HEPATITIS B VIRUS X PROTEIN INDUCES CELLULAR SENESCENCE AND AUTOPHAGY

by

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Abstract

Hepatitis B virus (HBV) is a significant global threat to human health due to its ability to cause chronic infections that can lead to hepatocellular carcinoma (HCC). While the process by which HBV increases the risk of HCC is unclear, evidence suggests that the hepatitis B X protein (HBx) may be a contributing factor. Cellular senescence is an important barrier to tumorigenesis, blocking the proliferation of cells that harbor excessive DNA damage or contain activated oncogenes. Autophagy is a non-proteasomal degradative pathway used by cells to recycle cytoplasmic contents under periods of nutrient starvation. This pathway is induced in response to a wide range of cellular stress factors, and has also been characterized as an effector mechanism for the establishment of cellular senescence. In this study, retroviral transduction of HepG2 cells with HBx resulted in the induction of cellular senescence and autophagy. The mechanism by which HBx can induce senescence is unclear. However, an increase in the accumulation of DNA damage was observed. HBx did not modulate the levels of the anti-apoptotic proteins Bcl-2, Bcl-xL, or Mcl-1, which can inhibit autophagy through interactions with the autophagy regulator Beclin 1. As well, the activity and phosphorylation status of JNK/SAPK, an inducer of autophagy via Bcl-2 phosphorylation, was unchanged. These results suggest that senescence may act as a barrier to HBx-induced oncogenesis, and may offer some explanation as to why HBx does not function as a more potent oncogene. Also, we propose that HBx modulates autophagy through a mechanism other than Bcl-2 phosphorylation or expression over the time course of this study.

List of Abbreviations and Symbols Used

3MA	3-methoxyamphetamine
53BP1	p53 binding protein 1
Ambra-1	activating molecule in Beclin-1-regulated autophagy-1
AMPO	amphotropic
ASPP	apoptosis stimulating protein of p53
ATCC	American Type Culture Collection
ATF3	activating transcription factor 3
Atg	autophagy-related
ATM	ataxia telangiectasia mutated
ATRA	all *trans* retinoic acid
Baf A1	Bafilomycin A1
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BH	Bcl-2 homology domain
Bif-1	Bax-interacting factor-1
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
bZip	basic leucine zipper
C/EBPα	CCAAT-enhancer-binding protein α
$CaCl_2$	calcium chloride
°C	Celsius
cccDNA	covalently closed circular DNA
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CHK2	checkpoint kinase 2
CMA	chaperone mediated autophagy
CpG	cytosine methylated prior to guanosine
CREB	cAMP responsive element binding
CUL4	cullin ubiquitin ligase 4
DDB1	DNA damage binding protein 1
DDR	DNA damage response
DHBV	duck hepatitis B virus
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
Dox	doxycycline
DTT	dithiothreitol
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
eIF2α	eukaryotic initiation factor-2 α
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase

FCS	fetal calf serum
GAPDH	glyceraldehydes 3-phosphate dehydrogenase
GCN2	general control nonderepressible-2
GFP	green fluorescent protein
H_2O_2	hydrogen peroxide
HBeAg	hepatitis B e antigen
HBSS	Hanks balanced salt solution
HBV	hepatitis B virus
HBx	hepatitis B virus X protein
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMGA2	high-mobility group A protein-2
HRP	horseradish peroxidase
HSV-1	herpes simplex virus 1
IκB	inhibitor of kappa B
ICER	induced cAMP early expressor
IFN	interferon
IGF-II	insulin-like growth factor II
IgG	immunoglobulin G
IL	interleukin
Jak	Janus activated kinase
JNK	c-Jun N-terminal protein kinase
KSHV	Kaposi's sarcoma-associated herpesvirus
LB	lysogeny broth
LC3	microtubule-associated protein light chain 3
MAPK	mitogen-activated protein kinase
Mcl-1	myeloid leukemia cell 1
MeOH	methanol
MHC	major histocompatibility complex
mL	milliliter
μg	microgram
γ-HV68	murine γ herpesvirus 68
μL	microliter
MMULV	Moloney Murine Leukemia Virus
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
Na_3VO_4	sodium orthovanadate
NaCl	sodium chloride
NaF	sodium fluoride
$NaHPO_4$	sodium phosphate
NF-κB	nuclear factor kappa B
NP-40	nonyl phenoxypolyethoxylethanol
ORF	open reading frame
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline

PBS-T	PBS with 0.1% Tween 20
PCNA	proliferating cell nuclear antigen
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
pgRNA	pre-genomic RNA
PI3K	phosphatidylinositol-3 kinase
PIP3	phosphatidylinositol-3 phosphate
PKB	protein kinase B
PKC	protein kinase C
PKR	protein kinase RNA-activated
PMSF	phenylmethanesulfonyl fluoride
polybrene	hexadimethrine bromide
PRR	pattern recognition receptors
PTEN	phosphatase and tensin homolog
puro	puromycin
RARβ2	retinoic acid receptor β2
Ras	rat sarcoma
rcDNA	relaxed circular DNA
RLU	relative light units
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
rpm	rotations per minute
RT	reverse transcriptase
RT-PCR	reverse transcriptase PCR
S.A. β-Gal	senescence-associated β-galactosidase
SAHF	senescence-associated heterochromatin foci
SAPK	stress-activated protein kinase
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
siRNA	short interfering RNA
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptors
Src	sarcoma
STAT	signal transducers and activators of transcription
TE	Tris-EDTA
TF	transcription factor
TGF-β	transforming growth factor β
TLR	Toll-like receptor
TNF-α	tumor-necrosis factor α
Tris HCl	tris(hydroxymethyl)aminomethane hydrochloric acid
Ulk	unc51-like kinase
UPR	unfolded protein response
UV	ultraviolet radiation
UVRAG	UV radiation resistance associated gene

V	volts
VPS	vacuolar protein sorting
WHV	woodchuck hepatitis virus
WHx	woodchuck hepatitis virus X protein
X-gal	bromo-chloro-indolyl-galactopyranoside
XPB	xeroderma pigmentosum B
XPD	xeroderma pigmentosum D

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Chapter 1: Introduction

1.1 Cellular Senescence

Cellular senescence is a form of permanent cell cycle arrest, characterized by heterochromatin modifications to the host genome and radical changes in gene expression that results in what is now termed the senescence phenotype [reviewed in (Campisi and d'Adda di Fagagna, 2007; Kuilman et al., 2010; Pazolli and Stewart, 2008)]. First reported by Dr. Leonard Hayflick in 1965 as an observation that cultured primary cells failed to divide indefinitely *in vitro*, senescence is now recognized as a response to many cellular perturbations, including telomere shortening, excessive DNA damage, and oncogenic replicative stress. Senescence is understood to have both beneficial and detrimental effects, acting as a potent barrier to the development of cancers, but also interfering with tissue regeneration. While there have been few studies examining whether viruses might modulate or otherwise affect the senescence response of their cellular hosts, this is now emerging as an intriguing possibility.

1.1.1 Inducers of Senescence

As noted previously, senescence was initially characterized as a failure of primary cells to grow indefinitely in culture, which is now termed replicative senescence and is the result of shortened telomere signaling (Herbig et al., 2004). Telomeres are repeating segments of DNA found at the ends of chromosomes that cannot be completely replicated during cellular division, leading to a gradual decay over multiple cell cycles (Harley, Futcher, and Greider, 1990). This process is counteracted in some cells by telomerases, enzymes that can extend telomeres by reverse transcription from an RNA template

[reviewed in (Collins and Mitchell, 2002)]. Critically shortened telomeres induce senescence through a DNA damage response (DDR) mechanism (d'Adda di Fagagna et al., 2003; Herbig et al., 2004). While cells will have multiple telomeres (2 per chromosome), it requires only one or a few critically shortened telomeres to induce cellular senescence (Hemann et al., 2001).

Senescence is induced by DNA damage, particularly when the damage is in the form of DNA double stranded breaks (Di Leonardo et al., 1994; Gire et al., 2004). This process is understood to occur in a p53-dependent manner, with p53 transcriptionally up-regulating $p21^{WAF1}$, an inhibitor of the cell cycle. Cells that have senesced due to DNA damage often maintain DNA damage foci composed of many proteins, including ataxia-telangiectasia mutated (ATM), checkpoint kinase 2 (CHK2), and p53 binding protein 1 (53BP1), over long periods of time [reviewed in (Campisi and d'Adda di Fagagna, 2007)]. The mechanism by which cells determine whether to continue with DNA damage repair or commit to senescence still remains unclear.

Cellular senescence can also be triggered in response to excessive mitotic stress in the form of oncogene expression, a process now known as oncogene-induced senescence. This response was first observed via over-expression of oncogenic *ras* (Serrano et al., 1997), but has subsequently been observed with other pro-proliferation proteins, such as E2F1 (Dimri et al., 2000). The mechanism by which excessive mitotic signaling triggers senescence is unclear, but may involve activation of the cell cycle inhibitor $p16^{INK4a}$. It has also been suggested that oncogene-induced senescence signaling involves the DDR, as interference with DDR prevents senescence in these experiments (Bartkova et al., 2006; Di Micco et al., 2006). Other factors that have been shown to induce senescence

include experimental treatments that interfere with chromatin regulation (Bandyopadhyay et al., 2002; Ogryzko et al., 1996), chronic interferon-β (IFN-β) or transforming growth factor-β (TGF-β) signaling (Moiseeva et al., 2006; Vijayachandra, Lee, and Glick, 2003), and improper *in vitro* cell culturing conditions (Ramirez et al., 2001).

1.1.2 Molecular Mechanisms and Markers of Senescence

The senescence phenotype is characterized by cell cycle arrest, resistance to certain apoptotic stimuli, and changes in gene expression, including the secretion of pro-inflammatory factors [reviewed in (Campisi and d'Adda di Fagagna, 2007; Kuilman et al., 2010)]. However, each of these responses is somewhat cell-type dependent, as variations in responses to apoptotic signals, arrested cellular DNA content, and gene expression, particularly regarding $p16^{INK4a}$, have all been reported. The mechanism by which senescence occurs is also cell-type dependent, however, it generally involves the engagement of p53 and expression of $p21^{WAF1}$, followed by delayed expression of $p16^{INK4a}$ (see Fig. 1.1). Senescence induced by DDR involves activation of p53 and expression of $p21^{WAF1}$, leading to cell cycle arrest (Di Leonardo et al., 1994). However, multiple studies have shown that experimental inhibition of p53 or $p21^{WAF1}$ can lead to re-activation of the cell cycle and continued proliferation after the induction of p53-dependent senescence in some circumstances (Beauséjour et al., 2003; Brown, Wei, and Sedivy, 1997). Activation of $p21^{WAF1}$ commonly leads to the delayed activation of $p16^{INK4a}$ expression (Stein et al., 1999). $p16^{INK4a}$ can also be up-regulated in a p53-independent fashion, and is commonly expressed during the establishment of oncogene induced senescence, although the mechanism by which this occurs is currently poorly understood. Expression of $p16^{INK4a}$ leads to the development of senescence-associated

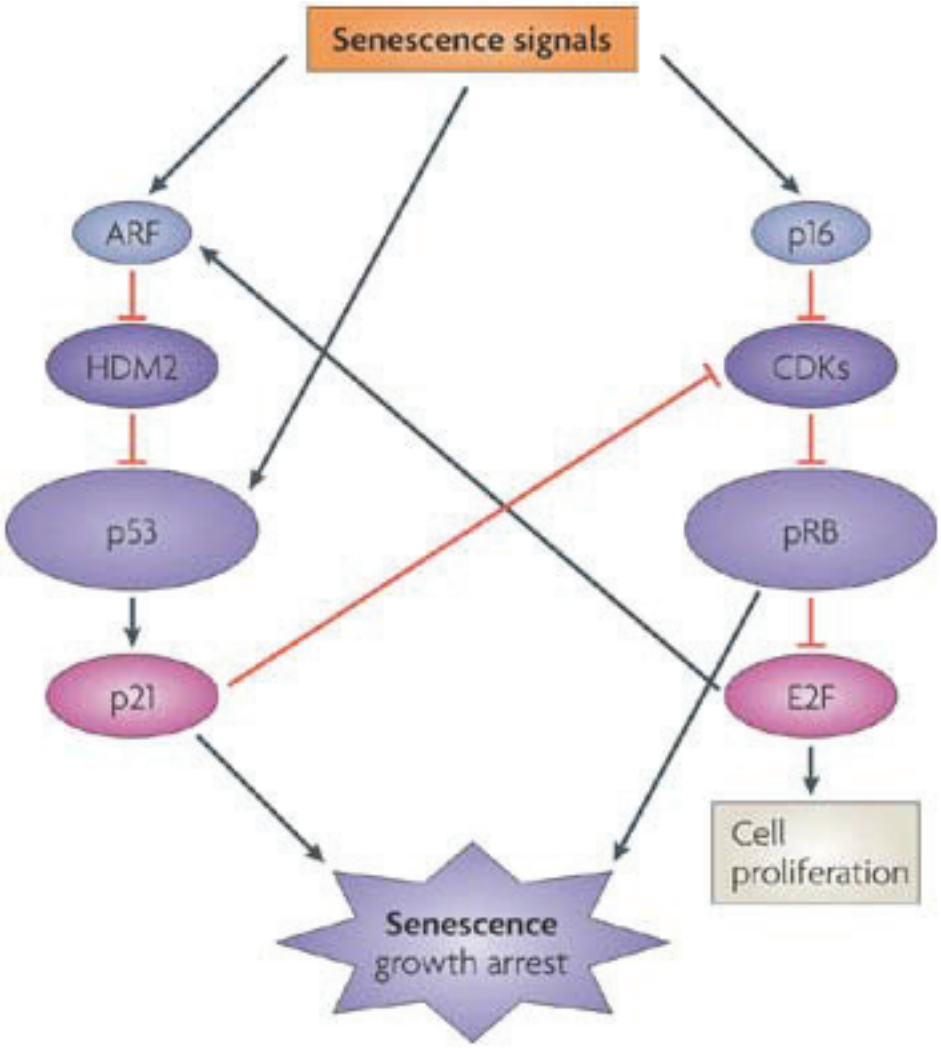

Figure 1.1 - Overview of Signaling Pathways Leading to Senescence - Senescence signaling activates the p53 and $p16^{INK4a}$ signaling pathways. DNA-damage response (DDR) signaling often initially activates p53, while oncogene induced senescence activates p16INK4a. p16INK4a activation can be delayed during the induction of senescence. p21WAF1 expression can independently activate RB through its effects on cyclin-dependent kinases. Reprinted by permission from Macmillan Publishers Ltd.: Nature Reviews Molecular Cell Biology, Vol. 8 Iss. 9, Campisi, J., d'Adda di Fagagna, F., Cellular senescence; when bad things happen to good cells, Copyright 2007.

heterochromatin foci (SAHFs) that silence the expression of multiple genes involved in cell cycle progression (Narita et al., 2003). Once established, SAHFs do not require the continued expression of $p16^{INK4a}$; this form of cell cycle arrest is not experimentally reversible (Beauséjour et al., 2003). Both $p21^{WAF1}$ and $p16^{INK4a}$ inhibit cell cycle progression by maintaining retinoblastoma (RB) in a hypo-phosphorylated state, which inhibits the E2F family of transcription factors from transcribing factors necessary for cell cycle progression. Senescence can be induced by activation of either $p21^{WAF1}$ or $p16^{INK4a}$, depending on the stimuli and cell type, and there have been isolated reports that senescence may be inducible by processes independent of both pathways [reviewed in (Campisi and d'Adda di Fagagna, 2007)].

Apart from a general lack of proliferation, multiple different markers are associated with cellular senescence; however, most develop with delayed kinetics, often first appearing several days after the initial cell cycle arrest. Senescence-associated β–galactosidase staining is one such marker, and is associated with an increase in lysosomal activity that is commonly observed within senescent cells (Dimri et al., 1995). Detection of SAHFs or persistent DNA damage foci, through the use of dyes or by immunostaining, can also be evidence of senescence. Changes in gene expression are also commonly used as markers of senescence. Prolonged increases in p53, $p21^{WAF1}$, and/or, $p16^{INK4a}$, maintenance of pRB in a hypo-phosphorylated state, and decreases in proteins associated with proliferation, including cyclin A and B, and proliferating cell nuclear antigen (PCNA), are all highly indicative of a senescence state [reviewed in (Kuilman et al., 2010)].

1.1.3 Senescence and Carcinogenesis

Senescence has been widely suggested to act as a barrier to the development of cancer, preventing cells with significant DNA damage or excessive mitogenic signaling from proliferating. There is some experimental data suggesting that transgenic mice that have blunted senescence responses have increased rates of cancer (Braig et al., 2005; Chen et al., 2005). However, avoidance of senescence alone is insufficient for the malignant transformation of cells, as additional genomic mutations are required. It has also been suggested that senescence may in fact increase the risk of certain cancers due to increased secretion of inflammatory factors [reviewed in (Pazolli and Stewart, 2008)]. Senescence has a complex inter-relationship with apoptosis, as interference with apoptosis causes some cells to senesce, and likewise interference with senescence can trigger increases in apoptosis (Rebbaa et al., 2003; Seluanov et al., 2001). It is unclear how cells determine when to senesce and when to undergo apoptosis, but the difference appears to be at least partially cell-type dependent.

1.2 Macroautophagy

Macroautophagy (hereafter referred to as autophagy) is a tightly regulated and evolutionarily conserved mechanism for the sequestration, lysosomal degradation, and recycling of discrete intracellular portions of eukaryotic cells. It facilitates the removal of materials not typically degraded by the ubiquitin-proteasomal pathway. Microautophagy, on the other hand, is a degradative process that occurs through invaginations at the lysosomal membrane. Chaperone mediated autophagy (CMA) is another pathway where targeted proteins are unfolded and shuttled directly through the lysosomal membrane. These last two processes reviewed elsewhere (Cuervo, 2004; Dice, 2007; Yang et al.,

2005) and will not be discussed here. Regulators of autophagy include hormones and growth factors that suppress the process during cellular growth, and it is also responsive to intracellular levels of nutrients, oxygen, and energy. The pathway acts as a defense mechanism against inducers of cellular stress (Pattingre et al., 2008; Wullschleger, Loewith, and Hall, 2006; Yang et al., 2005). Perturbations in autophagy have been correlated with numerous pathological conditions, including oncogenesis and cancer progression, neurodegenerative disorders, liver disease, myopathy, and cardiac disease (Levine and Kroemer, 2008; Meijer and Codogno, 2006; Mizushima et al., 2008). Autophagy has also been shown to play an important role in the pathogenesis of several viral infections and has been suggested to act as both an inducer and effector of innate and adaptive immune responses against intracellular pathogens. (Portions of the autophagy section of the introduction were previously published in: Lin, L.-T., P. W. H. Dawson, and C. D. Richardson. 2010. Viral interactions with macroautophagy: a double-edged sword. Virology 402:1-10.)

1.2.1 Molecular Mechanisms of Autophagy

The functional core of autophagy is the *de novo* synthesis of a double membrane-bound vesicle capable of fusing with an endosome or lysosome, which leads to the catabolic degradation of its encapsulated contents [reviewed in (Longatti and Tooze, 2009; Xie and Klionsky, 2007; Yang et al., 2005; Yoshimori and Noda, 2008)]. In mammals, this process begins with the expansion of a small, flat membrane sac of uncertain origin (termed the isolation membrane or phagophore). As autophagy-related (Atg) proteins are recruited to its surface, this membrane sac elongates and curves until the ends merge to form a double-membrane-bound vesicle (autophagosome). Atg

proteins associated with the outer membrane are then recovered or disassociate, and the completed structure fuses with an either an endosome (amphisome), or lysosome (autolysosome). An overview of this process is provided in Figure 1.2. Most of the currently identified molecular components necessary for this process can be roughly categorized into one of five groupings based upon their co-interactions; the Ulk1 (Atg 1) serine/threonine kinase complex, transmembrane mAtg 9 and interacting proteins, the Beclin 1 (Atg 6) – hVps34 complex, or one of two ubiquitin-like conjugation systems involving either Atg 12 or LC3 (Atg 8). While these groupings will be presented as functioning in a sequential fashion, it is important to note that complex interrelationships exist, and functions may occur in tandem rather than as part of a linear progression (Longatti and Tooze, 2009). An overview of proteins important in autophagosome formation and the regulation of autophagy are presented in Table 1.1.

While the initial stages of autophagosome formation within mammalian cells is an area of ongoing research, the process initially requires the activities of unc51-like kinase (Ulk1), or in some circumstances its paralog Ulk2, which are both homologs for the yeast protein Atg1 (Chan et al., 2009). During initiation Ulk1 will form a protein complex that recruits other Atg proteins to the nascent phagophore, which simultaneously affects the intracellular cycling of mAtg9, a transmembrane protein that is understood to be involved in lipid transport and protein recycling with the expanding membrane (Webber, Young, and Tooze, 2007). The exact source of lipids used in this expansion process remains controversial, with the endoplasmic reticulum, plasma membrane, and mitochondria all suggested to be potential lipid donors. The mammalian target of rapamycin (mTOR)

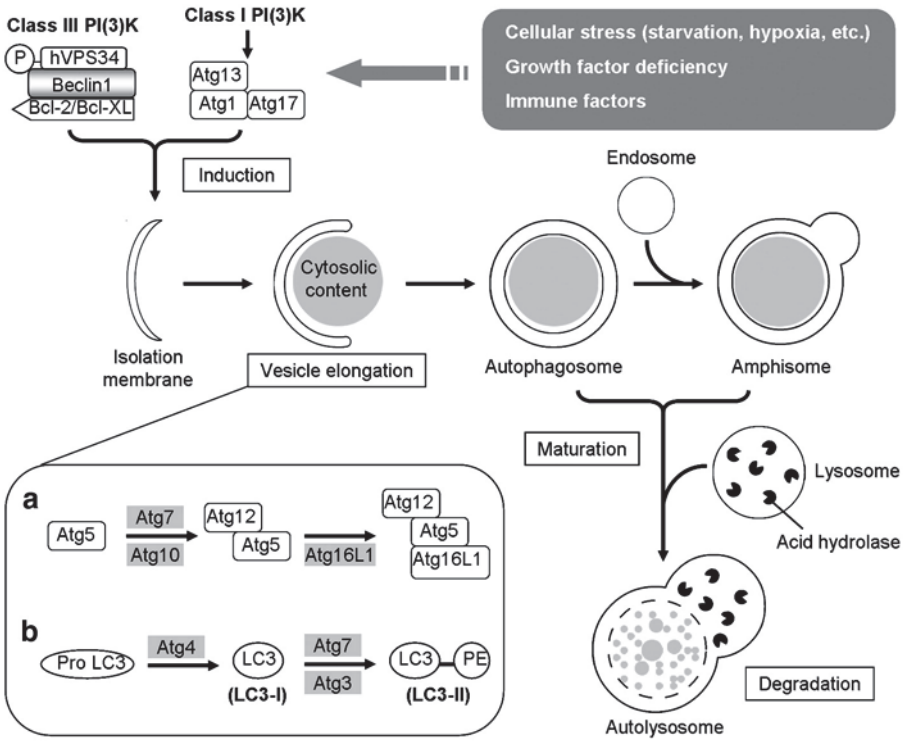

Figure 1.2 - Overview of the Autophagy Process - In response to cellular stimuli such as starvation and immune signals, the class I PI(3)K (phosphoinositide 3-kinases)-induced Atg1 complex and a class III PI(3)K complex involving Beclin-1 activate downstream ATG proteins in a series of steps that guide the induction, elongation, maturation, and degradation of the autophagosome. Two ubiquitin-like conjugation systems involving Atg12 (a) and LC3 (b) direct the vesicle elongation of the isolation membrane, which forms a crescent shape to sequester the cytoplasmic cargo. Upon completion, the autophagosome then undergoes the maturation step through a series of remodeling processes including fusion with endosomes/lysosomes. Fusion with lysosome helps the autophagosome mature into an autolysosome in which the autophagic vacuole along with its content is degraded. Reprinted from Virology, Vol. 402, Lin, L., Dawson, P., and Richardson, C., Viral interactions with macroautophagy: A double-edged sword, Pg. 1-10, Copyright (2010), with permission from Elsevier.

Table 1.1 - Significant Genes in the Mammalian Autophagy Pathway - Reprinted from Virology, Vol. 402, Lin, L., Dawson, P., and Richardson, C., Viral interactions with macroautophagy: A double-edged sword, pg. 1-10, Copyright (2010), with permission from Elsevier.

Gene	Important Interactions	Protein Function / Chacteristics
Formation of Autophagosomes		
ULK1 (ATG1)	Atg13, FIP200 (Atg17)	Ser/Thr kinase activity important for function; target(s) unknown. Downstream of mTOR signalling. Potentially involved in Atg9 cycling.
Beclin-1 (ATG6)	hVps34, Bcl-2/ Bcl-xL, UVRAG	Structural regulator of class III PI3 kinase hVps34. Contains BH3-like domain that is downregulatory when occupied.
hVPS34	Beclin-1, mTOR	Class III PI3 kinase; resulting PtdIns(3)Ps recruit Atg16L multimer/Atg18 to phagophore. Conflictingly activates mTor in response to amino acids.
ATG9	Atg2, Atg18	Transmembrane protein. Transits between phagophores and trans-golgi/late endosomes. Possible role(s) in protein recycling and/or membrane transit.
ATG12	Atg5, Atg16L	Covalently bound to Atg5 via mechanism similar to ubquitination.
ATG7	LC3, Atg12	Functionally similar to E1 ubiquitin activating enzyme (E1-like). Activates C-terminal glycine of both Atg12 and LC3.
ATG10	Atg12, Atg5	Functionally similar to E2 ubiquitin conjugating enzyme (E2-like). Accepts activated Atg12 and conjugates to internal lysine of Atg5.
ATG5	Atg12, Atg16L	Covalently bound to Atg12; conjugation allows Atg5 to associate with Atg16L.
ATG16L	Atg5-Atg12	Associates with Atg12-Atg5 and dimerizes. Present on outer surface of expanding phagophore; aids membrane curvature and LC3 recruitment (E3-like). Recycled.
ATG4	LC3	Cysteine protease; exposes C-terminal glycine on LC3 prior to lipidation. Subsequently recycles LC3 from outer membrane of autophagosome.
ATG3	LC3, Atg7	Functionally similar to E2 ubiquitin conjugating enzyme (E2-like). Conjugates LC3 with phosphatidylethanolamine (PE) phospholipid.
MAP1LC3 (ATG8)	Atg4	Experimental marker of induction. Cytosolic form (LC3-I) conjugated to PE, becoming membrane-associated (LC3-II). Possible role(s) in membrane expansion, autophagosome transit, and lysosomal fusion. Partially recycled by Atg4.
Regulation of Autophagy		
PI3K (class I)		Produces PtdIns(3)p that activate the Akt/PKB-mTor pathway.
PTEN		Phosphatase that counteracts PI3K by dephosphorylating PtdIns(3)p.
AKT/PKB	PDK1, Tsc 2	Ser/Thr kinase. Activated by PDK1 in presence of PtdIns(3)p. Inactivates Tsc 2.
REDD1/REDD2		Transcriptionally up regulated in response to hypoxia. Inactivates mTor pathway.
AMPK	LKB1, Tsc2	Activates Tsc2, leading to the induction of autophagy when the AMP/ATP ratio is high.
TSC2	Tsc1, Rheb, Akt/PKB, AMPK	GTPase-activating protein (GAP) with Tsc1; inactivates Rheb. Akt/PKB interferes with function, as does Erk1/2. AMPK enhances activity.
Rheb	Tsc1/Tsc2, mTor	Small GTPase. Activates mTor via binding kinase domain in GTP-dependent fashion. Tsc1/Tsc2 GAP activity converts to inactive, GDP-bound form.
mTOR	Rheb, raptor, mLST8	Key regulator of cellular growth. Autophagy induced when mTor inactivated. Ser/Thr kinase. Forms two protein complexes; mTORC1 associated with autophagy.
Anti-apoptotic Bcl-2 family	Beclin-1	Inhibit autophagy via binding with BH3 motif on Beclin-1. JNK1-mediated phosphorylation disrupts interaction and associated inhibition.
BH3-only Bcl-2 family	Anti-apoptotic Bcl-2 family	Competitively bind with anti-apoptotic Bcl-2 family members, interfering with their association with Beclin-1. Stimulate autophagy.
JNK1	Anti-apoptotic Bcl-2 family	Phosphorylates anti-apoptotic Bcl-2 family members, inhibiting interaction with Beclin-1. Activity induces autophagy.
UVRAG	Bif-1, Beclin-1	Interacts with Beclin-1's coiled-coil domain, strengthening Beclin-1/hVps34 interactions; promotes autophagy. Possible additional role in lysosome fusion.
p53		Controversial/contradictory role(s) in autophagy. P53-dependent autophagy observed experimentally. However, cytosolic p53 is inhibitory (mechanism unknown).
DRAM		Transmembrane lysosomal protein transcriptionally induced by p53. Stimulates autophagy. Necessary for both p53-dependent autophagy and apoptosis.

regulates Ulk1 through an uncharacterized mechanism that suppresses the initiation process.

After initiation, the structural regulator protein Beclin 1, a homolog of the yeast protein Atg6, is recruited to the expanding membrane. Beclin 1 regulates the activity of a class III phosphatidylinositol-3 kinase (PI3K), hVps34, through its interactions with multiple protein co-factors [reviewed in (Pattingre et al., 2008; Sinha and Levine, 2008)]. Once activated, hVps34 phosphorylates lipids in the phagophore membrane, which allows for the recruitment of additional proteins, such as the Atg16L complex. This complex is composed of the Atg16L protein and a covalently conjugated heterodimer of two proteins, Atg5 and Atg12, created through a novel ubiquitin-like conjugation system involving Atg 7 (E1-like) and Atg10 (E2-like). When fully assembled and associated with the phagophore membrane, the Atg16L complex functions as an E3-like protein in a second ubiquitin-like conjugation reaction involving the microtubule-associated protein light chain 3 (MAP1-LC3 or LC3), phosphatidylethanolamine (PE), Atg 7 (E1-like), and Atg 3 (E2-like). This lipidation localizes the LC3 protein to the expanding phagophore membrane. Functionally, LC3 is necessary for membrane elongation and vesicle closure, and may have a role in the microtubule-based transport of completed autophagosomes (Noda, Fujita, and Yoshimori, 2009; Tanida, Ueno, and Kominami, 2004).

Upon completion of the autophagosome most Atg proteins disassociate via an uncharacterized mechanism(s), which renders the vesicle capable of fusing with an endosome or lysosome. LC3 is an exception however, since only the accessible portion present on the outer membrane of the autophagosome is de-conjugated and recycled by the Atg 4 family of cysteine proteases. The maturation (fusion and degradation) of the

autophagosome is dependent upon components common to other endocytic trafficking pathways, including Rab GTPases and soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), however, the mechanism(s) that determines the specificity of this process are not currently well understood [reviewed in (Eskelinen, 2005; Noda, Fujita, and Yoshimori, 2009)].

1.2.2 Cellular Regulation of Autophagy

Central in the regulation of autophagy are two key proteins: mTOR and Beclin-1 (Pattingre et al., 2008; Sinha and Levine, 2008; Wullschleger, Loewith, and Hall, 2006). mTOR, a conserved serine/threonine kinase, is a component of protein complexes that integrate cellular signals relating to growth factors, nutrient and energy status, and cellular stress (Wullschleger, Loewith, and Hall, 2006). Important activators of mTOR include the class I PI3K-Akt/PKB signaling pathway and high concentrations of specific amino acids; high AMP/ATP ratios and hypoxia inactivate this pathway (Arsham and Neufeld, 2006; Beugnet et al., 2003; Pattingre et al., 2008; Wullschleger, Loewith, and Hall, 2006). Activated mTOR suppresses autophagy, enhancing the accumulation of cellular bulk by limiting lysosomal digestion. Downstream of mTOR, Beclin-1 is at the heart of a regulatory complex for the class III PI3K hVps34, whose activity is essential during autophagosome formation. Activators, such as UV radiation resistance associated gene (UVRAG), Bax-interacting factor-1 (Bif-1), and activating molecule in Beclin-1-regulated autophagy-1 (Ambra-1) associate with the Beclin-1 complex and enhance PtdIns(3)P production, while the Bcl-2 family anti-apoptotic proteins such as Bcl-2 and Bcl-xL bind to Beclin-1 and act in an inhibitory fashion (Pattingre et al., 2008; Pattingre et al., 2005; Sinha and Levine, 2008). The Bcl-2-related inhibition of autophagy is

abrogated by the stress-activated c-Jun N-terminal protein kinase 1 (JNK1) phosphorylation of Bcl-2 and Bcl-xL, as well as competition from other BH3 binding domain-containing proteins, providing one of several direct mechanistic links between autophagy and apoptosis (Sinha and Levine, 2008; Wei et al., 2008; Wei, Sinha, and Levine, 2008).

Many other cellular factors have been shown or are hypothesized to regulate autophagy, many of which have importance in viral infections. The eukaryotic initiation factor-2 alpha (eIF2α) and the starvation-responsive general control nonderepressible-2 (GCN2) eIF2α kinase are both indispensable for starvation-induced autophagy (Kouroku et al., 2007; Tallóczy et al., 2002; Tallóczy, Virgin, and Levine, 2006). Immune signaling molecules can modulate autophagy; type II interferon-γ (IFN-γ) and tumor-necrosis factor-α (TNF-α) are stimulatory, while the TH2-type cytokines interleukin-4 (IL-4) and IL-13 are suppressive (Deretic, 2005; Levine and Deretic, 2007). Certain pathogen-associated molecular patterns (PAMPs) trigger autophagy through their cognate pattern recognition receptors (PRRs), including Toll-like receptor 3 (TLR3), TLR4, and TLR7, although the molecular mechanism(s), physiological function(s), and range of PRRs that induce this pathway are all areas of continuing research (Delgado et al., 2009; Delgado and Deretic, 2009; Orvedahl and Levine, 2009). The p53 protein possesses a dual role in the regulation of autophagy dependent upon its localization; cytoplasmic p53 represses autophagy and must be degraded for autophagy to proceed, whereas nuclear p53 appears to induce it (Maiuri et al., 2009; Tasdemir et al., 2008). Many additional cellular factors, including extracellular signal-regulated kinase (Erk1/2) activation, intracellular release of calcium, increases in reactive oxygen species (ROS), and endoplasmic reticulum (ER)

stress have also been shown to trigger autophagy [reviewed in (Codogno and Meijer, 2005; Meijer and Codogno, 2004; Yang et al., 2005)].

1.2.3 Experimental Methods for Measuring Autophagy

The level of autophagy that is occurring in a given cell or lysate can be experimentally determined via multiple experimental approaches, including electron microscopy, LC3 lipidation (aggregation and modification), and protein degradation studies, amongst others. However, a few general cautions are advisable regarding these methods [reviewed in (Klionsky et al., 2008; Klionsky, Cuervo, and Seglen, 2007; Mizushima, 2004; Mizushima and Yoshimori, 2007)]. First, as autophagy is a process with numerous components, steps, and phases, it is important to clarify whether a given assay measures a step within this pathway (such as LC3 lipidation) or its overall physiological performance (the aim of protein degradation studies). Assays that measure the accumulation of autophagy proteins or structures, such as LC3 immunoblotting and LC3-GFP puncta formation, can be difficult to interpret, as an accumulation may be indicative of either an increase in the induction of the pathway, a blockage within the pathway prior to maturation, or a combination of these two possibilities. To resolve these difficulties, studies often employ inhibitors that block maturation (a similar accumulation in the both the presence and absence of the inhibitor is considered evidence of a block within the maturation process), or combine the results of multiple different experimental approaches. Second, autophagy is a responsive cellular process capable of significant fluctuation. Most assays capture this dynamic process at a single static moment in time. This can pose challenges, as cellular populations are frequently asynchronous, and viral proteins may have different effects on autophagy over time and at different levels of

expression. Finally, as different cell types have been shown to display differences in their autophagy responses, and is also a pathway frequently modified in transformed cells, caution should be used when comparing results between cell systems.

