the growth of viruses without GPT. 200 µl of the collected supernatant was used to infect the cells in the first well and was diluted in series by a factor of 6. At 48 hpi, virus was collected from a well that had the highest ratio of recombinants to parental virus, which was analyzed by GFP and RFP expression. Collected virus was passaged again as described. After fourth passage, plaques were picked to initiate the virus purification process. For plaque picks, confluent BHK21 cells in 12-well plates were infected with serial dilutions of recombinant virus for 1 h at 37℃. The virus was aspirated, and the cells were overlayed with Type VII Agarose mixed with 2X MEM supplemented with FBS and Pen-Strep. At 48 hpi, plaques were detected by fluorescent microscopy and picked with a plugged sterilized Pasteur pipettes, immediately inoculating them in 1 ml growth media (DMEM supplemented with 10% FBS and 1% Pen-Strep). In order to release the virus, freeze/thaw cycle at -80℃ was repeated 3 times, and this solution was directly used to infect BHK21 cells for next round of plaque picking, repeating the process as needed to isolate a purified recombinant ΔE3L vaccinia virus.

5.4.3. Amplification and Virus Titre Calculation. Confluent BHK21 monolayer in a six-well plates were infected with 300 µl of virus. At 48 hpi, virus was collected by 3 rounds of freeze-thaw cycle at -80℃. Amplification was repeated as required. To determine virus titre, BHK21 cells were seeded in 12-well plates. Virus stock was titrated through 10-fold serial dilutions, which were then used at 200 µl per well to infect the cells in triplicates. At 1 hpi, 500 µl of fresh DMEM supplemented with 10% FBS and 1% Pen-Strep was added to each well.

Plaques were counted using a fluorescent microscope at 24 hpi. Expression and sequences of the inserted genes, HBx and APAP, were confirmed with western blot and DNA sequencing respectively.

5.5. Transfections – siRNA

Huh7 or HeLa cells (1.0×10^5) were seeded in six-well plate, 96-well plate, or 10 cm Petri dish. Cells were transfected with 100 nM of small interfering RNA (siRNA) or scrambled siRNA (Dharmacon), using Attractene reagent (Qiagen) according to the manufacturer's protocol. Further experiments were carried out either at 48 h post-transfection (hpt) for transfection or at 72 hpt for infection. The target sequence for p65 siRNA was 5'-GGAUUGAGGAGAAACGUAA-3.' The target sequence for p50 siRNA was 5'-GAUGGGAUCUGCACUGUAA-3.'

5.6. NF-κB Activation and Mammalian Two Hybrid (MTH) Assay

Huh7 or HepG2 cells were plated at 30% density 24 h prior to transfection in 24-well plates. Cells in each well were co-transfected with 150 ng of total sample constructs and 50 ng of reporter plasmids, pNF-κB-SEAP for NF-κB activity or pG5-SEAP for protein interactions in MTH system, using Attractene. After 48 h incubation, positive control samples of NF-κB assay were treated with IL-1 (10 ng/ml) for additional 3 hours at 37°C. For the MTH assay, cells with the empty vectors were the negative control while those transfected with pM3-VP16 were the positive control. The activity of secreted alkaline phosphatase (SEAP) in the cell supernatant was measured using GreatEscape Chemiluminescence Detection Kit (Clontech) and GENios Pro Microplate Reader (Tecan). Results were analyzed using Microsoft Office Excel. All experiments were performed in triplicates with at least 3 repeats that had a standard deviation

(SD) of less than 10%. The readout of chemiluminescence activity was converted into ratios based on the vector control.

5.7. Immunoprecipitation (IP) and CoIP

Huh7 cells were plated at 50% confluency in 10 cm Petri dish and were incubated for 24 h. For each plate, cells were transiently transfected with total of 4 µg DNA. At 48 hpt, cells were harvested in PBS, and whole cell lysates were extracted with a non-denaturing lysis buffer supplemented with cOmplete ULTRA Protease Inhibitor Cocktail Tablets (Roche). Pull-down of the protein complex was performed first with overnight incubation at 4℃ using anti-Flag antibody (Abcam), followed by 2-hour incubation at room temperature with protein A/G agarose beads (pierce). Immunoprecipitated samples were diluted with 1X SDS sample buffer for analysis with western blotting. As a negative control, cells were transfected with empty vectors.

5.8. Western Blotting

Cells were washed twice with ice-cold PBS and harvested with 1X SDS sample buffer. SDS-PAGE was performed using 4-15% Mini-PROTEAN TGX precast gels with Tris/Glycine/SDS running buffer (Bio-Rad). All samples were run with Benchmark Pre-stained Protein Ladder and MagicMark XP Western Protein Standard (Life Technologies). Separated protein samples were transferred to nitrocellulose membrane of iBlot Transfer Stack by running iBlot Dry Blotting System (Life Technologies) for 7 min at 25V. Blocking and antibody incubations were performed in 5% skim milk TBS with 0.2% Tween (TBS-T) by shaking at 70-100 RPM. All washes were performed with TBS-T. Western blots were developed using Western Lighting ECL (Perkin-Elmer). Amersham Hyperfilm (GE Healthcare) was used to capture chemiluminescence.

5.9. Fluorescence Microscopy

Huh7 cells were plated at 30% density in a 24-well plate. After 24 h incubation at 37℃, cells were transfected with pAmCyanC1 HBx constructs and/or pAsRedC1 p65 plasmids that encode fluorescent proteins. Cells transfected with empty vectors were the negative control. At 24 hpt, cells were visualized under Carl Zeiss fluorescence microscope (Axiovert 200) equipped with X-Cite 120 that provide broad-spectrum excitation. All images were observed with 40X objective and were processed using Adobe Photoshop software.

5.10. Cytokine Assay - Real-Time PCR Array and RT-PCR

Confluent HeLa cells in 6-well plates were infected with vvΔE3L-HBx, vvΔE3L-APAP, vvΔE3L, or mock at an MOI of 5 and collected at 12 h post-infection (hpi). RNA was extracted using RNeasy Mini Kit (Qiagen) and was digested with Turbo DNase (Life Technologies) in order to remove residual genomic DNA contaminations. With Maxima Reverse Transcriptase (Thermo Scientific), 1 μg RNA was reverse transcribed into cDNA, which was then used for further experiments as a part of either real-time PCR (qPCR) or reverse transcription PCR (RT-PCR). For real-time qPCR, the RT^2 Profiler PCR Array System - Common Human Cytokines was used to quantify cytokine mRNA expression. The qPCR of the cDNA was performed with RT^2 SYBR green qPCR Master Mix (SABiosciences) on StepOne Real Time PCR system (Life Technologies). The results were standardized and analyzed using the PCR Array Data Analysis excel spreadsheet provided by the manufacturer. Cytokines with a threshold cycle (Ct) value greater than 35 were excluded from this analysis. For RT-PCR, cDNA was amplified by PCR using GoTaq Green Master Mix (Promega). Expression of IFNβ and TNFα were analyzed with agarose gel electrophoresis (1% agarose, TBE buffer, 120V), with glyceraldehyde-3-phosphoate

dehydrogenase (GAPDH) as the loading control. As the negative controls, cells were transfected with scrambled siRNA, mock-transfected, mock-infected, or infected with vvΔE3L recombinant virus. This viral vector, which does not contain HBx gene, also served as the positive control because it already had a cytokine expression profile previously established with the same experiment system used in this study. The TNFα forward primer sequence was 5′-ATGAGCACTGAAAGCATGATCCGGG-3′, and the reverse primer sequence was 5′-TGGTAGGAGACGGCGATGC-3′. The IFNβ forward primer sequence was 5′-TGTCTCCTCCAAATTGCTCTC-3′, and the reverse primer sequence was 5′-TCCTTGGCCTTCAGGTAATG-3′. The GAPDH forward primer sequence was 5′-AAGGTGAAGGTCGGAGTCAA-3′, and the reverse primer sequence was 5′-TTACTCCTTGGAGGCCATGT-3′.

5.11. Chromatin Immunoprecipitation (ChIP)

SimpleChIP Enzymatic Chromatin IP Kit with Magnetic Beads (Cell Signaling) was used. Confluent Huh7 cells in 10 cm Petri dish were transiently transfected using Attractene. At 48 hpt, ChIP was performed by using Flag, p65, IgG, and Histone H3 antibodies as outlined in the manufacturer's protocol. Samples were amplified by PCR using GoTaq Green Master Mix (Promega) with primers that span the promoter region, from - 276 to +185, of IFNβ gene. IgG antibody was used as a negatively control. Positive control samples, pulled down with Anti H3 antibody, was probed with primers supplied with the ChIP kit. The IFNβ sense primer sequence was 5'-TAGTCATTCACTGAAACTTTA-3', and antisense primer sequence was 5'-AGCTGCTTAATCTCCTCA-3'. Isolated DNA segments were sequenced at the NML Genomics Core using above IFNβ primers for further confirmation.

Chapter 6: Results

6.1. HBx Activates NF-κB Signal Transduction Pathway

HBx was previously shown to activate NF-κB (Hong et al., 2012). First, the HBx motif that is responsible for activating NF-κB pathway were characterized. Using SEAP-based NF-κB signal transduction reporter system (Clontech), HBx N1-N3 truncation mutants (Figure 1A) were screened for their capacity to induce NF-κB activation. As shown by Figure 1C and 1D, HBx significantly up-regulated NF-κB activity in both Huh7 and HepG2 cells. Such activation was comparable to the positive control, IL-1, which had 4-10 times higher NF-κB activity than the vector control. In comparison, N2 mutant had more than 2-fold reduction in its ability to activate NF-κB, compared to the wild-type in both cell lines. This suggested that the amino acid region 1-61 contained the motif important for NF-κB activation. Using bioinformatics approach with the available HBx sequences from 8 genotypes of HBV, a highly conserved serine-proline motif ^{39}SPSP^{42} was identified and mutated for further analysis (Figure 1B). As expected, the mutant with S39 and S41 substituted with alanines (APAP mutant) was also associated with significant reduction of NF-κB activity, similar to the N2 mutant. These results suggested that ^{39}SPSP^{42} motif is important for activating NF-κB pathway.

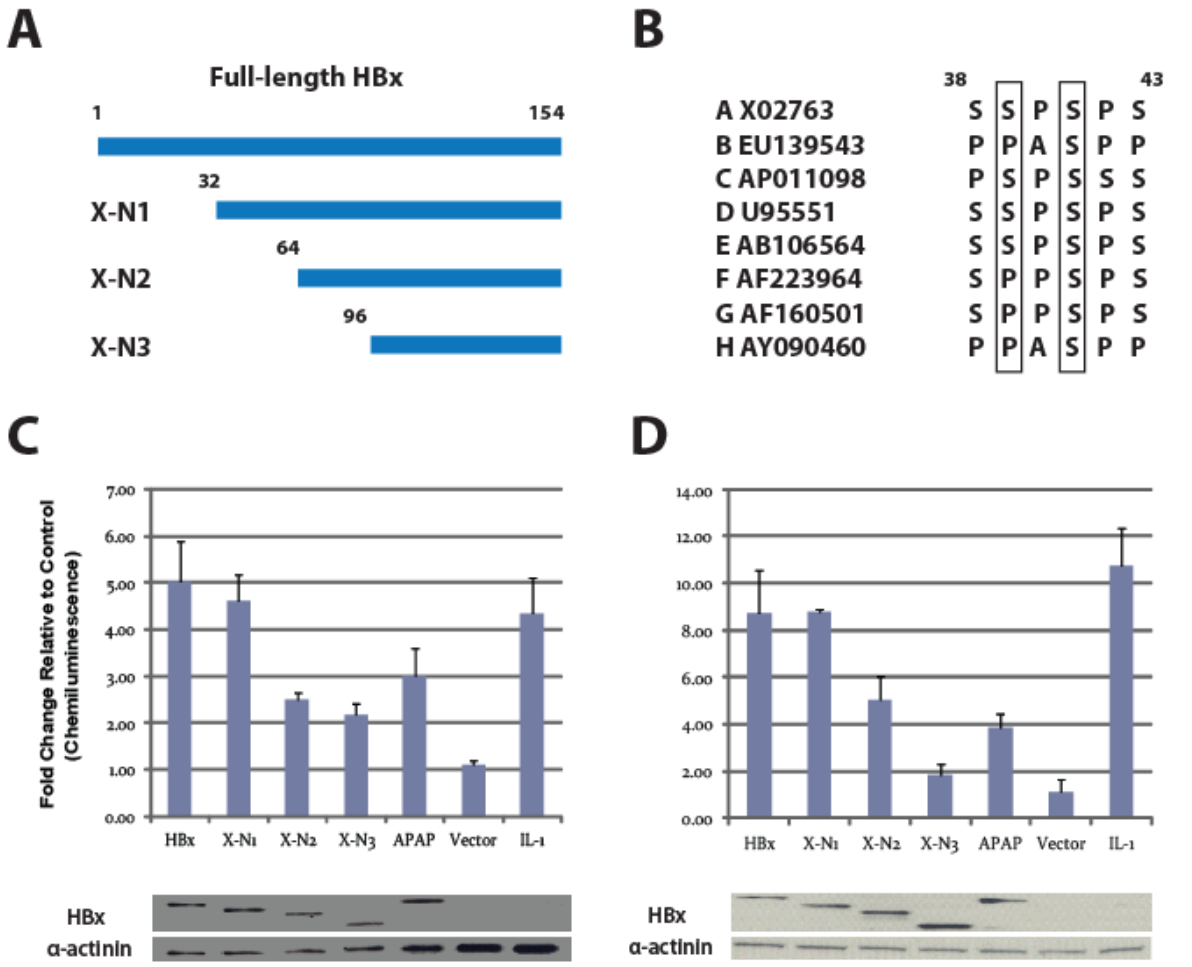


Figure 1. Characterization of HBx motif important for NF-κB activation. (A) HBx truncation mutants N1-N3 were constructed by sequential deletions from the N-terminus. (B) Serine-proline motif amino acid sequences from eight HBV genotypes were aligned. Highlighted amino acids at positions 39 and 41 were subsutituted with alanines via site-directed mutagenesis in order to make HBx APAP mutant. (C) NF-κB activation SEAP reporter assay of HBx and its mutants. Huh7 cells were co-transfected with pVP16 HBx constructs and pNF-κB-SEAP reporter plasmid. After 48 h incubation, positive control samples were treated with IL-1 (10 ng/mL) for 3 h. At 51 hpt, supernatants were collected and subjected to SEAP chemiluminescence analysis. Vector pVP16 supplied by the manufacturer was used as a negative control. Assays were performed in triplicates, and protein expressions were confirmed by western blot. Results are representative of three independent experiments (SD < 10%). (D) NF-κB assay was performed in HepG2 cells using the same protocol as above.

6.2. HBx Interacts with a Subunit of NF-κB Transcription Factor p65

To identify any links between HBx-p65 interaction and NF-κB activation, the same HBx mutants from previous experiments were used for comparison purposes. Another SEAP-based reporter experiment, mammalian two hybrid (MTH) assay was performed, in order to test if NF-κB activation by HBx correlates with its ability to physically interact with p65. In the MTH assay, protein of interest is tethered to pM or pVP16 vectors, which carry a DNA binding domain (BD) and an activation domain (AD) respectively. When two proteins expressed from the two vectors interact, BD and AD form a transcriptional activation complex on the pG5-SEAP reporter plasmid, inducing SEAP production. As shown in Figure 2A and 2B, wild-type HBx associated with p65 strongly in both Huh7 and HepG2 cells. HBx N2 and APAP, however, were not able to interact with p65, which were comparable to the vector control. The patterns of MTH SEAP activity, which represented protein-protein interactions in this case, was closely associated with the patterns of NF-κB activity in previous experiments. This observation suggests that in both Huh7 and HepG2 cells, the physical interaction between HBx and p65 may play a critical role in NF-κB activation.