Our studies have relied upon immunoblot analysis of the levels of the autophagy marker protein LC3-II, a widely used and well-characterized assay for the measurement of this pathway. The LC3 protein is initially translated as pro-LC3, which is then immediately proteolytically cleaved by Atg4 cysteine proteases to become LC3-I, a cytosolic protein with a diffuse localization (Tanida, Ueno, and Kominami, 2004). New LC3-I is continuously being synthesized at a basal level, but is transcriptionally up-regulated upon the induction of autophagy (Tanida, Ueno, and Kominami, 2004). As mentioned previously, during phagophore expansion LC3-I is localized to the expanding membrane via lipid conjugation to PE. This conjugated form of the protein is referred to as LC3-II, and can be distinguished from LC3-I on immunoblots due to a downward mobility shift. LC3-II protein levels are closely correlated to the amount of autophagosome membrane present within a cell, and are therefore frequently employed as an indicator of autophagy [reviewed in (Klionsky et al., 2008; Klionsky, Cuervo, and Seglen, 2007; Mizushima, 2004)]. Upon maturation the portion of LC3-II accessible on the outer membrane of the autophagosome is converted back into LC3-I through the actions of Atg4. However, the portion of LC3-II on the inner membrane is subject to lysosomal degradation. In this way, LC3-II is both produced and degraded throughout autophagy, which can complicate its use as a marker. As alluded to earlier, either an induction of autophagosome formation or a block in their maturation will result in an accumulation of LC3-II. Therefore, it is advisable to use inhibitors of autophagosome

maturation or lysosomal protein degradation as a control within these experiments (Klionsky et al., 2008; Mizushima and Yoshimori, 2007). Furthermore, LC3-II levels can decline over time, particularly if there is interference with the transcription or translation of pro-LC3. While different approaches have been suggested for the interpretation of LC3 immunoblots, the most commonly accepted method is a direct comparison of LC3-II levels, normalized against a loading control. Comparison of the ratio of LC3-I to LC3-II in different treatments is no longer common in the scientific literature, as LC3-I is extremely labile under lysis conditions and not a reliable marker of the absence of autophagy signals. While β–actin is most commonly used as a loading control in LC3 immunoblots, other proteins, including GAPDH (Alirezaei et al., 2008; Chang et al., 2007; Jackson et al., 2005; Kyei et al., 2009; Panyasrivanit et al., 2009), have also been employed.

1.2.4 Role of Autophagy in Antiviral Immunity

Autophagy is widely recognized as an important defense against intracellular pathogens, and many viruses have been shown to evade, subvert, or exploit autophagy, seemingly to insure their own replication or survival advantage. Studies to date have suggested that while some viruses hijack autophagy as a mechanism of membrane genesis, many appear to suppress or inhibit this pathway. An overview of potential mechanisms by which autophagy may function in antiviral host defense is presented in Figure 1.3. While this section will provide a general overview, viral interactions with autophagy are extensively reviewed elsewhere (Deretic and Levine, 2009; Kirkegaard, Taylor, and Jackson, 2004; Lee and Iwasaki, 2008; Lin, Dawson, and Richardson, 2010; Orvedahl and Levine, 2008).

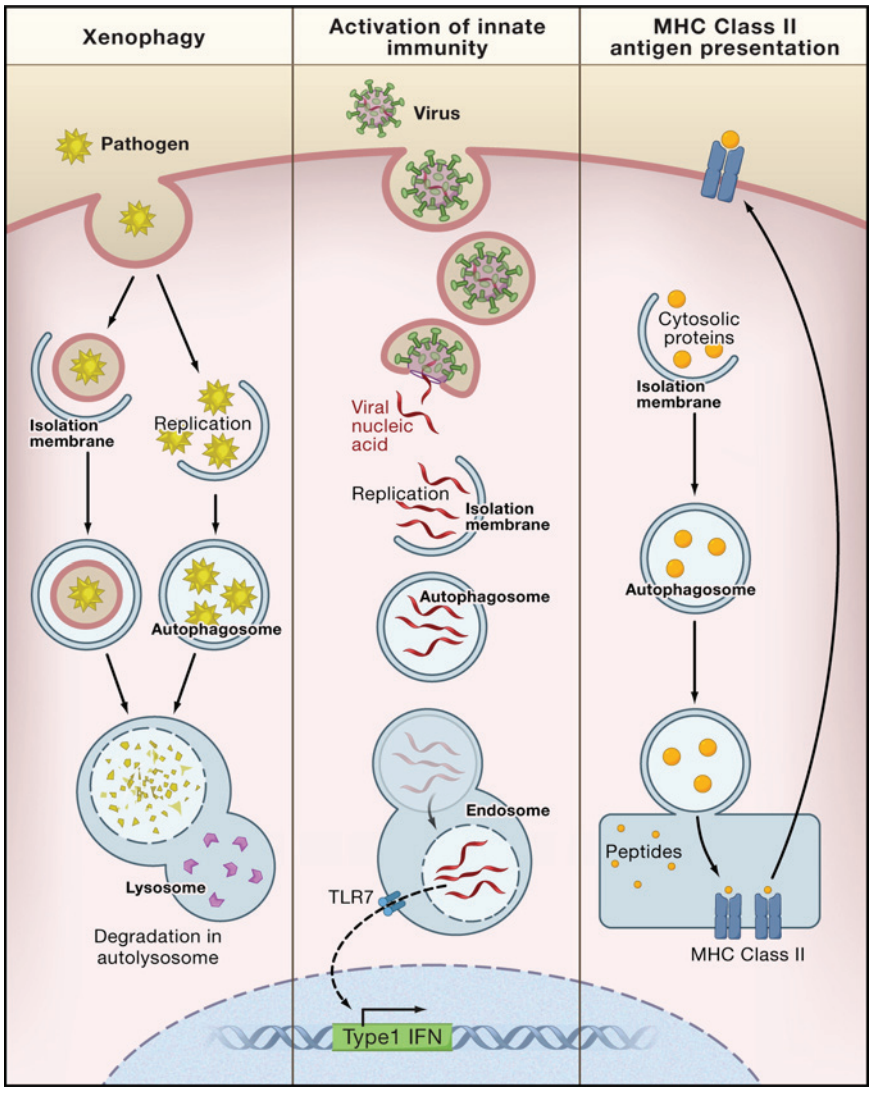

Figure 1.3 - Potential Roles of Autophagy in Host Defense - Autophagy has been suggested to act in host cellular defense via three mechanisms. First, autophagy may directly eliminate intracellular pathogens by sequestering them for lysosomal degradation, a process termed xenophagy. Second, autophagy may aid in the detection of pathogens by delivering PAMPs to endosomal compartments where they can be detected by toll-like receptors (TLRs), triggering innate immune responses.. This could result in a positive feedback mechanism for increased cytoplasmic sampling during times of possible infection. Third, autophagy has been suggested to allow endogenous antigens to be loaded into class II major histocompatibility complexes (MHC) by delivering cytoplasmic material to the lysosome for degradation, promoting adaptive immune responses. Reprinted from Cell, Vol. 132, Levine, B., Kroemer, G., Autophagy in the Pathogenesis of Disease, Pg. 27-42, Copyright (2008), with permission from Elsevier.

Multiple single stranded RNA viruses, including poliovirus, group B Coxsackieviruses, Dengue virus, and hepatitis C virus (HCV), exploit aspects of the autophagy pathway, presumably as a means of increasing intracellular membrane surfaces that will subsequently support viral replication complexes (Lin, Dawson, and Richardson, 2010). Poliovirus is often cited as the classic example of this type of modulation, as this virus induces the cellular accumulation of multi-membrane bound vesicles that contain the autophagy protein LC3 (Jackson et al., 2005; Taylor and Kirkegaard, 2007). Poliovirus has also been postulated to couple autophagy with vesicular secretory processes as a mechanism for viral egress. While these viruses appear to induce autophagy and are negatively affected by interference with this pathway, lysosomal degradation does not increase in all cases, suggesting that autophagosome maturation is incomplete or inhibited in these infections [reviewed in (Lin, Dawson, and Richardson, 2010)].

Autophagy can act as a defense against some intracellular pathogens by sequestering them within autophagosomes ultimately destined for lysosomal destruction. This process has been termed xenophagy (‘to eat what is foreign’), and is understood to be an important response to multiple bacterial pathogens, including *Mycobacterium tuberculosis* (Gutierrez et al., 2004), group A *Streptococcus* (Nakagawa et al., 2004), *Shigella flexneri* (Ogawa et al., 2005), *Legionella pneumophila* (Amer and Swanson, 2005), and *Yersinia pestis* (Pujol et al., 2009). Furthermore, many of these same bacteria possess mechanisms for avoiding being sequestered and degraded. Due to its importance with regards to bacterial pathogens, xenophagy has been widely speculated to also function in the control of viral pathogens (Lee and Iwasaki, 2008; Orvedahl and Levine,

2009). While multiple viruses have been shown to suppress autophagy, there has been little direct evidence of viral xenophagy. It has been reported that mutant herpes simplex virus 1 (HSV1) that lack ICP34.5, a gene that inhibits autophagy in addition to preventing PKR transcriptional repression, show an increased localization to autophagosomes (Alexander and Leib, 2008; Alexander et al., 2007; Tallóczy, Virgin, and Levine, 2006). However, the importance of ICP34.5's repression of autophagy has been disputed, with different effects observed *in vivo* and *in vitro* (Alexander et al., 2007; Orvedahl et al., 2007). Bacteria are often targeted by xenophagy through host driven modifications, such as ubiquitination, that then interact with adapter molecules that allow for their selective degradation [reviewed in (Fujita and Yoshimori, 2011)]. At this time, no comparable process has been demonstrated with a viral pathogen. Autophagy may still result in the degradation of virion or viral components in a non-specific manner, and autophagy is up-regulated in response to activation of a number of pattern recognition receptors (PRR), including toll-like receptors (TLRs) [reviewed in (Delgado et al., 2009; Delgado and Deretic, 2009; Orvedahl and Levine, 2009)]. However, I would argue that without a mechanism to target virion or viral components to the autophagosome, viral xenophagy is likely to be both an inefficient and ineffective response to viral infection.

Apart from xenophagy, autophagy may have other important antiviral effects. As a non-specific process, autophagy continuously samples and delivers cytoplasmic materials to lysosomes and endosomes, and may function as a form of cytoplasmic surveillance. This surveillance, through the delivery of cytoplasmic materials to late endosomes containing TLRs, is suggested to aid in the detection of viral pathogen-associated molecular patterns (PAMPs) (Delgado et al., 2009; Lin, Dawson, and

Richardson, 2010; Orvedahl and Levine, 2009). Detection of viral PAMPs then triggers antiviral host cell responses, such as the production of interferons (IFNs) and inflammatory cytokines. As the activation of multiple TLRs has been suggested to increase autophagy, detection of PAMPs might also result in a positive feedback loop, increasing cytoplasmic immunological surveillance at times when a pathogen is first detected. Autophagy has also been suggested to have a novel role in major histocompatibility complex (MHC) class II antigen presentation in professional antigen presenting cells. While MHC class II complexes typically display only exogenous antigens that have been internalized and lysosomally degraded, autophagy can deliver endogenous materials to the lysosome. In cells transformed with Epstein-Barr virus (EBV) (Paludan et al., 2005), or transduced with an LC3-tagged influenza matrix protein I (Schmid, Pypaert, and Münz, 2007), autophagy was observed to enhance antigen presentation, suggesting that endogenous antigens can be displayed in MHC class II complexes through this mechanism. These studies raise the exciting possibility that autophagy may be targeted as a mechanism of enhancing viral antigen presentation and vaccine performance (Schmid, Pypaert, and Münz, 2007).

Ongoing research is increasingly showing that viruses have developed mechanisms to subvert or suppress autophagy, and viral replication is frequently affected by experimental manipulation of autophagy levels. However, these viral modulators often have pleiotropic effects and affect other cellular processes, such as protein translation and apoptosis, which has presented a challenge in deciphering the importance of autophagy regulation amongst their other effects. One mechanism by which autophagy is frequently suppressed is interference with the autophagy regulator Beclin 1. HSV1 encodes a

protein, ICP34.5, that binds directly to Beclin 1 and inhibits autophagy, and this effect was essential for the virus's pathological effects *in vivo* (Orvedahl et al., 2007). Kaposi's sarcoma-associated herpesvirus (KSHV) has been shown to encode multiple proteins that modulate autophagy, including a viral Bcl-2 homolog (vBcl-2) (Pattingre et al., 2005), as does the murine γ herpesvirus 68 (γ–HV68) (Sinha et al., 2008). These vBcl-2 homologs lack the phosphorylation loop that regulates cellular Bcl-2 binding interaction with Beclin-1, rendering them resistant to regulatory modulation and potent inhibitors of autophagy and apoptosis.

1.2.5 Role of Autophagy in Cell Survival and Death

Autophagy is a highly conserved mechanism that enhances eukaryotic cell survival in response to multiple different cellular stressors and insults. The pathway was first characterized as a response to starvation, and it is now known that autophagy is directly regulated in part by changes in intracellular amino acids and energy levels (Codogno and Meijer, 2005). When faced with periods of starvation or low oxygen, autophagy allows for the catabolic digestion of a fraction of the cytoplasmic contents, providing resources necessary for the maintenance of life. While autophagy has been regarded as primarily a non-selective process, it can selectively remove damaged organelles, such as mitochondria and peroxisomes, which would otherwise be sources of cellular stress (Narendra et al., 2008). Autophagy has also been suggested to allow rapid cellular remodeling of cytoplasmic contents, which may be important in allowing cells to better respond to change (Cecconi and Levine, 2008). Transgenic mice that are deficient in autophagy typically die, either during later embryonic development, or during the neonatal transition period following birth where the mice must adapt to the loss of access

to the maternal nutrient supply [reviewed in (Cecconi and Levine, 2008; Levine and Yuan, 2005)].

However, it has also been theorized that autophagy can act as a form of programmed cellular death distinct from that of apoptosis, at least under certain conditions. In situations where apoptosis is experimentally inhibited, or when large numbers of cells are committed for removal (such as during development), cells will display the characteristics of what is termed type-II programmed cell death [reviewed in (Baehrecke, 2005; Codogno and Meijer, 2005; Maiuri et al., 2007b)]. These cells have excessive vacuolization with extensive organelle degradation, however, the cytoskeleton is maintained until late in the process, and DNA fragmentation is rarely observed. There is considerable debate regarding autophagy's role in cell death, which can best be summarized as a question of whether cell death occurs 'due' to autophagy, or 'with' autophagy. One piece of evidence supporting the position that autophagy has a role in programmed cell death is the observation that autophagy is important in the removal of cells during embryo development (Fimia et al., 2007; Qu et al., 2007). Also, unlike necrosis, type-II programmed cell death is not associated with inflammation, suggesting that autophagy in the absence of 'true' programmed cell death may fulfill a very similar role, limiting the damage normally associated with necrotic cell death when apoptosis is either inhibited or insufficient (Levine and Yuan, 2005). However, there is also evidence that autophagy is a pro-survival mechanism that remains active in dying cells. Phosphorylation of the anti-apoptotic Bcl-2 proteins at multiple sites will relieve their inhibition of both autophagy and apoptosis. However, in cells placed in starvation media, mono-phosphorylation was sufficient to trigger autophagy, but subsequent

phosphorylation events were necessary in order to induce apoptosis (Wei et al., 2008; Wei, Sinha, and Levine, 2008). This suggests that regulatory mechanisms that are shared by both apoptosis and autophagy may activate autophagy initially as an attempt to respond to the cellular stressor, and subsequently induce apoptosis if the stress is not resolved.

1.2.6 Autophagy and Tumorigenesis

Autophagy has a paradoxical relationship with tumorigenesis, with roles in both the prevention and progression of cancer [reviewed in (Brech et al., 2009; Levine, 2006; Morselli et al., 2009)]. There is considerable evidence that autophagy can function as a tumor suppressor. The autophagy regulatory gene Beclin 1 has been identified as a haplo-insufficient tumor suppressor gene that is monoallelically deleted in 40 to 75% of all sporadic breast, ovarian, and prostate cancers (Liang et al., 1999). Furthermore, transgenic mice that have only a single functional Beclin 1 gene have increased rates of spontaneous malignancies (Qu et al., 2003; Yue et al., 2003), as do mice that are deficient in Atg4c (Mariño et al., 2007). There is also a strong correlation between autophagy and the activities of various known tumor suppressors and oncogenes; numerous proteins that stimulate or enhance autophagy, (such as phosphatase and tensin homolog (PTEN), UVRAG, and Bif-1) are tumor suppressors, while numerous proteins that act to inhibit or suppress autophagy (such as PI3K and protein kinase B (PKB/Akt)) are known oncogenes [reviewed in (Brech et al., 2009; Maiuri et al., 2009)]. The mechanism by which autophagy functions to limit oncogenesis is unclear, however, it has been suggested that autophagy may limit DNA damage through the removal of damaged mitochondria (Narendra et al., 2008) and peroxisomes that would otherwise result in

increased reactive oxygen species (ROS) –related damage (Karantza-Wadsworth et al., 2007). Autophagy may also act as an effector mechanism of cellular senescence, which is a barrier to tumor development (Young et al., 2009).

Autophagy may also promote tumor formation, and some tumor cells have been observed to have elevated levels of autophagy. It has been suggested that autophagy may help tumor cells survive periods of low nutrients and oxygen common within tumor environments prior to angiogenesis (Levine, 2006). In this way, defects that reduce autophagy would enhance the initial stages and rate of oncogenesis, but mutations that enhance autophagy would be beneficial during later stages of tumor development. Increased levels of autophagy have also been suggested to interfere with some cancer therapeutics, and may render some cancers more aggressive [reviewed in (Kondo et al., 2005)]. Furthermore, the relationship between chemotherapeutics and autophagy is complex; some drugs, such as tamoxifen and rapamycin are strong inducers of autophagy, while others, such as 3MA and bafilomycin A1, inhibit autophagy (Kondo and Kondo, 2006).

1.2.7 Autophagy and Cellular Senescence

Autophagy has recently been characterized as an effector mechanism involved in the establishment of cellular senescence. In a recent landmark study, Young *et al.* demonstrated that expression of oncogenic *ras* triggered an increase in autophagy, particularly during the transition period when the senescence phenotype is first established (Young et al., 2009). Furthermore, interference with autophagy through knockdown of Atg5 or Atg7 resulted in a modest delay in the establishment of the senescence phenotype, and delayed the production of the pro-inflammatory cytokines

interleukin (IL) 6 and 8. Interference with Atg5 or Atg7 also increased the number of fibroblast cells able to bypass senescence to a similar degree as interference with RB, suggesting that cells that are defective in autophagy are substantively more likely to bypass senescence in the presence of a strong oncogenic signal. Multiple Atg genes were transcriptionally up-regulated during senescence; ULK3, an Atg1 homolog consistently up-regulated during senescence, but previously not associated with the regulation of autophagy, was observed to form puncta and co-localize with autophagosomes during senescence-associated autophagy, suggesting that this pathway might be differentially regulated during cellular senescence. The exact role of autophagy in the establishment of the senescence phenotype is unclear from this initial study, but it has been suggested to function as a means of rapid cellular remodeling, transitioning the cell from a proliferative to senescent state [reviewed in (White and Lowe, 2009; Young and Narita, 2010)]. Autophagy has also been suggested to have an yet unclear role in the production of senescence-associated IL6 and IL8 (Narita, Young, and Narita, 2009), which may reinforce the establishment of senescence in an autocrine-type fashion.

1.3 Hepatitis B Virus (HBV)

Hepatitis B, a disease of the liver similar in many respects to fecal-oral hepatitis (hepatitis A), but is instead spread via contact with blood and other bodily fluids. It was first described as early as the 1800s but, it was not until repeated outbreaks in the 1930s and 40s, when the disease was correlated with exposure to vaccines containing human sera, that the medical community began to search for a causative agent (Seeger, Zoulim, and Mason, 2007). In the 1960s Dr. Baruch Samuel Blumburg discovered the “Australia” antigen in the sera of multiply-transfused aboriginals in Australia (Blumberg et al., 1967).

He subsequently showed the agent to be both transmissible and associated with serum-derived hepatitis. When purified from infected sera, this antigen was present as 22 nm spherical and rod-like particles, but was also associated with 42 nm Dane particles (Dane, Cameron, and Briggs, 1970). These Dane particles were later shown to comprised of the viral nucleocapsid, DNA polymerase, and circular DNA genome of a virus belonging to a previously uncharacterized virus family now known as the *Hepadnaviridae*. This family of viruses is currently composed of two genera, the mammalian Orthohepadnaviruses, and the avian Avihepadnaviruses, and is unique due to the extremely small size of its viral genomes (approximately 3200 base-pairs) and novel replicative strategy (Seeger, Zoulim, and Mason, 2007).

Research into the molecular properties of HBV has been hampered by the lack of appropriate research model systems. Currently, only highly differentiated primary human hepatocytes (*in vitro*) and chimpanzees (*in vivo*) are naturally receptive to HBV infection, and both models are expensive and difficult to access (Seeger, Zoulim, and Mason, 2007). However, the related mammalian woodchuck hepatitis virus (WHV) and avian duck hepatitis B virus (DHBV) have been widely used as substitutes for HBV, and have provided valuable information regarding hepadnaviruses replication and the viral lifecycle. The majority of HBV research employs the exogenous expression of the HBV genome, either as the whole genome or as individual HBV genes, in a variety of primary, immortalized, and transformed cell lines. Transgenic mice have also been developed; however, these models have been of limited use due to the absence of virus cell receptors and deficiencies for viral replication. Despite these challenges, research continues with

the aim of better understanding this important viral pathogen and how it can be controlled.

1.3.1 Hepatitis B Epidemiology, Prognosis, and Treatment

Despite the availability of effective vaccines since the early 1980s, Hepatitis B remains a significant global threat to human health. It is currently estimated that approximately 360 million people are chronically infected with the disease, and that upwards of 2 billion individuals have been infected during their lifetime (de Franchis et al., 2003; Kew, 2010). Regions with the highest rates of infection include Pacific Asia, sub-Saharan Africa, and the Amazon basin; in these regions up to 8% of the population are chronically infected, and 70 to 98% show serological signs of previous exposure to the virus, although these numbers are declining (Custer et al., 2004). In endemic regions, most transmission occurs either perinatally (mother to child) or during early childhood (Kew, 2010). Until recently, perinatal transmission was common within Asian countries, with rates varying from 20 to 90%, depending largely on the disease status of the mother. However, immunoprophylaxis with hepatitis B immune globulin, combined with infant vaccination, has reduced these rates to 5 to 10% of HBV-seropositive pregnancies (Jonas, 2009). Transmission within sub-Saharan Africa typically occurs during early childhood (Kew, 2010). In regions with low rates of infection most transmission is between adults, and is frequently associated with high-risk activities (ie. intravenous drug use, unprotected sex) or accidental occupational exposure (ie. healthcare workers).

After transmission, the virus has an incubation period of 1 to 6 months, and is resolved in a majority (~65%) of individuals without any obvious clinical symptoms of the disease (Liang, 2009; McMahon et al., 1985; Seeger, Zoulim, and Mason, 2007).

However, a subset of individuals will develop an acute hepatitis, characterized by nausea, abdominal pain, fatigue, and in a minority of patients, jaundice (Seeger, Zoulim, and Mason, 2007). A small minority of patients (less than 1%) will develop fulminant hepatitis, which can result in liver failure and death, and frequently requires liver transplantation (Hoofnagle et al., 1995; Liang, 2009). While most adults infected with HBV clear the virus, approximately 5 to 10% develop a chronic, frequently lifelong, infection with severe long-term health consequences. Patients with chronic hepatitis B often go long periods of time with mild to no clinical symptoms, followed by periodic reactivation and more acute morbidity (Liang, 2009; Seeger, Zoulim, and Mason, 2007). The major risks associated with chronic HBV infection are progressive liver damage, leading to liver fibrosis and cirrhosis, and a dramatically increased incidence rate of hepatocellular carcinoma (HCC). Approximately 1/3 of individuals with chronic HBV will suffer a severe adverse outcome due to their disease, although these numbers may be in flux due to the advent of long-term nucleoside analog therapies. Unfortunately, while only a minority of adult transmission results in chronic infection, this is not the case when transmission occurs during early childhood; 70 to 90% of children are infected perinatally, and 50% of children infected under the age of five, will go on to develop chronic HBV infections [reviewed in (Kew, 2010)].

There are currently two treatment options for chronic HBV; pegylated interferon α (IFNα), which has direct antiviral effects and stimulates T-cell immunity, and nucleoside analogs, such as Ribavirin, Lamivudine, and Tenofovir Disoproxil, which interfere with viral DNA synthesis and replication (de Franchis et al., 2003; Seeger, Zoulim, and Mason, 2007). While these therapies can result in a sustained resolution of

infection in a minority of cases (20 to 25% in the case of pegylated IFNα (Niederau et al., 1996)), their secondary aim is a reduction of viremia, morbidity, and progression towards end-stage liver disease (Lim et al., 2009). IFNα therapy, while somewhat effective, is strongly associated with numerous adverse side effects; up to 95% of patients will experience at least some flu-like symptoms (fever, muscle pain, headache, fatigue, and nausea), 20 to 30% will experience psychiatric effects (severe depression, suicidal tendencies, sudden mood changes), and a minority will experience other serious side effects [reviewed in (Negro, 2010)]. As a result, adherence to the recommended 48-week course of IFNα therapy is low. Nucleoside analogs also have adverse effects, and due to HBV's error-prone polymerase, the development of drug resistant strains of the virus have been noted (Zoulim, 2011). Therefore, the development of more effective treatment options with fewer adverse side effects remains a research priority.

Chronic HBV infection is strongly correlated with HCC, and infected individuals are 50 to 100 times more likely to develop HCC over their lifetime. Due to this correlation, HBV is regarded as second only to tobacco as a human carcinogen (Kew, 2010). However, the exact mechanism by which chronic infection promotes tumorigenesis still remains controversial. HBV is associated with 55% of the global cases of HCC, but the percentage is closer to 90% in areas where HBV is endemic (Kew, 2010). HCC is the 6th most common cancer worldwide, has the 3rd highest annual cancer mortality rate, and has the shortest average survival time of any cancer, with most individuals succumbing to the disease within 12 months of diagnosis (Llovet, Burroughs, and Bruix, 2003; Parkin et al., 2005). HCC is highly refractory to treatment. However, efforts to control HBV infections have been shown to be effective in reducing its

incidence. In the two decades since Taiwan introduced universal vaccination against HBV, the incidence rate of HCC has fallen by 70%, and is predicted to continue to decline in the future (Chang et al., 2009).

1.3.2 Hepatitis B Virus Structure and Genome Organization

Sera from individuals infected with HBV contain three distinct particle types, referred to as spheres, filaments, and Dane particles; however, only the Dane particles contain the viral genome and are infectious. The sphere and filamentous particles are composed of HBV envelope proteins and host-derived membrane, are present at high levels during acute infection (up to 10,000x more units relative to Dane particles), and have been hypothesized to act as a form of 'immunological chaff', binding and removing potentially neutralizing antibodies from circulation (Seeger, Zoulim, and Mason, 2007). The Dane particle is spherical, approximately 42 to 47 nm in diameter, and composed of an outer lipid membrane and inner nucleocapsid, which contains the hepatitis B polymerase (pol) protein and a partially double-stranded DNA genome (Gerlich and Robinson, 1980). The outer lipid membrane contains three virally encoded proteins, the small (S), middle (M), and large (L) envelope proteins at a ratio of approximately 4:1:1. The nucleocapsid is composed of the core (C) protein and is assembled in an icosahedral arrangement. The genome is present in the virion as relaxed partially double stranded circular DNA (rcDNA) that is modified within infected host cells into mature covalently closed circular DNA (cccDNA), from which gene transcription can occur (Köck and Schlicht, 1993).

The HBV genome is the smallest amongst human viruses, at approximately 3,200 nucleotides, and every base pair is involved in encoding at least one HBV protein. It

contains four overlapping open reading frames (ORFs), termed S, C, P, and X, that encode for seven viral proteins (see Fig. 1.4) [reviewed in (Liang, 2009; Seeger, Zoulim, and Mason, 2007)]. The S ORF encodes three envelope proteins (S, M, and L) from three alternate in-frame initiation codons, corresponding to the ORF's pre-S1, pre-S2, and S (see Fig. X). Therefore, while all three envelope proteins contain a common C-terminus region, the M and L proteins have additional sequences, corresponding to pre-S2, or pre-S1 and preS2, at their N-terminus. The C ORF encodes two proteins; core (C) and e-antigen (HBeAg). The C protein self assembles into a capsid-like structure in the cytoplasm of cells in which it is expressed. The HBeAg contains a signal peptide directing its translation to the endoplasmic reticulum (ER), where it is post-transcriptionally modified and secreted from the cell. The exact function of HBeAg in HBV infection is controversial, but it has been suggested that it may interfere with immune responses to prolong infection (Milich and Liang, 2003), and high HBeAg antigen levels correlate with poor clinical outcomes (Liang, 2009; Liaw et al., 2010). The P ORF encodes for HBV's only enzyme, the hepatitis B polymerase (pol), which is responsible for transcription of pre-genomic mRNA (pgRNA) into the rcDNA viral genome. The pol protein is approximately 800 amino acid residues in length, and has three distinct domains; an N-terminal protein (TP) domain that regulates encapsulation and is important in initiation of DNA synthesis, a reverse transcriptase (RT) domain responsible for DNA synthesis, and a ribonuclease H domain that degrades pgRNA to facilitate positive strand synthesis. The final ORF encodes the X protein (HBx), a multifunctional non-structural protein that is expressed at low levels during HBV

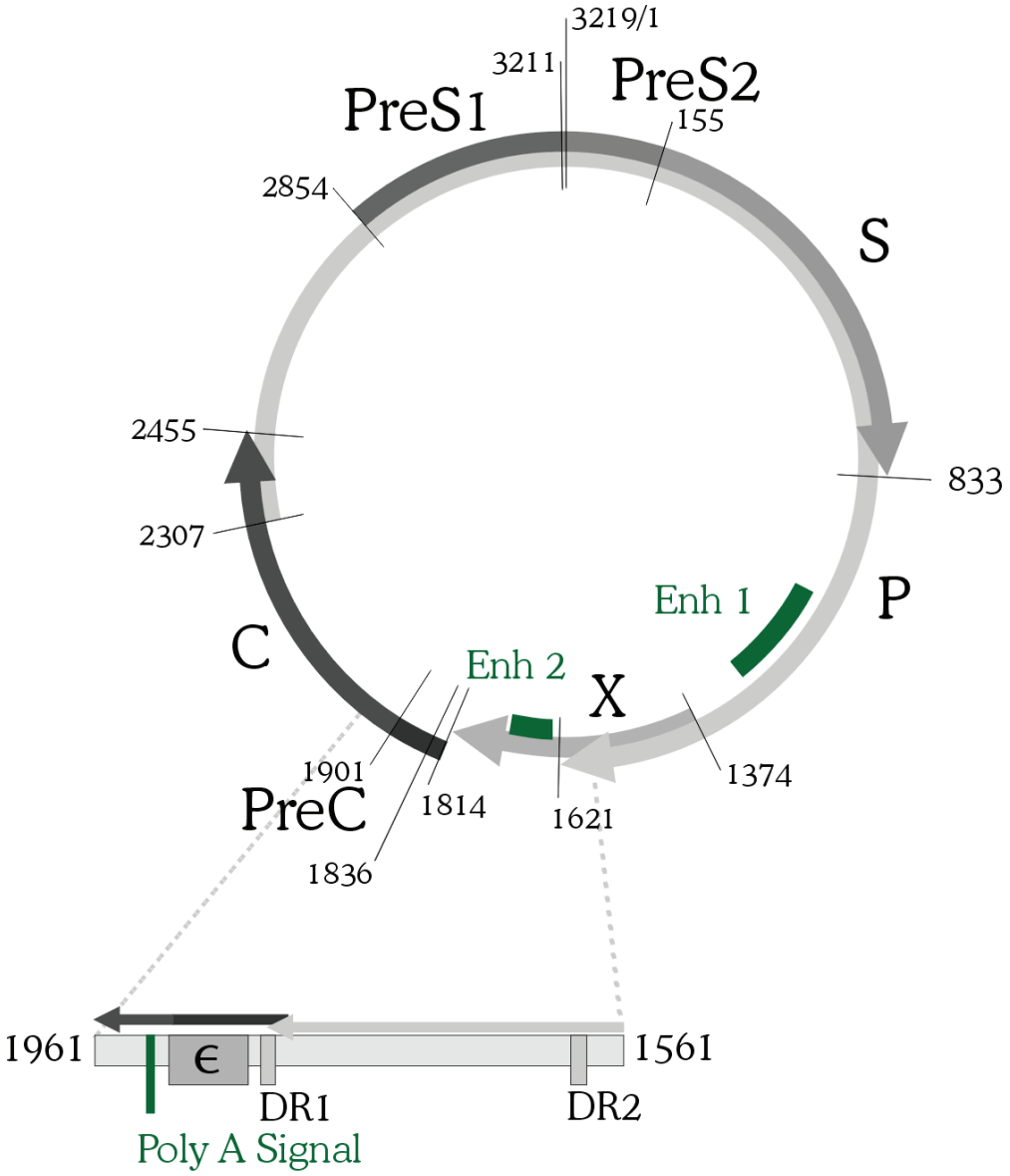

Figure 1.4 - Schematic of the Hepatitis B Virus (HBV) Genome - The HBV genome encodes for 7 proteins from 4 open reading frames (ORFs). Enh1 and Enh2 refers to HBV enhancer regions that regulate gene transcription. DR1 and DR2 refers to the direct terminal repeats involved in HBV replication. The envelope proteins L, M, and S, are encoded from the PreS1, PreS2, and S regions. X refers to hepatitis B virus X protein (HBx). P refers to the HBV polymerase. C refers to core and PreC encodes the hepatitis B e antigen (HBeAg). All regions of the HBV genome are translated into at least one viral protein. Adapted by permission from John Wiley & Sons, Inc.: Fundamentals of Molecular Virology, Acheson, N.H., "Hepadnaviruses", Copyright 2007.

infection. It has been reported to important for viral replication and possess weak oncogenic properties. HBx will be reviewed in in depth in subsequent sections.

1.3.3 Hepatitis B Virus Lifecycle

The initial step in the HBV lifecycle is attachment to a cell capable of supporting replication. The primary cellular host of HBV is the hepatocyte. However, other cell types have been postulated to support replication at much lower levels [reviewed in (Seeger, Zoulim, and Mason, 2007)]. An overview of the HBV lifecycle is provided in Figure 1.5. While the recognition of host receptor(s) is understood to involve the pre-S1 region of the L envelope protein (Klingmüller and Schaller, 1993), the exact identity of these host factors remains to be determined. Following attachment, virions undergo host cell entry and nucleocapsid disassembly via processes that are currently poorly understood, but theorized to involve endocytosis processes. HBV rcDNA genome is then transported into the nucleus via an unknown mechanism, and repaired to form cccDNA (Köck and Schlicht, 1993; Levrero et al., 2009). This process involves the completion of the double-strand synthesis, removal of the HBV polymerase and an RNA primer, and ligation of the DNA strands; the HBV polymerase is not required in any of these processes, and are understood to be performed by host cell factors. Once formed, the cccDNA serves as a template for at least 4 viral RNA transcripts 3.5 (pgRNA or pre-C/C mRNA), 2.4 (pre-S mRNA), 2.1 (S mRNA), and 0.8 kb (X mRNA), which in turn specify the production of HBV viral proteins.

Virion assembly begins in the cytoplasm with HBV polymerase binding to a stem loop structure in the 5' direct repeat 1 region (DR1) of the pgRNA, which in turn leads to its encapsidation by the core proteins (Pollack and Ganem, 1993; Will et al., 1987). Upon

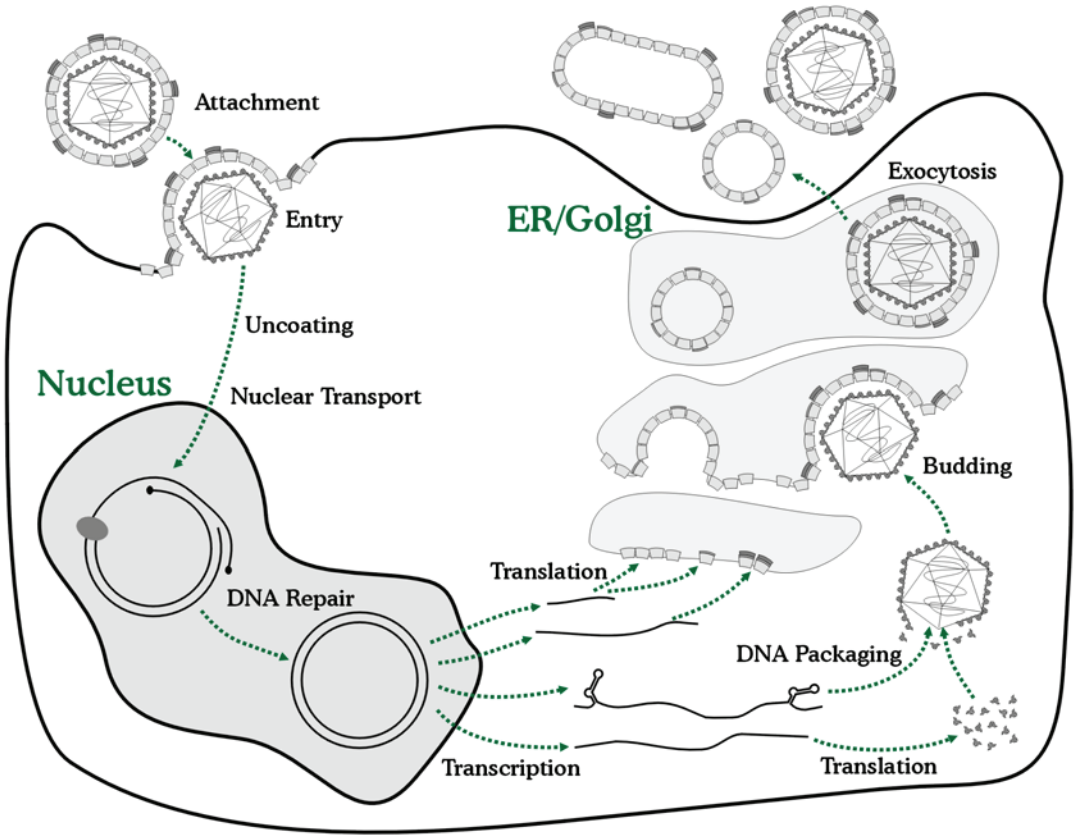

Figure 1.5 - Hepatitis B Virus (HBV) Lifecycle - The hepatitis B virus (HBV) lifecycle begins with virion attachment to the surface of a host cell, typically a hepatocyte, through unknown host receptor proteins. After virion entry and uncoating, the relaxed circular DNA (rcDNA) genome is transported into the nucleus and converted to mature covalently closed circular DNA (cccDNA) by host cell factors. Pre-genomic RNA (pgRNA) is packaged with the HBV polymerase in core nucelocapids, and HBV reverse transcription occurs. Assembled nucleocapids can be either uncoated, adding additional viral episomes to the nucleus, or associate with envelope proteins and host lipid and egress from the cell. Reproduced by permission from John Wiley & Sons, Inc.: Fundamentals of Molecular Virology, Acheson, N.H., "Hepadnaviruses", Copyright 2007.

encapsidation, the pgRNA is converted into the rcDNA form (see Fig. 1.6) [reviewed in (Seeger, Zoulim, and Mason, 2007)]. First, the polymerase serves as its own primer and reverse transcribes a short section of the 5' stem loop structure, becoming covalently attached to the growing strand. This short segment and the polymerase are transferred, via an uncharacterized mechanism, to the 3' DR1, and negative-sense DNA is synthesized while the pgRNA template itself is degraded. Upon completion of the negative-sense strand, the polymerase and an RNA primer created from the 5' end of the pgRNA translocate to the 5' direct repeat 2 region (DR2), and the polymerase proceeds to synthesize part of the positive-sense DNA strand. At this stage the viral genome is said to be in its relaxed circular form (rcDNA). This process is reviewed in Figure 1.6.

Completed nucleocapsids either acquire a viral envelope and are released from the cell, or disassemble and release their genomes, which are then transported into the nucleus and repaired, becoming an additional viral episome; in this way, a single infected cell may contain multiple HBV episomes (Zoulim, 2005). The S, M, and L envelope proteins are translationally inserted into the ER membrane during their synthesis. A portion of the S and M protein will aggregate in the ER membrane and bud into the ER lumen, forming spherical and filamentous particles that are eventually secreted from the cell via vesicular transport (Huovila, Eder, and Fuller, 1992). However, envelopment of nucleocapsid is understood to occur at a post-ER / pre-Gogli membrane location via an unknown mechanism (Bruss, 2004). How assembled Dane particles are ultimately released from the cell is an area of continuing research, but may involve transport through the trans-Golgi network.

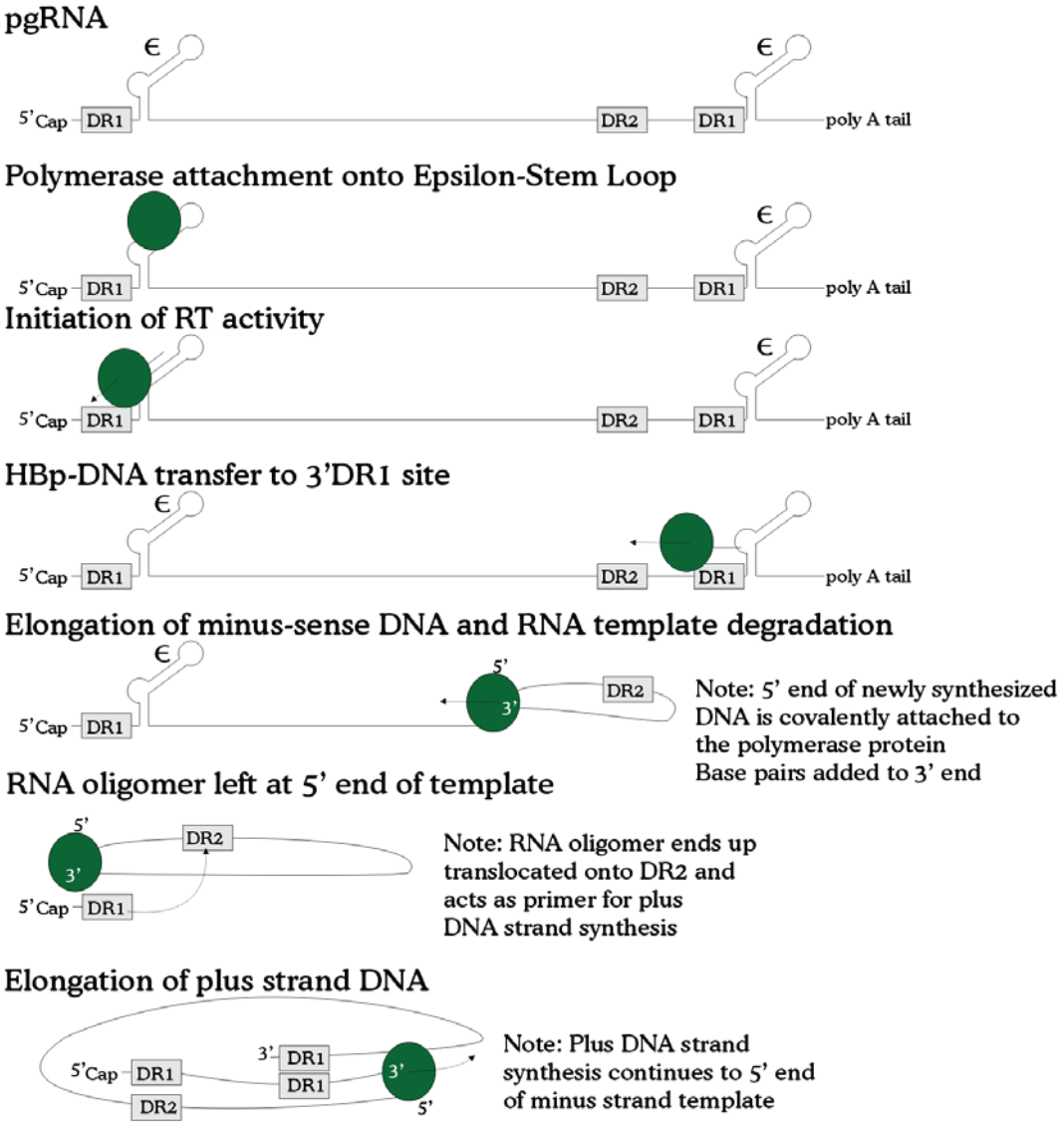

Figure 1.6 - Overview of Hepatitis B Virus (HBV) Reverse Transcription - After being packaged within HBV core nucleocapsids, the HBV polymerase initiates reverse transcription through association with the 5' epsilon-stem loop, where it produces a short primer sequence. It is then transferred to the 3' end of the pgRNA, where it synthesizes the negative-sense DNA strand while digesting the RNA template. Upon completion, the polymerase and an RNA primer derived from the 5' direct repeat (DR) 1 are transferred to the negative strand 5' DR2 and positive sense strand synthesis begins. For further detail, consult section 1.3.3. Reproduced by permission from John Wiley & Sons, Inc.: Fundamentals of Molecular Virology, Acheson, N.H., "Hepadnaviruses", Copyright 2007.

1.3.4 Hepatitis B X Protein (HBx)

The hepatitis B X protein (HBx) is the only protein expressed from the HBV genome that is not incorporated into secreted virions. Named 'X' because it had no sequence homology with any other known protein (Miller and Robinson, 1986), HBx is the smallest HBV protein consisting of 154 amino acid residues. It is highly conserved, both amongst different HBV serotypes and within all mammalian hepadnaviruses (Seeger, Zoulim, and Mason, 2007). During natural infection, the HBx protein is thought to localize primarily to the cytoplasm, with a much smaller fraction present within the nucleus (Dandri et al., 1998; Henkler et al., 2001; Zhang et al., 2000). These HBx populations are understood to have different protein half-lives, with cytoplasmic HBx being degraded fairly rapidly (half life of ~20 minutes) and nuclear HBx being turned over at a slower rate (half life of ~3 hours). When over-expressed, the proportion of HBx that is cytoplasmic increases substantially and segregates into two fractions, mitochondrial-associated and non-mitochondrial (Henkler et al., 2001).

HBx is a multifunctional protein that has been suggested to have pleiotropic effects within the host cell. As stated somewhat cynically in a review on the subject, "if there is a protein that is not among the list of HBx interactors, then this protein has probably not yet been tested" (Murakami, 2001). HBx is generally thought not to bind to DNA directly, although it may associate with the HBV genome (Belloni et al., 2009). Instead, HBx modulates the transcription of many genes through protein-protein interactions with both the transcriptional machinery and multiple cell signaling pathways. Additionally, HBx has been shown to interact with multiple host proteins and organelles, including p53, the proteasome, heat shock protein 60 and 70, and the mitochondria, and it

has effects on processes as diverse as cellular fate, protein degradation, epigenetic gene regulation, DNA repair, cell cycle regulation, and proliferation [reviewed in (Kew, 2011; Murakami, 2001; Tang et al., 2006)]. HBx has also been reported to have both autocrine and paracrine effects through the increased production of secreted factors such as transforming growth factor β (TGF-β) and insulin-like growth factor II (IGF-II). There is considerable debate whether HBx functions to enhance or prevent apoptosis [reviewed in (Kew, 2011)], with results varying depending on the cell type, experimental methods, level of expression, and HBx variant employed. As will be discussed in proceeding sections, the exact role of HBx in the HBV lifecycle is unclear, and HBx has been suggested to have a role in the development of HCC.

1.3.4.1 HBx and the HBV Lifecycle

While the X protein is highly conserved amongst all Orthohepadnaviruses, its exact role in viral replication and its effects on the cell remain controversial. While the HBx homolog, WHx, has been demonstrated to be essential for WHV replication *in vivo* (Chen et al., 1993; Zoulim, Saputelli, and Seeger, 1994), multiple reports have indicated that HBx may be dispensable *in vitro*, but only in certain cell lines. Mutations in HBV replicon systems that silence HBx expression result in a substantial decrease but not total suppression of HBV replication in the HepG2 cell line. However, HBx silencing has been shown to have no effect on HBV replication in the Huh7 cell line (Blum et al., 1992; Leupin et al., 2005; Zheng et al., 2003). Multiple mechanisms have been proposed regarding how HBx might function within HepG2 cells, but few suggestions have been made as to why it is dispensable in the Huh7 line. Two independent studies have reported that HBx increases HBV mRNA transcription in HepG2 cells (Leupin et al., 2005; Tang

et al., 2005), which is supported by the observation that HBx auto-regulates its own transcription, and can activate factors that bind to and enhance HBV gene transcription [reviewed in (Murakami, 2001)]. One report attributed the increase in viral mRNA to HBx's binding interaction with the DNA Damage Binding (DDB) protein DDB1, reporting that interference with DDB1 could decrease HBV replication, but only in the HepG2 cell line (Leupin et al., 2005). Furthermore, a study involving transgenic mice containing either wild-type or HBx-deficient HBV genomes found that viral mRNA production was inhibited in the absence of HBx (Xu et al., 2002). However, a contradictory study has claimed that HBx has no effect on HBV mRNA transcription in the HepG2 cells, but instead is required for the induction of DNA replication (Bouchard et al., 2003). This observation is further supported by a report that HBx stimulates the phosphorylation of core proteins, which in turn regulates the reverse transcription of HBV pgRNA to rcDNA during the encapsulation process (Melegari, Wolf, and Schneider, 2005).

1.3.4.2 HBx and Hepatocellular Carcinoma (HCC)

As previously discussed, chronic HBV infections are strongly correlated with an increased incidence of HCC. Unfortunately, the mechanism by which HBV infection increases or influences liver carcinogenesis remains unclear. One widespread theory is that HBx may act as a 'weak' oncogene, an idea supported by the observation that the avian hepadnaviruses, which lack HBx homologs, are not associated with any increased incidence of liver cancer. However, the mechanism by which different mammalian hepadnaviruses induce carcinogenesis may differ widely; WHV has been shown to activate N-*myc* expression in a majority of woodchuck tumors through the incidental

integration of viral DNA at specific, preferential sites (Fourel et al., 1990), which is not the case in HBV infection, where viral DNA integration is largely random in nature. The HBx gene is frequently integrated in HBV-associated HCC tumors, while other HBV genes are only rarely detected (Paterlini et al., 1995; Poussin et al., 1999). One study observed that 85% of HCC tumors expressed the HBx protein at detectable levels, and that the sera of 70% of HCC patients, but only 5% of chronic HBV patients, contained antibodies specific for HBx (Hwang et al., 2003). It has also been noted that c-terminal truncation of the HBx gene is common in integrants detected in HCC tumors, suggesting that incidental truncation during integration may enhance HBx's oncogenic effects (Poussin et al., 1999).

While there have been multiple attempts to determine whether HBx has oncogenic properties on its own, the results to date have been largely contradictory. Two independent studies have reported that HBx, under the regulation of its endogenous promoter region, dramatically increases the incidence of cancer in transgenic mice, with approximately three-quarters of animals eventually developing tumors, often in the liver (Kim et al., 1991; Yu et al., 1999). However, two other studies have reported the exact opposite findings; that HBx, under the regulation of a α1-antitrypsin promoter to generate expression in a liver-specific context, does not produce a noticeable increase in tumorigenesis in mice (Lee et al., 1990; Madden, Finegold, and Slagle, 2000). It has also been reported that transgenic mice expressing HBx have higher rates of HCC when exposed to hepatocarcinogens, or when expressed in concert with other known oncogenes, such as *ras* or *myc* (Kim et al., 2001; Madden, Finegold, and Slagle, 2001; Slagle et al., 1996). This suggests that HBx's effect may not be sufficient to induce

tumorigenesis on its own, but instead may increase the oncogenic potential of other carcinogenic factors. Multiple *in vitro* studies have reported that HBx has transformative properties in at least some immortalized cell lines (Gottlob et al., 1998; Höhne et al., 1990; Schaefer et al., 1998; Tarn et al., 1999). However, HBx does not transform primary cells, and was unexpectedly observed to interfere with the transformative properties of other oncogenes in these cell lines (Schuster, Gerlich, and Schaefer, 2000), an effect that is difficult to reconcile with the *in vivo* observations.

Given the large number of cellular processes that HBx has been purported to modulate, it is difficult to understand how this protein could not be involved in hepatocellular carcinogenesis. However, it should be noted that the timeframe between initial chronic HBV infection and the development of HCC is long, often decades, and only a minority of chronically infected individuals develop cancer. Therefore, many researchers speculate that any effect HBx has on hepatocellular carcinogenesis must be subtle and progressive in nature, and perhaps suppressed in the vast majority of infected cells.

1.3.4.3 HBx and Transcriptional Transactivation

cDNA microarray analysis of cells expressing HBx has demonstrated that HBx dramatically modulates the transcription of approximately 150 to 500 different genes (Chen et al., 2008; Ng et al., 2004; Song et al., 2004; Zhang et al., 2009). Furthermore, the promoter regions of multiple genes have been shown to be responsive to HBx expression, including p53, IGFII, and TGF-β1, as well as HBV's own enhancer regions [reviewed in (Murakami, 2001; Tang et al., 2006)]. While some of this activity can be attributed to HBx's activation of cytoplasmic cell signaling pathways, nuclear HBx has

been shown to interact with both the basal transcriptional machinery as well as multiple transcriptional regulators. Most studies agree that HBx cannot bind directly to cellular DNA and it is therefore not a direct transcriptional transactivator, but instead acts on transcription through protein-protein interactions with other factors. Several studies have reported that HBx can bind directly to RPB5, a subunit of RNA polymerase II (Cheong et al., 1995; Haviv, Vaizel, and Shaul, 1996; Lin et al., 1998). This has been suggested to competitively displace RMP, a transcriptional co-repressor, and influence interactions with transcription factors (TF) TFIIB, TFIIF, and TFIIH, possibly stabilizing initiation complex formation, as well as affected nucleotide excision repair through TFIIH. HBx is also understood to interact directly with multiple transcription factors, particularly those belonging to the basic leucine zipper (bZip) family, including cAMP response element binding (CREB), activating transcription factor 3 (ATF3), CCAAT-enhancer-binding protein α (C/EBPα), induced cAMP early expressor IIγ (ICERIIγ), and ICERIIr [reviewed in (Murakami, 2001)]. These interactions have been mapped to the N-terminal/central region of HBx, and are theorized to increase the dimerization and affinity for DNA of these transcriptional activators and repressors.

1.3.4.4 HBx and Epigenetic Regulation

Another mechanism by which HBx may alter gene expression is through changes to the epigenetic regulation of their promoter regions. DNA methylation, whereby a methyl group is conjugated to a cytosine that precedes a guanosine (a CpG dinucleotide) via the actions of a DNA methyltransferase (DNMT), can silence the expression genes with rich CpG 'islands' within their promoter region (Chen and Li, 2004; Herman and Baylin, 2003). Interestingly, HBx has been shown to transcriptionally up-regulate the

expression of two DNMTs (DNMT1 and DNMT3), and HBV-associated HCC frequently displays changes in the epigenetic regulation of tumor suppressor genes (Jung et al., 2007; Jung, Park, and Jang, 2010; Zhao et al., 2010; Zhu et al., 2007; Zhu et al., 2010). Expression of the retinoic acid receptor β2 (RARβ2) is down-regulated, via hypermethylation, in the presence of HBx, which in turn interferes with retinoic acid's ability to inhibit growth in these cells (Jung, Park, and Jang, 2010). The promoter region of $p16^{INK4A}$ has been shown to be hypermethylated in several studies, both *in vitro* and within patient isolates, suppressing its transcription and subsequent regulation of cell-cycle progression (Jung et al., 2007; Zhu et al., 2007; Zhu et al., 2010). It has also been suggested that the suppression of $p16^{INK4A}$ may have a positive-feedback effect on DMNT1 expression, which is enhanced by the transcription factor E2F1 when $p16^{INK4A}$ levels are suppressed (Jung et al., 2007). Third, the apoptosis stimulating protein of p53 (ASPP) family helps to regulate apoptosis by enhancing p53's ability to bind DNA and transcriptionally transactivate pro-apoptotic genes (Samuels-Lev et al., 2001). Both ASPP1 and ASPP2 are diminished in HCC samples isolated from HBV-positive patients with hypermethylation of their respective promoter regions, and experimental down-regulation *in vitro* was found both to enhance growth in soft agar and decrease apoptosis in the presence of serum starvation (Jung, Park, and Jang, 2010).

1.3.4.5 HBx and Cellular Signaling Pathways

In addition to HBx's nuclear effects on gene transcription, cytoplasmic HBx is suggested to modulate the activities of a number of cellular signaling kinase pathways. The extracellular signal-related kinases (ERKs) of the mitogen-activated protein kinase (MAPK) family form part of a signaling cascade that regulates gene transcription, cell

proliferation, and cellular fate in response to extracellular stimuli, such as growth factors, and activation by other cell signaling pathways [reviewed in (Chang and Karin, 2001; Pearson et al., 2001)]. When activated, MAPK proteins transit to the nucleus where they activate a broad range of transcription factors. While there is a consensus that HBx expression activates the Ras-Raf-MAPK pathway (Benn and Schneider, 1994; Benn et al., 1996; Bouchard et al., 2003; Bouchard, Wang, and Schneider, 2001; Chung, Lee, and Kim, 2004; Henkler et al., 1998; Klein et al., 1999; Nijhara et al., 2001a; Nijhara et al., 2001b; Yoo et al., 2003; Yun et al., 2004), the exact mechanism by which this occurs remains controversial. Several groups have reported that HBx may not activate Ras directly, but instead activates Src (Bouchard et al., 2003; Bouchard, Wang, and Schneider, 2001; Klein et al., 1999; Xia, Shen, and Zheng, 2005), a protein tyrosine kinase with important roles in cellular differentiation, proliferation, and survival [reviewed in (Thomas and Brugge, 1997)], which can in turn activate Ras. It has also been suggested that the HBx activates Src through the release of mitochondrial calcium (Bouchard et al., 2003; Bouchard, Wang, and Schneider, 2001; Xia, Shen, and Zheng, 2005) However, there has been at least one report indicating that Ras can be activated by HBx in the absence of calcium signaling (Benn and Schneider, 1994). HBx's activation of Src has effects apart from the activation of the Ras-Raf-MAPK pathway; it has been reported to interfere with cell-cycle regulation via the bypassing of G1 cell-cycle arrest (Bouchard et al., 2001), and may have a role in regulating HBV reverse transcription via phosphorylation of core proteins (Klein et al., 1999). The transcriptional activator nuclear factor kappa B (NF-κB) has also been widely noted to be activated by cytoplasmic HBx, likely through the phosphorylation and degradation of its inhibitor, IκB, via signaling

pathways such as Ras-Raf-MAPK and PKB/AKT (Clippinger, Gearhart, and Bouchard, 2009; Doria et al., 1995; Liu et al., 2010; Lucito and Schneider, 1992; Su and Schneider, 1996; Um et al., 2007). Furthermore, HBx's activation of NF-κB has been suggested to be important for the suppression of apoptosis, since interference with NF-κB activation enhances HBx's pro-apoptotic effects (Clippinger and Bouchard, 2008; Clippinger, Gearhart, and Bouchard, 2009; Um et al., 2007).

The protein kinase B (PKB)/Akt pathway is activated in the presence of HBx. PKB/Akt is normally activated via PI3K activity at the plasma membrane in response to extra-cellular growth factors, and functions to regulate apoptosis, through Bad, and cellular metabolism, through mTOR and glucose transport (Manning and Cantley, 2007). Activation of PKB/Akt has been shown to be a key suppressor of autophagy through its regulation of mTOR. Two studies have reported that HBx over-expression results in an activation of both general PI3K activity (as measured by the production of phosphatidylinositol-3 phosphate (PIP3)) and PKB/Akt, which in turn leads to an inhibition of apoptosis through phosphorylation of Bad (Lee et al., 2001; Shih et al., 2000). Treatment with PI3K inhibitors (Lee et al., 2001; Shih et al., 2000), or co-expression of the phosphatase PTEN (Kang-Park et al., 2006), results in a substantial increase in apoptosis in HBx expressing cells, suggesting that this pathway might be important for HBx's suppression of apoptosis. However, the mechanism(s) by which HBx activates PI3K remains unclear, as is the relative importance of PBK/Akt signaling to HBV replication outside of its effects on apoptosis.

The c-Jun N-terminal kinases (JNKs), also referred to as the stress-activated protein kinases (SAPKs), are activated in response to a wide array of cellular stressors

and inflammatory cytokines, triggering changes in gene transcription and the regulation of apoptosis [reviewed in (Vlahopoulos and Zoumpourlis, 2004)]. When activated through phosphorylation, JNKs transits to the nucleus to phosphorylate transcription factors, such as c-Jun and AP-1, as well as other proteins such as p53. JNKs also phosphorylate cytoplasmic proteins, such as the anti-apoptotic Bcl-2 proteins Bcl-2 and Bcl-xL, which influence the regulation of autophagy and apoptosis. While the actions of JNKs are complex, activation of JNKs is believed to enhance cell survival, and multiple viruses have been shown to encode proteins that enhance their activation [reviewed in (Tibbles and Woodgett, 1999)]. Humans have three JNK genes, termed JNK1, JNK2, and JNK3; JNK1 and JNK2 are expressed ubiquitously, while JNK3 is primarily expressed in the brain, heart, and testes. These genes are subject to alternative splicing arrangements that result in distinct protein isoforms (46 and 55 kDa), and splicing determines JNK substrate specificity. Multiple independent studies have reported that HBx expression, *in vivo* and *in vitro*, results in an increase in JNK and downstream transcriptional activity (Benn et al., 1996; Diao et al., 2001; Henkler et al., 1998; Murata et al., 2009; Nijhara et al., 2001b; Tanaka et al., 2006). Our lab has previously reported that HBx expression activates JNK, that JNK activation is dependent on the upstream JNK activator SEK1, and that this effect suppresses Fas-mediated apoptosis in hepatic cell lines (Diao et al., 2001). However, it is currently unknown whether HBx's observed activation of JNK results in an increase in anti-apoptotic Bcl-2 protein phosphorylation.

There have also been a limited number of reports that HBx activates members of the protein kinase C (PKC) and Janus activated kinase (Jak) cell signaling families. The PKC family of serine threonine kinases are regulated through calcium signaling and

phospholipids, and have long term effects on a wide range of cellular processes, depending largely upon their cellular context [reviewed in (Mellor and Parker, 1998)]. HBx up-regulates PKC signaling (Cross, Wen, and Rutter, 1993; Kekulé et al., 1993; Luber et al., 1993), and associates with the PKC-binding X associated protein 3 (XAP3) and ηPKC in *in vitro* binding assays (Cong et al., 1997). While PKC signaling activates NF-κB, HBx's activation of NF-κB has been reported to be PKC independent (Lucito and Schneider, 1992). Jaks are typically activated by cytokines and growth factors via their respected receptors, and in turn phosphorylate signal transducers and activators of transcription (STATs), which translocate to the nucleus and promote gene transcription (Kisseleva et al., 2002). There has been a single report indicating that stable HBx expression results in an activation of Jak1, but not Jak2 or Tyk2, with enhanced STAT phosphorylation (particularly STATs 3 and 5) and associated transcription (Lee and Yun, 1998).

1.3.4.6 HBx and the Tumor Suppressor p53

Often referred to as the 'watchman' or 'guardian' of the genome, the tumor suppressor protein p53 integrates and responds to a wide range of cellular signals relating to DNA damage, oncogene activation, and metabolic stress. In response to these signals p53 modulates gene transcription, and can activate DNA repair, induce cell cycle arrest, or initiate apoptosis (May and May, 1999; White, 1996). p53 has been predicted to be functionally defective in 50% of human cancers, and is the most commonly mutated tumor suppressor gene in human cancer (Hollstein et al., 1991; White, 1996). Multiple proteins encoded by DNA viruses, including simian virus 40 large T antigen, adenovirus E1B 55K, human papillomavirus E6, and Epstein-Barr virus EBNA-5, have been shown

to suppress the activities of p53 in order to enhance viral replication (May and May, 1999).

HBx has been shown to bind p53 at its C-terminal domain using the same region responsible for its transcriptional activation activities (Elmore et al., 1997; Takada et al., 1997; Truant et al., 1995; Ueda et al., 1995; Wang et al., 1994). This binding interaction has been reported to have multiple effects: First, HBx binding prevents p53 from translocating from the cytoplasm to the nucleus, interfering with p53-mediated gene-transcription and apoptosis (Elmore et al., 1997; Takada et al., 1997; Ueda et al., 1995). Second, HBx binding interferes with p53's ability to activate gene transcription at the level of the DNA (Lin et al., 1997; Truant et al., 1995; Wang et al., 1994). However, contradictory reports indicate that this is due to either to interference with p53's ability to bind DNA (Lin et al., 1997; Wang et al., 1994), or to initiate transcription after DNA binding has already occurred (Truant et al., 1995). Third, binding interferes with p53's ability to suppress the transcription of certain genes, such as α-fetoprotein, which is associated with liver regeneration and a marker commonly associated with HCC (Ogden, 2000). Fourth, HBx has been reported to interfere with p53's interaction with xeroderma pigmentosum B (XPB), an important factor involved in p53-mediated nucleotide excision repair through TFIIH (Wang et al., 1994).

Studies have indicated that HBx and p53 each act to regulate the protein levels of the other in complex ways, forming a feedback loop between them. HBx suppresses the transcription of p53 through interference with its promoter region, specifically an E-box binding element that up-regulates its expression (Lee and Rho, 2000). Similarly, p53 can suppress HBx's expression through interference with HBx's own auto-regulation via

HBV enhancer I promoter region (Lin et al., 1997). General HBV gene transcription, which is strongly influenced by HBx, is also suppressed by p53 in the presence of genotoxic stress, but is ameliorated by the over-expression of additional HBx (Doitsh and Shaul, 1999). Finally, nuclear p53 has recently been shown to help regulate HBx levels by influencing its proteasome-mediated degradation, as over-expression of p53 reduced HBx protein levels, while siRNA interference with p53 increased them (Park et al., 2009). This suggests that host cell mutations that impair p53 might increase HBx's oncogenic potential by amplifying its levels and associated actions on transcription and signaling pathways.

1.3.4.7 HBx and DNA Damage Repair

HBx has been reported to interfere with DNA damage repair through both p53 –dependent and p53–independent repair mechanisms. *In vitro* binding assays have shown that HBx can bind with xeroderma pigmentosum B (XPB) and D (XPD) (Jia, Wang, and Harris, 1999; Qadri, Fatima, and AbdeL-Hafiz, 2011), which interact with the transcription factor II H (TFIIH) and are involved in the process of nucleotide excision repair; HBx also associates with damaged DNA in the presence of a nuclear protein lysate (Capovilla, Carmona, and Arbuthnot, 1997). This binding interaction has been reported to sensitize cells to ultraviolet radiation (UV) treatment, increasing levels of apoptosis. While initially it was reported that HBx's interference with XPB occurred through its binding interactions with p53 (Wang et al., 1994), subsequent reports have indicated that HBx can interfere with DNA repair in both p53 –competent and p53–incompetent cell lines (Groisman et al., 1999; Jia, Wang, and Harris, 1999). This result might be due, in part, to HBx's interactions with DNA damage binding protein 1

(DDB1). Studies have identified the region of HBx that is responsible for DDB1 binding (Becker et al., 1998; Li et al., 2010; Sitterlin, Bergametti, and Transy, 2000), and mutations in this region have been reported to reduce HBx-associated apoptosis. However, interference with DDB1, or HBx's (or WHx's) binding interaction with DDB1, has been reported to interfere with HBV/WHV replication (Leupin et al., 2005; Sitterlin et al., 2000; Sitterlin, Bergametti, and Transy, 2000). The reason for this effect is unclear, however, it has been suggested that DDB1 may help to stabilize and regulate HBx protein levels (Bergametti, Sitterlin, and Transy, 2002), modulate HBx's translocation to the nucleus (Bontron, Lin-Marq, and Strubin, 2002; Sitterlin, Bergametti, and Transy, 2000), or have an unexplained role in regulating HBV reverse transcription (Leupin et al., 2005). DDB1 is also an essential component of the cullin ubiquitin ligase 4 –DDB1 (CUL4-DDB1) ubiquitin ligase system, which in concert with other CUL4-associated factors (DCAFs) functions as an E3-ubiquitin ligase (Lee and Zhou, 2007). Multiple viruses have been shown to encode proteins that hijack CUL systems to ubiquitinate and remove unwanted host cell factors [reviewed in (Barry and Früh, 2006)]. A recent study has suggested that HBx may function as a DCAF protein, redirecting CUL4-DDB1 ubiquitination to cellular targets (Li et al., 2010). Our lab is also interested in investigating whether HBx plays a role in DDB1-mediated ubiquitination and subsequent degradation of cellular proteins. Experiments are currently underway to elucidate whether HBx antagonizes the ubiquitination pathway by binding to pro-survival proteins, such as PKB/AKT, JNKs, and Bcl-xL, in order to protect them from degradation.