HBx-p65 interaction was further characterized by identifying the p65 motif that binds to HBx. Similar approaches descrbied above were taken. First, p65 C-terminus truncation mutants were screened to identify the region important for interacting with HBx, and this region was missing in both C1 and C2 mutants (Figure 3A). Using a bioinformatics approach, we predicted a negative charge-rich DXD motif (^{531}DED^{533}) in p65 that was important for interacting with HBx (Figure 3B). As shown in Figure 3C, DED3A mutant, which had its aspartic (D) and glutamic (E) acids replaced with alanines, had a significantly reduced (> 2-fold) interaction with HBx compared to the wild-type. The physical interactions between HBx and

p65 were confirmed by co-immunoprecipitation in Huh7 cells (Figure 3D). The western blot analysis of immunoprecipitated complex showed that the bands corresponding to HBx were undetectable for samples transiently transfected with HBx APAP or p65 DED3A mutants, indicating their interaction with wild-type counterparts are minimal. The direct association between HBx and p65 likely occur through the ^{39}SPSP^{42} and ^{531}DED^{533} motifs.

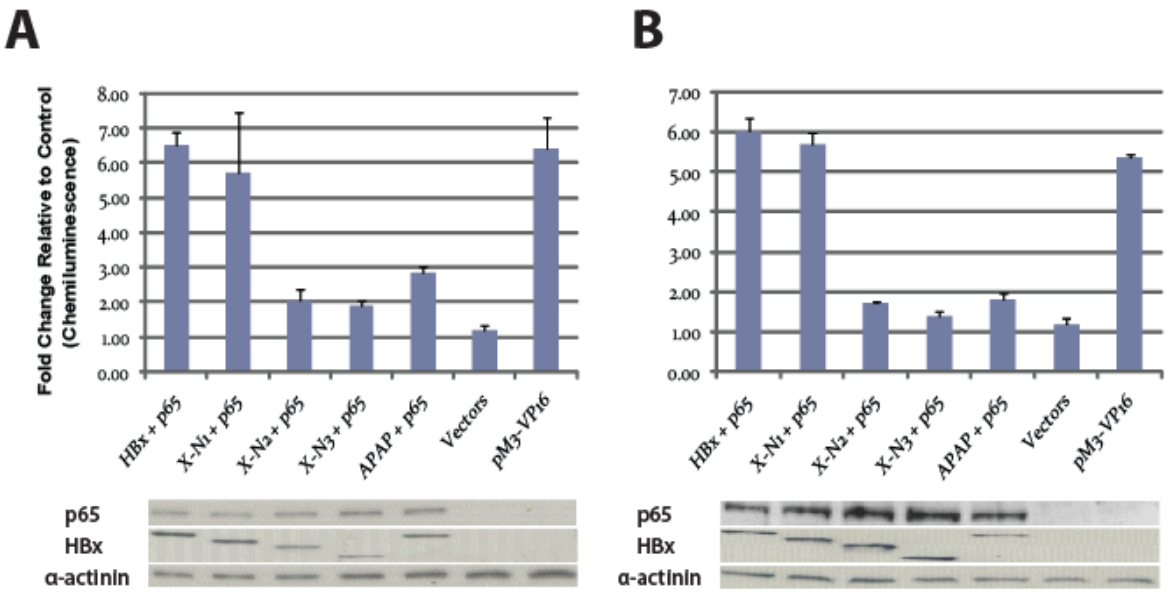


Figure 2. Ability of HBx and its mutants to interact with p65 is linked to their NF-κB activation potential. (A) Mammalian two hybrid (MTH) SEAP assay was performed to study HBx-p65 interaction. Huh7 cells were co-transfected with three plasmids: pVP16 HBx constructs, pM p65, and pG5-SEAP reporter plasmid. After 51 h incubation, supernatants were collected and subjected to SEAP chemiluminescence analysis. A positive control plasmid, pM3-VP16, was provided by the manufacturer. Vectors, pVP16 and pM, were used as a negative control. Western blots show stable protein expressions. Assays were performed in triplicates. Results are representative of three independent experiments (SD < 10%). (B) MTH assay was conducted in HepG2 cells using the same protocol as above.

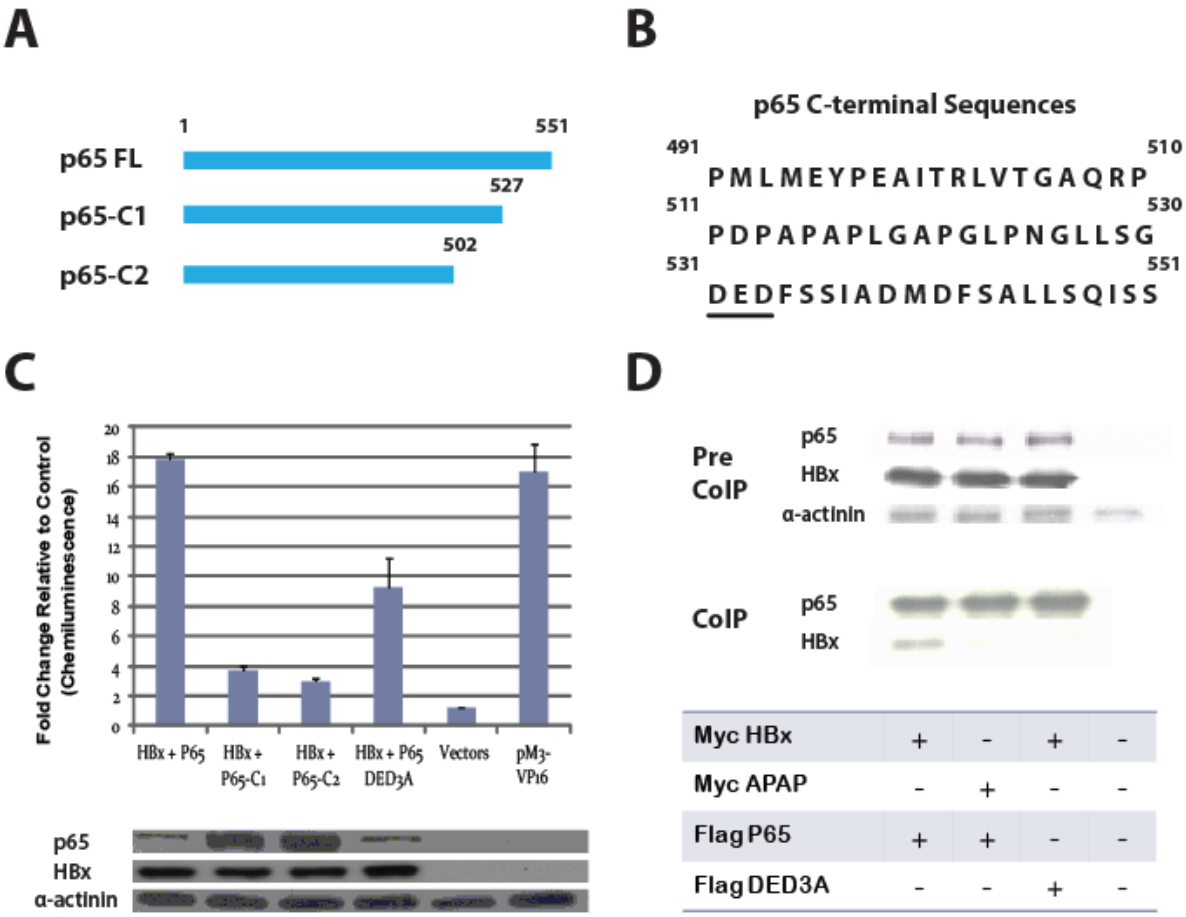


Figure 3. Characterization of interaction between HBx and p65. (A) C-terminus of p65 was sequentially deleted to make C1-C2 truncation mutants. (B) Amino acid sequences of p65 that contains the motif important for interacting with HBx. Underlined DXD motif was substituted with three alanines to make p65 DED3A mutant. (C) Mammlian two hybrid (MTH) assay was performed as previously described after co-transfecting the Huh7 cells with pVP16 HBx, pM p65 constructs, and pG5-SEAP reporter plasmid. Protein expressions were confirmed by western blot. Results are representative of three independent experiments (SD < 10%). (D) Co-Immunoprecipitation (CoIP) was used to confirm the physical interactions between HBx and p65. Huh7 cells were co-transfected with the plasmids as shown in the table and harvested at 48 hpt. Protein complex was pulled down with anti-Flag antibody, and samples were analyzed by western blot.

6.3. HBx Competes with p50 to Interact with p65

Since both HBx and p50 interacts with p65, the two proteins may compete with one another. Similar to the construction of p65 truncation mutants, C terminus of p50 was deleted in order to create a mutant (p50 C1) that could not interact with p65 (data not shown). First mammalian two hybrid (MTH) assay using Huh7 cells showed that p50, but not p50 C1 truncation mutant, was able to interfere with HBx binding to p65 (Figure 4A). Addition of p50 reduced HBx-p65 interaction almost by half, while C1 mutant did not have any effect. We have previously observed that HBx does not bind to p50 (data not shown). To verify whether or not p65-p50 interaction is responsible for hampering the association between p65 and HBx, a second MTH assay was performed. In this experiment, p50 was able to interact with p65, but C1 truncation mutant could not, as shown by the level of SEAP activity that was comparable to the vector control (Figure 4B). The results indicated that the inhibition of HBx-p65 interaction by p50 may depend on its ability to associate with p65, which in turn may block the binding of HBx through steric hindrance. To confirm the competition between HBx and p50, immunoprecipitation was performed. In the absence of p50 over-expression, the protein complex that was pulled down through Flag-tagged HBx had a significant amount of p65 compared to the negative control (Figure 4C). In contrast, expression of p50 prevented HBx from forming a complex with p65. Collectively, it can be concluded that HBx and p50 compete to interact with p65.

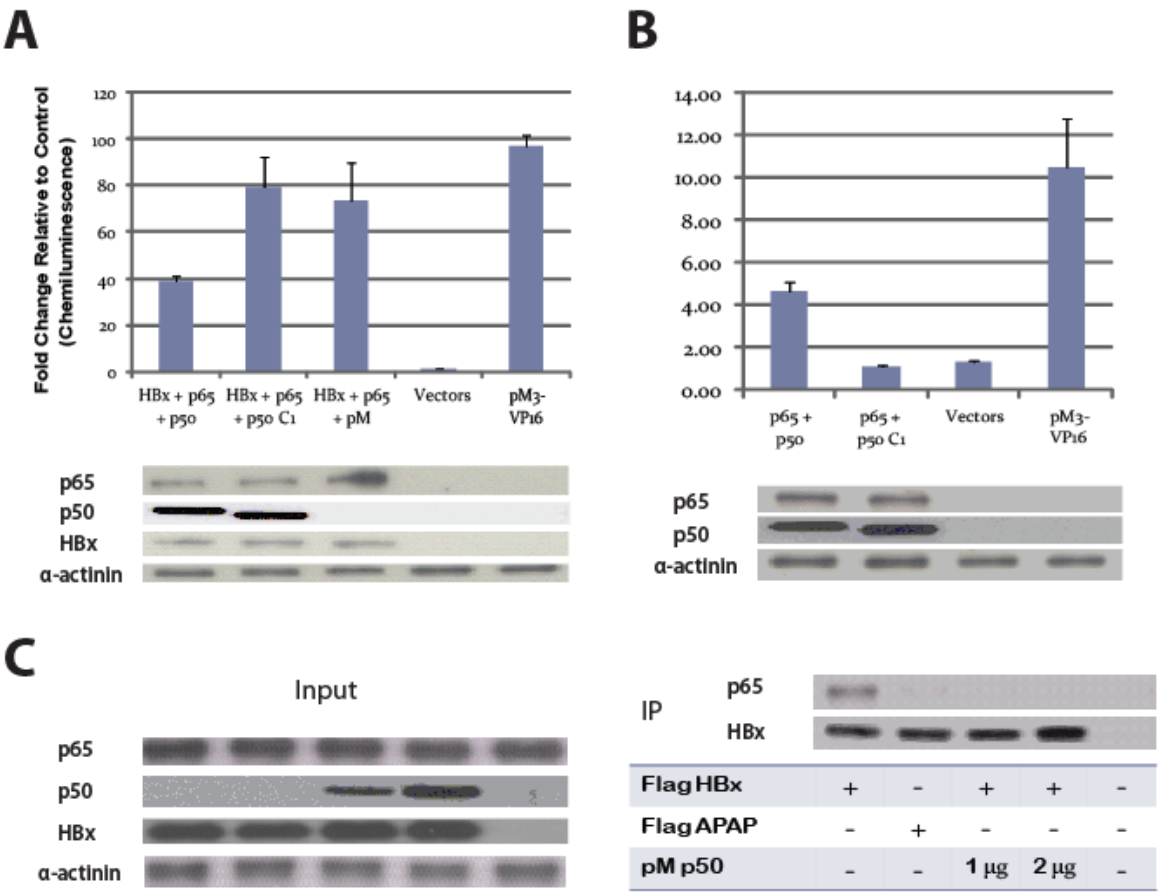


Figure 4. HBx and p50 compete to interact with p65. (A) Mammlian two hybrid (MTH) assay was performed in Huh7 cells as explained previously in order to investigate the effect of p50 on HBx-p65 interaection. Cells were co-transfected with up to four plamids: pVP16 HBx, pM p65, pM p50, and pG5-SEAP. (B) MTH SEAP assay was conducted in Huh7 cells as described before. Cells were co-transfected with pM p65, pM p50, and pG5-SEAP reporter plasmid. Protein expressions were verified with western blot. Assays were performed in triplicates. Results are representative of three independent experiments (SD < 10%). (C) Immunoprecipitation was performed to confirm the competition between HBx and p50. Huh7 cells were co-transfected with Flag HBx constructs and 1 or 2 µg of pM p50, as shown in the table. Cells were harvested at 48 hpt, and protein complex was immunoprecipitated with anti-Flag antibody. Samples were analyzed and input protein expressions were confirmed by western blot.

6.4. HBx Synergizes with p65, but is Antagonized by p50, in Activating NF-κB

Given the striking similarity between the ability of HBx to activate NF-κB and to interact with p65, manipulation of HBx-p65 interactions may affect NF-κB activity. Based on previous results, HBx and p65 may exhibit synergism, while p50 might antagonize the effect of HBx on NF-κB activation. SEAP-based NF-κB reporter assay was conducted by transiently transfecting Huh7 cells with appropriate constructs. When both proteins were over-expressed, HBx and p65 had a synergistic effect on NF-κB activation, which was greater than the sum of individual effects (Figure 5A). Addition of p50 had an opposite outcome, reducing the NF-κB activity that was up-regulated by HBx-p65 complex (Figure 5B). Such pattern of SEAP activity is similar to the interruption of HBx-p65 interaction by p50 as shown by previously mammalian two hybrid (MTH) and immunoprecipitation (IP) experiments. This observation further supports the hypothesis that NF-κB activation is linked to interaction between HBx and p65. Suggesting that p50 represses NF-κB is probably not accurate, although it is possible if p50 forms a homodimer. Since p50 can bind to p65 and form a heterodimer that is transcriptionally active, p50-containing dimer may simply have a lower NF-κB activation potential compared to the protein complex consisting of HBx. This notion is supported by the results in Figure 5B that provide a direct comparison between HBx-p65 and p50-p65. NF-κB activation by the former complex was at least twice as much as p50-p65 heterodimer. It also shows that the latter protein complex containing p50 can significantly up-regulate NF-κB activity, almost by six fold compared to the negative control. Taken together, the data illustrates that HBx and p65 can synergistically activate NF-κB activation. Also, p50 probably does not repress, but rather antagonizes the function of HBx by competing for p65 and forming a heterodimeric complex that has a lower transcriptional activity.