1.3.4.8 HBx and Bcl-2 Family of Proteins

The Bcl-2 family of proteins are important regulators of apoptosis that respond to cellular signals to control the release of pro-apoptotic executioners, such as cytochrome C, from the mitochondria. As discussed previously, anti-apoptotic Bcl-2 proteins can also act to regulate autophagy through binding interactions with the autophagy regulatory protein Beclin 1 (Erlich et al., 2007; Maiuri et al., 2007a; Pattingre et al., 2005). All Bcl-2 proteins contain at least one Bcl-2 homology (BH) domain, and can be classified as either anti- or pro- apoptotic based on their structure; anti-apoptotic Bcl-2 proteins such as Bcl-2, Bcl-xL, and Mcl-1 contain four BH domains, while pro-apoptotic family members have either three (Bax, Bak, and Bok) or one (Bik, Bim, Bid, and Bad) (Strasser, O'Connor, and Dixit, 2000; White, 1996). Bcl-2 proteins are regulated both at the level of transcription, and through post-translational modifications, such as phosphorylation and proteolytic cleavage. The anti-apoptotic family members Bcl-2 and Bcl-xL each possess a non-structured loop region between their BH3 and BH4 domains that contains multiple phosphorylation sites that are phosphorylated by the stress activated MAPK family members JNK1 (Maundrell et al., 1997), and, to a lesser extent, p38 (De Chiara et al., 2006). Multiple viruses encode for anti-apoptotic Bcl-2 homolog proteins, including adenovirus E1B-19K, Epstein-Barr virus BHRF1, and Kaposi sarcoma Herpes virus (KSHV) vBcl-2 [reviewed in (Castanier and Arnoult, 2011)], which suppress apoptosis, and in the case of KSHV, autophagy (Liang, E, and Jung, 2008; Pattingre et al., 2005).

Multiple independent investigators have demonstrated that HBx can modulate the levels of Bcl-2 family proteins, however, there is little consensus as to which ones, or to what effect. Two groups have reported that two hepatic cell lines, stably transfected with

HBx, showed dramatically enhanced levels of Bcl-2 mRNA and protein relative to a control, and that RNA interference (RNAi) against HBx can abrogate this effect (Ye et al., 2008; Zhang et al., 2009). However, two other studies observed that Bcl-2 was undetectable in their hepatic cell lines, regardless of the status of HBx (Cheng et al., 2008; Hu et al., 2011). Bcl-xL mRNA and protein levels were reported in one study to be reduced in the presence of HBx (Miao et al., 2006). On the other hand, two contradictory reports have demonstrated that HBx has no noticeable effect on Bcl-xL protein levels in their experimental systems (Cheng et al., 2008; Hu et al., 2011). Another study regarding Bcl-xL status has reported that in cells containing the entire HBV genome, RNAi treatment targeting HBx caused a reduction in Bcl-xL mRNA and protein levels, suggesting that HBx may stabilize Bcl-xL transcription in the presence of other HBV proteins (Chan and Ng, 2006). There have also been contradictory reports regarding the status of Mcl-1; one study found that HBx dramatically increased Mcl-1 mRNA and protein levels through an activation and up-regulation of cyclooxygenase-2 (COX-2; (Cheng et al., 2008)), while another found Mcl-1 levels unchanged in the presence of HBx alone (Hu et al., 2011). However, this second study observed that in cells treated with cisplatin, a DNA-damaging chemotherapeutic, HBx substantively increased the degradation of Mcl-1, sensitizing the cells to apoptosis. HBx's effect on levels of the pro-apoptotic Bcl-2 proteins is also currently unclear. Bid has been reported to be transcriptionally down-regulated upon over-expression of HBx, and was observed to be reduced in a large percentage of HCC tissue samples, but no reference was given regarding their HBV-infection status (Chen et al., 2001). Another study found that Bax was transcriptionally up-regulated by HBx, and that this sensitized hepatocytes to

TRAIL-mediated apoptosis (Liang et al., 2007). However, at least one other study has reported that Bax, Bak, and Bid protein levels are all unchanged by the expression of HBx (Miao et al., 2006). Although the lack of clear consensus makes it difficult to draw direct conclusions, in the majority of these studies at least one anti-apoptotic Bcl-2 protein was either up-regulated or stabilized, suggesting that HBx's general effect is to increase the activity of this family of proteins.

Limited reports have indicated that HBx may have direct binding interactions with some Bcl-2 proteins. HBx possesses a tentative BH3 domain, based on sequence analysis (Lu and Chen, 2005). While a yeast two-hybrid study found that no direct interaction between Bcl-2, Bcl-xL, or Bax could be shown, a subsequent study that employed a mammalian cell-based system similar to that of a yeast two-hybrid found HBx interacted with Bcl-2 and Bcl-xL, but not Bad, Bak, Bik, or Bax (Lu and Chen, 2005; Terradillos et al., 2002). Data from our own laboratory has suggested that over-expressed HBx co-localizes and co-immunoprecipitates with Bcl-xL, but not Bcl-2 (Dr. Christopher Richardson, unpublished data). However, it has also been reported that HBx can bind with Bax, inhibiting its interactions with 14-3-3ε and enhancing translocation to the mitochondria and induction of apoptosis (Kim et al., 2008). The relative importance of these interactions, or how they might affect the regulation and localization of Bcl-2 proteins, requires further investigation.

1.3.4.9 HBx and Autophagy

The first indication that HBV might modulate autophagy was the discovery that a key autophagy regulatory gene, Beclin 1, is significantly transcriptionally up-regulated in HCC tissue samples relative to their adjacent, non-HCC samples (Song et al., 2004).

While not all of the samples in this initial study were obtained from HBV-positive patients, the sample used in the initial microarray screening was, as were some of the 10 samples used in the subsequent Northern and RT-PCR studies (80% were indicated to be either HBV or HCV positive). Therefore, the authors speculated that the up-regulation might be the result of HBV viral protein expression, and the same laboratory subsequently published a follow-up report confirming their suspicions (Tang et al., 2009). In this study, Tang *et al.* reported that transfection with HBx increased Beclin 1 mRNA and protein levels, which in turn 'sensitized' cells to autophagy signals. Autophagy levels, quantified by LC3-GFP puncta scoring (cells with 5 or more puncta were counted as positive), were comparable when cells were treated with normal culturing media. However, a difference emerged when the cells were exposed to starvation media for 4 to 8 hours. This effect was susceptible to siRNA treatment against Beclin 1, and a similar effect was noted in cells transfected with plasmid containing a 1.3x copy of the HBV genome, as well as in the HepG2.2.15 model of HBV infection (Sells, Chen, and Acs, 1987), when compared with its parental cell line, HepG2.

A subsequent report by *Sir et. al.* (Sir et al., 2010) reinforced the observation that HBx over-expression can up-regulate autophagy, but to a different effect and via a different mechanism. This group found that HBx did not result in changes in Beclin 1 levels, but instead partially co-localized and co-immunoprecipitated with Vps34, a PI3K associated with Beclin 1 (Kihara et al., 2001). HBx, without additional autophagy stimuli, was found to induce an increase in LC3-GFP puncta and LC3-II immunoblot protein levels, but without a corresponding increase in long-lived protein degradation, suggesting that HBx could stimulate autophagosome formation while simultaneously partially

blocking maturation and lysosomal degradation. Similar results were obtained with plasmids containing a 1.3x copy of the HBV genome, with or without a silencing mutation in the HBx gene. Interestingly, suppression of autophagy, via inhibitors or siRNA treatment, interfered with HBV DNA production, but only in the HepG2, not the Huh7.5, cell line. As HBV protein and mRNA levels were largely unchanged in these experiments, the authors proposed that autophagy affects HBV DNA replication, via an unknown mechanism, at a stage subsequent to pgRNA and polymerase packaging with HBV core proteins, but preceding reverse transcription.

A third HBV autophagy study (Li et al., 2011) has suggested that while HBV does up-regulate the autophagy pathway, it is the expression of the small envelope protein (SHB) that is responsible for the effect. Using plasmids containing a 1.3x copy of the HBV genome and various silencing mutations for specific HBV genes, this study showed that HBV, via SHB, induces autophagy, as measured by LC3 –immunoblotting, LC3-GFP puncta formation, and EM imaging, but that autophagy-specific protein degradation, as measured via p62 immunoblotting, is unchanged. Furthermore, this response is triggered by ER stress and the unfolded protein response (UPR) [reviewed in (Ron and Walter, 2007)], and requires all three UPR signaling pathways (PERK phosphorylation of eIF2α, ATF6 cleavage and nuclear translocation, and IRE1 splicing of XBP1 mRNA). SHB partially co-localized with LC3-labeled puncta, and co-immunoprecipitated with the LC3-II isoform of the protein. Interference with autophagy (via inhibitors, or siRNA knockdown of Beclin 1 or ATG5) or the UPR (via siRNA knockdown of PERK, ATF6, or IRE1) substantively inhibited HBV particle production, while induction of autophagy, via rapamycin or starvation, increased production. From

these data the authors proposed that HBV uses autophagy during particle envelopment, and induces the pathway without also increasing lysosomal protein degradation. While these authors concluded that SHB was responsible for the reported effects, they did note that over-expression of HBx could induce autophagy, and it should be noted that HBx did have a noticeable effect in some of their experiments.

While the existing literature agrees that HBV can up-regulate autophagosome formation, there is little consensus on how this effect is achieved; this may be due to differences in experimental methods, cell lines, or the HBx gene itself. Interestingly, both papers that reported an HBx–associated induction of autophagy have attributed it to changes at the level of the Beclin 1 autophagy regulator, either directly, or through its associated PI3K, Vps34. However, HBx's reported modulation of the anti-apoptotic Bcl-2 proteins (Chan and Ng, 2006; Cheng et al., 2008; Hu et al., 2011; Miao et al., 2006; Ye et al., 2008; Zhang et al., 2009), important regulators of Beclin 1 activity (Erlich et al., 2007; Maiuri et al., 2007a; Pattingre et al., 2009; Wei et al., 2008), might also have a role in these effects.

1.3.4.10 HBx, Cell Cycle Progression and Cellular Senescence

While numerous studies have examined HBx's effects on cell cycle regulation and proliferation, there is currently little consensus on what exactly these effects are. There are reports that HBx can either limit cellular proliferation (Friedrich et al., 2005; Huang et al., 2004; Kuo et al., 2008; Kwun and Jang, 2004; Leach et al., 2003; Park et al., 2000; Qiao et al., 2001; Wu et al., 2006), induce cells to enter the cell cycle but then 'stall' during S phase or the transition from G_1 to S phase (Chen et al., 2008; Gearhart and Bouchard, 2010; Gearhart and Bouchard, 2011; Lee et al., 2002; Park et al., 2000;

Wu et al., 2006), or remove barriers to cell cycle entry and increase proliferation (Ahn et al., 2002; Benn and Schneider, 1995; Leach et al., 2003; Lee et al., 2002; Madden and Slagle, 2001; Ng et al., 2004). Likewise, there have been multiple reports that HBx can modulate the levels of $p16^{INK4a}$, $p21^{WAF1}$, $p27^{KIP1}$, and the cyclins A, B1, and D1 [reviewed in (Gearhart and Bouchard, 2010; Kew, 2011)]. Several groups have also reported that deregulation of the cell cycle enhances HBV DNA synthesis (Huang et al., 2004) [reviewed in (Gearhart and Bouchard, 2010)], presumably by increasing the intracellular levels of deoxyribonucleotides necessary for pgRNA reverse transcription.

While contradictory reports do exist, most studies to date have indicated that HBx down-regulates the expression of $p16^{INK4a}$, likely through the hypermethylation of its promoter region (Gearhart and Bouchard, 2010; Jung et al., 2007; Kim et al., 2010; Park et al., 2011; Zhu et al., 2007; Zhu et al., 2010). The situation is significantly more complicated regarding $p21^{WAF1}$, a potent cyclin-dependent kinase inhibitor that acts to induce cell-cycle arrest. HBx was initially reported to interfere with the p53-mediated up-regulation of $p21^{WAF1}$, via inhibition of p53 transcriptional transactivation (Wang et al., 1995). However, multiple successive studies, examining either HBx alone or in the context of whole HBV gene expression, have observed that HBx up-regulates the expression of $p21^{WAF1}$ (Chin et al., 2007; Friedrich et al., 2005; Gearhart and Bouchard, 2010; Gearhart and Bouchard, 2011; Oishi et al., 2007; Park et al., 2000; Qiao et al., 2001). It has also been suggested that $p21^{WAF1}$ expression is up-regulated in the presence of both p53 and HBx, but is suppressed by HBx when p53 is absent via a p53-independent mechanism involving interference with the Sp1 transcriptional transactivator (Ahn et al., 2001; Ahn et al., 2002). Kwun and Jang found that natural variants of HBx

isolated from patients had very different effects on $p21^{WAF1}$ expression, with p53 status being important for some, but not other mutants (Kwun and Jang, 2004). Variants with a key mutation at residue 101 (proline to serine) triggered strong expression of $p21^{WAF1}$ regardless of p53 status, while a mutation at residue 130 (lysine to methionine) strongly repressed $p21^{WAF1}$. While this study only examined cellular proliferation over a 72-hour period, when HBx was able to induce $p21^{WAF1}$ expression, proliferation was suppressed in both the HepG2 and Hep3B cell lines. It has also been reported HBx can have contradictory effects depending on its level of expression (Leach et al., 2003); high HBx concentrations suppressed $p21^{WAF1}$ and increased DNA synthesis, while low level expression induced both $p21^{WAF1}$ and $p27^{KIP1}$, and decreased DNA synthesis. In general, HBx appears to increase the expression of $p21^{WAF1}$, however, this frequently does not result in a complete arrest of the cell cycle.

Studies that have examined HBx's effects on proliferation *in vivo* have also produced contradictory results. There are several reports of HBx transgenic mice that have increased levels of hepatocellular proliferation, particularly those mice that also had increased incidence rates of HCC [reviewed in (Madden and Slagle, 2001)]. However, it has also been reported that some transgenic mice have lower levels of proliferation and regeneration, even after partial liver resection or treatment with liver-damaging agents, both of which had a strong proliferative effect in control animals (Madden and Slagle, 2001; Wu et al., 2006). Hepatocytes from these mice showed impaired DNA replication, possibly due to a block in cell cycle progression at the G_1/S transition. These observations match with the *in vitro* studies, in that it appears that HBx can be both pro- and anti- proliferative under different circumstances.

Two recent papers from Dr. Kyung Lib Jang's laboratory have reported that HBx can interfere with the induction of cellular senescence in the presence of hydrogen peroxide (H_2O_2) or all *trans* retinoic acid (ATRA) *in vitro*. In the initial report by Kim *et. al*., HepG2 cells stably transfected with HBx showed increased resistance to H_2O_2 – induced senescence, as measured by SA β-gal staining and BrdU incorporation 72-hours post treatment (Kim et al., 2010). Transient transfection of HBx had a dose-dependent effect on $p16^{INK4a}$ (decreased), $p21^{WAF1}$ (increased), and phosphorylation of RB (increased), but the study did not examine HBx's effects on senescence in a dose-dependent fashion. HBx's protection against senescence was dependent on suppression of $p16^{INK4a}$, and the $p16^{INK4a}$ promoter was highly methylated in the presence of HBx; interestingly, treatment with H_2O_2 had reduced HBx's methylation of the $p16^{INK4a}$ promoter at multiple sites when examined 72-hours post treatment. This laboratory's subsequent report by Park *et. al.* had very similar findings (Park et al., 2011); HBx expression overcame cellular senescence in the presence of ATRA in HepG2 and Hep3B cells, but not in Huh7 cells, which do not express either $p16^{INK4a}$ or $p21^{WAF1}$ and do not undergo senescence in response to ATRA treatment. The promoters of both $p16^{INK4a}$ and $p21^{WAF1}$ were reported to be hypermethylated in the presence of HBx, and over-expression of either $p16^{INK4a}$ or $p21^{WAF1}$ was sufficient to sensitize the HBx-expressing cells to the ATRA treatment. However, while a significant difference in SA β-gal staining was noted in the HepG2 cells stably expressing HBx, the difference was both negligible (~30% in HepG2 and ~20% in Hep3B at highest dose tested) and highly dose-dependent when HBx was transiently transfected into cells. Furthermore, this same laboratory has published a early report stating that HBx expression causes the

hypermethylation of the retinoic acid receptor-β2 (RAR-β2) promoter region, rendering HepG2 cells insensitive to retinoic acid-induced cell growth inhibition, and over-expression of RAR-β2 alone was sufficient to restore sensitivity to this compound (Jung, Park, and Jang, 2010); Park *et. al.* cite this early paper as evidence of HBx's ability to modulate gene transcription via hypermethylation, but provide no explanation why they chose to use ATRA as their senescence inducer given this earlier result. Interestingly, an earlier report examining the HepG2.2.15 model of HBV infection found that treatment with ATRA, DMSO, or sodium butyrate, as well as serum deprivation, all had strong negative effect on proliferation with an accumulation of a majority of cells in the G_1 phase of mitosis (Huang et al., 2004). While aspects of these studies may be criticized, collectively they suggest that HBx can induce a bypass of cellular senescence, but only under certain conditions.

While they do not employ the term 'cellular senescence', there have been several reports that show that HBx expression can have a profound effect on cellular proliferation, and may be evidence of an HBx-induced senescence phenotype. A recent study has reported that induction of HBx expression in a tetracycline-responsive (Tet-off) Chang liver cell line triggered a dramatic inhibition of cellular proliferation with a concurrent rise in p53 levels and decline in proliferating cell nuclear antigen (PCNA) and β-catenin levels (Kuo et al., 2008). While there was some evidence of an initial increase in apoptosis, HBx-expressing cells failed to proliferate, and were similar in number after 72 hours of HBx expression. Further microarray analysis and western blotting revealed that after 96 hours of HBx expression the levels of cyclins B1, D1, and E, and the cyclin-dependent kinases (CDKs) CDK2 and CDK4, were all substantively reduced. A report by

Gearhart *el al.* showed that in primary rat hepatocytes HBx could trigger entry into G_1, but prevented progression into S phase of mitosis, and that when plated at sub-confluent levels HBx expressing cells failed to proliferate to any noticeable degree relative to a control treatment (Gearhart and Bouchard, 2010). Finally, a 2000 study by Park *et al.* found that HBx expression in Hep3B cells increased $p21^{WAF1}$ levels and strongly inhibited cellular proliferation (Park et al., 2000); while the cell number of a control treatment increased approximately 20 times in 7 days, cells expressing one of two HBx constructs only increased by a factor of approximately 2 to 3 times.

Although there is considerable confusion within this area of HBx research, it does appear clear that HBx can modulate multiple factors associated with cellular proliferation. Differences in experimental methods, including cell type, mode and level of expression, and the HBx variant employed, are all likely to be contributing factors to the variation in responses that have been observed to date. Collectively, these studies suggest that HBx may either induce or bypass cellular senescence, depending upon the context of its expression.

1.4 Research Objectives and Hypothesis

HBV remains a serious threat to global health due to its propensity to cause chronic, often life-long, infections that dramatically increase the risk of HCC. Although the role of HBx in hepatocellular carcinogenesis is unclear, there is evidence that HBx may have oncogenic properties. The Richardson laboratory is interested in investigating how HBx modulates cellular activities and how these modulations might affect the course of hepatocellular carcinogenesis.

While refining methods for expressing the HBx protein without the use of transfection reagents, we serendipitously observed that retroviral transduction of HBx into the HepG2 and Huh7 cell lines caused a proportion of cells to adopt a dramatically enlarged and flattened morphology. These transduced cells failed to proliferate, yet remained adherent over extended periods of time under standard tissue culture conditions. Furthermore, previous research in our laboratory had shown that HBx expression could render HepG2 cells resistant to starvation and Fas-mediated apoptosis (Diao et al., 2001). Based on these and other observations, we hypothesize that HBx can induce cellular senescence when over-expressed by retroviral transduction. While there is no clear consensus regarding HBx's effects on the cell cycle, we were encouraged by reports that HBx could have anti-proliferative effects, and that it modulates proteins involved in cell cycle arrest. Therefore, one of the principle aims of this study was to examine whether the observed HBx-induced phenotype exhibited markers of cellular senescence.

Autophagy, a cellular stress response mechanism, plays an important role in host defense (reviewed in section 1.2.4), and may act as a tumor suppressor pathway (reviewed in section 1.2.6). Since autophagy may act as an effector mechanism during the establishment of cellular senescence, we became interested as to whether HBx expression would modulate the activity of this process. At the time this study was initiated, there was a single report examining HBx's effects on the autophagy pathway. These investigators reported that HBx expression 'sensitized' cells to autophagy stimuli (Tang et al., 2009). Their paper reported that HBx triggered an increase in the transcription and protein levels of Beclin 1, an important autophagy regulatory protein that is targeted for modulation by several viral pathogens. Significantly, HBx was also previously reported to have effects

upon the expression levels of the Bcl-2 anti-proteins. As well, HBx activates the JNK/SAPK pathway, which positively regulates autophagy through the phosphorylation of the Bcl-2 anti-apoptotic proteins. We therefore hypothesize that HBx modulates autophagy through effects on the Bcl-2 anti-apoptotic proteins and their regulation of Beclin 1. This section had four stated aims, namely: 1) to characterize HBx's effects on autophagy within our experimental model, 2) to examine whether HBx expression effects the expression or phosphorylation of the Bcl-2 anti-apoptotic proteins, 3) to explore whether Bcl-2 protein interactions with Beclin 1 are affected by HBx expression, and 4) to investigate whether the activity of the JNK/SAPK pathway, which can phosphorylate the Bcl-2 anti-apoptotic proteins, is modulated by expression of HBx. It is our hope that a better understanding of these processes relating to autophagy and senescence might aid in the development of novel therapeutic approaches for the prevention and treatment of HCC.

Chapter 2: Materials and Methods

2.1 Reagents

The inhibitors bafilomycin A1 and chloroquine were obtained from Sigma-Aldrich (Oakville, ON) and reconstituted in dimethyl sulfoxide (DMSO) and deionized water, respectively. Bafilomycin A1 was used at a final concentration of 100 nM, while chloroquine was used at a final concentration of 50 μM. Doxycycline (Sigma-Aldrich) was solubilized in deionized water and used at final concentrations of 50, 100, and 1,000 ng/mL. Poly-D-lysine hydrobromide (Sigma-Aldrich) was reconstituted in sterile tissue culture grade water, as per manufacture's instructions. Puromycin (Sigma-Aldrich) was used at a final concentration of 2 μg/mL with HepG2 cells, and 1.2 μg/mL with Huh7 cells. Hexadimethrine bromide (polybrene) was purchased from Sigma-Aldrich and was prepared in deionized water.

2.2 Cell Culture

Huh7, a human hepatocellular carcinoma cell line, was kindly provided by Dr. Stanley Lemon (University of Texas Medical Branch, Galveston, TX). The human hepatocellular carcinoma cell line HepG2 was obtained from two sources, purchased from the American Type Culture Collection (ATCC, Manassas, VA), and our laboratory stocks (designated HepG2 CDR). Except where otherwise noted, all HepG2 cells employed in this study were those obtained from the ATCC. The Amphotropic Phoenix cell line (Phoenix AMPO), a retrovirus packaging system derived from a human embryonic kidney cell line, 293T and elements of the Moloney Murine Leukemia Virus (MMULV), was obtained from Dr. Craig McCormick (Dalhousie University, Halifax,

NS). The HepG2.2.15 cell line, an *in vitro* model of HBV infection, was obtained from Dr. Lorne Tyrrell (University of Alberta, Edmonton, AB). Permission to use the doxycycline-inducible cells lines (Goldring et al., 2006), designated HepG2 Tet-ON and Huh7 Tet-ON, was obtained from Dr. Chris Goldring (University of Liverpool, Liverpool, UK) and the cells were acquired from Dr. Craig McCormick. The HepG2 Tet-ON and Huh7 Tet-ON cell lines were maintained in Ham's F12 media (Wisent Inc., St. Bruno, QC) containing 15% non-heat inactivated fetal calf serum (FCS) (Wisent). All other cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) (Wisent) supplemented with 10% FCS (Wisent), as well as 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM additional L-Glutamine (Wisent). All cells were maintained in 75cm^2 tissue culture vessels (BD Biosciences, Mississauga, ON) in a 37°C incubator with 5% CO_2. Where indicated, Williams' media E (Invitrogen, Burlington, ON) containing 10% FCS, DMEM (Wisent) containing no FCS, and Hank's Balanced Salt Solution (Invitrogen) containing no FCS, were used as experimental treatments.

2.3 Preparation of Cellular Extracts

To prepare whole cell lysates for use in Western immunoblot experiments, cells were first washed once in phosphate buffered saline (PBS; Wisent), mechanically scraped in incomplete 1x laemmli sample buffer [2% (w/v) sodium dodecyl sulfate (SDS), 50 mM tris(hydroxymethyl)aminomethane hydrochloric acid (Tris HCl), pH 6.8, 10% (v/v) Glycerol, and 1x Protease Inhibitor Cocktail (Roche Applied Sciences, Laval, QC)], and lysed on ice for 15 minutes. Lysates were sonicated using a Microson Ultrasonic Cell Disruptor (Misonix, Farmingdale, NY), and an aliquot taken for subsequent protein quantification. The remaining lysate was then aliquoted into vials, and bromophenol blue

and dithiothreitol (DTT) were added to give final concentrations of 0.2 mg/mL and 100 mM, respectively. Samples were boiled at 95°C for 3 minutes, and then frozen at -20°C for later analysis. Lysates were only exposed to a single freeze-thaw cycle in subsequent experiments.

Lysates destined for immunoprecipitation studies were prepared by washing cells once with PBS, mechanically scraping the cell in immunoprecipitation buffer [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 100 mM sodium chloride (NaCl), 10 mM β-Glycerophosphate, 1% (v/v) tergitol-type nonyl phenoxypolyethoxylethanol (NP-40), 2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM sodium fluoride (NaF), 1 mM sodium orthovanadate (Na_3VO_4), 1 mM phenylmethanesulfonyl fluoride (PMSF), 2 mM DTT, and 1x Protease Inhibitor Cocktail (Roche)], passing the material multiple times through a 25-gauge syringe needle, and holding the resulting lysate on ice for 15 minutes. Cellular debris was removed through centrifugation at 14000 rpm, 4°C using a Centrifuge 5417R (Eppendorf, Mississauga, ON), and the resulting supernatant was held at 4°C prior to immunoprecipitation.

To prepare lysates for luciferase activity assays, cells were first washed once with PBS, scraped in 1x Cell Culture Lysis Reagent from the Luciferase Assay System kit (Promega, Madison, WI), and frozen at -80°C for subsequent analysis. Lysates were thawed once immediately prior to analysis.

Protein quantification was performed using a DC Protein Assay kit (Bio-Rad, Mississauga, ON), as per manufacture's instructions. Briefly, 5 μL of either cell lysate, diluted to fall within the linear range of the assay, or a BSA protein standard corresponding to 2.5 to 30 μg/mL (Bio-Rad) was loading in triplicate on a 96-well plate.

25 μL of an alkaline copper tartrate solution (Working Reagent A), followed by 200 μL of a dilute Folin reagent (Reagent B), was added to each well. After a 15-minute incubation at room temperature, absorbance was measured at 750 nm using a Synergy HT Multi-detection Microplate Reader (Biotek Instruments, Winooski, Vermont), and protein concentrations, relative to the BSA standard, were calculated using Microsoft Excel (Microsoft Corp., Redmond, WA).

2.4 Cloning of pRetro-Tight-Pur:Hbx

To generate a retroviral vector that placed HBx under the control of a doxycycline-responsive transcriptional transactivator, the HBx gene was cloned into the pRetroX-Tight-Pur vector (Clonetech, Mountain View, CA). Experimental details including oligonucleotide design, annealing temperature prediction, and restriction digest enzyme selection were determined with the assistance of Geneious Pro software (Biomatters Ltd., Auckland, New Zealand). All oligonucleotide primers were synthesized by ACGT Corporation (Toronto, ON). The HBx gene was isolated by polymerase chain reaction (PCR) from the pEco63 vector (ATCC) that contains an EcoR1-digested ccc-DNA sequence of an *adw* strain of HBV. For this reaction, forward (5' ATA AGC GGC CGC ACC ATG GCT GCT AGG CTG TAC 3') and reverse (5' AGA CCC TAC GAA TTC ACA GTG GGA CTA GTA CAA GAG AT 3') primers were used in combination with a high fidelity PfuUltra II Fusion HS DNA polymerase kit (Stratagene, La Jolla, CA), and the following PCR cycling conditions: 94°C for 2 min., 10 cycles [94°C for 20 sec., 55°C for 30 sec., 72°C for 30 sec.], then 25 cycles [94°C for 20 sec., 67°C for 30 sec., 72°C for 30 sec.], followed by 72°C for 7 min. Products of this reaction were separated using electrophoresis in 0.8% ultra-pure Tris-Borate-EDTA agarose, and a

portion of the gel was stained with ethidium bromide (Invitrogen) to allow for visualization of the DNA fragments. A DNA fragment corresponding to the predicted size of the desired product was cut from the un-stained portion of the agarose gel, and extracted using a Qiaex II Gel Extraction kit (Qiagen, Mississauga, ON), as per manufacture's instructions.

The HBx fragment and the target pRetroX-Tight-Pur vector (Clontech, Mountain View, CA) were restriction digested with EcoR1 and Not1 restriction enzymes (New England Biolabs, Pickering, ON) in NEB buffer EcoR1 buffer for 1 hour at 37°C, then purified using ultra-pure agarose gel electrophoresis and Qiaex II Gel Extraction (Qiagen), as described above. The insert and vector were treated with a T4 DNA ligase enzyme (New England Biolabs) at 16°C overnight, then electroporated into electro-competent Top10 *E. coli* using a GenePulser Xcell Electroporation System (Bio-Rad). Bacteria were selected on LB-agar plates containing 100 μg/mL ampicillin (Sigma-Aldrich) at 37°C overnight, and individual colonies were picked, re-plated, and screened by colony PCR. Briefly, bacteria were placed in 20 μL deionized water, boiled for 5 min. at 95°C, centrifuged at 14,000 rpm for 5 min. (Centrifuge 5418, Eppendorf), and 5 μL of the resulting supernatant was used in a PCR reaction that would produce a 960 bp fragment only if the insert was correctly orientated within the vector [forward (5' CTG GAA AGA TGT CGA GCG GA 3') and reverse (5' GGC AGA TGA GAA GGC ACA AGA 3') primers, *Taq* DNA Polymerase (Invitrogen, Burlington, ON), 94°C for 2 min., 30 cycles [94°C for 20 sec., 55°C for 30 sec., 72°C for 30 sec.], 72°C for 7 min.]. Screened bacterial colonies were grown in liquid LB media with 100 μg/mL ampicillin, plasmid isolated with a QIAprep Spin Miniprep kit (Qiagen), and partially sequenced

[primer (5' ATC TGA GGC CCT TTC GTC TTC ACT 3')] (ACGT Corporation) to verify their construction. One partially sequenced clone was amplified through growth in liquid LB media with ampicillin, the plasmid isolated with a QIAprep Spin Maxiprep kit (Qiagen) and designated the pRetroX-Tight-Pur:HBx vector.

2.5 Retrovirus Production and Infection

To produce retroviral particles for gene transduction, Phoenix AMPO cells were transiently transfected with suitable retroviral vectors, and the resulting retrovirus-containing supernatants were collected and processed. Briefly, Phoenix AMPO cells were transfected using either a ProFection® calcium-phosphate transfection kit (Promega) or laboratory generated calcium-phosphate transfection reagents. All retrovirus production was performed in 10 cm^2 tissue culture dishes. Regardless of the source of transfection reagents, 20 μg of plasmid DNA was combined with H_2O or Tris-EDTA (TE) to give a final volume of 500 μL, and calcium chloride ($CaCl_2$) to give a final $CaCl_2$ concentration of 250 mM. The resulting solution was then mixed vigorously, via repeated pipetting, with an equal volume of a 2x HEPES –buffered saline solution. This solution was next incubated at room temperature for 30 minutes (ProFection® reagents), or added immediately (laboratory-generated reagents) in a drop-wise fashion to the target cells. The laboratory-generated calcium-phosphate transfection reagents consisted of 2.5 M $CaCl_2$ solution, TE buffer [1 mM Tris HCl, 0.1 mM EDTA] pH 7.6, and 2x HEPES –buffered saline [140 mM NaCl, 1.4 mM sodium phosphate ($NaHPO_4$), 50 mM HEPES]. Transfection efficiency could be qualitatively monitored via GFP expression resulting from pBMN-IP:GFP –transfection, and was consistently observed to be high (greater than 90% cells expressing GFP). The pBMN-IP and pBMN-IP:GFP retroviral vectors were

kindly provided by Dr. Craig McCormick. The pBMN-IP:HBx plasmid was developed and generously provided by David Cyr, and was partially sequenced (ACGT Corporation) to verify its construction. The pRetroX-Tight-Pur-Luc vector was from Clontech (Mountain View, CA).

Transfected Phoenix AMPO media was replaced the next day, then harvested after 48 or 72 hours. When media was collected at the 48-hour time-point, it could be replaced and harvested again after 24 hours; no difference in control GFP transfection was noted between 48 hour, 72 hour, or 48 +24 hour supernatants. Collected retrovirus-containing supernatant was filtered through a 0.45 μm Millex syringe filter (VWR, Mississauga, ON), then used immediately or frozen at -80°C.

Cells destined for retroviral transduction were trypsinized and seeded at low density (less than 50% confluency) in 6-well tissue culture plates, then allowed to re-adhere and rest for 24 hours. Polybrene was added to retroviral supernatants to give a final concentration of 4 μg/mL, and cell media was replaced with the appropriate retrovirus-containing media. Cells were then centrifuged at 1300 x *g*, 23°C for 1.5 hours in an Avanti J-26 XPI centrifuge equipped with a JS-5.3 rotor (Beckman Coulter, Mississauga, ON), and returned to a 37°C incubator overnight. Retroviral supernatants were replaced with fresh media, and the qualitative efficiency of transduction was assess in pBMN-IP:GFP control treatments; GFP expression was faintly detectable at 48 hours post-infection, and clearly visible at 72 hours. Cells were trypsinized, pooled, and placed in 10 cm^2 tissue culture dishes containing puromycin antibiotic selection media at 72 hours post-retroviral infection. Antibiotic selection was monitored using a non-

transduced control treatment, and after 48 hours of puromycin selection cells were trypsinized and re-plated into fresh media.

2.6 Validation of HepG2.2.15 Cell Line

To validate the integration and transcription of HBV genes in the HepG2.2.15 cell line, genomic DNA, isolated using a DNeasy Blood and Tissue kit (Qiagen), and total RNA, isolated using TRIzol Reagent (Invitrogen), were analyzed using HBx-specific PCR primers by Ryan Noyce. Complementary DNA (cDNA) was isolated from 2 μg of DNase I-treated total RNA using a M-MLV Reverse Transcriptase (Invitrogen) and Random Hexamers (Invitrogen). Subsequently, both genomic DNA and cDNA were used as a template in PCR with HBx-specific primers [HBx forward (5' ATG GCT GCT AGG CTG TGC TGC 3'), HBx reverse (5' AGG CAG AGG TGA AAA AGT TGC ATG G 3'), GAPDH forward (5' CGG AGT CAA CGG ATT TGG TCG TA 3'), GAPDH reverse (5' AGC CTT CTC CAT GGT GGT GAA GCC 3'), *Taq* DNA Polymerase (Invitrogen), 94°C for 2 min., 25 cycles [94°C for 20 sec., 58°C for 30 sec., 72°C for 30 sec.], 72°C for 7 min.]. Products were separated by agarose electrophoresis, and visualized on a Kodak 4000MM imaging system (Mandel Scientific, Guelph, ON).