The importance of p50-p65 interaction in dampening the effects of HBx on NF-κB activation was analyzed further. As shown by Figure 5C, p50 was able to reduce by half the NF-κB activity that was induced by HBx. In comparison, p50 C1 truncation mutant, which could neither interact with p65 nor interfere with HBx-p65 interaction in previously assay, had no effect on HBx. Interestingly, the p50 C1 mutant did have an inhibitory effect on p65 (Figure 5D), suggesting that p50 may have p65-independent mechanisms to impede NF-κB activation (Figure 5D). In the presence of HBx, however, the antagonism by p50 seems to occur because it can compete with HBx for p65 interaction.

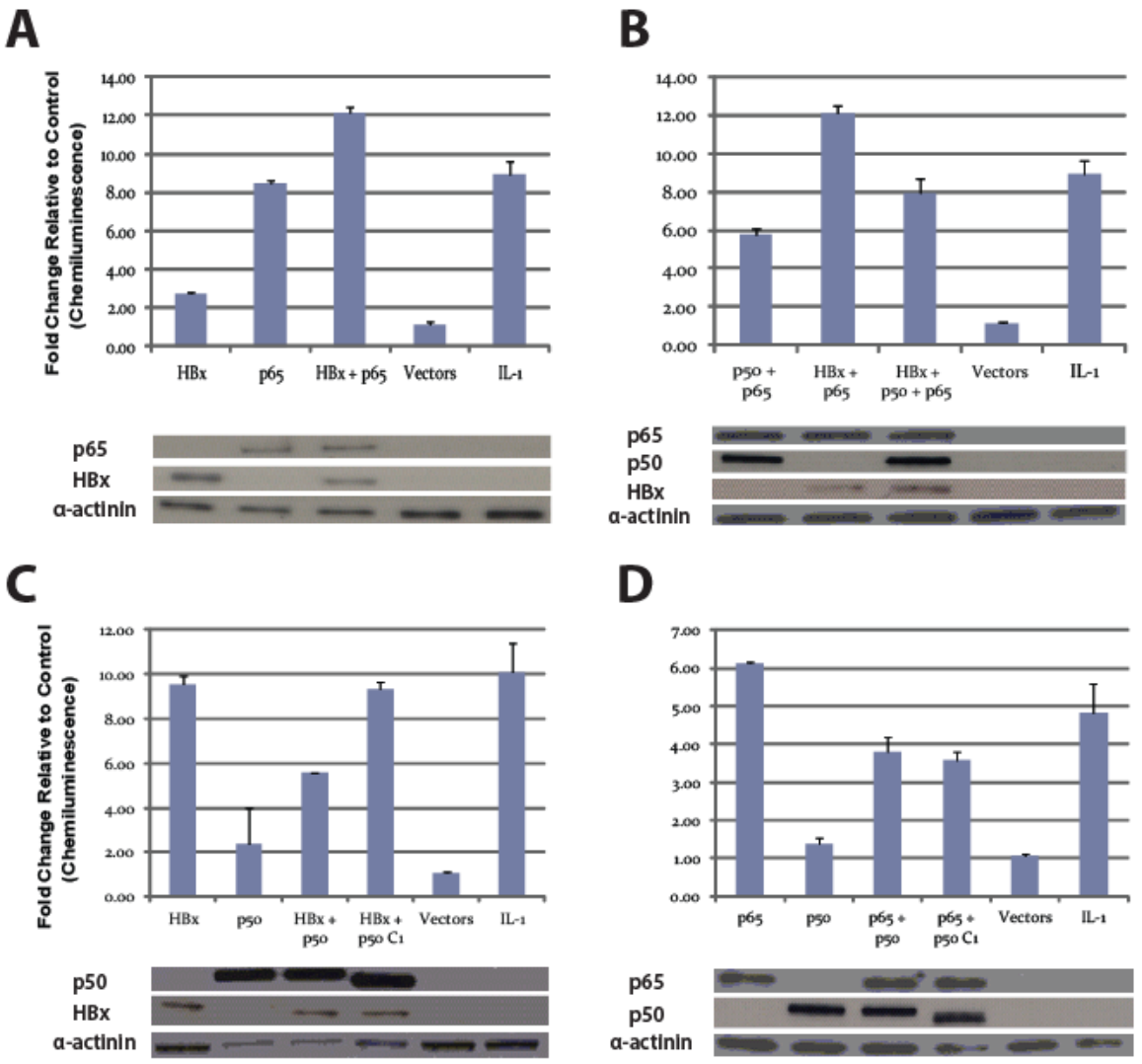


Figure 5. HBx synergizes with p65, but is antagonized by p50, when activating NF-κB pathway. (A) NF-κB activation SEAP assay was performed to identify any synergistic effect betwen HBx and p65. Huh7 cells co-transfected with pVP16 HBx, pM p65, and pNF-κB-SEAP. Experiment was carried out as previously described. (B) NF-κB assay was conducted to study the effect of p50 on NF-κB activity induced by HBx-p65 complex. Huh7 cells were co-transfected with combinations of pVP16 HBx, pM p65, pM p50, pNF-κB-SEAP plasmids. Same protocol was used. (C) NF-κB assay was carried out to analyze how p50 may repress HBx-mediated NF-κB activation After co-transfecting Huh7 cells with pVP16 HBx, pM p50 constructs, and pNF-κB-SEAP, same procedure as before was followed. (D) NF-κB assay was performed to exmaine the influence of p50 on p65-induced NF-κB activation. Huh7 cells were co-transfected as indicated, and previously outlined protocol was used. Western blots show stable protein expressions. Assays were performed in triplicates. Results are representative of three independent experiments (SD < 10%).

6.5. HBx is Functionally Analogous to endogenous p50 but not p65.

As previously mentioned, both HBx and p50 may interact with p65 to form a transcriptionally active protein complex, but each with different activation potential. It is widely accepted that p50 is a subunit of the most abundant prototypical NF-κB dimer that is essential for the activation of many genes. HBx and p50 are not homologues as they do not share any structural similarities. Nevertheless, it might be possible for HBx to replace p50 in certain situations as a functional analogue. In order to test this theory, endogenous p50 was down-regulated by siRNA through a knockdown experiment, followed by NF-κB SEAP assay to examine if HBx can still activate NF-κB pathway in the absence of p50. Consistent with current knowledge, down-regulation of p50 led to a dramatic decrease in NF-κB activation by most samples, including the positive control IL-1 that was rendered ineffective as a potent activator of NF-κB pathway (Figure 6A). The only exception was HBx. Based on the SEAP result, HBx was able to restore the NF-κB activity back to the level that was significantly higher than the negative control. As such, the viral protein may compensate for the loss of p50 to activate NF-κB pathway. Considering the evidence supporting that HBx-p65 has a higher transcriptional activity than p50-p65, one may expect HBx to exhibit greater NF-κB activation potential without p50. That may not necessarily be the case. The results support the previous finding that illustrates the existence of antagonism between HBx and p50. By definition, antagonism in this case refers to the phenomenon where the overall NF-κB activation is less than the sum of individual activations. HBx and p50 both activate NF-kB, but to a different degree. Hence, assuming there is abundance of the limiting factor, p65, to which both HBx and p50 bind, the two proteins may have some additive effects. Bear in mind, the competition would still exist, but minimal. Taking away p50 would relieve that competition, but also diminish the NF-κB activity

stimulated by p50. Based on this dual effects (positive and negative) of p50, a small, insignificant change in HBx-mediated NF-κB activation after p50 down-regulation makes sense. Unfortunately, the abilities of p50 and HBx to activate gene transcription from NF-κB-SEAP reporter plasmid have not been compared in a physiologically relevant setting. In previous biochemical experiments, p50 was over-expressed, and the amount of protein may not reflect the endogenous level during authentic HBV infections. Due to this shortcoming, the degree of functional analogy between HBx and p50 remains unclear. Nevertheless, p50 knockdown NF-κB assay, performed with proper controls, provide evidence that HBx can functionally replace p50. The results also demonstrate that p50 is not required for HBx to activate NF-κB pathway.

In order to directly assess the importance of p65 in HBx-mediated NF-κB activation, the same experiment as above was carried out with knockdown of p65 with siRNA transfection. As predicted, the negative impact of p65 knockdown was universal across all samples. Compared to the negative controls in which HBx and IL-1 were able to significantly up-regulate NF-κB activation, samples treated with p65-specific siRNA showed minimal NF-κB activity (Figure 6B). Such data can be interpreted in two ways: p65 might be critical for all NF-κB activations or specifically for those induced by HBx. The important functions of p65 as a NF-κB transcription factor are widely recognized, but this can neither be assumed nor generalized for every single activity of NF-κB signaling. Some genes under certain conditions can be activated by p65-free NF-κB dimers, raising the question if HBx can function independently of p65. Based on the NF-κB activation measured in a cellular environment that consists of HBx and APAP mutant, the findings effectively rule out the possibility that HBx can activate NF-κB pathway in a p65-independent manner. This is consistent with the data obtained from previous experiments. To reiterate, HBx-mediated NF-κB activation requires p65, but not p50. This

process seems to be governed by the direct interactions between HBx and p65, as well the antagonistic influences of p50.

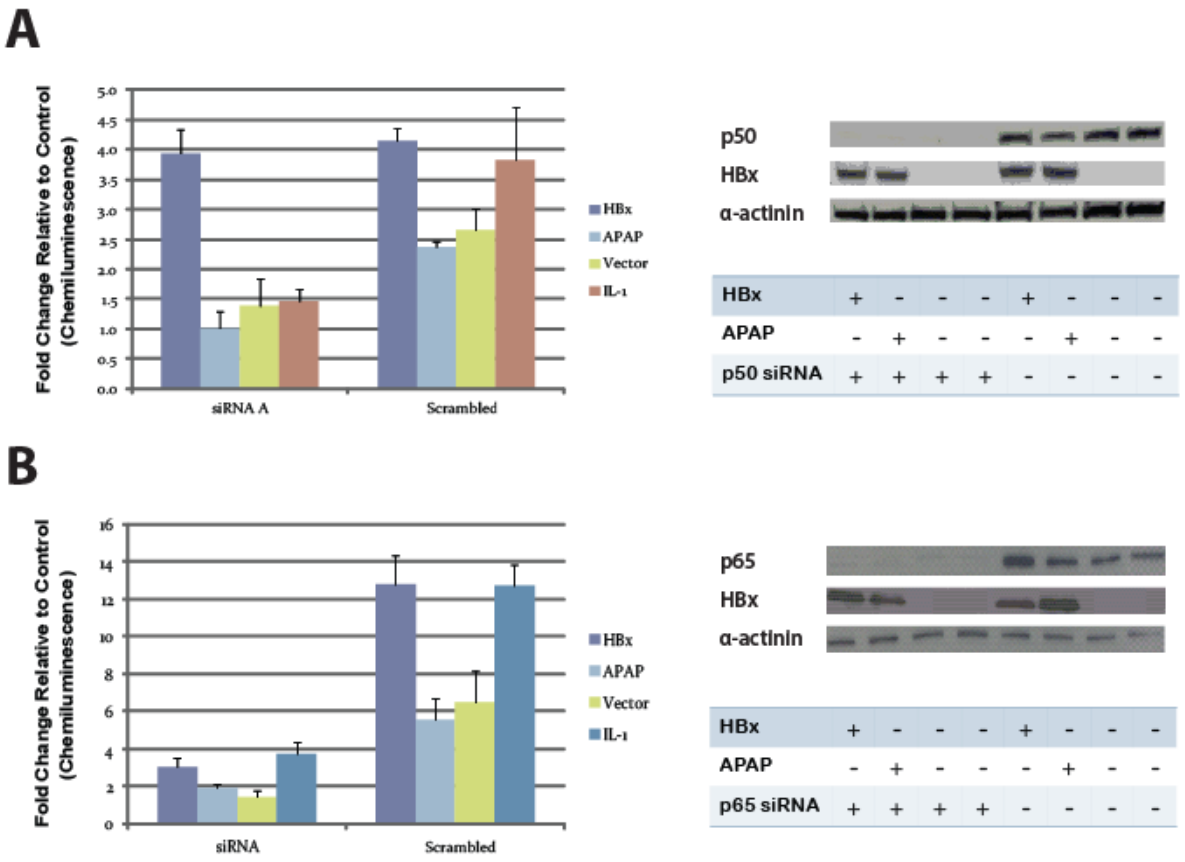


Figure 6. HBx can functionally replace endogenous p50, but not p65. (A) p50 Knockdown NF-κB SEAP Assay was performed to test if HBx can function as a p50 analogue. Huh7 cells (1.0×10^5) seeded in 96-well plate were transfected with 100 nM p50-specific or scrambled siRNA for 48 h. Cells were co-transfected with pVP16 HBx constructs and pNF-κB-SEAP reporter plasmid for another 48 h. Positive control samples were treated with IL-1 (10 ng/mL) for 3 h. At 99 h after initial transfection, supernatants were collected and subjected to SEAP chemiluminescence analysis. Vector pVP16 was used as a negative control. Protein expressions and efficiency of p50 siRNA knockdown were verified with western blot. Assays were performed in triplicates. Results are representative of three independent experiments (SD < 10%). (B) p65 Knockdown NF-κB Assay was performed as described in Huh7 cells to determine if p65 is required for HBx-mediated NF-κB activation.

6.6. HBx Promotes IκBα Phophorylation and p65 Nuclear Localization

Next, the molecular mechanisms associated with HBx-mediated NF-κB activation was attempted. It was previously reported that HBx interacts with and promotes IκBα phosphorylation in a dose-dependent manner, facilitating the accumulation of NF-κB transcription factor p65 in the nucleus (Su & Schneider, 1996). Other studies have also demonstrated that HBx induces nuclear translocation of p65 via different mechanisms (Lim et al., 2011; Liu et al., 2013). The importance of HBx-p65 interaction on these effects has not been investigated. First, Huh7 cells were transfected with an eukaryotic vectors expressing HBx and/or p65 fused with cyan fluorescent protein (CFP) or red fluorescent protein (RFP) respectively. Direct observations under fluorescence microscope revealed that when transfected alone, HBx localized primarily in the cytoplasm and p65 in the nucleus of the cells (Figure 7A). For most cell types, the majority of p65 remains sequestered in the cytoplasm, but in Huh7 cells, which is a carcinoma cell line, p65 can be found mainly in the nucleus (Shukla et al., 2011) due to the constitutive NF-κB activation. For samples co-transfected with HBx and p65 constructs, there was a significant difference in p65 localization depending on HBx-p65 interaction. As shown in Figure 7A, over-expression of HBx and p65 wild-type proteins was correlated with significant nuclear accumulation of p65. In contrast, if either of the proteins expressed by the cells was a mutated form (i.e., either HBx APAP or p65 DED3A mutant), p65 was primarily localized in the cytoplasm. This suggests that the HBx-p65 interaction plays a role in NF-κB activation in part by affecting the cellular localization of p65. This argument is somewhat obscured due to the initial containment of p65 in the nucleus. However, the constant shuttling of p65 in and out of the nucleus implies an existence of active cellular process responsible for p65

migration. Furthermore, introduction of APAP mutant in the cells prevented p65 nuclear translocation via unknown mechanisms. Collectively, it can be deduced that HBx-p65 interaction has a part in the active process that modulates p65 localization, ultimately facilitating its translocation to nucleus.