Total DNA from HepG2.2.15 culture supernatant and cell lysate, and control HepG2 cell lysate was isolated and analyzed by Dr. Liang-Tzung Lin to assess the presence of HBV genomic DNA through Taqman real-time PCR (Applied Biosystems, Carlsbad, CA). Briefly, total DNA from cell lysate was obtained using a DNeasy Blood and Tissue kit (Qiagen). To extract DNA from culture supernatants, viral particles were first precipitated using 40% (w/v) polyethylene glycol-8000 (Sigma-Aldrich) at 4°C overnight and centrifugation at 6800x *g* for 30 minutes. The resulting pellet was then

harvested for DNA using a DNeasy Blood and Tissue kit (Qiagen). The isolated total DNA extracts were subsequently amplified using the Taqman Universal Master Mix (Applied Biosystems) as per the manufacture's protocol, with custom primers and probe designed against HBV core [HBV core forward (5' AGT GTG GAT TCG CAC TCC T 3'), HBV core reverse (5' GAG TTC TTC TTC TAG GGG ACC TG 3'), HBV core probe (5' *FAM* CCA AAT GCC CCT ATC TTA TCA ACA CTT CC *MGB* 3')]. The amount of HBV genomic DNA was quantified based on a standard curve derived from the amplification of 10-fold dilutions of the HBV-genome containing plasmid, pEco63.

2.7 Luciferase Assay

The luciferase activity of cells transduced with pRetroX-Tight-Pur-Luc retrovirus was assayed using a Luciferase Assay System (Promega), as previously described in Lin *et al.* (Lin et al., 2010). Briefly, cells treated for 24 hours with 0, 100, or 1000 ng/mL doxycycline were lysed in a luciferase lysis buffer, mixed with a luciferase assay substrate, and measured using a Promega GloMax 20/20 Luminometer (Promega). Activity was reported as relative light units (RLU) and graphed on a log scale using GraphPad Prism software (GraphPad Software, La Jolla, CA).

2.8 Bright-field and Immunofluorescence Microscopy

Bright-field images of live cells in culture were obtained using a Leica DMI 4000 B phase contrast microscope (Leica Microsystems Inc., Concord, ON). To visualize 53BP1 puncta, cells were seeded on poly-D-lysine –treated 22 mm coverslips and cultured overnight to allow for re-attachment. Cells were fixed in 4% paraformaldehyde, permeablized with 90% methanol (MeOH), and blocked for 1 hour with 3% FCS.

Coverslips were immersed in a 1 in 200 dilution of 53BP1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, incubated with an Alexa Fluor 488 – conjugated secondary antibody (Invitrogen) for 1 hour, and counterstained with 10 μg/mL Hoechst 33258 (Invitrogen) for 10 minutes. Coverslips were mounted to glass microscope slides using a Glycerol-based fluorescence-mounting medium containing 0.01% p-phenylenediamine, an anti-fade agent. Slides were visualized using a Zeiss Axiovert 200 microscope equipped with a Hamamtsu Orca camera located in the Cellular Microscopy Digital Imaging facility (Dalhousie University, Halifax, NS).

2.9 Senescence-Associated β-Galactosidase Staining

The day prior cells were trypsinized and re-seeded in 6-well tissue culture plates (BD Biosciences) and allowed to re-adhere overnight. Cells were stained using the Senescence β–Galactosidase Staining kit (Cell Signaling Technologies, Danvers, MA). Briefly, cells were washed with PBS, fixed for 10 minutes with 1x Fixative Solution, washed twice with PBS, and stained overnight at 37°C (no additional CO_2) in 1x Staining Solution containing 1 mg/mL bromo-chloro-indolyl-galactopyranoside (X-gal). Bright-field images of stained cells were obtained using a Leica DMI 4000 B phase contrast microscope (Leica Microsystems).

2.10 Antibodies

A polyclonal antibody against HBx protein was previously generated in our laboratory as described in Diao *et al.*(Diao et al., 2001). Briefly, a maltose-binding protein –HBx fusion protein was expressed in *Escherichia coli*, purified by affinity chromatography, and used to immunize rabbits; resulting antiserum was purified by

Table 2.1 - Commercial Antibodies and Immunoblot Dilutions

Target	Company (Catalog#)	Immunoblot Dilution
LC3	Nanotools (0260-100/LC3-2G6)	1 in 200
LC3B	Sigma-Aldrich (L7543)	1 in 5,000
Beclin 1	Santa Cruz Biotechnology (sc-11427)	1 in 200
Bcl-2	Cell Signaling Technologies (2870)	1 in 1,000
Bcl-xL	Cell Signaling Technologies (2764)	1 in 2,000
Mcl-1	Cell Signaling Technologies (5453)	1 in 1,000
p-Bcl-2 (Ser 70)	Cell Signaling Technologies (2827)	1 in 1,000
p-Bcl-2 (Thr 56)	Cell Signaling Technologies (2875)	1 in 1,000
SAPK/JNK	Millipore / Chemicon (AB8910)	1 in 500
p-SAPK/JNK	Cell Signaling Technologies (4668)	1 in 1,000
p-c-Jun	Cell Signaling Technologies (9261)	1 in 1,000
Rb	Santa Cruz Biotechnology (sc-102)	1 in 500
HMGA2	Santa Cruz Biotechnology (sc-30223)	1 in 200
p53	Santa Cruz Biotechnology (sc-136)	1 in 1,000
p21	Santa Cruz Biotechnology (sc-6246)	1 in 500
p16 INK4A	Cell Signaling Technologies (4824)	1 in 1,000
Cyclin A	Santa Cruz Biotechnology (sc-596)	1 in 500
GAPDH	Ambion (AM4300)	1 in 20,000
53BP1	Santa Cruz Biotechnology (sc-22760)	Not Applicable
Alexa Fluor 488	Invitrogen (A11008)	Not Applicable
Rabbit IgG	Santa Cruz Biotechnology (sc-2027)	Not Applicable
Rabbit HRP	Invitrogen (G-21234)	1 in 5,000
Mouse HRP	Jackson IR (115-035-146)	1 in 5,000
Clean-Blot IP	Pierce (21230)	1 in 1,000

affinity chromatography with His-tagged HBx and nickel-sepharose beads. This HBx antibody was employed in immunoblotting experiments at a dilution of 1 in 500. Commercial antibodies used in this thesis were obtained from Sigma-Aldrich, Santa Cruz Biotechnology, Nanotools Antibodies (Teningen, Germany), Cell Signaling Technologies, Millipore (Billerica, MA), Invitrogen, and Jackson ImmunoResearch (West Grove, PA), and are listed in Table 2.1.

2.11 Immunoprecipitation

Immunoprecipitation lysates were standardized to 250 μg of total lysate protein and 1 mL volume in immunoprecipitation buffer. All lysates were pre-cleared through the addition of 1 μg normal rabbit IgG control sera and 20 μL re-suspended Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) for 30 minutes at 4°C with gentle mixing. After 5 minutes centrifugation at 2,500 rpm, the supernatants were transferred to new vials, and either 2 μg of Beclin 1, 10 μL of Bcl-xL, or 2 μg of normal rabbit IgG sera were added. Vials were gently mixed for 1 hour at 4°C, 20 μL re-suspended protein A/G beads added, and then incubated with mixing overnight at 4°C. Agarose beads were pelleted and washed 5 times with immunoprecipitation buffer, then boiled for 3 minutes in 2x protein sample buffer [100 mM Tris-HCl (pH 8.0), 20% glycerol, 4% SDS, 2% β-mercaptoethanol, 0.001% bromophenol blue]. To avoid detection of denatured heavy and light chain IgG added during the immunoprecipitation process, a native IgG-specific Clean-Blot® IP Detection Reagent was employed as the secondary antibody in all immunoblotting experiments.

2.12 SDS-polyacrylamide Gel Electrophoresis

For immunoblot analysis of HBx, $p21^{WAF1}$, $p16^{INK4A}$, HGMA2, Bcl-2, Bcl-xL, Mcl-1, p-Bcl-2 (Ser70), and p-Bcl-2 (Thr56), discontinuous denaturing SDS-PAGE gels comprised of 15% resolving and 5% stacking polyacrylamide gels were cast using the Mini Protean 3 gel electrophoresis system (Bio-Rad). For Beclin 1, SAPK/JNK, p-SAPK/JNK, and p-c-Jun, 12% resolving polyacrylamide gels, and for p53, RB, and Cyclin A, 9% resolving polyacrylamide gels were employed. LC3 immunoblots using the antibody obtained from Sigma-Aldrich employed 9-15% gradient gels cast using the Mini Protean system and a Pharmacia LKB gradient maker. Samples were standardized, in terms of both total cell lysate protein amount and loading volume, prior to being loaded on the gel. Protein samples were separated using a Mini Protean 3 apparatus (Bio-Rad) at a constant voltage of 80 V for the first 30 minutes, then 130 V until the dye front emerged from the bottom of the gel (approximately 1 hour and 50 minutes).

2.13 Western Immunoblot Analysis

Polyacrylamide gels were transferred onto polyvinylidene fluoride (PVDF) membranes (Pierce) using either a X-Cell II semi-dry transfer apparatus (Invitrogen) at 385 milliamperes (mAMP), or a Mini Trans-Blot wet transfer apparatus (Bio-Rad) at 350 mAMP, for a period of 1 hour. Blots were then treated with methanol (MeOH) for 10 seconds, dried at room temperature for 15 minutes, and re-activated in MeOH for 5 minutes. All blots were blocked in 5% non-fat skim milk in PBS containing 0.1% Tween-20 detergent (PBS-T) for either 1-hour at room temperature, or overnight at 4°C. Blots were washed 3-times with PBS-T, placed in the appropriate primary antibody, in 5% BSA / PBS-T or 5% FCS / PBS-T at the dilution indicated on Table 2.1, and gently

agitated. Blots were then washed again in PBS-T, and placed in an appropriate αIgG secondary antibody conjugated with horseradish peroxidase (HRP). After a final wash in PBS blots were developed via chemiluminescence, and imaged on a Kodak 4000MM imaging system (Mandel Scientific).

Chapter 3: Results

3.1 Acknowledgements

I would like to acknowledge the following individuals for their contributions to the experimental results. David Cyr supplied the pBMN-IP:HBx retroviral vector necessary for the generation of retroviral particles for HBx transduction. Andrew Leidal provided both reagents and advice regarding the recognition and characterization of cellular senescence. Liang-Tzung Lin validated the HBV-model HepG2.2.15 cell line, and assisted with the luciferase quantification assay. Gary Sisson provided cloning advice and guidance. Dr. Chris Goldring of the University of Liverpool provided the HepG2 and Huh7 doxycycline–inducible (Tet-ON) cell lines. Dr. Ryan Noyce supplied advice regarding protein immunoprecipitation, western blotting, and experimental design.

3.2 HepG2 and Huh7 Cells Transduced With HBx Display Changes in Morphology and Growth Over Time

Due to the lack of accessible, effective *in vitro* models of HBV infection, most studies examining the effects of individual HBV proteins have relied upon transient transfection and gene over-expression experiments to evaluate their effects upon the cell and the infectious cycle. Unfortunately, transfection reagents often have cytotoxic or deleterious off-target effects that can make it difficult to differentiate between the effects of the viral protein of interest, and those of the experimental treatment itself (Jacobsen, Calvin, and Lobenhofer, 2009). Furthermore, certain cell lines, such as the hepatocellular carcinoma cell line HepG2, are difficult to transfect efficiently. To avoid these issues, retrovirus particles were derived from either pBMN-IP:HBx, control pBMN-IP:GFP, or pBMN-IP ('Empty') retroviral plasmids and used to infect HepG2 and Huh7 cells. The

HBx protein could be detected by western blotting in infected HepG2 cells, but not Huh7 cells, as early as two days post-infection (Fig. 1A). At time points up to 4 days post-infection, the morphology of HepG2 and Huh7 cells transduced with the HBx retrovirus closely resembled that of both non-transduced and control pBMN-IP -transduced cells (data not shown). To enhance the proportion of cells expressing HBx, transduced cells were given a rest period of three days and then subjected to puromycin selection for an additional two-day period. We initially found that while a fraction of both treatments were resistant to puromycin, the proportion was consistently less in HBx-transduced samples. When left in culture, the control pBMN-IP-transduced HepG2 and Huh7 cells quickly grew to confluency (Fig. 3.1B), and appeared to possess a morphological phenotype typical for their respected cell type. However, the pBMN-IP:HBx-transduced HepG2 and Huh7 cells failed to show noticeable population growth over time, and gradually came to adopt an enlarged, flattened morphology, often with long pseudopodia-like cytoplasmic extensions. The majority of these cells remained adherent in culture over extended periods of time, and could re-adhere after trypsinization. While both cell types showed similarities in their response, far fewer Huh7 cells adopted the altered morphology, and to a much greater extent, than was observed in the HepG2 cell line. These observations, particularly the lack of detectable population growth, suggested that retroviral transduction of HBx might induce a cellular senescence response in a proportion of transduced HepG2 and Huh7 cells.

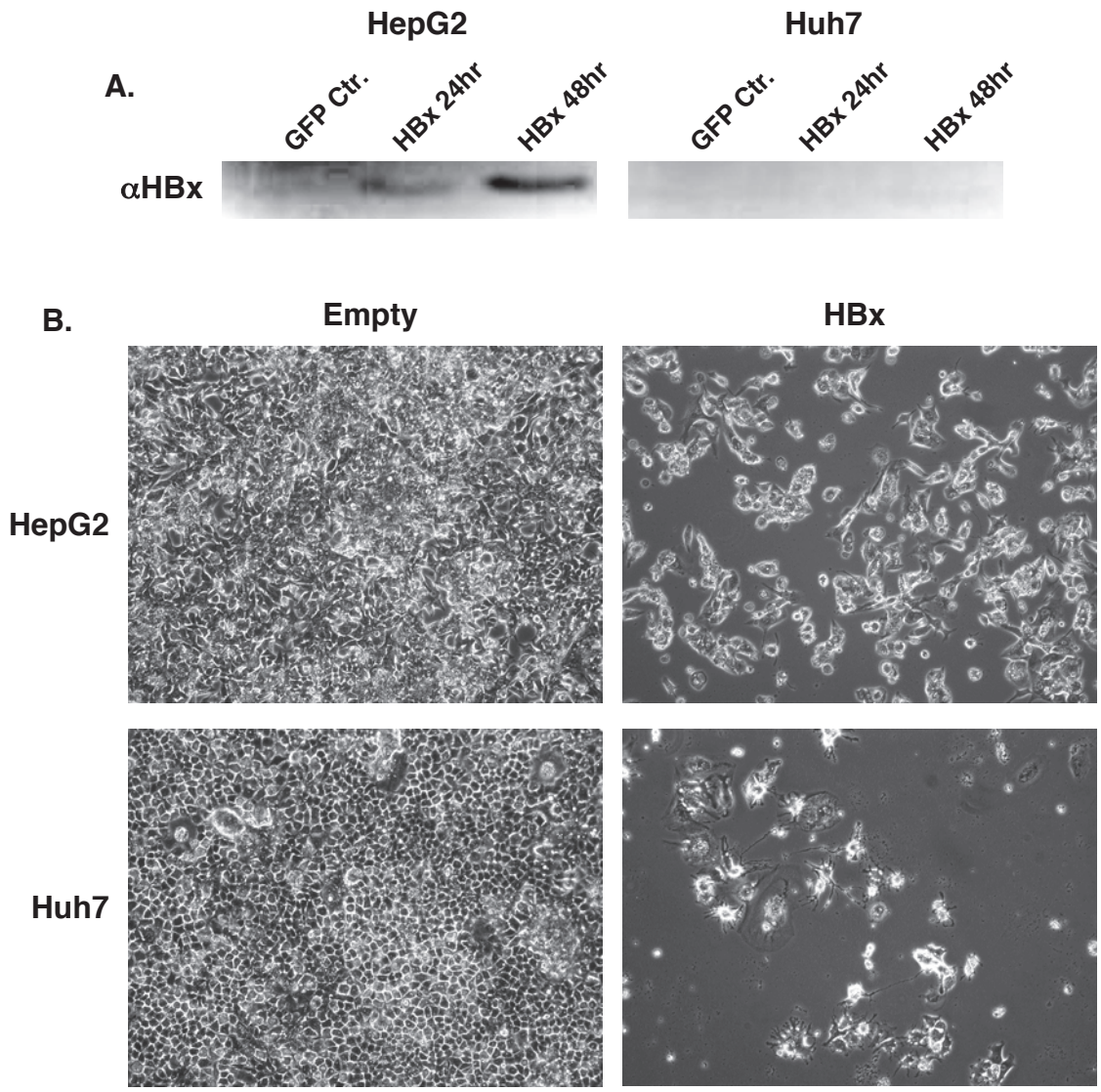

Figure 3.1 - HepG2 and Huh7 Cell Lines Transduced with HBx Adopt Altered Morphology and Have Suppressed Population Growth - A) Cells were transduced with either pBMN-IP:HBx (HBx) or pBMN-IP:GFP -derived (GFP) retrovirus, then lysed at 24 or 48 hrs. HBx was detectable as early as 24hrs in HepG2 cells, but was not detected in the Huh7 cells. **B)** Cells were transduced with either pBMN-IP:HBx-derived (HBx) or pBMN-IP-derived (Empty) retrovirus, allowed 3 days to recover, then subjected to 2 days of puromycin selection. Selected cells were re-plated in growth media for 4 days prior to live-cell imaging (9 days post-transduction). Images are 10x magnification. While control (Empty) treatments grew to confluency, HBx-expressing HepG2 or Huh7 populations showed no sign of expansion. HBx-expressing cells displayed a flattened, enlarged morphology, often with pseudopodia-like cytoplasmic extensions.

3.3 Expression of HBx in HepG2 Cells Induces Cellular Senescence

To examine whether the observed phenotype in HBx-transduced cells was due to the induction of a cellular senescence response, we investigated several markers of the senescence state (Campisi and d'Adda di Fagagna, 2007). For all senescence experiments, HepG2 or Huh7 cells were transduced with pBMN-IP:HBx or control pBMN-IP:(empty) retrovirus, selected with puromycin, and then maintained in culture for 8 days to allow any phenotype to develop prior to analysis. The first senescence marker investigated was β-Galactosidase activity, a lysosomal enzyme whose expression is up-regulated in senescent cells (Dimri et al., 1995; Itahana, Campisi, and Dimri, 2007). β-Galactosidase expression was detected using a senescence β-Galactosidase staining kit (Cell Signaling Technologies), as per manufacture's instructions. Both HepG2 and Huh7 cells transduced with HBx showed a larger proportion of cells staining positive for enhanced β-Galactosidase activity relative to their controls (Fig. 3.2). However, the effect was observed to be more uniform in the HepG2 cell line, where the majority of HBx-transduced cells stained positive for enzymatic activity. Furthermore, while some of the HBx-transduced Huh7 cells did demonstrate strong β-Galactosidase staining, multiple attempts to detect the HBx protein by western blot in these treatments were unsuccessful (Fig. 3.6). For these reasons we focused our subsequent senescence investigation using the HepG2 cell line.

Cell lysates from transduced HepG2 cells were collected and analyzed by western immunoblot for changes in senescence-associated protein levels. HBx transduction

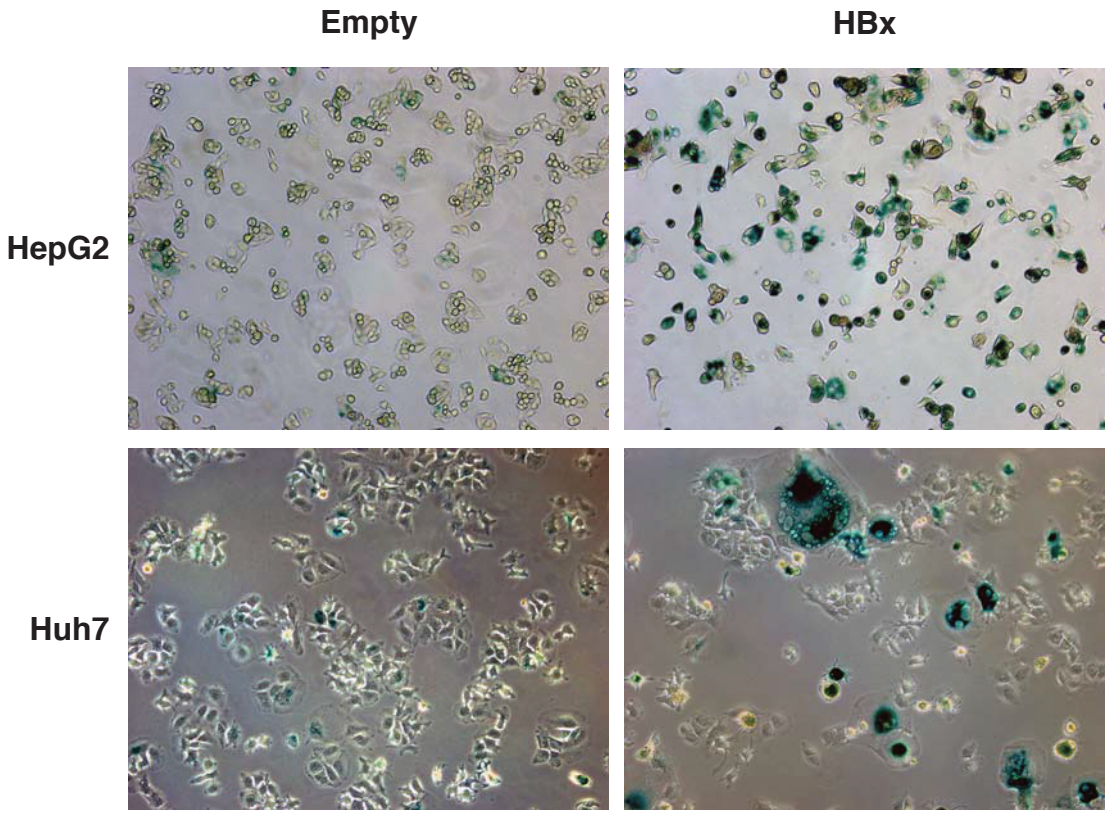

Figure 3.2 - HepG2 and Huh7 cell lines transduced with HBx possess enhanced senescence-associated β-Galactosidase staining. Cells transduced with pBMN-IP:HBx-derived (HBx) or pBMN-IP-derived (Empty) retrovirus were puromycin-selected and maintained in culture for 8 days (13 days post-infection). Cells were then re-plated, fixed, and β-Galactosidase stained overnight using a senescence β-Galactosidase staining kit (Cell Signaling Technologies). Representative images are 10x magnification. Both HepG2 and Huh7 HBx-expressing cells displayed greater levels of senescence-associated staining relative to controls, but the difference was more pronounced in the HepG2 cell line.

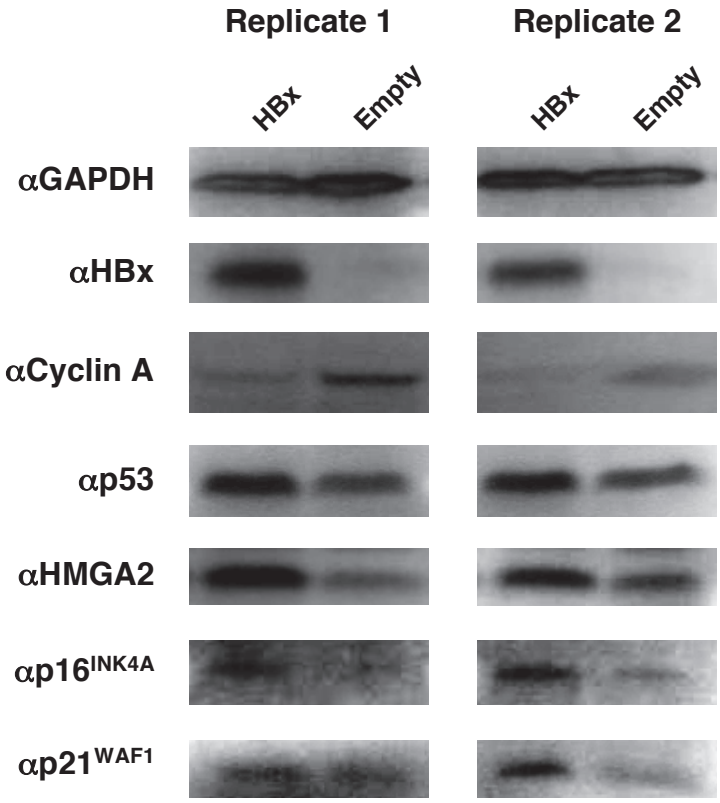

Figure 3.3 - HBx induces senescence in HepG2 cells, evident as changes in senescence-associated protein levels. HBx-expressing HepG2's were harvested in 1x sample buffer 13 days post-retroviral transduction. Protein lysates were quantified by a DC (detergent competent) protein assay (Biorad) and analyzed by western blot. Elevated levels of senescence markers HMGA2, p53, $p16^{INK4A}$, and p21 were detected in HBx-transduced cells. Cyclin A, which is absent in senescent cells, was detected only in the pBMN-IP control (Empty) treatment. An αGAPDH antibody was employed as a loading control. The HBx protein was verified present in lysates from all HBx-transduced cells. Representative data from experiment performed in triplicate.

resulted in substantive increases in HMGA2 and p53 levels, and yielded detectable increases in $p21^{WAF1}$ and $p16^{INK4a}$ (Fig. 3.3), proteins whose levels are elevated in senescent cells (Campisi and d'Adda di Fagagna, 2007). Furthermore, cyclin A, an essential protein for cell cycle progression that is absent in senescent cells, was present in control pBMN-IP transduced lysates but only faintly detectable in pBMN-IP:HBx transduced cells. The HBx protein was verified by western blot to be present in all pBMN-IP:HBx lysates. On the basis of the observed changes in senescence-associated protein levels, β-Galactosidase staining pattern (Fig. 3.2), and lack of noticeable cell growth (Fig. 3.1), we concluded that HBx transduction induces senescence in a proportion of HepG2 cells.

3.4 HBx Expression in HepG2 Cells Leads to the Accumulation of DNA Damage

Numerous factors can trigger cellular senescence (Campisi and d'Adda di Fagagna, 2007), including a shortening of telomeres (Harley, Futcher, and Greider, 1990; Martens et al., 2000), expression of certain strong oncogenes (Serrano et al., 1997; Zhu et al., 1998), interference with chromatin modification (Bandyopadhyay et al., 2002; Ogryzko et al., 1996), and severe DNA damage (Di Leonardo et al., 1994). Since it has been reported that HBx expression can interfere with DNA damage repair (Becker et al., 1998; Groisman et al., 1999), we investigated whether HBx-expressing senescent HepG2 cells possessed increased levels of DNA damage accumulation relative to control cells. At 13 days following retroviral transduction, HepG2 cells were trypsinized, seeded onto treated cover slips, and immunostained with an α53BP1 (Santa Cruz) antibody, a marker for foci containing DNA damage (Rappold et al., 2001). When visualized, 53BP1

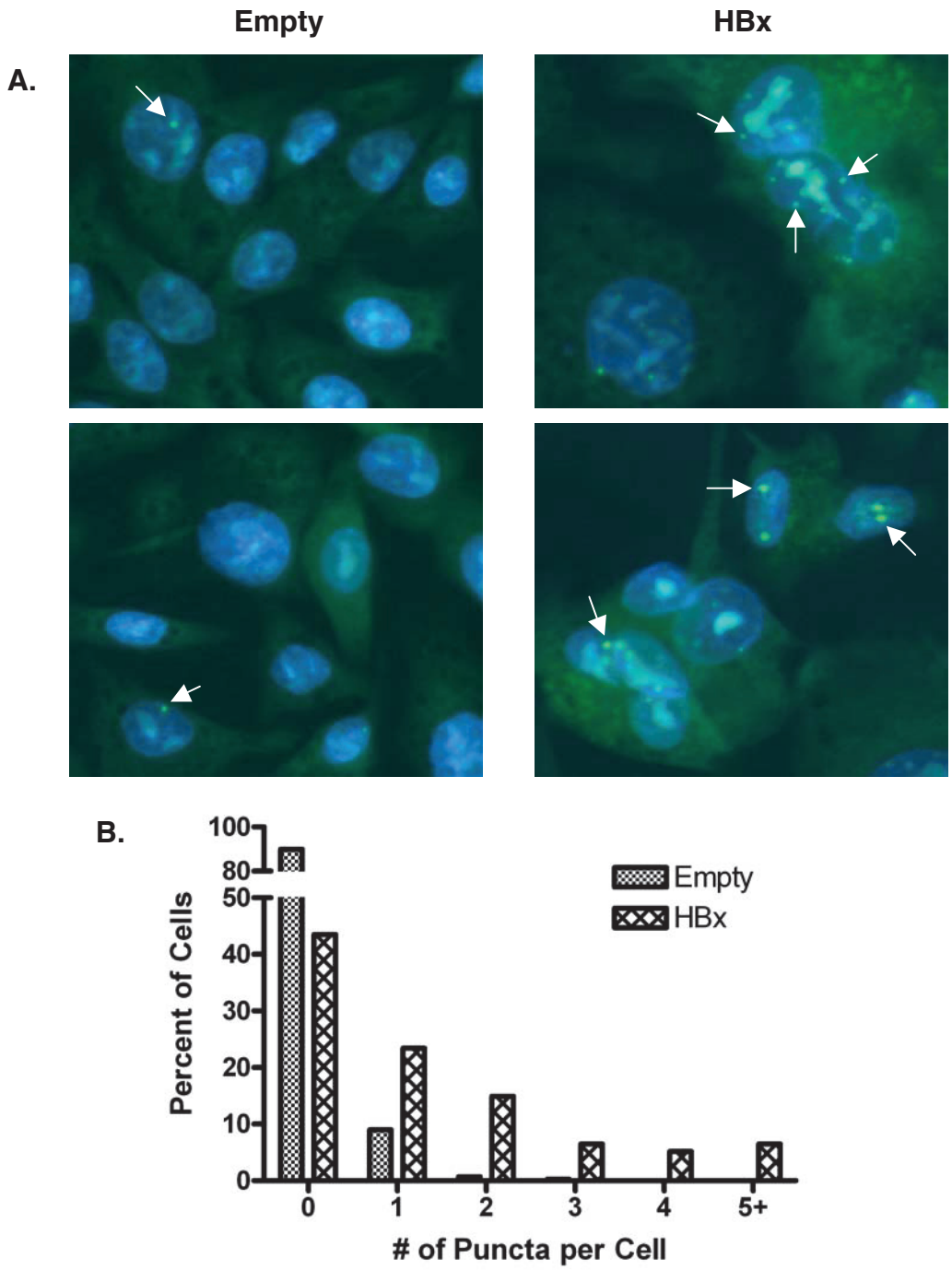

Figure 3.4 - HBx induces an accumulation of DNA damage evident as 53BP1-staining puncta. A) Representative images of 53BP1 immuno-stained HepG2 cells. Cells were retrovirally transduced, puromycin-selected, and maintained in culture for 8 days (13 days post-infection). These cells were re-plated on cover slips, fixed and permeablized, and immuno-stained with α53BP1 (Santa Cruz) and Alexa Fluro 488 fluorescent secondary antibodies. Images are x40 magnification. Puncta indicated by arrows. **B)** Distribution of number of 53BP1 puncta present in each cell. 288 control (Empty) and 154 HBx expressing cells analyzed. n=1.

nuclear-localized puncta (Fig. 3.4A) could be detected in both the control pBMN-IP transduced and HBx-transduced HepG2 cells, but at different frequencies (Fig. 3.4B). A greater proportion of the HBx-expressing HepG2 cells contained 53BP1 puncta relative to the control, and HBx-expressing cells were observed to be more likely to contain multiple 53BP1 puncta than the control cells. However, it should be noted that a significant proportion (~40%) of HBx-expressing HepG2 cells had no detectable 53BP1 puncta, making it difficult to conclude that an accumulation of DNA damage alone was responsible for the observed senescence phenotype. These data suggest that transduction of HepG2 cells with HBx results in the accumulation of DNA damage within a subpopulation of cells, either through interference with DNA repair or an increase in the rate at which DNA damage occurs.

3.5 Autophagy in HepG2 and Huh7 Cells Can Be Measured by LC3 Immunoblotting

Recent studies have implicated autophagy as an effector mechanism in the establishment of senescence. Researchers have demonstrated that disruption of autophagy can delay oncogene-induced senescence, as well as increase both the proportion of cells that remain proliferative or undergo apoptosis (Gamerdinger et al., 2009; Patschan et al., 2008; Young et al., 2009). Interestingly, several contradictory studies have also reported that HBV modulates autophagy through the actions of the HBx protein (Sir et al., 2010; Tang et al., 2009). To clarify whether HBx modulates autophagy in our experimental model, it was first necessary to establish the appropriate experimental conditions for the cell lines under investigation. Initially, HepG2 and Huh7 cells were exposed to 'feeding' media that suppressed autophagy, consisting of DMEM supplemented with 10% FCS, or

'starvation' media that induced autophagy, consisting of Hanks Balanced Salt Solution (HBSS). Protein lysates from these cells were analyzed by Western immunoblot for variations in the level of LC3-II, an indicator of autophagy activity. Surprisingly, repeated experiments revealed that both cell types responded unexpectedly to these treatments, with HBSS suppressing autophagy at time points as short as 30 minutes, and DMEM resulting in high background levels of LC3-II (Fig. 3.5A). Upon further investigation it was discovered that Williams' media E, a media specifically developed for the *in vitro* culture of primary hepatocytes, and DMEM without FCS, could be employed to establish 'fed' and 'starved' media conditions, respectively, in both the HepG2 and Huh7 cell lines (Fig. 3.5B). It should be noted that, while uncommon, these media conditions have been employed in other autophagy studies (Itakura et al., 2008; Liu et al., 2009; Takahashi et al., 2009; Yin, Ding, and Gao, 2008). To determine whether qualitative differences in LC3-II levels would appear linear over a range of protein quantities, lysates from HepG2 cells, treated with Williams' media E, were analyzed by Western immunoblot at three different protein concentrations corresponding to 3, 6, and 9 μg of total cell lysate protein. LC3-II levels increased in a linear fashion (Fig. 3.5C), demonstrating that qualitative differences in LC3-II levels could be demonstrated using these methods. Collectively, these data suggest that LC3 western blotting can be used to detect differences in LC3-II levels in HepG2 and Huh7 cell lines.

3.6 HBx Induces Autophagy in HepG2 Cells

We next investigated whether the HBx protein could modulate autophagy, as determined by changes in LC3-II levels, when transduced into cell lines of hepatic origin. HepG2 and Huh7 cells were transduced using either control pBMN-IP or pBMN-IP:HBx

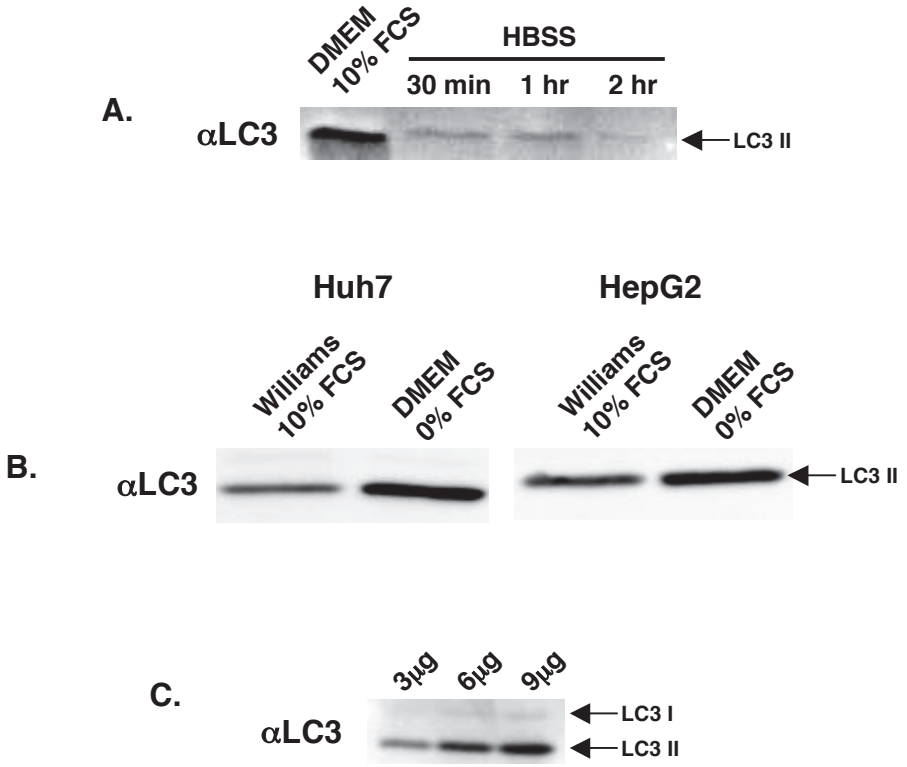

Figure 3.5 - Western blotting for LC3 protein in HepG2 and Huh7 cells detects changes in autophagy in response to media conditions. A) Huh7 cells present high basal LC3-II levels in typical 'full' media conditions (DMEM, 10% FCS), but barely detectable levels when exposed to nutrient poor 'starvation' media (HBSS, 0% FCS). HepG2 cells showed similar results (data not shown). **B)** Further investigation revealed that a 'rich' media originally developed for culturing hepatocytes (Williams, 10% FCS) and DMEM without FCS could serve as effective 'fed' and 'starved' media conditions for the HepG2 and Huh7 cell lines. After 2 hours, exposure to Williams media produced low basal levels of LC3-II in both HepG2 and Huh7 cells, while DMEM without FCS induced a strong response. **C)** Qualitative differences in LC3-II levels could be visualized by LC3 western blotting. HepG2 cells, treated for 2 hours with Williams Media, were lysed and quantified prior western blotting. The α LC3 westerns in **A)** and **B)** employed LC3 primary antibody from Nanotools Antibodies, while **C)** used LC3 primary antibody obtained from Sigma-Aldrich.

derived retrovirus, allowed three days to recover, then puromycin selected for an additional two days. These cells were trypsinized, cultured at low density (less than 25% confluency) overnight in Williams' media E, and then treated with either fresh 'fed' (Williams' media E, 10% FCS) or 'starved' (DMEM, 0% FCS) media for two hours prior to lysis. In both the Huh7 (Fig. 3.6) and HepG2 (Fig. 3.7) cell lines, transduction with HBx resulted in qualitatively higher levels of LC3-II relative to controls, indicating that the autophagy pathway was up-regulated by HBx transduction. This pattern was evident whether the cells were 'starved' or 'fed'. Interestingly, while HBx could be easily detected in transduced HepG2 cells by Western immunoblot, this was not the case in the Huh7 cell line, even when the maximal amount of cell lysate was analyzed. We concluded that both cell lines showed a potential up-regulation in autophagy in response to HBx-transduction, however, it was felt that the lack of detectable HBx in the Huh7 cell line would present a significant challenge in determining whether the observed effect was a direct effect of the HBx protein; for this reason subsequent autophagy investigations focused upon the effect of HBx in HepG2 cells.

Autophagy inducers can increase LC3-II levels by either enhancing the induction of autophagy, or by blocking the maturation and degradation of autophagosomes (Klionsky et al., 2008; Mizushima and Yoshimori, 2007; Tanida, Ueno, and Kominami, 2008). To distinguish between these two possibilities, cells expressing HBx were treated with bafilomycin A1 or chloroquine, compounds that disrupt lysosomal acidification and autophagic protein degradation. LC3-II accumulated to a similar extent in both the HBx and control cell lines when exposed to either compound (Fig. 3.8). These data suggest that HBx does not block autophagosome maturation in the HepG2 cell line.

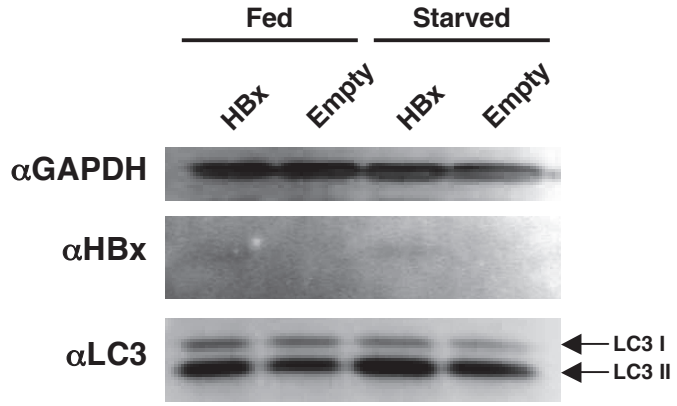

Figure 3.6 - HBx transduction induces autophagy in Huh7 cells as detected by LC3 western blotting. Huh7's transduced with HBx or 'empty' control retrovirus were puromycin-selected and subsequently lysed 6 days post-infection. While qualitative differences in LC3-II levels between HBx and Control were detectable under both Fed (Williams, 10% FCS) and Starved (DMEM, 0%FCS) conditions, HBx was not detectable in any lysate. Representative data from experiment performed in duplicate.

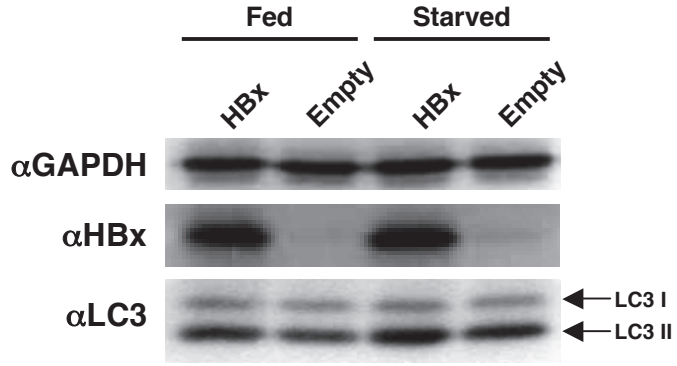

Figure 3.7 - HBx induces autophagy in HepG2 cells as detected by LC3 western blotting. HepG2's transduced with HBx or 'empty' control retrovirus were puromycin-selected and subsequently lysed 6 days post-infection. Qualitative differences in LC3-II levels between HBx and Control were detectable under both Fed (Williams, 10% FCS) and Starved (DMEM, 0%FCS) conditions. HBx protein could be detected in the HBx-transduced cell lysate. Representative data from experiment performed in triplicate.

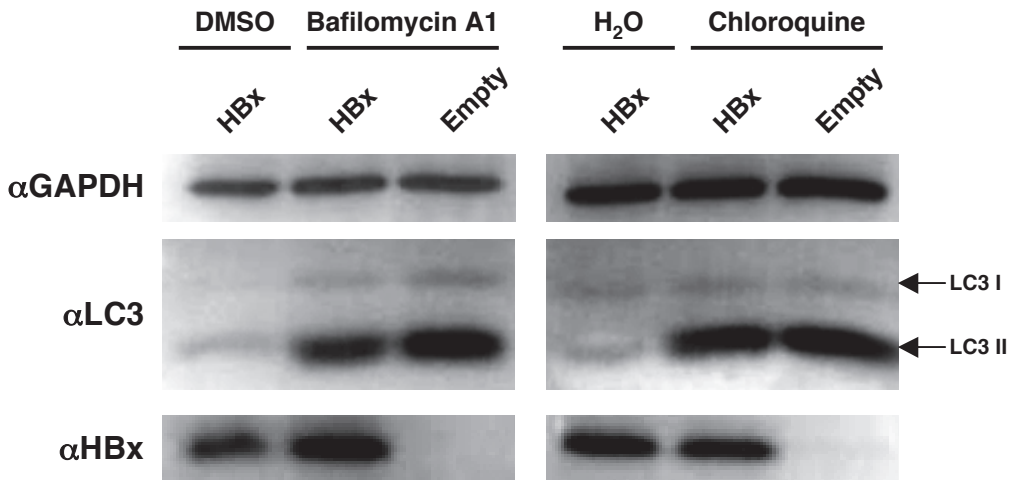

Figure 3.8 - HBx does not block autophagosome maturation in HepG2 cells. Treatment of HepG2 cells with Bafilomycin A1 (100nM, 4Hr) or Chloroquine (50μ M, 4Hr), compounds that block lysosomal acidification and subsequent LC3-II degradation, both resulted in an accumulation of LC3-II in HBx and control (Empty) transduced cells. The substantive qualitative difference between compound-treated and untreated HBx-transduced cells indicated that HBx-associated accumulations of LC3-II are not the product of a block to autophagosome maturation. Transduced HepG2 cells were puromycin selected, lysed 6 days post-infection, and quantified by DC protein assay (Biorad) prior to western blot analysis.

3.7 Autophagy Levels in the HBV-model HepG2.2.15 Cell Line Can Not Be Directly Compared With Those in the HepG2 Cell Line

To investigate whether full-length HBV genomes, producing endogenous levels of HBV proteins, could up-regulate autophagy in a manner similar to that observed in HBx over-expressing cells, we attempted to compare the autophagic response of the HepG2.2.15 model of HBV infection (a HepG2 derived cell line that contains two sequentially integrated copies of the HBV genome, producing all HBV proteins and secreting HBV virion) (Sells, Chen, and Acs, 1987) with that of its parental cell line, HepG2. Preliminary studies conducted during the refinement of the LC3 Western blotting assay observed that HepG2.2.15 cells, verified to contain HBV mRNA transcripts (Fig. 3.9A and B), had lower levels of LC3-II in comparison to a control HepG2 cell lysate (data not shown). However, upon further investigation it was discovered that a contradictory result could be obtained if HepG2 cells obtained directly from the American type culture collection (ATCC) were used in place of those continuously cultured in our own laboratory (data not shown). To clarify these results, HepG2 cells, both from our own laboratory collection (CDR) and those obtained directly from ATCC, and HepG2.2.15 cells were seeded to similar densities (less than 25% confluency), cultured overnight in Williams' media E, treated with fresh Williams' media E for 2 hours the next morning, and lysed. While both HepG2 cells obtained from ATCC and HepG2.2.15 cells contained comparable levels of LC3-II, the HepG2 cells from our laboratory collection displayed elevated levels of LC3-II (Fig. 3.9). From these results we found it difficult to conclude whether autophagy was modulated by HBV in the HepG2.2.15 cell line, and suggest that further direct comparisons between HepG2.2.15's

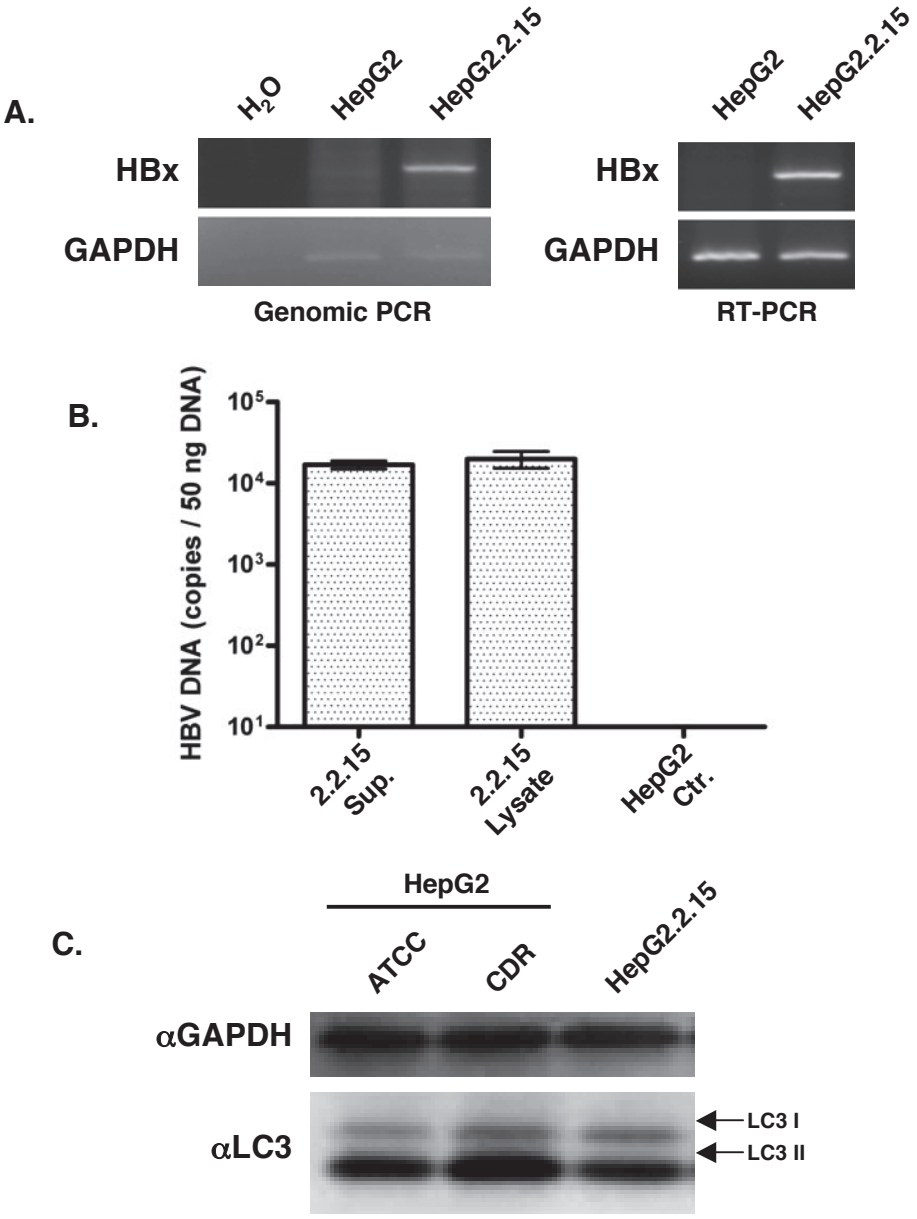

Figure 3.9 - The HepG2.2.15 cell line cannot be directly compared with its parental HepG2 cell line as a means of examining HBV's regulatory effects on autophagy. The HepG2.2.15 cell line is a widely employed *in vitro* model of HBV infection. To validate the model **A)** genomic and mRNA HBx, and **B)** HBV core DNA within and released from the cell line, were measured via genomic, RT, and real-time PCR, respectively. **C)** Lysates from HepG2 cells obtained from two sources (ATCC and CDR) and HepG2.2.15 cells were analyzed via LC3 western blotting. While LC3-II levels were comparable in the ATCC HepG2 and HepG2.2.15 cell lysates, they were elevated in CDR HepG2 lysates, suggesting that deviations in autophagy responses may have developed between the HepG2 and HepG2.2.15 cell lines since the development of the latter in the 1980's.

and the parental HepG2 cell line with regards to autophagy would be unadvisable. With the exception of data presented in Fig. 3.5A and B, all experiments involving the HepG2 cell line employed cells obtained directly from ATCC.

3.8 HBx Can Be Expressed in Doxycycline-Inducible HepG2 and Huh7 Cell Lines

In order to enable future studies examining whether HBx's observed induction of autophagy occurs at expression levels more closely reflecting those of endogenous expression, we obtained two cell lines with doxycycline–inducible gene expression systems (Tet-ON) initially derived from the HepG2 and Huh7 cell lines (Goldring et al., 2006). To ensure that these cells could show variable LC3-II levels in response to autophagy stimuli, both lines were seeded at low densities (less than 25% confluency), cultured overnight in Ham's F12 supplemented with 15% FCS, and treated with either 'fed' (Williams' media E, 10% FCS) or 'starved' (DMEM, 0% FCS) media for 2 hours prior to lysis. Western blotting revealed that both Huh7 Tet-ON and HepG2 Tet-ON (Fig. 3.10A) cells had elevated levels of LC3-II in response to starvation, suggesting that these cell lines were suitable for use with autophagy studies.

Since the Tet-ON cell lines in question were created using a modified Tet-ON transactivator (Goldring et al., 2006), we investigated whether they were compatible with the "Retro-X Advanced Inducible Expression System" (Clontech Laboratories) vectors that we intended to use in this study. Both the Huh7 Tet-ON and HepG2 Tet-ON cells were transduced with pRetroX-Tight-Pur-Luc –derived retrovirus and selected with puromycin. These cells, along with control GFP-transduced and parental cell line-transduced treatments, were treated with varying concentrations of doxycycline (0, 100,

and 1000 ng/mL) and analyzed for luciferase activity. Luciferase activity increased approximately 75 times in the Huh7 Tet-ON and 110 times in the HepG2 Tet-ON cell lines at the highest doses of doxycycline tested, and both cell lines were judged to have low levels of luciferase activity in the absence of the drug (Fig. 3.10B). As expected, control GFP-transduced and parental cell line-transduced treatments displayed only low background luciferase activity. These data suggest that both the Huh7 Tet-ON and HepG2 Tet-ON cell lines were compatible with Retro-X advanced inducible vectors, with low levels of background gene expression in the absence, and high levels of expression in the presence of doxycycline.

To produce Huh7 Tet-ON and HepG2 Tet-ON –derived cell lines capable of expressing the HBx protein in a dose-dependent fashion, the HBx gene was cloned into the pRetroX-Tight-Pur vector. Huh7 Tet-ON and HepG2 Tet-ON cells were transduced with pRetroX-Tight-Pur:HBx –derived retrovirus, allowed to recover for three days, and selected with puromycin for 2 additional days. The resulting cells were termed Huh7 Tet-ON:HBx and HepG2 Tet-ON:HBx, and were examined for HBx expression via western blot after 24 hours exposure to 0, 50, or 100 ng/mL doxycycline. While HBx was strongly evident in the HepG2 Tet-ON:HBx cells at both 50 and 100 ng/mL doxycycline, HBx was only faintly detectable in the Huh7 Tet-ON:HBx cells at these same doses of doxycycline (Fig. 3.10C). No noticeable morphological differences, or signs of excessive cell death (detached or rounded cells), were detected after 24 hours of HBx expression. It should also be noted that HBx was not detectable in either cell line when doxycycline was absent from the media (Fig. 3.10C). We concluded that HepG2 Tet-ON:HBx cells express HBx when treated with doxycycline, and would be suitable for further studies

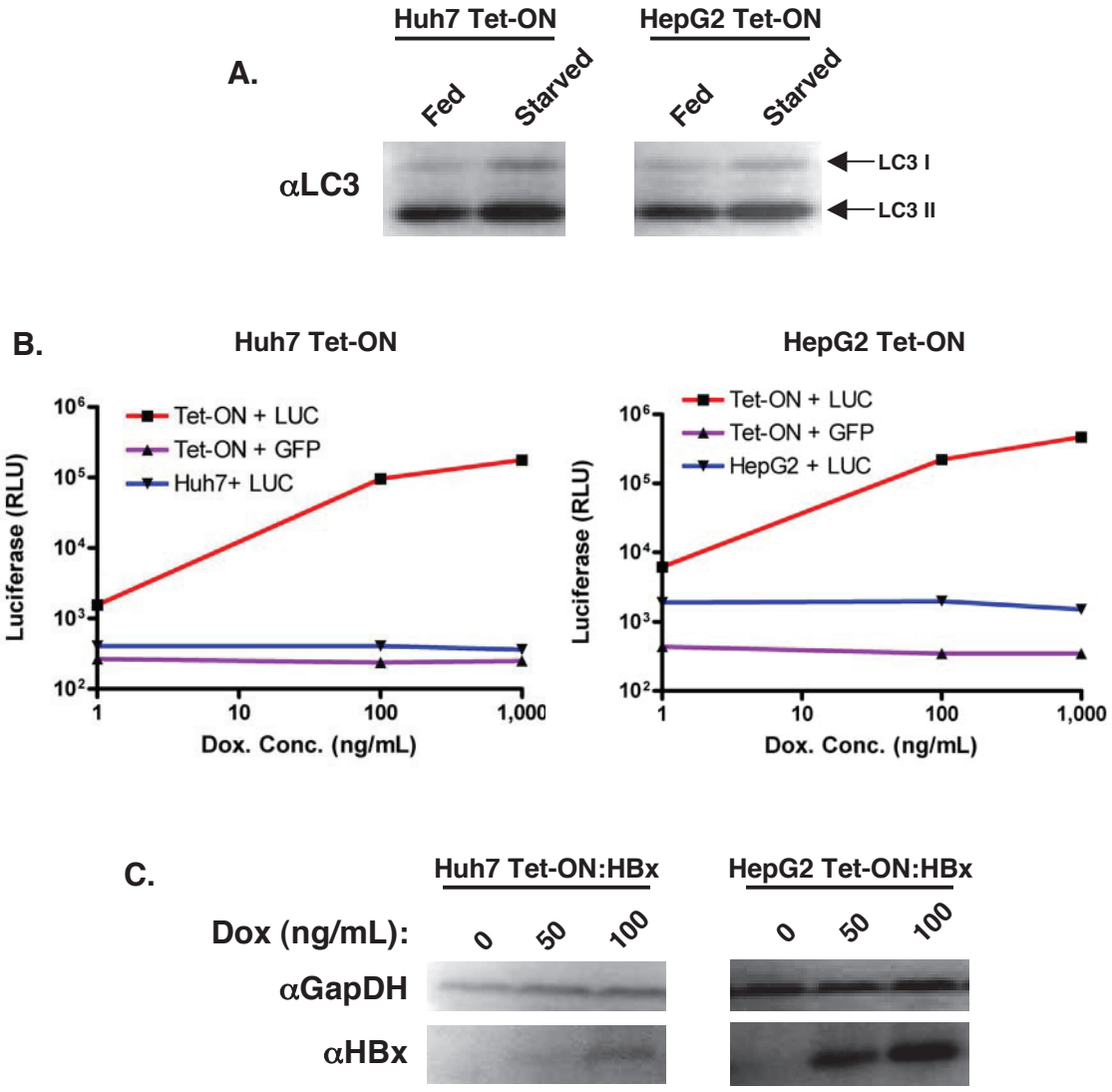

Figure 3.10 - Huh7 and HepG2 Tet-ON HBx cell lines produce HBx in response to doxycycline treatment. A) Tet-ON Huh7 and HepG2 cell lines (obtained from C. Goldring, University of Liverpool) were evaluated for their autophagy response through exposure to either 'fed' (Williams, 10% FCS) or 'starved' (DMEM, 0% FCS) media and subsequent analysis by western blot. LC3-II levels qualitatively increased in 'starved' media. **B)** Transduction with a pRetro-Tight-Puro-Luciferase plasmid (Clonetech) derived retrovirus produced a population expressing Luciferase in response to doxycycline treatment. An approximately 110-fold (Huh7) and 75-fold (HepG2) difference in Luciferase activity was detected between the control (0 ng/mL Dox.) and highest dose tested (1,000 ng/mL Dox.). n=2. **C)** Transduction with a pRetro-Tight-Puro-HBx derived retrovirus produced a population expressing HBx in response to doxycycline. Treatment of these cells with doxycycline produced HBx that was detectable by western blot, however, levels were low in the Huh7 Tet-ON cell line.

designed to examine the effects of HBx expression on senescence and autophagy in a dose-dependent fashion.

3.9 HBx Expression in HepG2 Cells Does Not Alter the Levels of Beclin-1 or Anti-Apoptotic Bcl-2 Proteins

The observed increases in autophagy within HepG2 cells in response to HBx over-expression led us to speculate how the HBx protein might modulate the activities of this pathway. Conflicting studies have indicated that HBx may (Song et al., 2004; Tang et al., 2009), or may not (Sir et al., 2010), affect the production of Beclin-1, an important autophagy regulatory protein. Interestingly, there have also been numerous contradictory reports that HBx expression modifies the production of anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-xL, and Mcl-1) (Chan and Ng, 2006; Cheng et al., 2008; Hu et al., 2011; Miao et al., 2006; Ye et al., 2008; Zhang et al., 2009), proteins that have been shown to down-regulate autophagy through binding interactions with Beclin 1 (Erlich et al., 2007; Maiuri et al., 2007a; Pattingre et al., 2005; Wei et al., 2008). To investigate whether HBx-transduction of HepG2 cells results in changes in Beclin 1 or anti-apoptotic Bcl-2 protein levels, HepG2 cells were transduced with HBx or control retrovirus, puromycin selected, and treated with 'Fed' (Williams' media E, 10% FCS) media for 2 hours prior to lysis. Beclin 1 (Fig 3.11A), Bcl-2, Bcl-xL, and Mcl-1 protein levels (Fig. 3.11B), were observed to be similar in both the HBx and control –transduced treatments in multiple replicate samples. These data suggest that HBx does not modulate autophagy in HBx-transduced HepG2 cells through changes in the levels of Beclin 1 or the Bcl-2 anti-apoptotic family of proteins.

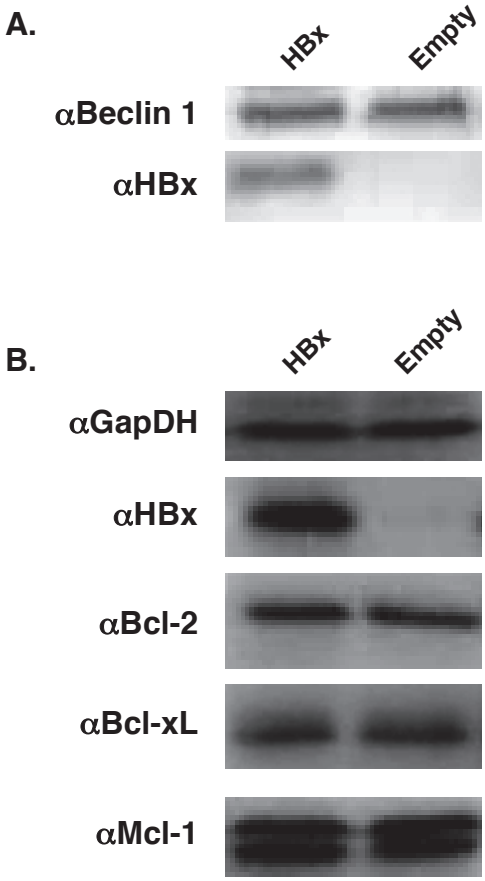

Figure 3.11 - HepG2 levels of Beclin 1, an important regulator of autophagy, as well as the interacting proteins Bcl-2, Bcl-xL, and Mcl-1, are unchanged by HBx. HepG2 cells transduced with HBx and puromycin selected were lysed 6 days post-infection, quantified by DC protein analysis (Biorad), and analyzed by western blotting. **A)** Beclin 1 levels were comparable in HBx and control (Empty) -transduced treatments. **B)** Bcl-2, Bcl-xL, and Mcl-1 levels were comparable in HBx-transduced and control (Empty) -transduced treatments. All cells were treated with 'Fed' (Williams, 10% FCS) media for 2 hours prior to lysis. HBx protein was detectable in all HBx-transduced lysates. Representative data from experiments performed in duplicate (Beclin 1) or triplicate (Bcl-2 proteins).

3.10 HBx Expression Does Not Lead to the Chronic Activation of the Stress-Activated SAPK/JNK Signal Transduction Pathway

While HBx-transduction of HepG2 cells did not result in detectable changes in Beclin 1, Bcl-2, Bcl-xL, or Mcl-1 protein levels (Fig. 3.11), the possibility remained that HBx might still modulate autophagy through Beclin 1 via alterations in its regulation by anti-apoptotic Bcl-2 proteins (Erlich et al., 2007; Maiuri et al., 2007a; Pattingre et al., 2005; Wei et al., 2008). To examine this hypothesis in the most direct manner possible, we investigated whether Beclin 1 interacted with a Bcl-2 anti-apoptotic protein, specifically Bcl-xL, in the presence or absence of HBx. While we were successful in our attempts to immunoprecipitate both endogenous Beclin 1 and Bcl-xL with αBeclin 1 and αBcl-xL antibodies, respectively, we were unable to detect any interactions between these two proteins, regardless of HBx status (Fig. 3.12). Unfortunately, as there was no positive control for this experiment, we were unable to draw any direct conclusions from the observation.

We next examined the effect of HBx on Beclin 1 regulation in an indirect manner, focusing on the phosphorylation status of the anti-apoptotic Bcl-2 family of proteins. Phosphorylation of Bcl-2 is the primary mechanism by which its interaction with Beclin 1 and suppression of autophagy are relieved (Pattingre et al., 2009; Wei et al., 2008). Regrettably, multiple attempts to detect phosphorylated Bcl-2 in the HepG2 cell line, using αphospho-Bcl-2 (Ser70) or αphospho-Bcl-2 (Thr56) antibodies (Cell Signaling Technologies), were unsuccessful, even when cells were treated overnight with 1 μg/mL nocodazole as a positive control (data not shown). Since it has been reported that HBx activates the c-Jun N-terminal protein kinase 1 (JNK1)

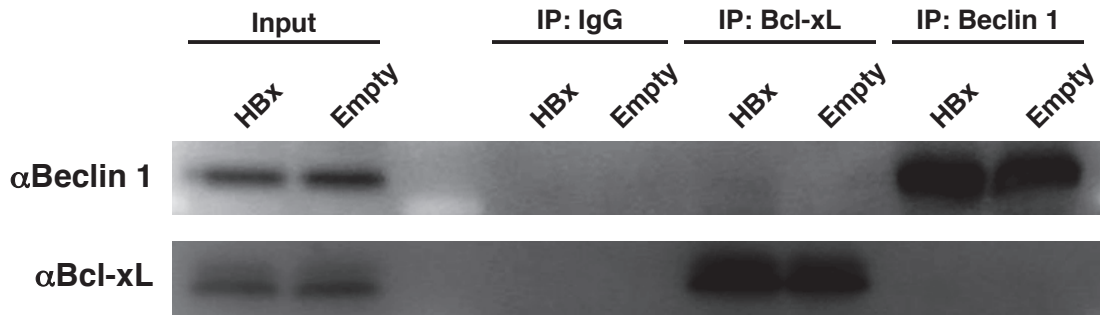

Figure 3.12 - Immunoprecipitation of endogenous Bcl-xL and Beclin 1 failed to detect a mutual interaction in HepG2 cells. Low confluency HepG2 cells, transduced with either HBx or a control (Empty) retrovirus 6 day prior, were cultured overnight in rich media (Williams, 10% FCS), lysed in immunoprecipitation buffer, and quantified by DC protein assay (Biorad). Approximately 250 μg of whole cell lysate protein were immunoprecipitated with rabbit IgG control (Santa Cruz), αBcl-xL (Cell Signaling Technologies) or αBeclin 1 (Santa Cruz) antibodies and Protein A/G -Agarose beads (Santa Cruz). Beads were then boiled in x2 sample buffer and samples analyzed by western blot. Samples immunoprecipitated with αBcl-xL or α Beclin 1 contained endogenous Bcl-xL or Beclin 1, respectively. However, neither Bcl-xL or Beclin 1 were observed to co-immunoprecipitate with the other protein.

(Benn et al., 1996; Diao et al., 2001; Henkler et al., 1998; Nijhara et al., 2001b; Tanaka et al., 2006), which has been shown to up-regulate autophagy activity via Bcl-2 phosphorylation (Pattingre et al., 2009; Wei et al., 2008), we compared the level of JNK/SAPK activity in HBx-transduced HepG2 cells relative to a control treatment. Puromycin-selected HBx and control pBMN-IP –transduced HepG2 cells at 6 days post-retroviral infection were treated with Williams' media E, 10% FCS for two hours, then lysed and analyzed by western blot with phospho-specific αSAPK/JNK antibody (Cell Signaling Technologies). Both the p46 and p54 phosphorylated isoforms of SAPK/JNK were present at similar levels in both HBx and control treatments (Fig 3.13A). To verify that the SAPK/JNK pathway could become activated in the HepG2 cell line, the experiment was repeated with the addition of a positive control where cells were exposed to 250, 500, or 750 μJ/cm^2 of ultraviolet radiation (UV) 30 minutes prior to lysis. Again, faint levels of phosphorylated SAPK/JNK were detected in both the HBx and 'empty' – transfected control (Fig. 3.13B), but UV treatment as low as 250 μJ/cm^2 resulted in a robust increase in the phosphorylation of both isoforms. Total levels of SAPK/JNK were found to be similar in all treatments. As a further measure of SAPK/JNK activation, we examined the phosphorylation status of a downstream target protein; c-Jun. c-Jun phosphorylation levels were comparable in the HBx and transfection control samples, but appreciably increased in UV-irradiated samples (Fig. 3.13B). From these data we found no evidence that HBx-associated increases in autophagy were due to regulatory changes at the level of Beclin 1, however, we could not definitively rule out the possibility of such an effect based on the evidence at hand. It is possible that HBx modulation of autophagy occurs via a different mechanism.

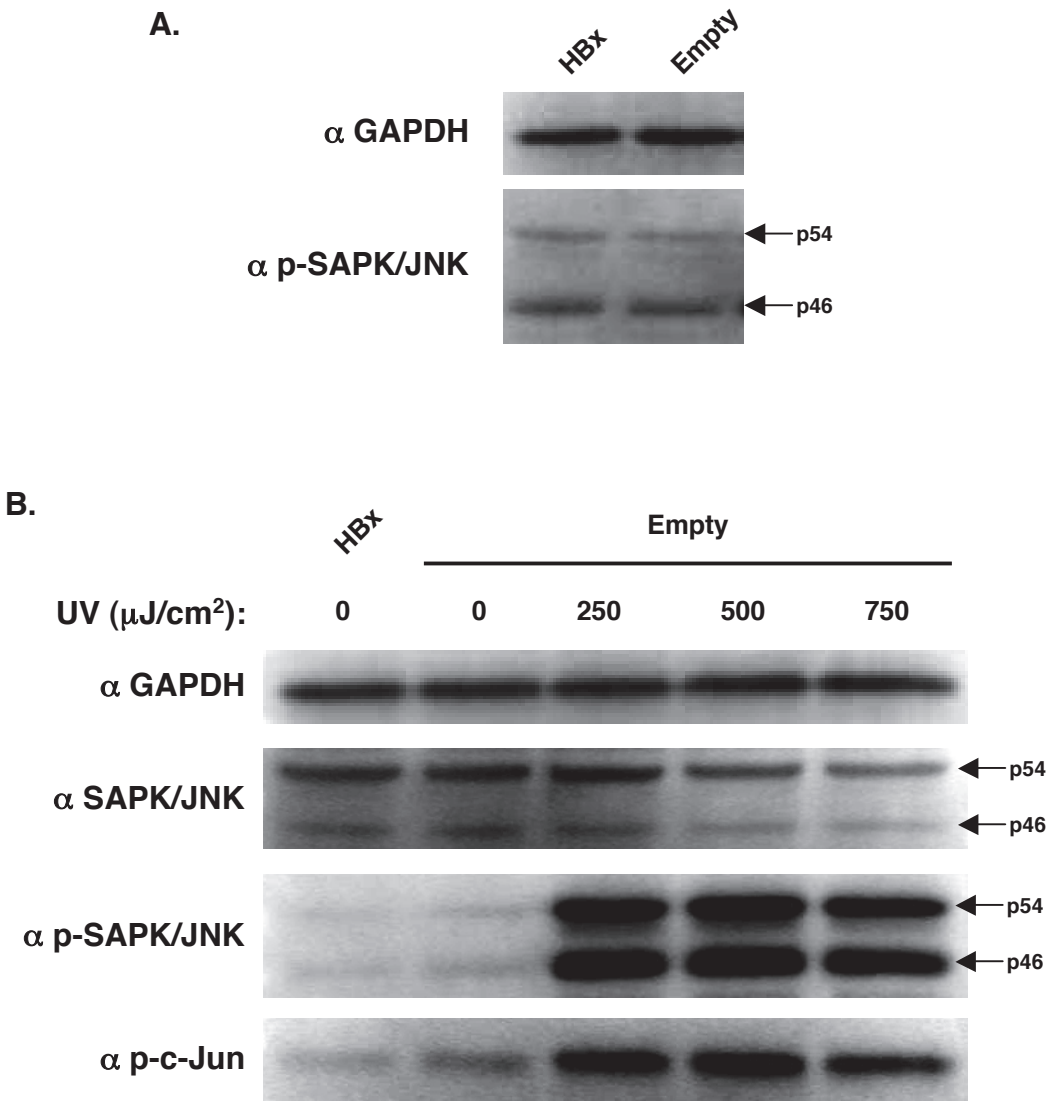

Figure 3.13 - SAPK/JNK activity in the HepG2 cell line is unchanged in response to HBx. HepG2 cells transduced with HBx and puromycin-selected were lysed 6 days post-infection, quantified by DC protein analysis (Biorad), and analyzed by western blot. **A)** Preliminary investigations showed that phospho-SAPK/JNK levels were comparible in HBx and control -transduced treatments. **B)** Treatment of control-transduced HepG2 cells with 250, 500, or 750 μJ/cm^2 ultraviolet radiation 30 minutes prior to lysis resulted in robust SAPK/JNK and downstream c-Jun phosphorylation. Total SAPK/JNK levels were similar in HBx and control-transduced cells. All cells were treated with Williams media E, 10% FCS for 2 hours prior to lysis.