Since migration of p65 into the nucleus is preceded by IκBα phosphorylation, the effect of HBx-p65 complex on this process was examined. As shown by the western blot analysis in Figure 7B, HBx and IL-1, but not APAP, were able to induce IκBα phosphorylation when endogenous p65 was present. Down-regulation of p65 abolished these effects on IκBα. The observed differences in IκBα posttranslational modification were not dependent on the amount of IκBα protein. These findings illustrate that p65 is required for HBx to promote IκBα phosphorylation, which may in turn facilitate p65 nuclear translocation. HBx-p65 interaction seems critical for various cytoplasmic processes leading up to NF-κB activation.

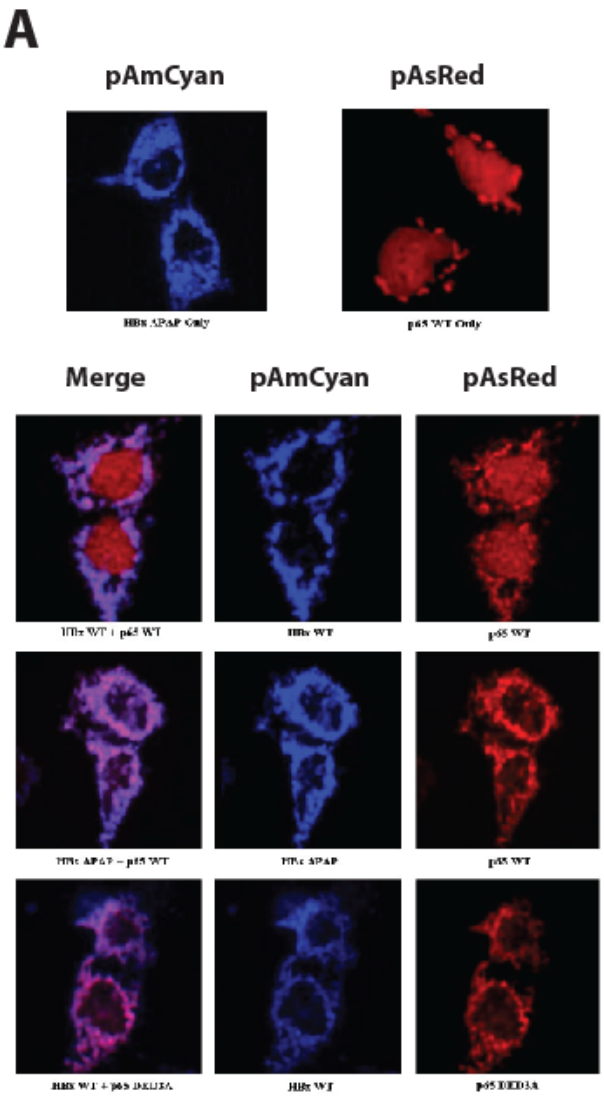


Figure 7. HBx promotes p65 nuclear localization and IκBα phosphorylation in a p65-dependent manner. (A) Flourescence Microscopy was used to visualize the cellular localization of HBx and p65 in Huh7 cells. As indicated, cells were transfected with one plasmid (upper panel) or co-transfected with pAmCyan HBx and pAsRed p65 constructs (lower panel). At 24 hpt, samples were directly observed under the Carl Zeiss Axiovert 200 microscope. All images were processed with Adobe Photoshop software and were shown at 400x magnification. (B) Western blot was used to examine the effect of HBx on IκBα phosphorylation. Huh7 cells seeded in 96-well plate were transfected with 100 nM p65- specific or scrambled siRNA. After 48 h incubation, cells were transfected with pVP16 HBx constructs for another 48 h. Positive control samples were treated with IL-1 (10 ng/mL) for 3 h. At 99 h after initial trasnfection, cells were collected for analysis with western blot. Protein expressions and p65 siRNA knockdown were verified.

6.7. HBx Up-regulates IFNβ and TNFα Expression

To understand the biological significance of the HBx-p65 interaction, cytokine assay was performed by infecting HeLa cells with recombinant ΔE3L vaccinia viruses expressing HBx or APAP mutant. This experiment aimed to analyze cytokine mRNA expressions using both real-time quantitative PCR (qPCR) and reverse transcription PCR (RT-PCR). RNA extracted from the infected cells were reverse transcribed into cDNA, which was then subjected to qPCR using a real time profiler PCR array system. A total of 84 common human cytokines that belong to IFN, IL, TNF, TGF, and platelet-derived growth factors/vascular endothelial growth factor superfamilies, were screened for any noticeable changes in expression. Cytokines detected with a threshold cycle value greater than 35 were excluded from the analysis. Many of the cytokines were regulated to a similar degree by the three recombinant viruses: ΔE3L HBx, ΔE3L APAP, and ΔE3L virus control. For example, TGF-β2 was up-regulated 4.2-fold, 4.0-fold, and 3.1-fold respectively, compared to mock-infected cells (Table 2). Several cytokines, including IL-21and TGFα, were unaffected by the recombinant viruses. Evidently, each virus was associated with a unique pattern of cytokine expression. Relative to both ΔE3L APAP and ΔE3L virus control, infection with ΔE3L HBx had remarkable modulatory effects on various cytokines involved in antiviral response, inflammation, and cell differentiation. Examples of these with values of fold changes in expression relative to either APAP or the virus control are shown in Figure 8A. Compared to the APAP substitution mutant, the wild-type HBx expressed by the recombinant virus was able to significantly up-regulate antiviral IFNβ (3.7-fold), proinflammatory TNF (11.4-fold), IL-19 (6.7-fold), and an embryonic morphogen NODAL (2.5-fold). Interestingly, expression of anti-inflammatory IL-4 was 7-fold lower in samples infected with ΔE3L HBx compared to ΔE3L APAP. It is noteworthy that NF-κB pathway can control the expression of

above proteins at the transcription level, except NODAL for which relevant data does not exist. Infection of HeLa cells with ΔE3L vaccinia virus was previously shown to up-regulate IFNβ, TNF, INHBA, and IL-6 quite significantly (Myskiw, Arsenio, van Bruggen, Deschambault, & Cao, 2009). As such, their relative expressions were used as quality control measure to ensure the validity of the experiments (Table 2). Taken together, HBx expression by ΔE3L vaccinia virus infection is associated with a unique cytokine profile that is different from that of APAP. This provides some evidence that HBx-p65 interaction may play a role in determining the specificity of gene regulation.

To confirm the regulatory effects of HBx on cytokine expressions and to assess potential involvement of NF-κB factors p65 and p50, RT-PCR was conducted. HeLa cells were mock-transfected or transfected with 100 nM p50, p65, or scrambled siRNA for 76 h. Then the cells were infected with the recombinant ΔE3L vaccinia viruses. RNA was extracted at 12 hpi. Expressions of IFNβ, TNFα, and GAPDH were determined by RT-PCR (Figure 8C). Consistent with the previous real-time qPCR data, HBx introduced into the cells through vaccinia viral vectors significantly induced both IFNβ and TNFα expressions compared to APAP and negative control. As expected, RT-PCR results showed no noticeable differences in the expression of the two cytokine genes between ΔE3L APAP and ΔE3L virus control. Indicated by the strengths of the band, the cytokine mRNAs were not detected when p65 was down-regulated, illustrating the requirement of p65. In the absence of p50, however, HBx was still able to up-regulate cytokine expression, providing further support on analogous functions of p50 and HBx. GAPDH was used as a loading control. Therefore, the findings suggest that HBx can regulate cytokine expression, IFNβ and TNFα at least, by modulating NF-κB pathway. In this process, p65, but

not p50, is required, and may determine the degree and/or specificity of HBx functions through a direct interaction.

Table 2. Real-Time Quantitative PCR Array Results Showing Regulation of Cytokine mRNA Expression by HBx in HeLa Cells.

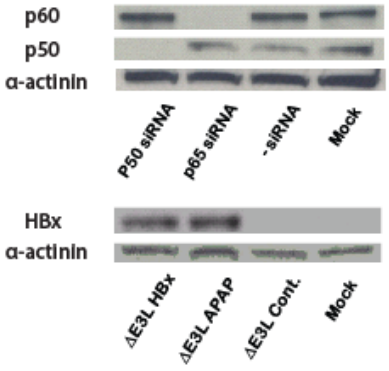
Cytokine		Fold Change in Expression of HeLa Cells Infected with Virus (Relative to Mock)		
		vvΔE3L HBx	vvΔE3L APAP	vvΔE3L
IFN Family				
	IFNB1	706.3381	189.0760	96.0170
	IFNA2	15.7678	10.7694	1.9812
	IFNA4	66.6883	28.8564	16.1582
	IFNA5	2.4544	1.5817	1.2115
	INFG	-1.6894	-2.4055	-1.5377
TNF Family				
	TNF	26.5449	2.3114	2.0557
	CD70	-1.0740	-1.1471	1.2051
	TNFSF10	-2.2275	-2.5064	-1.5356
	TNFSF11	1.8436	1.8178	1.8349
	TNFSF13B	4.8732	2.8442	-1.1309
	TNFSF8	-1.7208	-3.0760	2.4371
IL Family				
	IL6	117.1787	110.1362	16.5193
	IL12A	6.5828	7.8468	2.3392
	IL12B	1.1362	-1.8963	-2.9876
	IL16	-3.8391	-10.7937	-2.4697
	IL17A	4.1205	2.7192	3.6588
	IL17B	-10.7805	-6.5698	-12.1802
	IL17C	2.0280	1.6400	1.2553
	IL18	2.6456	2.2918	1.3200
	IL19	29.1026	4.3477	6.5785
	IL2	-1.1295	-3.8879	-1.2720
	IL20	8.6505	12.0977	3.8928
	IL21	1.3947	1.0180	-1.2934
	IL4	11.9789	89.5558	4.9085
	IL9	1.6514	-1.6758	-2.2100
TGF Family				
	INHBA	23.6824	20.8872	2.6061
	BMP4	-1.1773	1.2825	1.6689
	BMP5	2.2409	33.6019	18.6273
	BMP7	-1.0863	-5.0607	-1.6557
	GDF2	1.6253	-1.2785	-1.9235
	GDF5	-2.8124	-1.4131	-1.8711
	TGFA	1.5990	1.1235	1.2317
	TGFB1	1.3313	1.3704	1.1400
	TGFB2	4.2211	4.0213	3.1171
	NODAL	20.9792	8.3256	6.0390

A

Cytokine		Fold Change in Expression		
		HBx / APAP	HBx / Virus	APAP / Virus
IFN Family	IFNB1	3.7357	7.3563	1.7858
TNF Family	TNF	11.4841	12.9129	1.1244
IL Family	IL-19	6.6937	4.4239	-1.5130
	IL-4	-7.4761	2.4404	18.2450
TGF Family	NODAL	2.5198	3.4739	1.3786

Red >2 Fold Change Blue <-2 Fold Change

B



C

p50 siRNA	+	-	-	-	+	-	-	-	+	-	-	-	-
p65 siRNA	-	+	-	-	-	+	-	-	-	+	-	-	-
-'ve siRNA	-	-	+	-	-	-	+	-	-	-	+	-	-
T-Mock	-	-	-	+	-	-	-	+	-	-	-	+	-
I-Mock	-	-	-	-	-	-	-	-	-	-	-	-	+

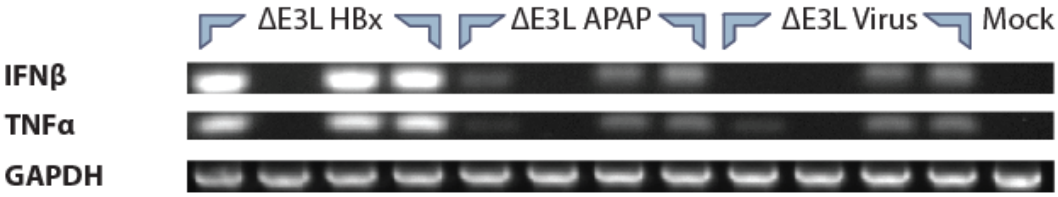


Figure 8. HBx modulates cytokine mRNA expressions within ΔE3L vaccinia virus-infected cells in a p65-dependent manner. A) Real-time qPCR array was used to assess the effect of HBx on the mRNA levels of common human cytokines. Confluent HeLa cells in six-well plates were mock-infected or infected with ΔE3L HBx, ΔE3L APAP or ΔE3L virus control at an MOI of 5. At 12 hpi, cells were collected to extract RNA, which was then digested with DNase and reverse transcribed into cDNA. Real-time qPCR was performed, and data was analyzed by excluding the cytokines displaying Ct value > 35. Results are shown as fold change in expression relative to either APAP mutant or the viruls control. (B) Western blot was performed to determine the efficiency siRNA knockdown, as well as the expression of HBx and APAP mutant from recombinant ΔE3L viruses. For the first blot, HeLa cells (1.0×10^5) were seeded in six-well plates and transfected with 100 nM p50, p65, or scrambled siRNA for 72 h. For the second western blot, confluenct HeLa cells in six-well plates were infected as previously exaplained. At 12 hpi, cells were collected, and protein expressions were deteremined by western blotting. (C) Reverse transcription PCR (RT-PCR) was used to further characterize the effects of HBx on cytokine expression. HeLa cells (1.0×10^5) seeded in six-well plates were transfected with 100 nM p50, p65, or scrambled siRNA for 72 h and then infected as described above. At 12 hpi, cells were collected, and RNA was extracted for RT-PCR. Results shown are representative of three independent experiments. (T-Mock = mock transfection; I-Mock = mock infection)

6.8. HBx-p65 Complex Binds to Promoters of IFNβ

HBx may regulate cytokine expression by altering specific stages of the transcription process. To further elucidate the mechanisms underlying the function of HBx in the nucleus, the chromatin immunoprecipitation (ChIP) assay was utilized to investigate the interaction between HBx and DNA. The experiment was also complemented with p65 knockdown and sequential ChIP in order to evaluate the importance of p65, particularly its interaction with HBx. Previous studies have shown that HBx-p65 is recruited to certain gene promoters to controls its expression (Bui-Nguyen et al., 2010). Moreover, both suppressive and stimulatory effects of HBx on IFNβ production has been documented on multiple accounts (Kumar et al., 2011; C. Wei et al., 2010), but the involvement of HBx-p65 complex has not been assessed. The promoter of IFNβ has a NF-κB consensus sequence 5'-GGGAAATTCC-3' (-139 to -130) to which both HBx and p65 may bind. As such, primers for the PCR analysis were designed to span over the nucleotide sequences from -276 to +185 so as to include the κB site. Huh7 cells were transfected first with siRNA for 48 h to reduce the level of p65, and then with Flag HBx or APAP plasmids. Cross-linked protein-DNA complexes were pulled down using Flag, p65, IgG, and Histone H3 antibodies. Sequential, second ChIP was performed on the chromatin eluted from Flag-immunoprecipitated samples using p65 antibody in order to verify that HBx-p65 complex, rather than HBx alone, is recruited to the gene promoter. As expected, p65 was associated with the chromatin isolated from all three samples (Figure 9A). The findings illustrated that HBx, but not APAP or the vector controls, was recruited to the IFNβ promoter, indicating the importance of interaction between HBx and p65, which may serve as a bridge that connects HBx and DNA. This was supported by the observation that the recruitment of HBx to the cytokine promoter was dependent on p65, as affirmed by re-ChIP showing a direction association between the IFNβ

gene and HBx-p65 complex (Figure 9A). Expression of IFNβ mRNA was consistent in all input samples, but no detectable level was observed with samples pulled down with nonspecific IgG antibody. For a positive control, RPL30 gene was probed from chromatin immunoprecipitated with histone H3 antibody. Taken together, the findings illustrate that HBx may also function in the nucleus, regulating the transcription of IFNβ and other cytokine genes by directly binding to their cis elements within the promoter region in a p65-dependent manner. Since HBx does not bind to DNA directly, p65 may play a critical role in bridging the function of HBx with transcription regulation.

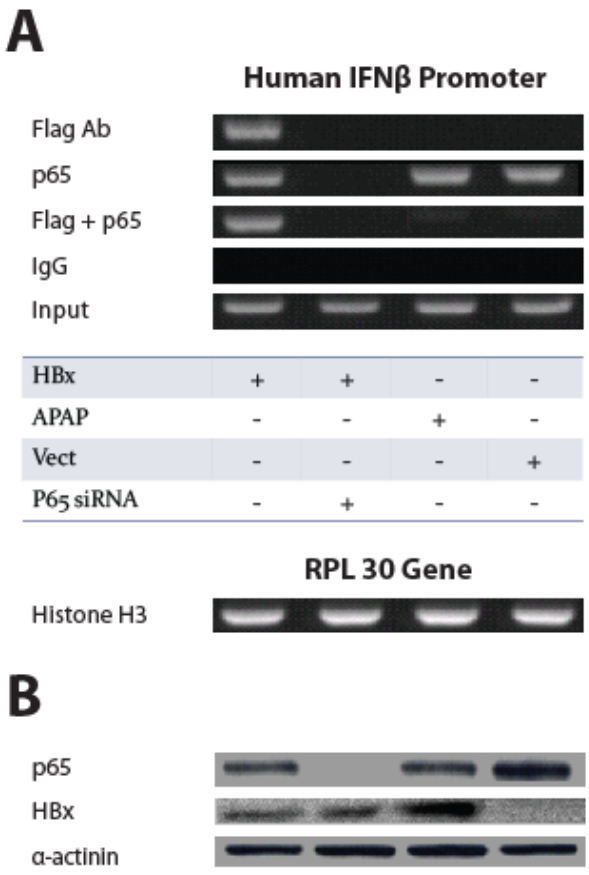


Figure 9. HBx-65 binds to the promoter region of IFNβ gene. (A) Chromatin immunoprecipitation (ChIP) were performed to study the recruitment of HBx-p65 complex to IFNβ chromatin. Huh7 cells (1.0×10^5) were seeded in 10 cm Petri dish and were transfected with 100 nM p65-specific or scrambled siRNA for 48 h. Cells were transfected with pFlag HBx plasmids for additional 48 h. Cross-linked samples were pulled down with Flag, p65, IgG, and H3 antibodies. Sequential ChIP was carried out with p65 antibody using chromatin eluted from anti-Flag pull-down samples. Isolated DNA were subjected to PCR with primers that amplify segments from -276 to +185 of IFNβ gene. RPL30 primers were used for the H3 positive control. Results are representative of three independent experiments. (B) Western blot was utilized to verify protein expression and p65 siRNA knockdown.

Chapter 7: Discussion

HBx plays a critical role during HBV pathogenesis as an oncoprotein. As numerous studies have demonstrated, HBx can modulate signal transduction, as well as gene expression within hepatocytes. A majority of them have focused on the function of cytoplasmic HBx, based on the common observation that most HBx resides in the cytoplasm. However, HBx can also localize to mitochondria and nucleus, where it might have important functions. One of the signaling pathways that have been implicated in HBV-associated HCC is the NF-κB pathway. The first report to document the ability of HBx to activate the NF-κB pathway was almost 20 years ago (Doria, Klein, Lucito, & Schneider, 1995). Since then a great deal of knowledge on the relationship between HBx and NF-κB pathway has been obtained, largely due to the extensive investigations into the cell biology of the signaling pathway. The NF-κB cascade has been independently linked to various devastating diseases including cancers and those that are immune-mediated. This signal pathway has not only a wide range of functions essential for the host, such as cell differentiation and immune responses, but also a dynamic network of crosstalk with heterologous signaling. HBx may extensively utilize NF-κB pathway. Its multiple layers of regulatory mechanisms are targets that HBx can manipulate. NF-κB is undoubtedly an appealing target of a foreign invader like HBV that affects various cellular activities. However, many aspects of HBx-mediated NF-κB regulation remain a mystery. Currently available data explains how HBx may constitutively activate the NF-κB pathway, but it does not account for the specificity associated with HBx functions. Molecular occurrences in the nucleus, particularly transcription regulation, may address this gap. NF-κB transcription factors, including p50 and p65, can form various dimers. Even a very small difference in their nuclear concentration, affinities for DNA target sequence, and interaction with coregulators can have profound

physiological impacts. As such, this study has focused on the relationship between HBx and p65, aiming to explore their roles in HBx-mediated NF-κB activation.

The interplay between HBx and the NF-κB pathway might be of tremendous importance in promoting oncogenesis. Since HBx does not bind to DNA, its interactions with other proteins seem to be the key to understanding its functions as well as underlying molecular mechanisms. This study has mapped interaction motifs important for HBx-p65 interaction. As hypothesized, mapping of amino acid sequences of HBx revealed a close association between NF-κB activation and its physical interaction with p65, suggesting a link between the two phenomena. A serine-proline motif ^{39}SPSP^{42} identified in this study has previously been reported to mediate the interaction between HBx and other proteins. One example is the amplified in breast cancer 1 (AIB-1/SRC3/NCoA3), a coactivator and an oncoprotein commonly over-expressed in various cancers (Hong et al., 2012). HBx and AIB-1 had a synergistic effect on NF-κB activation, and HBx-AIB-1 complex up-regulated the expression of a NF-κB controlled gene matrix metalloproteinase-9 (MMP-9) in HepG2 cells (Liu et al., 2012). In addition, peptidyl-prolyl cis/trans isomerase (Pin1) was shown to bind to the SPSP motif of HBx to augment its transcription activation (Pang et al., 2007). Pin1 was shown to directly bind to pThr254-Pro motif in p65 and inhibit its binding to IκBα, increasing its nuclear accumulation (Ryo et al., 2003). The relationship between HBx, p65, AIB-1, and Pin1 is unclear. It is possible that they are all recruited to the DNA binding site to form a transcriptionally active complex. HBx may change the dynamics of protein recruitment and alter the rate, initiation, and/or termination of transcription. AIB-1 bound to HBx may regulate p65 transcription activity by modifying the histones bound to the DNA. Moreover, interaction of Pin1 and p65 can be facilitated by HBx that binds to both. It would be beneficial and insightful to see if Thr254 and Asp531 (DED Motif) of

p65 are in close proximity in its three-dimensional structure. These functions of HBx may involve additional regulatory mechanisms. Ser41 in HBx is one of the phylogenetically conserved residues that is predicted to be phosphorylated and exposed on the surface of the protein (Hernández, Venegas, Brahm, & Villanueva, 2012). The interaction motif in p65 may also have a particular role. Originally identified from glycosyltransferase, a negative charge-rich DXD sugar-binding motif (^{531}DED^{533}) in p65 not only binds to HBx, but also may play a role in O-linked N-acetylglucosamine (O-GlcNAc) modifications of proteins (Li et al., 2001). O-GlcNAcylated has been reported in p65 and IKKβ, but is only predicted to occur in HBx Ser41 (Hernández et al., 2012). A study has suggested that hyper-O-GlcNAcylation of p65 may contribute to constitutive, oncogenic NF-κB activation (Ma, Vocadlo, & Vosseller, 2013). The role of HBx in O-GlcNAc modification has not been studied. Based on the current literature, it is not difficult to deduce that HBx-p65 interaction may lead to various posttranslational modifications and recruitment of coregulators that can contribute to the modulation of NF-κB pathway.

HBx and p50 seem to have antagonistic effects, which to the author's knowledge have never been reported before. The ability of p50 to interfere with HBx-p65 interaction coincided with its inhibition of NF-κB activity that was induced by HBx. Its physiological relevance and significance however remains to be investigated. The degree of competition between p50 and HBx for p65, as well as their collective influences on NF-κB pathway likely depends on the cellular environment and protein levels. Biochemical investigations using SEAP reporter assay may not reflect the natural HBV infection and are limited in that proteins of interests are over-expressed artificially. If there were plenty of the limiting factor p65, HBx and p50 may not compete as much, ultimately having an additive effect on NF-κB activation. In contrast, if there

is very little p65, the impact of the competition becomes more critical, and the level of NF-κB activity induced by HBx and p50 will average out based on their proportions, never exceeding the lower and upper limit of their transcription activation potential. This is a classic definition of antagonism, a phenomenon that was observed between HBx and p50 in this study. In a NF-κB assay, p50 C1 truncation mutant that could not interact with p65 had no effect on HBx, while p50 reduced HBx-mediated NF-κB activation. This illustrates that p50 requires the function similar to that of HBx - direct binding to p65 - in order to compete and to limit the influence of HBx on NF-κB pathway. Bound to p65, p50 and HBx may target the same gene but with different dynamics of cofactors; on the other hand, the two proteins may confer different transcriptional target specific altogether. Transcription factor p50 binds to p65 to make a prototypical NF-κB dimer that activates gene transcriptions (Oeckinghaus & Ghosh, 2009). Previous studies have shown evidence that HBx also joins p65 to form a transcriptionally active complex (Liu et al., 2012). As the findings illustrate, HBx may functionally replace p50 in activating NF-κB, but the two proteins may have different activation potential as well as specificity. This is not surprising given that various NF-κB dimers have specific cell distribution, as well as unique functions. Antagonistic effect of p50 on HBx might be accompanied by its ability to repress NF-κB activity in general. Such inhibition is radically different from antagonism because it implies that p50 has a directly opposite function from HBx. Homodimers consisting of p50 was shown to suppress the activation of NF-κB target genes by recruiting histone deacetylase 1 (HDAC-1) and histone methytransferase, EHMT1, both of which are posttranslational modifiers that can limit DNA access to the transcription factors (Ea et al., 2012; Elsharkawy et al., 2010). It was observed that p50 C1 mutant that could not affect HBx function was able to reduce p65-mediated NF-κB activation. This suggests that the

inhibitory effect of p50 on HBx likely involves antagonism based on p65 interaction, but does not rule out the possible contribution of p50 homodimers. This may depend on the rate of homodimer formation determined by amount of p50 available. Moreover, the limitation of the NF-κB SEAP reporter assay should be considered. The reporter plasmid contains a specific *cis* element to which NF-κB transcription factors bind. Since each κB binding sites have different affinities for NF-κB dimers and coregulators (Oeckinghaus & Ghosh, 2009), the findings cannot be generalized to every gene regulation controlled by NF-κB pathway. In natural infection setting, it is highly unlikely that HBx or p50 predominates over the other. Introduction of HBx into hepatocytes may increase the overall capacity of NF-κB activation. As such, activated state of NF-κB signaling can be maintained without reaching exhaustion, probably due to the buffering effect of p50 that antagonizes HBx function.

In the cytoplasm, HBx-p65 complex may influence p65 translocation by promoting post translational modification of IκBα. HBx was previously shown to increase the accumulation of p65 in the nucleus (Lim et al., 2011; Yanning Liu et al., 2013). Out data illustrated that HBx-p65 might be important for the migration of p65 into nucleus. This method, however, is limited in that it cannot quantify the amount of p65 in specific cellular compartments. Fractionation of nuclear and cytoplasmic compartments has been attempted, but with no success. It is unclear whether p65 translocation is facilitated by HBx or prevented by APAP mutant. The interpretation is further complicated because majority of p65, which should localize in the cytoplasm, is found in the nucleus of Huh7 cells in this study. Such atypical p65 localization pattern has been observed before and was attributed to unknown characteristics or defects of Huh7 hepatocarcinoma cell line (Shukla et al., 2011). Using a different cell line may help to resolve this issue. Also, the correlation is very clear based on the microscope images. If

interaction between HBx-p65 is abolished, p65 nuclear localization is also diminished. It is unlikely that HBx directly shuttles p65 or that p65 is sequestered in the nucleus by HBx because only a small proportion of HBx has been observed in the nucleus. Involvement of additional protein(s) essential for p65 nuclear transport mechanisms seems probable. A logical candidate is IκBα, an inhibitory protein that sequesters p65 in the cytoplasm by masking its nuclear localization signal (NLS). It was previously reported that HBx can induce IκBα phosphorylatoin in a dose-dependent manner (Su & Schneider, 1996). Similarly, western blot analysis showed that HBx, but not APAP, mutant promoted IκBα phosphorylation, providing adequate explanation for the results obtained from fluorescence microscopy. Based on the p65 knockdown study, phosphorylation of IκBα by HBx was dependent on p65. It was previously shown that p65 and IκBα form an autoregulatory loop, in which p65 induces the expression of IκBα gene to facilitate its production, and thus termination of NF-κB responses. With the experiment protocol utilized, the results did not show any noticeable changes in the amount of endogenous IκBα, suggesting that HBx may directly affect IκBα phosphorylation by interacting with p65. The underlying molecular mechanism is unclear. It is possible that p65 facilitates the interaction between HBx and IκBα, allowing HBx to recruit into close proximity the kinases, such as IKK complex that has the ability to phosphorylate IκBα (Lim et al., 2013). Another plausible explanation may involve the alteration of interaction between p65 and IκBα which by itself may influence the dynamics of protein modifications. Collectively, the findings suggest that HBx-p65 interaction facilitates IκBα phosphorylation and subsequent degradation, effectively promoting p65 translocation into the nucleus. This study provides not only confirmations for currently available data, but also additional layer of understanding by showing

that the effects of HBx on both p65 nuclear localization and IκBα phosphorylation depends on its ability to interact with p65.

The observed interaction between HBx and p65 is biologically relevant as it can regulate the transcription of a specific set of cytokines. This study marks the first comprehensive cytokine expression analysis of HBx in human cell line. The use of recombinant virus vector better reflects the natural infection setting than transfection. The effects of vaccinia virus infection on the NF-κB pathway in HeLa cells are well-characterized. This particular ΔE3L mutant of vaccinia virus lacks E3L protein that plays an important role in subverting innate immune responses by suppressing the expression of diverse cytokines controlled by NF-κB factors (Myskiw et al., 2009). In addition to E3L, other vaccinia viral proteins, such as M2L and C4, were shown to inhibit NF-κB activation (Ember, Ren, Ferguson, & Smith, 2012; Gedey, Jin, Hinthong, & Shisler, 2006). Compared to the APAP mutant and the virus control, HBx expressed in HeLa cells through vaccinia viral vector was able to activate or inhibit specific cytokine mRNA expression as shown by real-time PCR (qPCR) array analysis. Most remarkable changes were observed with IFNβ, TNF, IL-19, and IL-4, all of which have been shown to be regulated by NF-κB factors, involved in inflammation, and implicated in various cancers, including HCC. The observed up-regulation of IFNβ mRNA is somewhat contradictory to the effects of HBx. As a member of the Type I interferon, IFNβ has essential biological functions, such as proapoptotic effects, that contribute to both viral innate immunity and anti-tumor activity (Trinchieri, 2010). Previous studies have shown that HBx suppresses IFNβ production in multiple ways (Wei et al., 2010). This discrepancy can be explained by the cellular environment created in response to vaccinia virus infection. To elaborate, virus-induced IFNβ production in host cells involves recognition of viral pathogen-associated molecular patterns (PAMPs),

including dsDNA and dsRNA. E3L is a dsRNA-binding protein that confers resistance to Type I interferon (Myskiw et al., 2009). As such, ΔE3L vaccina virus is presumed to trigger immune responses through its dsRNA intermediate, as opposed to the dsDNA that is detected during HBV infection (Kumar et al., 2011). A previous study has thoroughly investigated the effects of HBx on IFNβ production stimulated by different nucleic acids by utilizing poly(dAT:dAT) and poly(I:C) as substitutes (Kumar et al., 2011). HBx was shown to inhibit dsDNA-mediated IFNβ activation and to up-regulate dsRNA-induced expression. This is consistent with our findings. The effect of HBx on NF-κB mediated gene expression is probably highly specific depending on the cellular context. As for the other cytokines, TNFα is a potent proinflammatory cytokine that can activate NF-κB pathway. HBx was shown to induce the expression of TNFα (Lou et al., 2013). Its pleiotropic functions in inflammation, cell survival, and angiogenesis have been implicated in numerous accounts of cancer studies (Balkwill, 2009). Commonly associated with advanced tumor stage and poor prognosis, interleukin-19 (IL-9) was shown to contribute to inflammation by stimulating liver cells to produce reactive oxygen species and promoting neutrophil chemotaxis (Hsing et al., 2012). This study is the first report to link HBx to IL-19 up-regulation. Lastly, interleukin-4 (IL-4) is an anti-inflammatory cytokine with a paradoxical role in cancer (Z. Li, Chen, & Qin, 2009). Specifically in hepatocytes, however, IL-4 was shown to induce apoptosis both *in vitro* and *in vivo* (Aoudjehane et al., 2007). Consistent with these results, previous studies have also shown that HBx down-regulates IL-4 in human T lymphocytes (Lou et al., 2013). These cytokines may contribute to development and progression of HBV-associated HCC. As shown by reverse transcription PCR (RT-PCR), HBx-mediated expression of IFNβ and TNFα were dependent on p65, but not p50. While providing evidence that HBx and

p50 are functionally analogous, these findings highlight the importance of p65 transcription factor and its interaction with HBx in modulating cytokine expressions.