Chapter 4: Discussion

4.1 Summary

Cellular senescence is a response to many stress factors, including DNA damage and oncogene activation, and provides multicellular organisms with some protection against tumorigenesis. Here, we are the first to report that HBx, a suspected oncogene, can induce senescence *in vitro*, as determined by an accumulation of S.A. β-galactosidase staining and increases in senescence-associated protein markers (Fig. 3.2 and 3.3). While the exact mechanism by which this process occurs requires further investigation, HBx expression was observed to result in an accumulation of DNA damage in the form of immunostained 53BP1 puncta (Fig. 3.4), which has been shown to be an inducer mechanism in senescence responses. Interestingly, different responses to HBx were noted in two hepatocellular carcinoma cell lines (Fig. 3.1 and 3.2), suggesting that this response may also be cell-type dependent.

Like senescence, autophagy is a cellular stress response that may limit carcinogenesis, and it is activated during the establishment of the senescence phenotype. In this study we observed that autophagy levels are increased in response to HBx expression, as determined by an accumulation of LC3-II in both fed and starved samples (Fig. 3.6 and 3.7). HBx was not observed to cause any noticeable inhibition of autophagosome maturation, since inhibition of lysosomal degradation through treatment with bafilomycin A1 or chloroquine resulted in a significant accumulation of LC3-II (Fig. 3.8). As HBx has been reported to modulate the levels and phosphorylation of Bcl-2 anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, we examined whether these effects might be involved in the observed modulation of autophagy through the Beclin 1 autophagy

regulator. Beclin 1 and Bcl-2 anti-apoptotic protein levels were unchanged by HBx (Fig. 3.11), and attempts to co-immunoprecipitate Beclin-1 and Bcl-xL were unsuccessful (Fig. 3.12). HBx was not observed to activate the JNK/SAPK pathway as previously reported, and no increase in JNK/SAPK or downstream c-Jun phosphorylation was detected (Fig. 3.13). We have concluded that while HBx induces autophagy, it is unlikely that this effect is mediated through the JNK/SAPK pathway. Finally, we have developed two cell lines that express HBx in a tetracycline-inducible fashion to enable future studies of HBx's effects in a dose-dependent fashion (Fig. 3.10).

HBx has been speculated to have an uncertain, but positive role in tumorigenesis (reviewed in section 1.3.4.2). However, this study has provided evidence that the tumor suppressor senescence pathway is activated in response to HBx expression, and may act to limit its oncogenic potential. Since autophagy has been shown to act as an effector mechanism during the establishment of senescence, this study also explored how HBx expression modulates autophagy. HBx expression up-regulates autophagy, and evidence provided suggests that this effect is not due to an activation of the JNK/SAPK pathway or changes in Bcl-2 anti-apoptotic protein levels. Furthermore, this study has laid foundations for further investigations regarding the effects of HBx level of expression on senescence and autophagy.

4.2 Why Has HBx-Induced Senescence Not Been Previously Reported in the Literature?

In this study we observed that HBx expression can induce cellular senescence in the HepG2 hepatocellular carcinoma cell line (Fig. 3.2 and 3.3). However, despite the fact that numerous other studies have examined HBx's effects on cellular proliferation

and the cell cycle, no other group has yet reported this effect. While this statement is technically accurate, a critical reading of the literature reveals that multiple studies have reported that HBx can be anti-proliferative and trigger changes in gene expression that are indicative of senescence (reviewed in section 1.3.4.10). These studies may not use the term 'senescence', however, their results match closely to that of our own (Gearhart and Bouchard, 2010; Kuo et al., 2008; Park et al., 2000). I would further speculate that part of the confusion regarding HBx's effects on apoptosis may be due to senescence, since this process can render cells highly resistant to certain apoptotic stimuli [reviewed in (Campisi and d'Adda di Fagagna, 2007)].

The degree of variation within the literature regarding HBx's effects upon the cell cycle is puzzling, and it is clear that HBx may actually increase senescence bypass in some circumstances (Kim et al., 2010; Park et al., 2011). The reasons for these discrepancies remain unclear, although studies have shown that it could be due to variations with the HBx protein sequence (Kwun and Jang, 2004), or differences in the levels of HBx expression (Leach et al., 2003). Interestingly, the variant of HBx employed in this study is typical of most HBx natural isolates, and it did not have either of the $p21^{WAF1}$ enhancing or suppressing functions. An important future direction for this project is to examine the effects of these natural mutations on the senescence response, with the prediction that mutants that block $p21^{WAF1}$ expression will interfere with the establishment of a senescence phenotype. Also, the development of the tetracycline-inducible system for HBx (Fig. 3.10) will allow us to examine whether HBx's induction of senescence is a dose-dependent effect, as has been suggested by the work in Leach *et. al.* (Leach et al., 2003).

Another puzzling aspect of our observation that HBx can induce senescence is the fact that numerous other groups have reported the creation of cell lines that stably express this protein. It has been our experience that the creation of HBx stable cell lines is challenging, an observation that has been reported elsewhere in the literature (Lee and Yun, 1998). I would speculate that successes are reported more frequently than failures. Stable cell lines that express HBx can be constructed, but this may be more difficult than the literature would initially suggest. Furthermore, the use of cell lines stably expressing HBx in senescence studies may lead to ambiguous results. In both Kim *et al.* and Park *et al*., HBx stable cell lines had considerably higher levels of senescence avoidance in the presence of senescence-inducing compounds relative to transient transfection experiments. This might imply that the process of creating stable cell lines naturally selects for a population of cells that are resistant to senescence (Kim et al., 2010; Park et al., 2011).

4.3 How Does HBx Expression Trigger Senescence?

The mechanism by which HBx expression results in senescence requires further investigation. HBx was observed to cause an increased accumulation of DNA damage foci, as detected by immunofluorescent staining for 53BP1 (Fig. 3.4). DNA damage has been reported to induce senescence in the literature (reviewed in section 1.1.1). However, not all cells had evidence of DNA damage, suggesting that other mechanisms may be involved in this effect. While unlikely, it is possible that HBx triggers senescence through mitotic stress, as it has been shown to activate a number of proliferation signaling pathways, including Ras and Src (reviewed in section 1.3.4.5). However, if this were the case we would have expected a burst of cellular proliferation following HBx expression,

which was not observed in our study. To completely rule this out, one future objective of this project is to directly monitor the proliferation of HBx expressing cells using a bromodeoxyuridine (BrdU) assay at various times after transduction or expression in our tetracycline-inducible cell lines.

While we did observe an increase in $p21^{WAF1}$ protein levels in HBx senescent cells (Fig. 3.3), the difference was slight, and less than has been observed in other HBx studies (Friedrich et al., 2005; Gearhart and Bouchard, 2010; Park et al., 2000). This could simply be due to differences in immunoblotting methods. However, it raises the possibility that HBx may not rely upon $p21^{WAF1}$ to arrest the cell cycle. We were surprised to observe an increase in $p16^{INK4a}$ levels (Fig. 3.3), as multiple independent reports have indicated that HBx expression triggers a hypermethylation and subsequent silencing of its promoter region (Jung et al., 2007; Kim et al., 2010; Zhu et al., 2010). One intriguing possibility is that while HBx expression may initially cause the hypermethylation and suppression of $p16^{INK4a}$, continued senescence signaling from other pathways might relieve this inhibition over time. Interestingly, Park *et. al.* found that while HBx alone resulted in hypermethylation of the $p16^{INK4a}$ promoter, when cells were also treated with the senescence inducing agent (ATRA) the overall level of methylation and inhibition decreased (Park et al., 2011). Subsequent experiments examining the methylation of the $p16^{INK4a}$ promoter, along with $p16^{INK4a}$ protein levels, at multiple times during the establishment of senescence would address this question.

As HBx has been reported to suppress p53 expression and activity (reviewed in section 1.3.4.6), we were surprised to observe that HBx expression could increase levels of p53 (Fig. 3.3), although we did not examine its cellular localization or activity directly.

As p53-dependent senescence has been reported to be experimentally reversible in some cases (reviewed in section 1.1.2), we would be interested in exploring whether HBx-induced senescence can be bypassed when HBx expression is withdrawn. This question could be examined using our tetracycline-inducible HBx cell lines. While $p16^{INK4a}$ protein was detected in HBx senescent cells, multiple attempts to detect RB were unsuccessful. As $p16^{INK4a}$ is understood to cause experimentally irreversible senescence through chromatin modifications via RB, it would also be interesting to investigate whether HBx senescent cells have increased levels of γH2AX-staining foci, a marker of these modifications.

Another possible avenue for further investigation is to examine the relationship between HBx's binding interactions with the CUL4-DDB1 ubiquitin ligase system and the establishment of cellular senescence. As this system has important roles in DNA repair, gene transcription, and regulation of signal transduction pathways, it is a distinct possibility that HBx-related modulations of DDB1 may play a role in the establishment of cellular senescence. To investigate this possibility, a HBx mutant that does not bind DDB1 (Arginine to Glutamic Acid at residue 96), or a DDB1 mutant defective for HBx binding (described in Li et. al., 2010), should be employed in further HBx senescence studies.

4.4 Why Were There Differences in HBx Responses Between the HepG2 and Huh7 Cell Lines?

Differences were observed in the responses of the HepG2 and Huh7 cell lines after transduction with HBx using both senescence and autophagy assays. While HBx was easily detected via immunoblotting in the HepG2 cell line, repeated attempts to

detect HBx in Huh7 cells were unsuccessful (Fig. 3.1, 3.6, and 3.7). Furthermore, while HBx expression could be induced in a HepG2 Tet-ON cell line, induction of HBx was observed to be almost undetectable in a Huh7 Tet-ON cell line (Fig. 3.10). It is unclear why HBx was not detectable in the Huh7 cell line, however, we have previously observed that over-expression of HBx is cytotoxic in this cell line, and that cells that express significant quantities of HBx may be rapidly removed from the cellular population. While a fraction of Huh7 cells transduced with HBx did show increases in S.A. β-gal staining, along with a dramatic change in cellular morphology (Fig. 3.1 and 3.2), a noteworthy proportion did not. From these data I would speculate that while some Huh7 cells transduced with HBx might undergo senescence, the majority of HBx expressing cells are removed through necrosis or apoptosis.

In our autophagy studies, transduction with HBx was observed to increase autophagy levels in the Huh7 cell line (Fig. 3.6). However, as HBx was undetectable in these cell lysates, it appears likely that the increase is due to an indirect effect of HBx expression. This could be an example of autophagy occurring 'with' apoptosis, representing a general response to cellular stress rather than a direct modulation of a regulatory mechanism. Another possibility is that if HBx transduction results in increases in cellular necrosis, the release of cellular debris and ROS into the media could trigger autophagy in adjacent cells. Therefore, the observation that HBx expression increases autophagy in the Huh7 cell line should be treated with caution.

4.5 Does Our Report That HBx Increases Autophagy Correlate With the Existing Literature?

The observation that HBx expression triggers a moderate increase in autophagy correlates well with existing reports in the literature. As similarly reported by Sir *et. al.* (Sir et al., 2010) and Li *et. al.* (Li et al., 2011), we observed that HBx expression, in the presence of full or starvation media, resulted in an increase in autophagy as measured by an increase in LC3-II protein accumulation (Fig. 3.7). This result contradicts Tang *et. al.* (Tang et al., 2009), who observed that HBx expression only 'sensitized' cells to autophagy signals. However, all three existing reports agree that HBx expression has an enhancing effect on the autophagy pathway. While Li *et. al.* proposed that the HBV small envelope protein has a more potent effect on the autophagy pathway, their study did observe a ~20% decline in autophagy when they employed HBV genomes in which HBx expression was silenced, which is similar to the level of increase that we observed in our HBx over-expression experiments.

Both Sir *et. al.* and Li *et. al.* reported that their observed increases in autophagy did not result in a corresponding increase in protein degradation. In our study, treatment with bafilomycin A1 or chloroquine, agents that inhibit lysosomal degradation, resulted in a substantial accumulation of LC3-II in both HBx expressing and control cells, suggesting that HBx does not pose a significant barrier to autophagosome maturation and degradation (Fig. 3.8). However, given the debate within the literature, further studies into HBx's effects on autophagosome maturation and associated long-lived protein degradation would be beneficial.

As HBx has been shown to have numerous different cellular effects, and autophagy is triggered in response to a wide range of different stimuli, there are multiple potential mechanisms by which HBx expression could result in an up-regulation in the

autophagy pathway. One intriguing possibility is that the observed increase in autophagy is due to the induction of cellular senescence, as autophagy is reported to act as an effector mechanism during the establishment of this phenotype (review in section 1.2.7). To investigate this possibility, future experiments would examine the expression level and localization of ULK3 in HBx expressing cells, as ULK3 has been suggested to differentially modulate autophagy in the place of ULK1 during the establishment of cellular senescence (Young et al., 2009). Furthermore, since deficiencies in autophagy delay the induction of senescence and increase the proportion of cells that are able to bypass cell cycle arrest, it is our intention to examine HBx's effects on senescence in HepG2 cells rendered deficient in their autophagy responses. In these experiments, RNA interference against Atg5 and Atg12 will be used to impair the autophagy response prior to transduction with HBx. Through the analysis of senescence responses at various time points and colony formation assays, these experiments will examine the importance of autophagy in the establishment of HBx-induced senescence.

4.6 Why is the HepG2.2.15 Cell Line of Limited use in Autophagy Research?

While the HepG2.2.15 cell line, an experimental model that employs two sequentially integrated copies of the HBV genome, has been a valuable tool for HBV research, our results indicate that it is of limited use in autophagy studies. While two previously published reports have drawn conclusions regarding HBV's effect based on comparisons made between HepG2.2.15 and its parental HepG2 cell line (Sir et al., 2010; Tang et al., 2009), it is our observation that this type of comparison is unreliable (Fig. 3.9). When parental HepG2 cells from two different sources (ATCC and laboratory

cultures) were compared, each displayed different levels of basal autophagy, suggesting that cell lines can diverge in their autophagy responses when continuously cultured over extended periods of time. As the HepG2.2.15 cell line was initially developed over two decades ago, it is possible that differences between it and its parental cell line may be in part due to divergence in their autophagy responses.

This observation does not preclude making comparisons within the HepG2.2.15 cell line itself. For example, RNA interference against autophagy genes has been used to investigate the impact of autophagy on HBV replication within this cell line (Li et al., 2011). However, as HBx has regulatory effects on endogenous HBV gene transcription, RNA interference against HBx will have effects beyond HBx expression alone. Therefore other models of HBV infection, such as HBV replicon plasmids or tetracycline-inducible expression systems, are likely better models for HBV autophagy research.

4.7 Does HBx Modulate Autophagy Through the Beclin 1 Autophagy Regulator?

As discussed previously (section 1.4), HBx might modulate autophagy through Beclin 1, either via changes in the expression levels of Beclin 1 or the Bcl-2 anti-apoptotic proteins, or through modulation of the phosphorylation of the Bcl-2 family of proteins. Immunoblotting experiments revealed that HBx expression had no noticeable effect on levels of Beclin-1 or the Bcl-2 anti-apoptotic proteins Bcl-2, Bcl-xL, or Mcl-1 (Fig. 3.11). This result both correlates with, and rejects, the current scientific literature, since for each of these proteins there is at least one report that indicates that its level is unchanged in the presence of HBx, and at least one report to the contrary (reviewed in sections 1.3.4.8 and 1.3.4.9). However, as autophagy levels were increased in the absence

of any changes in the expression levels of these proteins, it appears unlikely that this is a mechanism by which autophagy is modulated under the conditions and cell lines used here.

Attempts to detect phosphorylated Bcl-2 through immunoblotting were unsuccessful, even when cells were treated with nocodazole as a positive control. However, this experiment may be further refined through the use of different phospho-specific Bcl-2 antibodies, or the addition of a phosphatase inhibitor cocktail in the place of β-glycerophosphate and Na_3VO_4. The phosphorylation status of the Bcl-2 anti-apoptotic proteins might also be ascertained through immunoprecipitation of said proteins, followed by immunoblotting with general phosphorylated threonine and serine antibodies.

Beclin 1 is inhibited through binding interactions with the Bcl-2 anti-apoptotic proteins that are relieved through the phosphorylation of the Bcl-2 proteins. In order to ascertain the level of interaction between these proteins in the presence and absence of HBx, we attempted an immunoprecipitation of endogenous Beclin 1 and Bcl-xL (Fig. 3.12). While we were able to detect significant precipitation of both proteins, no mutual interaction was detected. It is not clear why Beclin 1 and Bcl-xL did not co-immunoprecipitate in this experiment, as it could either be indicative of a problem with the experimental method or a lack of this regulatory mechanism within this cell line. However, as a future direction, we would like to repeat these experiments with three modifications. First, as phosphorylation of Bcl-xL disrupts its binding interaction with Beclin 1, as a positive control we would like to add a general phosphatase to the cell lysates to see if an interaction can be experimentally induced. Second, we would like to

explore whether HBx could co-immunoprecipitate with the Beclin 1 complex. It has been previously reported that HBx does co-immunoprecipitate with the PI3K, VPS34, which is part of the Beclin 1 complex (Sir et al., 2010). This raises the possibility that HBx might interact directly with Beclin 1 in order to modulate autophagy. Third, through co-immunoprecipitation we would like to investigate whether the binding interactions of other members of the Beclin 1 complex, such as UVRAG and Bif-1, are affected by the presence of HBx. In addition to acting as a positive control for the immunoprecipitation reaction, these experiments could provide additional information, particularly if HBx is observed to co-immunoprecipitate with the Beclin 1 complex.

4.8 Why did HBx expression in the HepG2 cell line fail to increase JNK/SAPK phosphorylation?

During periods of cellular stress, the Bcl-2 anti-apoptotic proteins are phosphorylated at multiple residues by JNK1 in order to affect their regulation of both autophagy and apoptosis. As multiple reports indicate that HBx expression activates the JNK/ SAPK pathway (review in section 1.3.4.5), we examined whether HBx would have a similar effect in our experimental system. Immunoblotting experiments showed that HBx expression had no effect on the phosphorylation of the JNKs, nor did it have an effect on the phosphorylation of the downstream target protein, c-Jun (Fig. 3.13). Given that multiple reports have indicated that HBx can activate this pathway, we were surprised by this result. One possible explanation is that as most previous studies have employed transient transfection methods and examined HBx's effects at earlier time points, prolonged expression of HBx, as is the case in our system, has other effects. It is possible that with prolonged activation, feedback mechanisms within the pathway

eventually down-regulate its activities back towards basal levels. To explore this possibility, one future direction of this project is to examine HBx's effects on the JNK/SAPK pathway at earlier time points and more physiologically relevant doses using the tetracycline-inducible cell line models.

However, it appears unlikely that the modulation of autophagy that was observed in this study is due to the activation of JNK1 and its subsequent phosphorylation of Bcl-2 family members, at least at the time point examined, since up-regulation in autophagy was observed in the absence of increased JNK/SAPK or c-Jun phosphorylation. As mentioned previously, there are numerous other mechanisms by which HBx could modulate autophagy, including activation of MAPK pathways, the release of intracellular calcium, the induction of genotoxic stress, activation of the VPS34 PI3K, and increases in cellular ROS levels. Future research could focus on these other candidate mechanisms in order to expand our understanding of the mechanism by which HBx modulates autophagy.

4.9 Concluding Remarks

Cellular senescence is a response to many stress factors, including DNA damage and oncogene activation, which provides multicellular organisms with some protection against tumorigenesis. This study is the first to report that HBx, a suspected oncogene, can induce senescence in vitro. The clinical ramifications of this discovery are unclear, however, if HBV replication is impaired by senescence, it could provide a new model explaining why HBx expression is carefully maintained at low levels during HBV infection, and might provide some explanation as to why HBx isn't more carcinogenic.

While this study did demonstrate that HBx increases autophagy levels, the mechanism by which this occurs remains unclear. As levels of Bcl-2 anti-apoptotic proteins were unchanged, and no changes in JNK/SAPK activation were observed, this study found no evidence that HBx modulates autophagy through the Bcl-2 anti-apoptotic proteins. The possibility remains that HBx may modulate autophagy at the level of Beclin 1, or via another mechanism. As autophagy has been suggested to act as an effector mechanism during the establishment of the senescence phenotype, we are interested in further exploring the possible relationship between these two processes.

4.10 Future Experiments

1) Examine HBx's ability to induce senescence and autophagy in the tetracycline-inducible HepG2 and Huh7 cell lines in a dose dependent fashion.

2) Using RNA interference, disrupt the autophagy response in the HepG2 cell line and examine HBx's ability to induce senescence. Examination will focus on the time required to induce the senescence phenotype (time course study), and the ability of autophagy deficient cells to bypass senescence (BrdU and colony forming assays).

3) Re-examine Beclin 1 immunoprecipitation assays to ascertain if HBx associates with the Beclin 1 protein complex. If an interaction is detected we will examine its effect on other Beclin 1 binding interactions (UVRAG and Bif-1) and VPS34 activity via *in vitro* PIP3 assay.

4) Using tetracycline-inducible HepG2 and Huh7 cell lines, examine effects of HBx expression on JNK/SAPK activation at multiple time points, and in a dose dependent fashion.

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Appendix A: Viral Interaction with Macroautophagy: A Double-Edged Sword

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A.0 Abstract

Autophagy is an evolutionarily conserved eukaryotic mechanism that mediates the removal of long-lived cytoplasmic macromolecules and damaged organelles via a lysosomal degradative pathway. Recently, a multitude of studies have observed or suggested that individual viral infections may have complex interconnections with the

autophagic process. These observations strongly imply that the autophagy pathway has virus-specific roles in processes relating to viral replication, host innate and adaptive immune responses, virus-induced cell death programs, and viral pathogenesis. Autophagy can supply internal membrane structures necessary for viral replication or may simply prolong cell survival during viral infections and postpone the cell's inevitable death. It can influence the survival of both infected and bystander cells during the course of an infection. On the other hand, this process has also been linked to the recognition of viral signature molecules during innate immunity and has been suggested to help rid the cell of infection as an antiviral defense. The purpose of this review is to critically discuss currently known or suggested interactions between different viruses and the autophagic pathway, and survey the current state of knowledge and emerging themes within this field.

A.1. Introduction

Macroautophagy (hereafter referred to as autophagy) is a tightly regulated and evolutionarily conserved mechanism for the sequestration, lysosomal degradation, and recycling of discrete intracellular portions of eukaryotic cells, facilitating the removal of materials not typically degraded by the ubiquitin-proteosomal pathway. Regulators of this process include hormones and growth factors that suppress autophagy during cellular growth, as well as intracellular levels of nutrients, oxygen, and energy, allowing the pathway to act as a defense mechanism against inducers of cellular stress (Pattingre et al., 2008; Wullschleger, Loewith, and Hall, 2006; Yang et al., 2005). Perturbations in autophagy have been correlated with numerous pathological conditions, including oncogenesis and cancer progression, neurodegenerative disorders, liver disease,

myopathy, and cardiac disease, highlighting the importance of this pathway in human health and cellular homeostasis (Levine and Kroemer, 2008; Meijer and Codogno, 2006; Mizushima et al., 2008). Autophagy has been shown to play an important role in the pathogenesis of several viral infections and is suggested to act as both an inducer and effector of innate and adaptive immune responses against intracellular pathogens, including viruses. Currently, evidence suggests that viruses have evolved a diverse array of countermeasures to contend with this pathway; some inhibit autophagy and are negatively affected when this interference is abrogated, while others appear to subvert it to their own ends and respond positively when it is induced. However, still other viruses are seemingly unaffected by autophagy, and do not appear to regulate the pathway through any apparent mechanism(s). This review seeks to provide both a synopsis of currently known and suspected viral interactions with the autophagy pathway, and to stimulate a critical discussion concerning the central trends that have been suggested within this field of research.

A.2. Overview of the Mechanisms and Regulation of Autophagy

For the sake of brevity, only an overview of the mechanisms and regulation of the autophagy mechanism will be provided. The reader is referred to more detailed reviews concerning specific aspects of autophagy, such as the formation and maturation of the autophagosome (Longatti and Tooze, 2009; Mizushima, 2007; Noda, Fujita, and Yoshimori, 2009; Reggiori and Klionsky, 2005; Xie and Klionsky, 2007; Yang et al., 2005; Yoshimori and Noda, 2008), regulatory mechanisms of this pathway (Meijer and Codogno, 2004; Pattingre et al., 2008; Wullschleger, Loewith, and Hall, 2006; Yang et al., 2005), roles in innate antigen recognition and MHC antigen presentation (Delgado et

al., 2009; Delgado and Deretic, 2009; Levine and Deretic, 2007; Münz, 2006; Orvedahl and Levine, 2009; Schmid and Münz, 2005; Virgin and Levine, 2009), and the relationship between autophagy and regulated cell death (Codogno and Meijer, 2005; Ferraro and Cecconi, 2007; Kroemer and Levine, 2008; Maiuri et al., 2007; Scarlatti et al., 2009). The core process of autophagy is the de novo synthesis of a double membrane-bound vesicle capable of fusing with an endosome or lysosome, which ultimately leads to the catabolic degradation of the encapsulated cargo (Fig.1). In mammals, this process begins with the expansion of a small, flat membrane sac of uncertain origin (termed the isolation membrane or phagophore). As autophagy-related (Atg) proteins are recruited to its surface, this membrane sac elongates and curves until the ends merge to form a double-membrane-bound vesicle (autophagosome). Atg proteins are then recovered or disassociate from the autophagosome, and the completed structure fuses with an endosome (amphisome) or lysosome (autolysosome). A brief overview of mammalian genes of particular significance in the regulation and execution of autophagy is provided in Table 1.

Central in the regulation of autophagy are two key proteins: mTOR and Beclin-1 (Pattingre et al., 2008; Sinha and Levine, 2008; Wullschleger, Loewith, and Hall, 2006). The mTOR, a conserved serine/threonine kinase, is a component of protein complexes that integrate cellular signals relating to growth factors, nutrient and energy status, and cellular stress (Wullschleger, Loewith, and Hall, 2006). Important activators of mTOR include the class I PI3K-Akt/PKB signaling pathway and high concentrations of specific amino acids; high AMP/ATP ratios and hypoxia inactivate this pathway (Arsham and Neufeld, 2006; Beugnet et al., 2003; Pattingre et al., 2008; Wullschleger, Loewith, and

Hall, 2006). Activated mTOR suppresses autophagy, enhancing the accumulation of cellular bulk by limiting lysosomal digestion. Downstream of mTOR, Beclin-1 is at the heart of a regulatory complex for the class III PI3K hVps34, whose activity is essential during autophagosome formation. Activators, such as UV radiation resistance associated gene (UVRAG), Bax-interacting factor-1 (Bif-1), and activating molecule in Beclin-1-regulated autophagy-1 (Ambra-1) associate with the Beclin-1 complex and enhance PtdIns(3)P production, while the Bcl-2 family anti-apoptotic proteins such as Bcl-2 and Bcl-xL bind to Beclin-1 and act in an inhibitory fashion (Pattingre et al., 2008; Sinha and Levine, 2008). The Bcl-2-related inhibition of autophagy is abrogated by stress-activated c-Jun N-terminal protein kinase 1 (JNK1) phosphorylation and competition from other BH3 binding domain-containing proteins, providing one of several direct mechanistic links between autophagy and apoptosis (Sinha and Levine, 2008; Wei et al., 2008; Wei, Sinha, and Levine, 2008).

Numerous other cellular factors have been shown or are hypothesized to regulate autophagy, many of which have importance in viral infections. The eukaryotic initiation factor-2 alpha (eIF2α) and the starvation-responsive general control nonderepressible-2 (GCN2) eIF2α kinase are both indispensable for starvation-induced autophagy, suggesting that other eIF2α kinases with important roles in antiviral defense, such as double-stranded RNA (dsRNA)-dependent protein kinase (PKR) and PKR-like ER kinase (PERK), may also up-regulate this pathway in response to cellular stressors (Kouroku et al., 2007; Tallóczy et al., 2002; Tallóczy, Virgin, and Levine, 2006). Immune signaling molecules can modulate autophagy; type II interferon-γ (IFN-γ) and tumor-necrosis factor-α (TNF-α) are stimulatory, while the TH2-type cytokines interleukin-4 (IL-4) and

IL-13 are suppressive (Deretic, 2005; Levine and Deretic, 2007). Certain pathogen-associated molecular patterns (PAMPs) trigger autophagy through their cognate pattern recognition receptors (PRRs), including Toll-like receptor 3 (TLR3), TLR4, and TLR7, although the molecular mechanism(s), physiological function(s), and range of PRRs that induce this pathway are all areas of continuing research (Delgado et al., 2009; Delgado and Deretic, 2009; Orvedahl and Levine, 2009). The p53 protein possesses a dual role in the regulation of autophagy dependent upon its localization; cytoplasmic p53 represses autophagy and must be degraded for autophagy to proceed, whereas nuclear p53 appears to induce it (Maiuri et al., 2009; Tasdemir et al., 2008). Many additional cellular factors, including extracellular signal-regulated kinase (Erk1/2) activation (Pattingre, Bauvy, and Codogno, 2003; Shinojima et al., 2007; Wang et al., 2009), intracellular release of calcium (Gordon et al., 1993; Høyer-Hansen and Jäättelä, 2007), increases in reactive oxygen species (ROS) (Djavaheri-Mergny et al., 2007; Djavaheri-Mergny et al., 2006; Scherz-Shouval and Elazar, 2007; Scherz-Shouval et al., 2007), and endoplasmic reticulum (ER) stress (Ding et al., 2007; Kouroku et al., 2007; Ogata et al., 2006; Yorimitsu et al., 2006) have also been shown to trigger the pathway to autophagy.

While numerous different experimental approaches including electron microscopy, LC3 lipidation (aggregation and modification), and protein degradation studies amongst others can be employed to identify or quantify autophagy in higher eukaryotes, a few general cautions are advisable regarding the specific challenges these methods present within the context of viral studies. First, as autophagy is a process with numerous components, steps, and phases, it is important to clarify whether a given assay measures a step within this pathway (such as LC3 lipidation) or its overall physiological

performance (the aim of protein degradation studies). Since several viruses have been shown to modulate autophagy at multiple different stages to different effect, it is often necessary to combine assays examining both induction and maturation in order to make comprehensive observations regarding viral interactions with this pathway. Second, while autophagy is a responsive cellular process capable of fluctuation, most assays capture this dynamic process at a single static moment in time. This can pose challenges, as cellular populations are frequently asynchronous and the effects of a viral infection may vary during the course of its lifecycle. Finally, as different cell types have been shown to display differences in their autophagy responses, it can be difficult to make direct comparisons between different cellular models. Cell-type dependent effects have also been observed, and particular caution should be used with viruses that infect more than one cell type, such as HIV-I. For further information regarding the limitations or capacities of existing autophagy assays, readers are advised to consult the following excellent reviews (Klionsky et al., 2008; Mizushima, 2004).

A.3. Viral Interactions with the Autophagy Pathway

Many viruses have been shown to evade, subvert, or exploit autophagy, seemingly to insure their own replication or survival advantage, while others are apparently unaffected by this intrinsic pathway and fail to modulate it by any detectable way. The following discussion concerning viral infection, autophagy, and host immunity has been structured on four emergent themes that have been identified in the research published to date; autophagy as 1) a mechanism for membrane remodeling, 2) a digestive defensive response, 3) a means of cellular surveillance, and 4) a cellular fate-determining process.

A comprehensive summary of viruses for which studies have been undertaken is provided in Table 2.

A.3.1. Autophagy is a mechanism for remodeling internal membranes associated with viral replication.

For many viruses, the production of progeny virion is intimately associated with host cell membranes or cytoskeletal elements. As such, many viruses are known to subvert host endosomal and secretory pathways in order to induce host membrane alterations that can then support viral replication and assembly (Miller and Krijnse-Locker, 2008). A number of viruses have been shown to replicate in, or in close association with, multi-membraned vesicles that possess many of the characteristics of autophagosomes. Given the nature and location of these vacuoles, there is strong evidence that autophagosomes may serve as a site of viral replication during some infections and that the autophagy pathway might therefore be exploited by viruses to enhance virion production. Supporting this assertion, the membranes associated with viral replication are often derived from the ER, which has also been suggested as a source of the autophagic double-membrane (Mijaljica, Prescott, and Devenish, 2006). Indeed, the similarities between autophagic vacuoles and some virally-induced membrane alterations has lead to increased suspicion, and in some cases proof, of a connection between positive-stranded RNA viral replication and the autophagy mechanism.