The function of HBx-p65 in the nucleus is still poorly understood. Only a small percentage (~5%) of HBx localizes to the nucleus (Forgues et al., 2003), but as previously mentioned, even a small difference in its concentration can have a huge physiological impact. There are just a few studies that have illustrated the recruitment of HBx and/or p65 to the gene promoter (Bui-Nguyen et al., 2010; Liu et al., 2012). The findings of this study provides further evidence that HBx-p65 complex directly binds to DNA and broadens its target genes to include cytokines, in particular IFNβ. A study has shown that HBx facilitates the recruitment of p65 to metastasis associated 1 (MTA1) gene, increasing the amount of p65 bound to its promoter. Since HBx cannot bind to DNA, it seems somewhat counter-intuitive that it can promote p65 recruitment to a specific site. It is possible, however, for HBx to confer certain restrictions and specificity to the HBx-p65 dimer to favor its association with specific κB binding sites. One of the main functions of p50, which might be functionally analogous to HBx, is to determine the specificity of NF-κB dimers (Wan & Lenardo, 2009). Furthermore, HBx may function as an accessory protein that optimizes transcription by recruiting various host factors, including coactivators and coregulators. These proteins may directly affect p65 (e.g., posttranslational modification) or function in a p65-independent manner. Such molecular mechanisms explain the synergism exhibited by HBx and p65 in activating NF-κB pathway. In the nucleus, HBx may require p65 not only to reach its target genes, but for its important functions in transcription regulation. It seems plausible that HBx and p65 have overlapping, reciprocal functions that determine the specificity and degree of NF-κB activation.

To discuss some of the weaknesses of this study, the technical aspects of the experiments that contribute to the limitations of the findings can be outlined. Throughout the study, the use of human carcinoma cell lines, Huh7, HepG2, and HeLa, were inconsistent. Hepatocellular carcinoma cell line Huh7 was used the most because it had a significantly higher transfection efficiency than HepG2 when using the Attractene reagent. Although the NF-κB and MTH assays have been replicated with HepG2 cells, the findings on underlying molecular mechanisms may not necessarily apply to this cell line, given the multiple ways in which HBx can modulate NF-κB pathway (Huang et al., 2012; Lim et al., 2013). HeLa cells derived from cervical cancer cells were previously used in a cytokine assay (qPCR and RT-PCR) to characterize the gene expression profile associated with the recombinant vaccinia virus ΔE3L (Myskiw et al., 2009). This established system with appropriate negative and positive controls was utilized for HBx investigation in order to maximize the reliability of the data. It remains unknown whether or not the same pattern of cytokine gene expressions will be observed in hepatocellular carcinoma cells, such as Huh7. The NF-κB assay is flawed in that the *cis* element in the reporter plasmid cannot account for the numerous NF-κB binding sites located throughout human genome. As previously mentioned, different dimers have unique DNA binding specificities (Oeckinghaus & Ghosh, 2009), and without the empirical data, it might be nearly impossible to predict the genes that are targeted by HBx-p65 complex. Moreover, the results showed discrepancies in the level of NF-κB activity induced by HBx, suggesting there might be other factors involved. It may simply reflect the difference in the presence of endogenous NF-κB factors that can bind to the *cis* element of the reporter plasmid. In the western blot analysis, only the proteins fused to a tag were probed, and so the level of endogenous proteins may vary depending on the trial of an experiment. It is also possible that cellular background of cofactors may have influenced the

expression and/or stabilization of proteins. For example, the rate of HBx turnover can be regulated by the level of p65 (Shukla et al., 2011), and under certain conditions, HBx may exist as a homodimer with unique functions (Lee & Yun, 1998). Between each trial of an experiment, the levels of HBx were not compared because their absolute levels could not be determined using western blot. In addition, both pVP16 and pM vectors of the MTH assay contain nuclear localization signal and thus can dramatically change protein localizations within cells. Vaccinia viral vector system also has a pitfall in the context of this study due to its functions in regulating NF-κB signaling. The exact influences of the vaccinia virus on HBx-mediated NF-κB modulation could not be determined. Presumably, if it was during natural HBV infection or if introduced into the cells with a different viral vector system, HBx may induce a distinct NF-κB responses. Omission of IFNβ protein level confirmation in the RT-PCR experiment raises a doubt that amount of mRNA may not correlate with that of the protein, which indeed is often the case (Vogel & Marcotte, 2012). Lastly, this study lacks the data on the physiological relevance of certain biochemical data. The extent to which the observed phenomena (e.g., the competition between p50 and HBx-mediated Iκβα phosphorylation, and existence of HBx-p65 complex) will occur within human hepatocytes during HBV infection remains elusive. Further studies with animal models may help to address some of these issues. Despite these limitations, the findings of the experiments mentioned above are meaningful as they will promote better understanding of HBx-mediated oncogenesis.

The results obtained in this study overall may prove beneficial for the scientific communities that deal with HBV molecular virology, cancer cell biology, and/or novel therapeutics development. Focusing on one specific viral protein HBx, experiments were tailored in such a way to elucidate the molecular mechanisms underlying HBV pathogenesis that

lead to HCC. Previously reported importance of protein-protein interactions in HBx functions is further supported by this project. In addition, it seems HBx plays diverse, holistic roles in multiple cellular compartments, which suggests that host factors in various locations within the cell may also contribute to the pleotropic effects of HBx. Identification and investigation of these factors will help to enrich current data, which can collectively pinpoint the major players (e.g., signal transduction pathways and genes) that directly or indirectly influence the development and progression of HCC. Moreover, studying the oncogenesis induced by HBx will contribute to the advancement of cancer cell biology. As a viral oncoprotein, HBx may catalyze tumorigenesis by modulating specific cellular processes, thereby providing a unique opportunity to assess the causality of certain molecular changes. This study has illustrated an intimate interplay between HBx and NF-κB pathway, emphasizing the effects on regulatory mechanisms and cytokine gene expressions. Based on the observations, HBx-mediated oncogenesis may depend on NF-κB signaling, and an increase in p65 nuclear localization may precede HCC development. Also, the findings of the cytokine assay demonstrates the target gene specificities of the activated dimers of NF-κB transcription factors, thereby refining the current understanding that constitutive NF-κB activation is responsible for cancer development. Consistent with previous reports, inflammation due to certain cytokines may promote tumorigenesis during HBV infection. Lastly, with the use of APAP mutant, it can be inferred that physical interaction between HBx and p65 plays important roles in both the cytoplasm (e.g., modulation of signal pathway) and the nucleus (e.g., regulation of gene transcription). It is challenging to predict the scope of influences that HBx-p65 interaction has on HCC, but the potential implications for treatment strategies cannot be ignored. If HBx-p65 complex does indeed play an essential role, as supported by this study, the physical interaction between the two

proteins can be a promising target of novel therapeutics. To develop the appropriate drugs that disrupt the association between HBx and p65, their interaction motifs identified from the MTH assay will prove crucial. This approach may also provide valuable insights on how to manipulate p65 functions to control the NF-κB responses in such a way that impedes or blocks malignant cell transformation. In effect, targeting protein-protein interactions with precision and accuracy may help to prevent, treat, and even reverse not only HCC, but also other human cancers in general. Having focused on the interplay between HBx and NF-κB signal transduction pathway, this study has an enormous potential to influence the lives of many individuals, including patients, family, and scholars, who are directly or indirectly affected by HBV-associated HCC.

Chapter 8: Conclusion and Future Directions

This study has demonstrated the important role of HBx-p65 interaction in modulating the NF-κB pathway at multiple levels. In both Huh7 and HepG2 hepatocellular carcinoma cell lines, a serine-proline motif ^{39}SPSP^{42} in HBx was found to be critical for both NF-κB activation as well as HBx-p65 interaction, effectively establishing a link between the two phenomena. This is the first report to identify a negative charge-rich DXD motif (^{531}DED^{533}) in p65 that is important for interacting with HBx. The direct association between HBx and p65 was interrupted by p50 over-expression. In addition, p50 was found to antagonize, rather than repress, the effects of HBx on NF-κB activity. The results suggested that inhibitory effects p50 on HBx is linked to its ability to compete with HBx for p65. Interestingly, HBx was able to functionally replace p50 to activate NF-κB pathway, indicating that these proteins share a similar function, but with different capacity. To the author's knowledge, this is the first study to identify and elucidate this intriguing relationship between HBx and p50. The function of HBx-p65 in the cytoplasm was examined through two interlinked processes, p65 nuclear localization and IκBα phosphorylation. HBx-p65 interaction may have a direct influence on the translocation of p65, most likely by promoting IκBα phosphorylation. Furthermore, HBx expressed through a recombinant ΔE3L vaccinia virus system modulated a unique profile of cytokine mRNA expression in HeLa cells. This process required p65, but not p50. This study is the first of its kind to investigate HBx through a large-scale, comprehensive cytokine expression analysis. Last but not least, HBx-p65 complex was recruited to the IFNβ promoter region, indicating that HBx can regulate gene expressions as it is recruited to the DNA through p65. Collectively, the findings of this study provide compelling evidence that HBx-p65 interaction is essential for HBx-mediated NF-κB modulation. The effects of HBx-p65 complex are not exclusive to a

specific cellular compartment, but may occur in both cytoplasm and nucleus. It seems that HBx plays a rather holistic role through p65, affecting and manipulating every step of the NF-κB pathway to in order to meet its needs. All together, the findings of this study provide a strong support for the hypothesis that HBx modulates NF-κB activity by physically interacting with p65 in both cytoplasm and nucleus, ultimately leading to a unique profile of cytokine gene expression in human carcinoma cells.

Evidently, the interplay between HBx and NF-κB pathway, in particular p65, seems to be of paramount importance in HBV-associated HCC. However, there still are significant gaps in literature that need to be filled, and further explorations are warranted. With the obtained data, this study paves the way for subsequent investigations of HBx-p65 interaction. Given the importance of HBx-p65 interaction, it could be a very promising target for novel therapeutics against chronic HBV infection and HCC. Characterization of interaction motifs should provide insights on how to manipulate and/or prevent HBx-p65 interaction. To further our knowledge in molecular virology and cancer biology, further characterization of the DED motif in p65 and its role in O-GlcNAcylation in the presence of HBx may provide meaningful data related to oncogenesis. Cytokine expression analysis certainly requires verification due to certain limitations (e.g., the artificial expression system rather than authentic HBV infection). The nature and extent to which recombinant ΔE3L vaccinia viruses have influenced NF-κB pathway cannot be measured. Use of a different viral vector may unveil certain masked effects of HBx. For some of the cytokines up-regulated by HBx-p65, including IL-19, virtually none or very little data exist. Research into these specific cytokines in the context of HBV-associated HCC may provide valuable information. Finally, nuclear events that govern HBx-mediated gene regulation need more attention. ChIP assay can be extended to other cytokine genes in order to

assess the applicability of the findings. Also, studying HBx-p65 complex and its protein interactions in the nucleus may enhance our knowledge on how exactly HBx regulates gene expression. These studies will undoubtedly enhance our knowledge in HBV pathogenesis and related-diseases, including HCC.

References

Ahn, J. Y., Jung, E. Y., Kwun, H. J., Lee, C.-W., Sung, Y.-C., & Jang, K. L. (2002). Dual effects of hepatitis B virus X protein on the regulation of cell-cycle control depending on the status of cellular p53. *The Journal of General Virology*, *83*(Pt 11), 2765–72.

Aoudjehane, L., Podevin, P., Scatton, O., Jaffray, P., Dusanter-Fourt, I., Feldmann, G., … Conti, F. (2007). Interleukin-4 induces human hepatocyte apoptosis through a Fas-independent pathway. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *21*(7), 1433–44. doi:10.1096/fj.06-6319com

Aravalli, R. N., Steer, C. J., & Cressman, E. N. K. (2008). Molecular mechanisms of hepatocellular carcinoma. *Hepatology (Baltimore, Md.)*, *48*(6), 2047–63. doi:10.1002/hep.22580

Aspinall, E. J., Hawkins, G., Fraser, a, Hutchinson, S. J., & Goldberg, D. (2011). Hepatitis B prevention, diagnosis, treatment and care: a review. *Occupational Medicine (Oxford, England)*, *61*(8), 531–40. doi:10.1093/occmed/kqr136

Balkwill, F. (2009). Tumour necrosis factor and cancer. *Nature Reviews. Cancer*, *9*(5), 361–71. doi:10.1038/nrc2628

Barker, L. F., Shulman, N. R., Murray, R., Hirschman, R. J., Ratner, F., Diefenbach, W. C. L., & Geller, H. M. (1970). Transmission of Serum Hepatitis. *The Journal of the American Medical Association*, *211*(9), 1509–12. doi:10.1001/jama.1970.03170090025005

Beasley, R. P. (1982). Hepatitis B virus as the etiologic agent in hepatocellular carcinoma - epidemiologic considerations. *Hepatology*, *2*, 21S–26S.

Beck, J., & Nassal, M. (2007a). Hepatitis B virus replication. *World Journal of Gastroenterology : WJG*, *13*(1), 48–64.

Beck, J., & Nassal, M. (2007b). Hepatitis B virus replication. *World Journal of Gastroenterology : WJG*, *13*(1), 48–64.

Benn, J., & Schneider, R. J. (1994). Hepatitis B virus HBx protein activates Ras-GTP complex formation and establishes a Ras, Raf, MAP kinase signaling cascade. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(22), 10350–4.

Benn, J., & Schneider, R. J. (1995). Hepatitis B virus HBx protein deregulates cell cycle checkpoint controls. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(24), 11215–9.

Bisceglie, A. Di. (2009). Hepatitis B and hepatocellular carcinoma. *Hepatology*, *49*, S56–S60. doi:10.1002/hep.22962.Hepatitis

Block, T. M., Guo, H., & Guo, J.-T. (2007). Molecular virology of hepatitis B virus for clinicians. *Clinics in Liver Disease*, *11*(4), 685–706, vii. doi:10.1016/j.cld.2007.08.002

Bouchard, M. J., & Schneider, R. J. (2004). The enigmatic X gene of hepatitis B virus. *Journal of Virology*, *78*(23), 12725–34. doi:10.1128/JVI.78.23.12725-12734.2004

Bouchard, M. J., Wang, L., & Schneider, R. J. (2006). Activation of focal adhesion kinase by hepatitis B virus HBx protein: multiple functions in viral replication. *Journal of Virology*, *80*(9), 4406–14. doi:10.1128/JVI.80.9.4406-4414.2006

Bui-Nguyen, T., Pakala, S., & Sirigiri, R. (2010). NF-κB signaling mediates the induction of MTA1 by hepatitis B virus transactivator protein HBx. *Oncogene*, *29*(8), 1179–1189. doi:10.1038/onc.2009.404.NF-

Busca, A., & Kumar, A. (2014). Innate immune responses in hepatitis B virus (HBV) infection. *Virology Journal*, *11*, 22. doi:10.1186/1743-422X-11-22

Campbell, K. J., & Perkins, N. D. (2004). Post-translational modification of RelA(p65) NF-kappaB. *Biochemical Society Transactions*, *32*(Pt 6), 1087–9. doi:10.1042/BST0321087

Cha, M.-Y., Ryu, D.-K., Jung, H.-S., Chang, H.-E., & Ryu, W.-S. (2009). Stimulation of hepatitis B virus genome replication by HBx is linked to both nuclear and cytoplasmic HBx expression. *The Journal of General Virology*, *90*(Pt 4), 978–86. doi:10.1099/vir.0.009928-0

Cheong, J. H., Yi, M., Lin, Y., & Murakami, S. (1995). Human RPB5, a subunit shared by eukaryotic nuclear RNA polymerases, binds human hepatitis B virus X protein and may play a role in X transactivation. *The EMBO Journal*, *14*(1), 143–50.

Clippinger, A. J., & Bouchard, M. J. (2008). Hepatitis B virus HBx protein localizes to mitochondria in primary rat hepatocytes and modulates mitochondrial membrane potential. *Journal of Virology*, *82*(14), 6798–811. doi:10.1128/JVI.00154-08

Coffin, C. S., Mulrooney-cousins, P. M., Marle, G. Van, Roberts, J. P., Michalak, T. I., & Terrault, N. A. (2011). Hepatitis B Virus Quasispecies in Hepatic and Extrahepatic Viral Reservoirs in Liver Transplant Recipients on Prophylactic Therapy, 955–962. doi:10.1002/lt.

Cooray, S. (2004). The pivotal role of phosphatidylinositol 3-kinase-Akt signal transduction in virus survival. *Journal of General Virology*, *85*(5), 1065–1076. doi:10.1099/vir.0.19771-0

Dan, H. C., Cooper, M. J., Cogswell, P. C., Duncan, J. a, Ting, J. P.-Y., & Baldwin, A. S. (2008). Akt-dependent regulation of NF-{kappa}B is controlled by mTOR and Raptor in association with IKK. *Genes & Development*, *22*(11), 1490–500. doi:10.1101/gad.1662308

Digicaylioglu, M., & Lipton, S. a. (2001). Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. *Nature*, *412*(6847), 641–7. doi:10.1038/35088074

Doria, M., Klein, N., Lucito, R., & Schneider, R. J. (1995). The hepatitis B virus HBx protein is a dual specificity cytoplasmic activator of Ras and nuclear activator of transcription factors. *The EMBO Journal*, *14*(19), 4747–57.

Downward, J. (2003). Targeting RAS signalling pathways in cancer therapy. *Nat Rev Cancer*, *3*, 11–22. doi:10.1038/nrc969

Ea, C.-K., Hao, S., Yeo, K. S., & Baltimore, D. (2012). EHMT1 protein binds to nuclear factor-κB p50 and represses gene expression. *The Journal of Biological Chemistry*, *287*(37), 31207–17. doi:10.1074/jbc.M112.365601

Elgouhari, H. M., Abu-Rajab Tamimi, T. I., & Carey, W. D. (2008). Hepatitis B virus infection: understanding its epidemiology, course, and diagnosis. *Cleveland Clinic Journal of Medicine*, *75*(12), 881–9. doi:10.3949/ccjm.75a.07019

El-Serag, H. B. (2012). Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology*, *142*(6), 1264–1273.e1. doi:10.1053/j.gastro.2011.12.061

Elsharkawy, A. M., Oakley, F., Lin, F., Packham, G., Mann, D. A., & Mann, J. (2010). The NF-kappaB p50:p50:HDAC-1 repressor complex orchestrates transcriptional inhibition of multiple pro-inflammatory genes. *Journal of Hepatology*, *53*(3), 519–27. doi:10.1016/j.jhep.2010.03.025

Ember, S. W. J., Ren, H., Ferguson, B. J., & Smith, G. L. (2012). Vaccinia virus protein C4 inhibits NF-κB activation and promotes virus virulence. *The Journal of General Virology*, *93*(Pt 10), 2098–108. doi:10.1099/vir.0.045070-0

Fausto, N., Campbell, J. S., & Riehle, K. J. (2006). Liver regeneration. *Hepatology*, *43*(2 Suppl 1), S45–53. doi:10.1002/hep.20969

Feitelson, M. A., Arzumanyan, A., Reis, H. M. G. P. V, Clayton, M. M., Sun, B. S., & Lian, Z. (2011). Viral hepatitis B. In S. P. S. Monga (Ed.), *Molecular Pathology of Liver Diseases* (Vol. 5, pp. 553–567). Boston, MA: Springer US. doi:10.1007/978-1-4419-7107-4

Ferlay, J., Shin, H. R., Bray, F., Forman, D., Mathers, C., & Parkin, D. M. (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International Journal of Cancer*, *127*, 2893–2917. doi:10.1002/ijc.25516

Forgues, M., Difilippantonio, M. J., Linke, S. P., Ried, T., Nagashima, K., Feden, J., … Wang, X. W. (2003). Involvement of Crm1 in hepatitis B virus X protein-induced aberrant centriole replication and abnormal mitotic spindles. *Molecular and Cellular Biology*, *23*(15), 5282–92. doi:10.1128/MCB.23.15.5282

Gearhart, T. L., & Bouchard, M. J. (2011). The hepatitis B virus HBx protein modulates cell cycle regulatory proteins in cultured primary human hepatocytes. *Journal of Virology*, *155*(1), 363–7. doi:10.1016/j.virusres.2010.09.023

Gedey, R., Jin, X.-L., Hinthong, O., & Shisler, J. L. (2006). Poxviral regulation of the host NF-kappaB response: the vaccinia virus M2L protein inhibits induction of NF-kappaB activation via an ERK2 pathway in virus-infected human embryonic kidney cells. *Journal of Virology*, *80*(17), 8676–85. doi:10.1128/JVI.00935-06

Gilmore, T. D. (2006). Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene*, *25*, 6680–6684. doi:10.1038/sj.onc.1209954

Goldstein, S. T., Zhou, F., Hadler, S. C., Bell, B. P., Mast, E. E., & Margolis, H. S. (2005). A mathematical model to estimate global hepatitis B disease burden and vaccination impact. *International Journal of Epidemiology*, *34*, 1329–1339. doi:10.1093/ije/dyi206

Greten, T. F., Papendorf, F., Bleck, J. S., Kirchhoff, T., Wohlberedt, T., Kubicka, S., … Manns, M. P. (2005). Survival rate in patients with hepatocellular carcinoma: a retrospective analysis of 389 patients. *British Journal of Cancer*, *92*(10), 1862–8. doi:10.1038/sj.bjc.6602590

Han, J., Ding, L., Yuan, B., Yang, X., Wang, X., Li, J., … Ye, Q. (2006). Hepatitis B virus X protein and the estrogen receptor variant lacking exon 5 inhibit estrogen receptor signaling in hepatoma cells. *Nucleic Acids Research*, *34*(10), 3095–106. doi:10.1093/nar/gkl389

Herceg, Z., & Paliwal, A. (2009). HBV protein as a double-barrel shot-gun targets epigenetic landscape in liver cancer. *Journal of Hepatology*, *50*(2), 252–5. doi:10.1016/j.jhep.2008.11.013

Hernández, S., Venegas, M., Brahm, J., & Villanueva, R. a. (2012). The viral transactivator HBx protein exhibits a high potential for regulation via phosphorylation through an evolutionarily conserved mechanism. *Infectious Agents and Cancer*, *7*(1), 27. doi:10.1186/1750-9378-7-27

Hiscott, J., Kwon, H., & Génin, P. (2001). Hostile takeovers: viral appropriation of the NF-kappaB pathway. *The Journal of Clinical Investigation*, *107*(2), 143–51. doi:10.1172/JCI11918

Hong, A., Han, D. D., Wright, C. J., Burch, T., Piper, J., Osiowy, C., … He, R. (2012). The interaction between hepatitis B virus X protein and AIB1 oncogene is required for the activation of NFκB signal transduction. *Biochemical and Biophysical Research Communications*, *423*(1), 6–12. doi:10.1016/j.bbrc.2012.05.021

Hsing, C.-H., Cheng, H.-C., Hsu, Y.-H., Chan, C.-H., Yeh, C.-H., Li, C.-F., & Chang, M.-S. (2012). Upregulated IL-19 in breast cancer promotes tumor progression and affects clinical

outcome. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *18*(3), 713–25. doi:10.1158/1078-0432.CCR-11-1532

Huang, W.-C., Chen, W.-S., Chen, Y.-J., Wang, L.-Y., Hsu, S.-C., Chen, C.-C., & Hung, M.-C. (2012). Hepatitis B virus X protein induces IKKα nuclear translocation via Akt-dependent phosphorylation to promote the motility of hepatocarcinoma cells. *Journal of Cellular Physiology*, *227*(4), 1446–54. doi:10.1002/jcp.22860

Hutti, J. E., Pfefferle, A. D., Russell, S. C., Sircar, M., Perou, C. M., & Baldwin, A. S. (2012). Oncogenic PI3K mutations lead to NF-κB-dependent cytokine expression following growth factor deprivation. *Cancer Research*, *72*(13), 3260–9. doi:10.1158/0008-5472.CAN-11-4141

Hwang, E. W., & Cheung, R. (2011). Global epidemiology of hepatitis B virus (HBV) infection. *The North American Journal of Medicine and Science*, *4*(1), 7–13.

Iannacone, M., Sitia, G., Ruggeri, Z. M., & Guidotti, L. G. (2007). HBV pathogenesis in animal models: recent advances on the role of platelets. *Journal of Hepatology*, *46*(4), 719–26. doi:10.1016/j.jhep.2007.01.007

Jacobs, M. D., & Harrison, S. C. (1998). Structure of an IκBα/NF-κB Complex. *Cell*, *95*(6), 749–758. doi:10.1016/S0092-8674(00)81698-0

Kang, K.-F., Wang, X.-W., Chen, X.-W., Kang, Z.-J., Zhang, X., Wilbur, R. R., … Zhou, S.-F. (2013). Beclin 1 and nuclear factor-κBp65 are upregulated in hepatocellular carcinoma. *Oncology Letters*, *5*(6), 1813–1818. doi:10.3892/ol.2013.1307

Katz, S., Ayala, V., Santillán, G., & Boland, R. (2011). Activation of the PI3K/Akt signaling pathway through $P2Y_2$ receptors by extracellular ATP is involved in osteoblastic cell proliferation. *Archives of Biochemistry and Biophysics*, *513*(2), 144–52. doi:10.1016/j.abb.2011.06.013

Kawauchi, K., Araki, K., Tobiume, K., & Tanaka, N. (2009). Loss of p53 enhances catalytic activity of IKKbeta through O-linked beta-N-acetyl glucosamine modification. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(9), 3431–6. doi:10.1073/pnas.0813210106

Khattar, E., Mukherji, A., & Kumar, V. (2012). Akt augments the oncogenic potential of the HBx protein of hepatitis B virus by phosphorylation. *The FEBS Journal*, *279*(7), 1220–30. doi:10.1111/j.1742-4658.2012.08514.x

Kidd-Ljunggren, K., Holmberg, a, Bläckberg, J., & Lindqvist, B. (2006). High levels of hepatitis B virus DNA in body fluids from chronic carriers. *The Journal of Hospital Infection*, *64*(4), 352–7. doi:10.1016/j.jhin.2006.06.029

Kiernan, R., Brès, V., Ng, R. W. M., Coudart, M.-P., El Messaoudi, S., Sardet, C., … Benkirane, M. (2003). Post-activation turn-off of NF-kappa B-dependent transcription is regulated by acetylation of p65. *The Journal of Biological Chemistry*, *278*(4), 2758–66. doi:10.1074/jbc.M209572200

Kobayashi, M., Suzuki, F., Akuta, N., Hosaka, T., Sezaki, H., Yatsuji, H., … Kumada, H. (2007). Loss of hepatitis B surface antigen from the serum of patients with chronic hepatitis treated with lamivudine. *Journal of Medical Virology*, *79*(10), 1472–7. doi:10.1002/jmv.20994

Kumar, M., Jung, S. Y., Hodgson, A. J., Madden, C. R., Qin, J., & Slagle, B. L. (2011). Hepatitis B virus regulatory HBx protein binds to adaptor protein IPS-1 and inhibits the activation of beta interferon. *Journal of Virology*, *85*(2), 987–95. doi:10.1128/JVI.01825-10

Lavanchy, D. (2004). Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *Journal of Viral Hepatitis*, *11*(2), 97–107.

Lee, Y.-H., & Yun, Y. (1998). HBx Protein of Hepatitis B Virus Activates Jak1-STAT Signaling. *Journal of Biological Chemistry*, *273*(39), 25510–25515. doi:10.1074/jbc.273.39.25510

Levrero, M., & Belloni, L. (2010). Signaling Pathways in Liver Diseases. doi:10.1007/978-3-642-00150-5

Levrero, M., Pollicino, T., Petersen, J., Belloni, L., Raimondo, G., & Dandri, M. (2009). Control of cccDNA function in hepatitis B virus infection. *Journal of Hepatology*, *51*(3), 581–92. doi:10.1016/j.jhep.2009.05.022

Li, J., Rancour, D. M., Allende, M. L., Worth, C. a, Darling, D. S., Gilbert, J. B., … Young, W. W. (2001). The DXD motif is required for GM2 synthase activity but is not critical for nucleotide binding. *Glycobiology*, *11*(3), 217–29.