A link between poliovirus (PV)-induced double-membrane vesicles and autophagy has long been suggested, and is often cited as the classic example of viral exploitation of the autophagy pathway. Ultrastructural and biochemical analyses has revealed that PV induces massive rearrangements in intracellular membranes, resulting in

clusters of double membrane-bound vesicles capable of supporting viral replication complexes (Bienz, Egger, and Pasamontes, 1987; Rust et al., 2001; Schlegel et al., 1996; Suhy, Giddings, and Kirkegaard, 2000). These PV-induced vesicles display the classic autophagosomal marker LC3, which has been hypothesized to be directly recruited by viral proteins (Jackson et al., 2005; Taylor and Kirkegaard, 2007). Subsequent studies have further shown that additional secretory vesicle trafficking molecules are also recruited during the formation of these membranous replicative vesicles (Belov et al., 2007), and that the association of these vesicles with the microtubule network aids in the non-lytic release of PV virion particles (Taylor et al., 2009). Hence, it has been postulated that PV factors initiate elements of both the interrelated secretory trafficking and autophagy pathways to ultimately create a membranous structure capable of supporting both viral replication and virion egress. Besides PV, several other picornaviruses also appear to subvert the autophagy machinery to promote their replication. The group B Coxsackieviruses, CVB3 and CVB4, induce autophagosome formation, and biochemical inhibition of this pathway negatively impacts virion production (Wong et al., 2008; Yoon et al., 2008). Similarly, the enteropathogen EV71 has been shown to trigger autophagy in vitro, and that this induction can significantly increase viral yield (Huang et al., 2009). Apart from the Picornaviridae family, two Dengue virus (DENV) serotypes (DENV-2 and DENV-3) have also been shown to interact with the autophagy machinery to promote their replication (Khakpoor et al., 2009; Lee et al., 2008; Panyasrivanit et al., 2009). Interestingly, the stage of the autophagic process with which DENV is associated varies depending upon the viral serotype involved; DENV-2 translation/replication complex has been shown to specifically locate with pre-lysosomal fusion amphisomes, while DENV-3

requires further vesicle maturation and post-lysosomal fusion vacuoles (autolysosomes) to efficiently produce virions (Khakpoor et al., 2009; Panyasrivanit et al., 2009). The autophagy pathway also appears to be critical in the replication of another Flaviviridae member, namely the hepatitis C virus (HCV), which has also been shown to induce autophagosome formation (Ait-Goughoulte et al., 2008; Dreux et al., 2009; Sir et al., 2008; Tanida et al., 2009). In the case of HCV, autophagy is thought to be responsible for providing membranous support structures during the initial translation and de novo replication of HCV RNA following infection, but subsequently contributes little to virus maturation once an infection is stably established in the host cell (Dreux et al., 2009; Tanida et al., 2009). Finally, basal levels of autophagy has recently been shown to enhance macrophage-tropic human immunodeficiency virus (HIV) viral protein processing and virion production in vitro, suggesting a role for autophagy in HIV biosynthesis in this cell type (Kyei et al., 2009).

While these results are strongly suggestive that viruses can subvert or induce autophagy in order to create advantageous membrane alterations, other research has demonstrated that this effect is not universal. Not all viral infections that induce membrane alterations, including some which are closely related to the examples provided above, modulate or are affected by the autophagy pathway. The replication of human rhinovirus 2, a picornavirus that shares many similarities with PV, is not affected by drugs that either inhibit or induce autophagy, and does not elicit the formation of LC3-positive vesicles (Brabec-Zaruba et al., 2007). Contradictory observations have been made for the Coronaviridae members, murine hepatitis virus (MHV) and severe acute respiratory syndrome-coronavirus (SARS-CoV); some studies have suggested that

endogenous LC3 co-localizes with SARS-CoV and MHV vacuole-associated replicase proteins (Prentice et al., 2004a; Prentice et al., 2004b), yet other studies have had difficultly corroborating these results (de Haan and Reggiori, 2008; Snijder et al., 2006; Stertz et al., 2007). In line with these observations, MHV viral replication and release have been shown to be comparable in cells with both normal and defective autophagy mechanisms (Zhao et al., 2007). Likewise, the viral production kinetics of vaccinia virus, which has been shown to utilize double-membrane vesicles for its replication, was similar in wild-type, Atg5-/-, and Beclin-1-deficient cells (Zhang et al., 2006). Thus, subversion of autophagy as a mechanism for inducing membrane alterations may be either cell-type- or virus-dependent. These results suggest that considerable care should be exercised prior to concluding that autophagy is the mechanism responsible for membrane alterations observed under microscopy to ensure that they are biochemically and mechanistically connected to this pathway. While the evidence to date strongly indicates a positive role for autophagy in the replication of some viruses, the observed variation, particularly amongst closely related viruses, suggests that other mechanisms may exist for inducing similar alterations in host cells. One should approach these studies carefully with the correct diagnostic tools for autophagy.

A.3.2. Autophagy can be a mechanism for defense: the digestion or elimination of unwanted viral intruders.

As the previous section has indicated, viruses may induce autophagy for their own replicative advantage; yet, the same process may also confine viral replication complexes within vesicles as an innate defense against infection (Wileman, 2006). Furthermore, autophagy has been documented to function as a host cell defense against some

intracellular pathogens. This process, which has been termed xenophagy ('to eat what is foreign'), and results in the autophagic-lysosomal destruction of invading pathogens. Several bacterial pathogens including Mycobacterium tuberculosis (Gutierrez et al., 2004), group A Streptococcus (Nakagawa et al., 2004), Shigella flexneri (Ogawa et al., 2005), Legionella pneumophila (Amer and Swanson, 2005), and Yersinia pestis (Pujol et al., 2009) have been shown to either actively inhibit or be degraded through xenophagy. Subsequently, this process has been demonstrated to be particularly important in restricting bacterial escape and survival. Just as xenophagy restricts certain bacterial pathogens, autophagy may capture replicating viruses or newly assembled virions within their host cells, and eliminate them through sequestration and lysosomal degradation (Kirkegaard, Taylor, and Jackson, 2004; Levine, 2005; Wileman, 2007).

The initial hypothesis that autophagy might function as an antiviral defense mechanism was suggested when the autophagy effector and regulatory protein Beclin-1 was shown to be an antiviral effector in mice infected with the neurotropic Sindbis virus (SINV) (Liang et al., 1998). Over-expression of Beclin-1 protected mice from fatal SINV encephalitis, reducing neuronal apoptosis and decreasing SINV viral replication (Liang et al., 1998). The antiviral and anti-apoptotic effects attributed to Beclin-1 in SINV infection in vivo suggested that autophagy may function as a defense against other viral infections. Since these initial observations were first published, further evidence has shown that autophagy may function as a defense against viral infections.

Similar effects to those observed with SINV infection have been demonstrated in HSV-1 encephalitis (Orvedahl et al., 2007). In murine fibroblasts and neurons, mutant HSV-1 deficient in ICP34.5, a viral Beclin-1-interacting protein, but not wild-type virus,

induced autophagy upon infection in a PKR-dependent manner (Alexander et al., 2007; Orvedahl et al., 2007; Tallóczy et al., 2002; Tallóczy, Virgin, and Levine, 2006). This viral induction of autophagy resulted in the observed localization and xenophagic degradation of virions within autophagosomes (Alexander and Leib, 2008; Alexander et al., 2007; Tallóczy, Virgin, and Levine, 2006). However, the exact significance of increased mutant virions within double-membraned vesicles and xenophagy is questionable, since suppression of autophagy through the use of Atg5-/- mouse embryonic fibroblasts (MEFs) did not significantly alter the replication efficiency of either wild-type or ICP34.5 mutant HSV-1 in vitro (Alexander et al., 2007; Jounai et al., 2007). In the case of HSV-1, it is thought that while ICP34.5 expression can inhibit autophagy in infected cells, the prevention of PKR-mediated translational arrest, rather than regulation of autophagy, may be the pivotal determinant of HSV-1 replicative efficiency in cell culture (Alexander et al., 2007). In contrast to these in vitro observations, the suppression of autophagy in vivo by ICP34.5 appears to be critical in HSV-1 pathogenesis, since Beclin-1-binding-deficient ICP34.5 mutant viruses are neuroattenuated with regards to lethal encephalitis in mice (Orvedahl et al., 2007). Interestingly, this HSV-1 mutant's virulence can be reconstituted if the infection is conducted in mice deficient for the antiviral effector PKR (Orvedahl et al., 2007). The observed discrepancies between in vitro and in vivo results may be due to cell-type dependent differences, and/or the effects of other HSV-1 proteins on the autophagy pathway (Alexander and Leib, 2008).

The observation that a HSV-1 viral protein that abrogates autophagy is necessary in vivo to observe certain pathogenic effects, highlights the potential antiviral function of

this pathway. This may be particularly true in the case of neurotropic viruses, since modulation of autophagy has been suggested to influence the development of certain neurological diseases, (Itzhaki, Cosby, and Wozniak, 2008; Orvedahl and Levine, 2008). Along these lines, it has been noted that feline, simian, and human immunodeficiency viral infections in vivo can indirectly induce deficits in neuronal autophagy, and that this effect may contribute to the neuro-inflammatory pathology observed in these diseases (Alirezaei et al., 2008; Zhu et al., 2009). Thus, the dysregulation of autophagy by certain neurotropic viruses may not only interfere with their lysosomal clearance, but may also have a significant impact in terms of their pathogenic effect.

One remaining question though is whether xenophagy truly functions as an antiviral effector mechanism, since only HSV-1 viral particles have been observed microscopically within the confines of autophagosomes. Furthermore, this effect may be virus-specific rather than a general defensive mechanism of viral clearance. While it is tempting to speculate that xenophagy, as demonstrated within the field of bacteriology, exists as a general host defense mechanism for the clearance of all intracellular pathogens, including viruses, the limited amount of direct evidence available at this time suggests that caution is warranted. In particular, care should be exercised in differentiating between the incidental inclusion of virion or viral components within autophagosomes due to either their uptake by background or stress-induced activation of this pathway, and enhanced, autophagosome-driven clearance of these materials.

The HSV-1 ICP34.5 protein is known to antagonize PKR by dephosphorylating eIF2α (He, Gross, and Roizman, 1997), and, as discussed above, also blocks the induction of autophagy in a PKR-dependent fashion (Orvedahl et al., 2007; Tallóczy et

al., 2002; Tallóczy, Virgin, and Levine, 2006). As many viruses employ numerous countermeasures for disrupting the IFN and PKR antiviral systems, these viral disturbances may also have significance in modulating downstream autophagic processes. Indeed, viral regulation of autophagy in order to evade innate immunity has been linked to innate immune effector roles of autophagy other than that of digestion (discussed in section 3.3).

A.3.3. Autophagy may function in security surveillance: a watchdog for foreign molecules.

In addition to xenophagy, autophagy may function as an antiviral pathway in a less direct fashion, sampling and delivering cytoplasmic material to cellular compartments (endosomes and lysosomes). This process may play a significant role in the activation of innate and adaptive immune responses to foreign pathogens. Research has shown that autophagy is involved in the delivery of various antigens (viral, self, and tumor origin) to MHC class II antigen-presenting molecules, which can in turn lead to the activation of CD4+ T lymphocytes (Levine and Kroemer, 2008). As an example, the delivery of Epstein-Barr viral antigens to MHC-class-II-loading compartment (also known as late endosomes), prior to CD4+ T cell stimulation, has been shown to utilize the autophagy mechanism (Paludan et al., 2005). Constitutive autophagy in immune and epithelial cells has also been observed to participate in the delivery of LC3-tagged influenza matrix proteins to MHC class II-associated endosomal compartments, which resulted in enhanced antigen presentation to CD4+ T cells (Schmid, Pypaert, and Münz, 2007). Furthermore, the importance of autophagy-mediated class II antigen presentation was recently demonstrated in the regulation of HSV-1 pathogenesis by viral ICP34.5

(Leib et al., 2009). As well, it has been shown that the immunization of mice with influenza A (IFA)-infected cells exhibiting enhanced autophagy facilitates more robust MHC class I antigen presentation to the CD8+ T cells (Uhl et al., 2009). This evidence suggests that autophagy may contribute to MHC class I/II-specific viral antigen presentation, aiding the induction of adaptive immune responses. These studies also raise the exciting possibility to that it may be feasible to exploit autophagy to deliver viral antigens and enhance MHC class I/II presentation for the development of novel vaccines and adjuvant therapies (Schmid, Pypaert, and Münz, 2007).

The autophagy machinery has also been suggested to play a role in innate immunity by delivering PAMPs to their associated PRRs, including the TLRs. TLRs play critical roles in detecting bacterial and viral signatures and in eliciting appropriate host cell defenses such as IFNs and inflammatory cytokines against bacterial and viral infections (Takeuchi and Akira, 2007). It has been recently shown that the delivery of viral nucleic acids to endosomal TLRs in plasmacytoid dendritic cells (pDCs) can occur through the autophagosomes (Lee et al., 2007). While most TLRs reside on the cell surface, a subset involved in the detection of viral gene products, including TLR3, 7/8, and 9, are located in endosomal compartments and aid in the detection of endocytosed viral material (Barton, 2007). Autophagosomes could theoretically facilitate the sequestration, delivery, and detection of cytoplasmic viral nucleic acids, thereby helping to elicit a classical IFN response (Levine and Deretic, 2007). Indeed, autophagy has been shown to mediate ssRNA virus recognition through TLR7 during vesicular stomatitis virus (VSV) and Sendai virus (SENV) infection. It contributes to the production of interferon-α by pDCs in vitro and in vivo (Lee et al., 2007). Hence, this intrinsic pathway

may play an important role in the detection of viral antigens and in the induction of the subsequent IFN response in pDCs.

More recently, evidence supporting the involvement of autophagy in the TLR-related detection of viral PAMPs has increased with the finding that TLRs themselves can induce autophagosome formation. This suggests a potential feedback circuit involving autophagy in the pathogen-triggered immune response. Lipopolysaccharide (LPS)-induced autophagy has been demonstrated in both murine and human macrophages, and requires the TRIF (Toll-interleukin-1 receptor domain-containing adaptor-inducing IFN-β)-dependent TLR4 pathway, whose downstream components include the receptor-interacting protein (RIP1) and the p38 MAPK (Xu et al., 2007). While the physiological importance of this TLR-mediated induction is currently unclear, it suggests that TLR4 can induce autophagy, which may help to limit the spread of pathogenic infections. More importantly, it appears that there is cross-talk between the two pathways and that the autophagic machinery may be regulated by TLRs. TLR3 (dsRNA recognition), TLR7/8 (ssRNA recognition), and TLR9 (recognizes dsDNA with unmethylated CpG motifs) can identify viral signatures (Kawai and Akira, 2006). TLR4 can also recognize mouse mammary tumor virus (MMTV) envelope protein (Rassa et al., 2002) and the respiratory syncytial virus (RSV) fusion protein (Kurt-Jones et al., 2000). In addition, TLR2 recognizes the envelope proteins of HSV-1 (Kurt-Jones et al., 2004) and human cytomegalovirus (Compton et al., 2003), as well as the measles virus hemagglutinin (Bieback et al., 2002). As many of these TLRs share downstream adaptors TRIF and myeloid differentiation primary response gene (88) (MyD88) (Kawai and Akira, 2006), it is possible that apart from TLR4, other TLRs may also trigger autophagy.

On the other hand, autophagy has also been suggested to suppress the innate immune response to VSV infection. The retinoic-acid-inducible gene I (RIG-I)-mediated recognition of VSV in murine fibroblasts is inhibited by overexpression of Atg12-Atg5 (Jounai et al., 2007). Furthermore, MEFs deficient in Atg5 or Atg7 are resistant to VSV infection, more sensitive to polyinosinic:polycytidylic acid (poly:IC) treatment, and produce higher type I IFN responses following either treatment (Jounai et al., 2007; Tal et al., 2009). This evidence suggests that autophagy's effects are likely both cell type- and PRR-dependent, and further demonstrates the intricate nature of the relationship between autophagy and host cell innate immunity.

A.3.4. Viruses can modulate autophagy to determine cell fate by either postponing or hastening cell death.

Autophagy is involved with many biological pathways linked to cellular stress, and the signals that drive the activation of autophagy may ultimately decide the fate of the cell. Indeed, it has been suggested that autophagy possesses a dual role in mediating cell survival and cell death. As a survival mechanism, autophagy sustains cell viability during brief periods of cellular stress, either by providing metabolic substrates in times of shortage, or by removing damaged organelles in order to prevent apoptosis. On the other hand, autophagy has also been shown to induce cellular death under certain circumstances, which has been characterized as a form of programmed cell death (type II PCD) that differs from the more classical apoptotic (type I PCD) and necrotic forms of cell death (Levine and Yuan, 2005). Inhibition of autophagy can trigger apoptosis under starvation conditions (Boya et al., 2005), whereas cells deficient in the pro-apoptotic Bax and Bak proteins undergo autophagic cell death upon treatment with strong

pharmacological agents such as etoposide (Shimizu et al., 2004). More importantly, based on autophagy's dual nature with regards to cell fate, it has been suggested that this pathway might have important roles in the development of cancer and tumor suppression (Baehrecke, 2005; Gozuacik and Kimchi, 2007; Kondo et al., 2005; Levine and Yuan, 2005) (Brech et al., 2009; Eisenberg-Lerner et al., 2009; Maiuri et al., 2009; Morselli et al., 2009; Scarlatti et al., 2009).

Recent studies indicate that autophagy may function as a pro-survival mechanism during viral infections. Autophagy has been suggested to extend the survival time of human parvovirus B19-infected erythroid cells during viral expansion (Nakashima et al., 2006). Furthermore, protection against SINV-induced neuronal apoptosis and encephalitis in mice is conferred by concurrent overexpression of Beclin-1, but not anti-apoptotic Bcl-2 (Liang et al., 1998). The hepatitis B virus (HBV)-encoded transcriptional transactivator protein X (HBx) has been shown to up-regulate the expression of Beclin-1 and stimulate autophagy (Tang et al., 2009), an effect that was also observed in HBV-associated hepatocellular carcinomas (HCC) (Song et al., 2004). Interestingly, HBx has long been suggested to be tumorigenic (Kim et al., 1991), while Beclin-1 is commonly regarded as a haplo-insufficient tumor suppressor gene (Qu et al., 2003; Yue et al., 2003). Hence, autophagy could be up regulated by HBV to prolong cell survival, and this process may help initiate the development of HCC through an as yet uncharacterized mechanism.

As previously indicated, autophagy has been theorized to induce a novel form of programmed cell death, particularly under circumstances when apoptosis is impaired. Hence, while autophagy has been generally characterized as a pro-survival mechanism,

certain viruses may counter-intuitively block this process in order to prolong the survival of infected cells. The Kaposi's Sarcoma-Associated Herpesvirus (KSHV)-encoded viral Bcl-2 (vBcl-2) protein inhibits autophagy through its interaction with Beclin-1 (Pattingre et al., 2005) and similar effects have also been observed with the closely related murine γ-herpesvirus-68 (γHV-68)-encoded vBcl-2 molecule M11 (Ku et al., 2008; Sinha et al., 2008). However, in addition to their effects on autophagy, vBcl-2s from γ-herpesviruses also protect infected cells from apoptosis (Choi et al., 2001; Moore and Chang, 2003). While some authors have suggested that vBcl-2 help γ-herpesviruses escape autophagic degradation (Pattingre et al., 2005), given the powerful anti-apoptotic role of vBcl-2 it is difficult to gauge the relative importance of these effects. More recently, the KSHV viral FADD-like interleukin-1-β-converting enzyme (FLICE)/caspase-8-inhibitory protein (vFLIP) was shown to suppress autophagy by interfering with the processing of LC3, and that this effect abrogated autophagy-associated cell death in infected cells (Lee et al., 2009).

Interestingly, viral infections have also been documented to trigger autophagy specifically as mechanism of inducing cell death in uninfected bystander cell populations. During HIV infection, expression of HIV envelope glycoproteins (gp120 and gp41, collectively termed "Env") induces autophagy in uninfected bystander CD4+ T lymphocytes, which results in a novel cell death program with characteristics of both type I and type II PCD (Espert et al., 2006). Thus, it has been suggested that this autophagy-mediated phenomenon may be significant in contributing to general CD4+ T cell losses during the course of HIV infections (Espert et al., 2006; Espert et al., 2007; Levine and Sodora, 2006). Similarly, HIV gp120 could also enhance autophagy in uninfected

neuronal cells, leading to neuronal cell death and potentially contributing to HIV-associated dementia (Spector and Zhou, 2008). Interestingly, in plants, autophagy has been shown to protect bystander non-infected cells from cell death induced by Tobacco mosaic virus (TMV) infection (Liu et al., 2005).

The observed differential regulation of autophagy by viral infection in controlling cellular fate provides some indication to the potential complexity of this intrinsic pathway. It is important to note that it is inherently difficult to distinguish between autophagy as a mechanism of virally induced cell death and a cellular response to infection. Furthermore, there is potential that this may be a pathway whose biology is commonly modified in laboratory cancer cell lines used in virology research (Baird et al., 2008). For example, autophagy has been suggested to regulate the death of brain tumor and prostate cancer cell models infected with engineered adenoviruses (Alonso et al., 2008; Ito et al., 2006; Jiang et al., 2007; Rajecki et al., 2009; Ulasov et al., 2009; Yokoyama et al., 2008), but conflicting results have also suggested that this response may actually be an attempt at survival (Baird et al., 2008). Moreover, as characteristics of both type I and type II PCD are often seen in conjunction, it is often difficult to differentiate whether the observed phenomenon is an executioner mechanism or a futile attempt at cell survival. Nonetheless, from the above discussions it is clear that autophagy can contribute in the regulation of cellular fate, and as such may be specifically regulated by viruses to promote their life cycle.

A.4. Discussion

Questions regarding how viral infections interact with, and are impacted by, the autophagy pathway are increasingly gaining prominence within virology. While this

relatively new area of research has produced a number of exciting results, limits in our understanding of the mechanism, its regulation, and in the available investigative tools continue to present challenges, as would be expected in any emerging area of research. One complexity for researchers is that the regulation of autophagy is intimately interconnected with cellular growth and survival, as well as numerous cellular stress responses. Hence, considerable caution should be exercised before an effect on viral replication is directly attributed to this pathway. Furthermore, the natural complexity and diversity of viral pathogens and their host cells has resulted in numerous conflicting observations, making it difficult to draw clear and concise conclusions regarding the general role of autophagy in viral infections.

Despite these and other challenges, there is increasing evidence that this ancient cellular process is a significant factor in the fate of numerous different viral infections. During poliovirus infection, autophagy promotes viral replication through its most primitive function: the ability to induce membrane remodeling and vesicle formation. The processes of xenophagy and immune surveillance via cytoplasmic sampling suggest that autophagy may have evolved to act as a defense against intracellular pathogens that viruses must now contend with. Finally, autophagy's interconnections with cell death programs ingrain it in processes that ultimately determine cellular fate, suggesting that viruses may modulate it to influence cell survival and ensure their own reproductive advantage. Thus, autophagy may be implicated in the biological pathogenesis and/or replicative success of many viral infections through a wide variety of possible mechanisms, and can exhibit multiple distinct and cell type-dependent roles in the course of a single viral infection. This is best illustrated in the case of HIV, where the autophagy

machinery is: 1) down-regulated in virally-infected CD4+ T cells (Espert et al., 2009; Zhou and Spector, 2008), which may constitute a strategy for avoiding autophagosome-mediated degradation and/or endosomal detection in order to enhance virion production; 2) engaged in HIV-infected macrophages to promote virion production (Kyei et al., 2009); and 3) activated in bystander neurological and immunological naïve cells to commit cell death by type II PCD, thereby contributing to HIV pathogenesis (CD4+ T cell depletion and neuronal death associated-dementia) (Espert et al., 2006).

The revelation that autophagy can function in immune defense of the host cells, particularly relating to innate immunity, suggests reciprocal regulation (through TLRs and IFN response) aiding in the subsequent development of an adaptive immune response (through enhancement of MHC presentation) to clear pathogens in infected tissues. Further research has the potential to reveal unknown protein interactions and could lead to the development of new pharmacological therapies for treating various virally induced diseases and associated cancers. As new research tools emerge, continuing research will help to clarify the role of autophagy in viral replication, host immune responses, and viral pathogenesis. The intricacies of viral interactions with this ancient and highly conserved pathway strongly suggest that autophagy can function in a myriad of ways, some of which appear contradictory at first glance. Depending upon the circumstances, autophagy can be pro-survival or pro-death, can enhance viral replication or aid in the development of the antiviral response. Autophagy is a double-edged sword that can cut both ways. Clearly a virus would prefer to be on autophagy's good side!!

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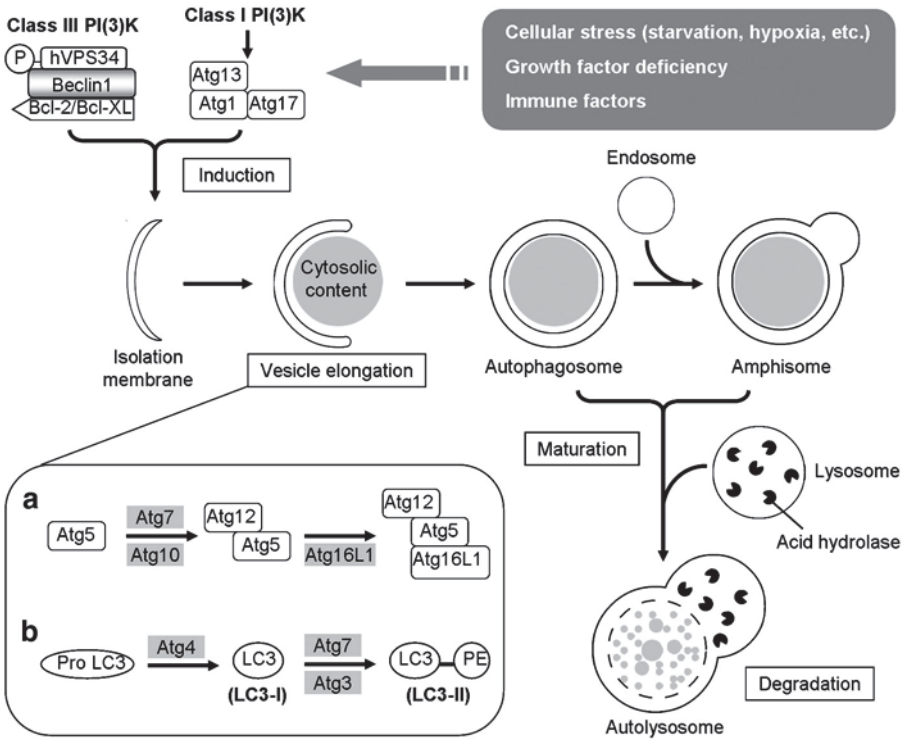

Figure A1 - Overview of the autophagy process. In response to cellular stimuli such as starvation and immune signals, the class I PI(3)K (phosphoinositide 3-kinases)-induced Atg1 complex and a class III PI(3)K complex involving Beclin-1 activate downstream ATG proteins in a series of steps that guide the induction, elongation, maturation, and degradation of the autophagosome. Two ubiquitin-like conjugation systems involving Atg12 (a) and LC3 (b) direct the vesicle elongation of the isolation membrane, which forms a crescent shape to sequester the cytoplasmic cargo. Upon completion, the autophagosome then undergoes the maturation step through a series of remodeling processes including fusion with endosomes/lysosomes. Fusion with lysosome helps the autophagosome mature into an autolysosome in which the autophagic vacuole along with its content is degraded.

Table A1. Significant genes in the mammalian autophagy pathway.

Gene	Important Interactions	Protein Function / Chacteristics
Formation of Autophagosomes		
ULK1 (ATG1)	Atg13, FIP200 (Atg17)	Ser/Thr kinase activity important for function; target(s) unknown. Downstream of mTOR signalling. Potentially involved in Atg9 cycling.
Beclin-1 (ATG6)	hVps34, Bcl-2/ Bcl-xL, UVRAG	Structural regulator of class III PI3 kinase hVps34. Contains BH3-like domain that is downregulatory when occupied.
hVPS34	Beclin-1, mTOR	Class III PI3 kinase; resulting PtdIns(3)Ps recruit Atg16L multimer/Atg18 to phagophore. Conflictingly activates mTor in response to amino acids.
ATG9	Atg2, Atg18	Transmembrane protein. Transits between phagophores and trans-golgi/late endosomes. Possible role(s) in protein recycling and/or membrane transit.
ATG12	Atg5, Atg16L	Covalently bound to Atg5 via mechanism similar to ubquitination.
ATG7	LC3, Atg12	Functionally similar to E1 ubiquitin activating enzyme (E1-like). Activates C-terminal glycine of both Atg12 and LC3.
ATG10	Atg12, Atg5	Functionally similar to E2 ubiquitin conjugating enzyme (E2-like). Accepts activated Atg12 and conjugates to internal lysine of Atg5.
ATG5	Atg12, Atg16L	Covalently bound to Atg12; conjugation allows Atg5 to associate with Atg16L.
ATG16L	Atg5-Atg12	Associates with Atg12-Atg5 and dimerizes. Present on outer surface of expanding phagophore; aids membrane curvature and LC3 recruitment (E3-like). Recycled.
ATG4	LC3	Cysteine protease; exposes C-terminal glycine on LC3 prior to lipidation. Subsequently recycles LC3 from outer membrane of autophagosome.
ATG3	LC3, Atg7	Functionally similar to E2 ubiquitin conjugating enzyme (E2-like). Conjugates LC3 with phosphatidylethanolamine (PE) phospholipid.
MAP1LC3 (ATG8)	Atg4	Experimental marker of induction. Cytosolic form (LC3-I) conjugated to PE, becoming membrane-associated (LC3-II). Possible role(s) in membrane expansion, autophagosome transit, and lysosomal fusion. Partially recycled by Atg4.
Regulation of Autophagy		
PI3K (class I)		Produces PtdIns(3)p that activate the Akt/PKB-mTor pathway.
PTEN		Phosphatase that counteracts PI3K by dephosphorylating PtdIns(3)p.
AKT/PKB	PDK1, Tsc 2	Ser/Thr kinase. Activated by PDK1 in presence of PtdIns(3)p. Inactivates Tsc 2.
REDD1/REDD2		Transcriptionally up regulated in response to hypoxia. Inactivates mTor pathway.
AMPK	LKB1, Tsc2	Activates Tsc2, leading to the induction of autophagy when the AMP/ATP ratio is high.
TSC2	Tsc1, Rheb, Akt/PKB, AMPK	GTPase-activating protein (GAP) with Tsc1; inactivates Rheb. Akt/PKB interferes with function, as does Erk1/2. AMPK enhances activity.
Rheb	Tsc1/Tsc2, mTor	Small GTPase. Activates mTor via binding kinase domain in GTP-dependent fashion. Tsc1/Tsc2 GAP activity converts to inactive, GDP-bound form.
mTOR	Rheb, raptor, mLST8	Key regulator of cellular growth. Autophagy induced when mTor inactivated. Ser/Thr kinase. Forms two protein complexes; mTORC1 associated with autophagy.
Anti-apoptotic Bcl-2 family	Beclin-1	Inhibit autophagy via binding with BH3 motif on Beclin-1. JNK1-mediated phosphorylation disrupts interaction and associated inhibition.
BH3-only Bcl-2 family	Anti-apoptotic Bcl-2 family	Competitively bind with anti-apoptotic Bcl-2 family members, interfering with their association with Beclin-1. Stimulate autophagy.
JNK1	Anti-apoptotic Bcl-2 family	Phosphorylates anti-apoptotic Bcl-2 family members, inhibiting interaction with Beclin-1. Activity induces autophagy.
UVRAG	Bif-1, Beclin-1	Interacts with Beclin-1's coiled-coil domain, strengthening Beclin-1/hVps34 interactions; promotes autophagy. Possible additional role in lysosome fusion.
p53		Controversial/contradictory role(s) in autophagy. P53-dependent autophagy observed experimentally. However, cytosolic p53 is inhibitory (mechanism unknown).
DRAM		Transmembrane lysosomal protein transcriptionally induced by p53. Stimulates autophagy. Necessary for both p53-dependent autophagy and apoptosis.

Table A2. Brief summary of known interactions between autophagy and viral infections.

Virus	Known Interaction(s) with Autophagy	Key References
Adenovirus	Contradictory results; autophagy may enhance or detract from engineered adenovirus-induced cell death; differences possibly cell-type/virus specific.	(Ito et al., 2006) (Baird et al., 2008)
Coronavirus	Contradictory evidence. May induce or subvert autophagy membrane remodeling. Autophagy may enhance viral replication.	(Prentice et al., 2004a) (Zhao et al., 2007)
Coxsackievirus	Autophagosome formation enhances CVB3/CVB4 replication *in vitro*. Autophagy affects virally-induced apoptosis.	(Wong et al., 2008) (Yoon et al., 2008)
Cytomegalovirus	Inhibits autophagy through unidentified mechanism.	(Chaumorcel et al., 2008)
Dengue Virus	Induces autophagy. Disruption reduces corresponding viral titers. Membrane co-localization observed with serotype-specific differences.	(Lee et al., 2008) (Panyasrivanit et al., 2009) (Khakpoor et al., 2009)
Drosophila C Virus	Induces formation of COPI-dependent vesicles. Interactions with autophagy mechanism unknown.	(Cherry et al., 2006)
Enterovirus 71	Induces/co-localizes with autophagic membranes. Induction increases viral titer. Mechanism unclear.	(Huang et al., 2009)
Epsten-Barr Virus	Autophagy may aid EBV MHC-II antigen presentation, but contradictory evidence exists. Possibly part of proliferation regulation.	(Paludan et al., 2005) (Lee and Sugden, 2008)
Hepatitis B Virus	Encodes transcriptional transactivator HBx, resulting in increased expression of Beclin-1. Sensitizes cells to autophagy signals.	(Tang et al., 2009)
Hepatitis C Virus	Induces autophagy; inhibits maturation. Knockdown of autophagy genes or ER stress response limits replication. Autophagy may aid HCV antigen presentation/TLR detection in some cell types.	(Sir et al., 2008) (Dreux et al., 2009)
Herpes Simplex Virus Type I	Viral encoded ICP34.5 antagonizes pathway through inhibition of PKR/eIF2a induction, as well as Beclin-1 binding. Autophagy effects of minimal importance *in vitro*, but essential for neurovirulence *in vivo*.	(Tallóczy, Virgin, and Levine, 2006) (Alexander et al., 2007) (Orvedahl et al., 2007) (English et al., 2009)
Human Immunodeficiency Virus	Autophagy-related mechanism part of envelope protein-induced bystander T-cell death and HIV dementia. Pathway disregulated in some infected cell types, but some components identified as necessary host co-factors.	(Espert et al., 2006) (Alirezaei et al., 2008) (Zhou and Spector, 2008) (Espert et al., 2009) (Kyei et al., 2009)
Influenza Virus	Induces autophagosome formation; inhibits maturation. Interference decreases viral yield. May enhance MHC-II antigen presentation.	(Gannagé et al., 2009) (Zhou et al., 2009)
Kaposi's Sarcoma-Associated Herpesvirus	Encodes viral homolog of Bcl-2 that inhibits autophagy through binding interactions with Beclin-1's BH3 domain. Viral FLIP suppresses autophagy through inhibition of Atg3.	(Pattingre et al., 2005) (Ku et al., 2008) (Lee et al., 2009)
Parvovirus	Induces formation of autophagosome-like vesicles, possibly to extend host cell survival during viral replication process.	(Nakashima et al., 2006)
Poliovirus	Induces formation of double membrane, LC3-positive vesicles. Aids viral replication and release. Viral 2BC protein triggers LC3 lipidation.	(Jackson et al., 2005) (Taylor and Kirkegaard, 2007)
Rhinovirus	Does not induce or modulate autophagy. Replication unaffected by induction or inhibition of pathway.	(Jackson et al., 2005) (Brabec-Zaruba et al., 2007)
Sendai Virus	In pDCs, autophagy enhances delivery of viral nucleic acids to endosomes for TLR7 recognition.	(Lee et al., 2007)
Sindbis Virus	*in vivo* up-regulation of autophagy via Beclin-1 over-expression reduces fatal encephalitis in mice.	(Liang et al., 1998)
Tobacco Mosaic Virus	Autophagy necessary to restrict virally-induced programmed cell death responses to the site of infection.	(Liu et al., 2005)
Vaccinia Virus	Induces vesicle formation, but mechanism independent of autophagy.	(Zhang et al., 2006)
Varicella-Zoster Virus	Induces autophagy. Role in infection unclear.	(Takahashi et al., 2009)
Vesicular Stomatitis Virus	Autophagy enhances endocytic detection in pDCs, but may diminishes cytosolic detection/antiviral responses in other cell types.	(Jounai et al., 2007) (Lee et al., 2007) (Shelly et al., 2009)

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