Li, S. K., Ho, S. F., Tsui, K. W., Fung, K. P., & Waye, M. Y. M. (2008). Identification of functionally important amino acid residues in the mitochondria targeting sequence of hepatitis B virus X protein. *Virology*, *381*(1), 81–8. doi:10.1016/j.virol.2008.07.037

Li, Z., Chen, L., & Qin, Z. (2009). Paradoxical roles of IL-4 in tumor immunity. *Cellular & Molecular Immunology*, *6*(6), 415–22. doi:10.1038/cmi.2009.53

Lim, K.-H., Choi, H. S., Park, Y. K., Park, E.-S., Shin, G. C., Kim, D. H., … Kim, K.-H. (2013). HBx-induced NF-κB signaling in liver cells is potentially mediated by the ternary complex of HBx with p22-FLIP and NEMO. *PloS One*, *8*(3), e57331. doi:10.1371/journal.pone.0057331

Lim, K.-H., Kim, K.-H., Choi, S. Il, Park, E.-S., Park, S. H., Ryu, K., … Seong, B. L. (2011). RPS3a over-expressed in HBV-associated hepatocellular carcinoma enhances the HBx-

induced NF-κB signaling via its novel chaperoning function. *PloS One*, *6*(8), e22258. doi:10.1371/journal.pone.0022258

Lin, Y.-J., Lee, M.-H., Yang, H.-I., Jen, C.-L., You, S.-L., Wang, L.-Y., … Chen, C.-J. (2013). Predictability of liver-related seromarkers for the risk of hepatocellular carcinoma in chronic hepatitis B patients. *PloS One*, *8*(4), e61448. doi:10.1371/journal.pone.0061448

Liu, Y., Lou, G., Wu, W., Shi, Y., Zheng, M., & Chen, Z. (2013). Interferon-α sensitizes HBx-expressing hepatocarcinoma cells to chemotherapeutic drugs through inhibition of HBx-mediated NF-κB activation. *Virology Journal*, *10*(1), 168. doi:10.1186/1743-422X-10-168

Liu, Y., Tong, Z., Li, T., Chen, Q., Zhuo, L., Li, W., … Yu, C. (2012). Hepatitis B virus X protein stabilizes amplified in breast cancer 1 protein and cooperates with it to promote human hepatocellular carcinoma cell invasiveness. *Hepatology (Baltimore, Md.)*, *56*(3), 1015–24. doi:10.1002/hep.25751

Liu, Y., & Wu, F. (2010). Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. *Environmental Health Perspectives*, *118*(6), 818–24. doi:10.1289/ehp.0901388

Lok, A. S. F. (2002). Chronic Hepatitis B. *New England Journal of Medicine*. doi:10.1056/NEJM200205303462202

Lou, X., Hou, Y., & Liang, D. (2013). Effects of hepatitis B virus X protein on human T cell cytokines. *Canadian Journal of Microbiology*, *59*(9), 620–6. doi:10.1139/cjm-2013-0259

Ma, Z., Vocadlo, D. J., & Vosseller, K. (2013). Hyper-O-GlcNAcylation is anti-apoptotic and maintains constitutive NF-κB activity in pancreatic cancer cells. *The Journal of Biological Chemistry*, *288*(21), 15121–30. doi:10.1074/jbc.M113.470047

Madden, C. R., & Slagle, B. L. (2001). Stimulation of cellular proliferation by hepatitis B virus X protein. *Disease Markers*, *17*(3), 153–7.

Mahoney, F. J. (1999). Update on diagnosis, management, and prevention of hepatitis B virus infection. *Clinical Microbiology Reviews*, *12*(2), 351–66.

May, P., & May, E. (1999). Twenty years of p53 research: structural and functional aspects of the p53 protein. *Oncogene*, *18*(53), 7621–36. doi:10.1038/sj.onc.1203285

McMahon, B. J. (2014). Chronic hepatitis B virus infection. *The Medical Clinics of North America*, *98*(1), 39–54. doi:10.1016/j.mcna.2013.08.004

Miyamoto, S., Schmitt, M. J., & Verma, I. M. (1994). Qualitative changes in the subunit composition of kappa B-binding complexes during murine B-cell differentiation. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(11), 5056–60.

Myskiw, C., Arsenio, J., van Bruggen, R., Deschambault, Y., & Cao, J. (2009). Vaccinia virus E3 suppresses expression of diverse cytokines through inhibition of the PKR, NF-kappaB, and IRF3 pathways. *Journal of Virology*, *83*(13), 6757–68. doi:10.1128/JVI.02570-08

Nayersina, R., Fowler, P., Guilhot, S., Missale, G., Cerny, A., Schlicht, H. J., … Chisari, F. V. (1993). HLA A2 restricted cytotoxic T lymphocyte responses to multiple hepatitis B surface antigen epitopes during hepatitis B virus infection. *Journal of Immunology (Baltimore, Md. : 1950)*, *150*(10), 4659–71.

Oeckinghaus, A., & Ghosh, S. (2009). The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harbor Perspectives in Biology*, *1*(4), a000034. doi:10.1101/cshperspect.a000034

Oeckinghaus, A., Hayden, M. S., & Ghosh, S. (2011). Crosstalk in NF-κB signaling pathways. *Nature Immunology*, *12*(8), 695–708. doi:10.1038/ni.2065

Ott, J. J., Stevens, G. a, Groeger, J., & Wiersma, S. T. (2012). Global epidemiology of hepatitis B virus infection: new estimates of age-specific HBsAg seroprevalence and endemicity. *Vaccine*, *30*(12), 2212–9. doi:10.1016/j.vaccine.2011.12.116

Ozturk, M., Arslan-Ergul, A., Bagislar, S., Senturk, S., & Yuzugullu, H. (2009). Senescence and immortality in hepatocellular carcinoma. *Cancer Letters*, *286*(1), 103–13. doi:10.1016/j.canlet.2008.10.048

Pang, R., Lee, T. K. W., Poon, R. T. P., Fan, S. T., Wong, K. B., Kwong, Y.-L., & Tse, E. (2007). Pin1 interacts with a specific serine-proline motif of hepatitis B virus X-protein to enhance hepatocarcinogenesis. *Gastroenterology*, *132*(3), 1088–103. doi:10.1053/j.gastro.2006.12.030

Papa, S., Zazzeroni, F., Pham, C. G., Bubici, C., & Franzoso, G. (2004). Linking JNK signaling to NF-kappaB: a key to survival. *Journal of Cell Science*, *117*(Pt 22), 5197–208. doi:10.1242/jcs.01483

Park, S. G., Chung, C., Kang, H., Kim, J.-Y., & Jung, G. (2006). Up-regulation of cyclin D1 by HBx is mediated by NF-kappaB2/BCL3 complex through kappaB site of cyclin D1 promoter. *The Journal of Biological Chemistry*, *281*(42), 31770–7. doi:10.1074/jbc.M603194200

Parkin, D. M., Bray, F., Ferlay, J., & Pisani, P. (2002). Global cancer statistics, 2002. *CA: A Cancer Journal for Clinicians*, *55*(2), 74–108.

Paterlini, P., Poussin, K., Kew, M., Franco, D., & Brechot, C. (1995). Selective accumulation of the X transcript of hepatitis B virus in patients negative for hepatitis B surface antigen with hepatocellular carcinoma. *Hepatology (Baltimore, Md.)*, *21*, 313–321. doi:S0270913995000449 [pii]

Prasad, S., Ravindran, J., & Aggarwal, B. B. (2010). NF-kappaB and cancer: how intimate is this relationship. *Molecular and Cellular Biochemistry, 336*(1-2), 25–37. doi:10.1007/s11010-009-0267-2

Qiao, L., Leach, K., McKinstry, R., Gilfor, D., Yacoub, a, Park, J. S., … Dent, P. (2001). Hepatitis B virus X protein increases expression of p21(Cip-1/WAF1/MDA6) and p27(Kip-1) in primary mouse hepatocytes, leading to reduced cell cycle progression. *Hepatology (Baltimore, Md.), 34*(5), 906–17. doi:10.1053/jhep.2001.28886

Quétier, I., Brezillon, N., Duriez, M., Massinet, H., Giang, E., Ahodantin, J., … Kremsdorf, D. (2013). Hepatitis B virus HBx protein impairs liver regeneration through enhanced expression of IL-6 in transgenic mice. *Journal of Hepatology*, *59*(2), 285–91. doi:10.1016/j.jhep.2013.03.021

Ryo, A., Suizu, F., Yoshida, Y., Perrem, K., Liou, Y.-C., Wulf, G., … Lu, K. P. (2003). Regulation of NF-kappaB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. *Molecular Cell*, *12*(6), 1413–26.

Seki, E., & Brenner, D. A. (2007). The role of NF-kappaB in hepatocarcinogenesis: promoter or suppressor? *Journal of Hepatology*, *47*(2), 307–9. doi:10.1016/j.jhep.2007.05.006

Shin, H. D., Park, B. L., Kim, L. H., Jung, J. H., Kim, J. Y., Yoon, J. H., … Lee, H.-S. (2003). Interleukin 10 haplotype associated with increased risk of hepatocellular carcinoma. *Human Molecular Genetics*, *12*(8), 901–6. doi:10.1093/hmg/ddg104

Shukla, R., Yue, J., Siouda, M., Gheit, T., Hantz, O., Merle, P., … Sylla, B. S. (2011). Proinflammatory cytokine TNF-α increases the stability of hepatitis B virus X protein through NF-κB signaling. *Carcinogenesis*, *32*(7), 978–85. doi:10.1093/carcin/bgr057

Su, F., & Schneider, R. J. (1996). Hepatitis B virus HBx protein activates transcription factor NF-kappaB by acting on multiple cytoplasmic inhibitors of rel-related proteins. *Journal of Virology*, *70*(7), 4558–66.

Sun, B., & Karin, M. (2008). NF-kappaB signaling, liver disease and hepatoprotective agents. *Oncogene*, *27*(48), 6228–44. doi:10.1038/onc.2008.300

Szpakowski, J.-L., & Tucker, L.-Y. (2013). Causes of death in patients with hepatitis B: A natural history cohort study in the United States. *Hepatology*, *58*, 21–30. doi:10.1002/hep.26110

Tergaonkar, V., Pando, M., Vafa, O., Wahl, G., & Verma, I. (2002). p53 stabilization is decreased upon NFkappaB activation: a role for NFkappaB in acquisition of resistance to chemotherapy. *Cancer Cell*, *1*(5), 493–503.

Terradillos, O., Billet, O., Renard, C. a, Levy, R., Molina, T., Briand, P., & Buendia, M. a. (1997). The hepatitis B virus X gene potentiates c-myc-induced liver oncogenesis in transgenic mice. *Oncogene*, *14*(4), 395–404. doi:10.1038/sj.onc.1200850

Thomas, M., Pim, D., & Banks, L. (1999). The role of the E6-p53 interaction in the molecular pathogenesis of HPV. *Oncogene*, *18*, 7690–7700. doi:10.1038/sj.onc.1202953

Tian, Y., Yang, W., Song, J., Wu, Y., & Ni, B. (2013). Hepatitis B virus X protein-induced aberrant epigenetic modifications contributing to human hepatocellular carcinoma pathogenesis. *Molecular and Cellular Biology*, *33*(15), 2810–6. doi:10.1128/MCB.00205-13

Trinchieri, G. (2010). Type I interferon: friend or foe? *The Journal of Experimental Medicine*, *207*(10), 2053–63. doi:10.1084/jem.20101664

Truant, R., Antunovic, J., Greenblatt, J., Prives, C., & Cromlish, J. a. (1995). Direct interaction of the hepatitis B virus HBx protein with p53 leads to inhibition by HBx of p53 response element-directed transactivation. *Journal of Virology*, *69*(3), 1851–9.

Venook, A. P., Papandreou, C., Furuse, J., & de Guevara, L. L. (2010). The incidence and epidemiology of hepatocellular carcinoma: a global and regional perspective. *The Oncologist*, *15 Suppl 4*, 5–13. doi:10.1634/theoncologist.2010-S4-05

Vogel, C., & Marcotte, E. M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nature Reviews. Genetics*, *13*(4), 227–32. doi:10.1038/nrg3185

Wan, F., Anderson, D. E., Barnitz, R. a, Snow, A., Bidere, N., Zheng, L., … Lenardo, M. J. (2007). Ribosomal protein S3: a KH domain subunit in NF-kappaB complexes that mediates selective gene regulation. *Cell*, *131*(5), 927–39. doi:10.1016/j.cell.2007.10.009

Wan, F., & Lenardo, M. J. (2009). Specification of DNA binding activity of NF-kappaB proteins. *Cold Spring Harbor Perspectives in Biology*, *1*(4), a000067. doi:10.1101/cshperspect.a000067

Wang, J., Basagoudanavar, S. H., Wang, X., Hopewell, E., Albrecht, R., García-Sastre, A., … Beg, A. a. (2010). NF-kappa B RelA subunit is crucial for early IFN-beta expression and resistance to RNA virus replication. *Journal of Immunology (Baltimore, Md. : 1950)*, *185*(3), 1720–9. doi:10.4049/jimmunol.1000114

Wang, J. J., Zheng, Y., Sun, L., Wang, L., Yu, P. B., Dong, J. H., … Ren, Y. C. (2011). TP53 codon 72 polymorphism and colorectal cancer susceptibility: A meta-analysis. *Molecular Biology Reports*, *38*, 4847–4853. doi:10.1007/s11033-010-0619-8

Wang, X. W., Forrester, K., Yeh, H., Feitelson, M. a, Gu, J. R., & Harris, C. C. (1994). Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and

association with transcription factor ERCC3. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(6), 2230–4.

Ward, J. W., & Averhoff, F. (2011). Refugees, mass casualties, and hepatitis B transmission. *The Journal of Infectious Diseases*, *204*(3), 338–9. doi:10.1093/infdis/jir371

Wei, C., Ni, C., Song, T., Liu, Y., Yang, X., Zheng, Z., … Zhong, H. (2010). The hepatitis B virus X protein disrupts innate immunity by downregulating mitochondrial antiviral signaling protein. *Journal of Immunology (Baltimore, Md. : 1950)*, *185*(2), 1158–68. doi:10.4049/jimmunol.0903874

Wei, Y., Neuveut, C., Tiollais, P., & Buendia, M. (2010). Molecular biology of the hepatitis B virus and role of the X gene. *Pathologie-Biologie*, *58*(4), 267–72. doi:10.1016/j.patbio.2010.03.005

Yan, H., Peng, B., Liu, Y., Xu, G., He, W., Ren, B., … Li, W. (2014). Viral entry of hepatitis B and D viruses and bile salts transportation share common molecular determinants on sodium taurocholate cotransporting polypeptide. *Journal of Virology*, *88*(6), 3273–84. doi:10.1128/JVI.03478-13

Yang, H.-I., Lu, S.-N., Liaw, Y.-F., You, S.-L., Sun, C.-A., Wang, L.-Y., … Chen, C.-J. (2002). Hepatitis B e antigen and the risk of hepatocellular carcinoma. *The New England Journal of Medicine*, *347*(3), 168–74. doi:10.1056/NEJMoa013215

Yano, M., Ohkoshi, S., Aoki, Y., Takahashi, H., Kurita, S., Yamazaki, K., … Aoyagi, Y. (2013). Hepatitis B virus X induces cell proliferation in the hepatocarcinogenesis via up-regulation of cytoplasmic p21 expression. *Liver International : Official Journal of the International Association for the Study of the Liver*, *33*(8), 1218–29. doi:10.1111/liv.12176

You, Z., Madrid, L. V, Saims, D., Sedivy, J., & Wang, C.-Y. (2002). c-Myc sensitizes cells to tumor necrosis factor-mediated apoptosis by inhibiting nuclear factor kappa B transactivation. *The Journal of Biological Chemistry*, *277*(39), 36671–7. doi:10.1074/jbc.M203213200

Zhang, J., Zou, S., & Giulivi, a. (2001). Epidemiology of hepatitis B in Canada. *The Canadian Journal of Infectious Diseases = Journal Canadien Des Maladies Infectieuses*, *12*(6), 345–50.

Zhang, T., Xie, N., He, W., Liu, R., Lei, Y., Chen, Y., … Wei, Y. (2013). An integrated proteomics and bioinformatics analyses of hepatitis B virus X interacting proteins and identification of a novel interactor apoA-I. *Journal of Proteomics*, *84*, 92–105. doi:10.1016/j.jprot.2013.03.028

Zhou, Y., Wang, S., Ma, J.-W., Lei, Z., Zhu, H.-F., Lei, P., … Huang, B. (2010). Hepatitis B virus protein X-induced expression of the CXC chemokine IP-10 is mediated through

activation of NF-kappaB and increases migration of leukocytes. *The Journal of Biological Chemistry*, *285*(16), 12159–68. doi:10.1074/jbc.M109.067629