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Subversion of cellular autophagy during virus infection: Insights from hepatitis B and hepatitis C viruses☆

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Abstract

Autophagy is a self-eating process, in which the damaged or excessed cell organelles and misfolded protein aggregates are removed from the cellular microenvironment. Autophagy is generally thought of as a pro-survival mechanism which is not only important for balancing energy supply at times of nutrient deprivation but also in the removal of various stress stimuli to ensure homeostasis. In addition to the target materials of "self" origin, autophagy can also eliminate intracellular pathogens and acts as a defense mechanism to curb infections. In addition, autophagy is linked to the host cell's innate immune response. However, viruses have evolved various strategies to manipulate and overtake host cell machinery to establish productive replication and maintain infectious process. In fact, replication of many viruses has been found to be autophagy-dependent and suppression of autophagy can potentially affect the viral replication. Thus, autophagy can either serve as an anti-viral defense mechanism or a pro-viral process that supports viral replication. Hepatitis B virus (HBV) and hepatitis C virus (HCV) are known to co-opt cellular autophagy process as a pro-viral tool. Both viruses also induce mitophagy, which contributes to the establishment of chronic hepatitis. This review focuses on the roles of autophagy and mitophagy in the chronic liver disease pathogenesis associated with HBV and HCV infections.

Keywords

Autophagy; Hepatitis B virus (HBV); Hepatitis C virus (HCV); Endoplasmic reticulum (ER) stress; Mitophagy

1. Introduction

According to World Health Organization (WHO), approximately 257 million people are infected with hepatitis B virus (HBV) globally, and an estimated 71 million people are chronic hepatitis C virus (HCV) infected carriers.[1,2] Infections caused by HBV and HCV are responsible for a substantial proportion of liver diseases worldwide and an annual death of around 1 million people around the world.[3–6] HBV and HCV are hepatotropic viruses that

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Conflict of interest

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share similar modes of transmission and consequences that lead to liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC).[2] About 80% of all HCC is HBV/HCV-associated.[3] In the areas with a high prevalence of HBV, coinfection of HBV and HCV is often reported.

1.1. HBV and HCV

Despite sharing a similar mode of transmission and pathological consequences, HBV and HCV are entirely different. HBV is a member of the *hepadnaviridae* family and deoxyribonucleic acid (DNA)-genome containing virus that replicates via an ribonucleic acid (RNA) intermediate, termed as pregenomic RNA.[7] At the time of infection, 3.2 kb long, partially doubled stranded and relaxed circular DNA (rcDNA) is converted to covalently closed circular DNA (cccDNA) by host cell machinery and stably remains in the nucleus for a long time.[8] In the nucleus, the cccDNA serves as a template for the synthesis of 5 major HBV transcripts that are initiated from distinct transcription start sites and terminate at a common polyadenylation site.[8] All HBV transcripts are responsible for coding various structural and non-structural proteins that eventually take part in the viral replication cycle. Steps of HBV life cycle are executed in both, nucleus and cytoplasm of the host cells.[8] In contrast, the replication of HCV is predominantly cytoplasmic. HCV contains positive-sense, single stranded RNA genome, which encodes a polyprotein of approximately 3010 amino acids that is subsequently cleaved by viral and host proteases resulting in the synthesis of 10 viral proteins that include the 3 structural proteins (core, E1, E2) and 7 non-structural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B).[9] HCV RNA replicates in the endoplasmic reticulum (ER)-derived membranous structures, the membranous web.[10] The assembly of HCV nucleocapsid and viral particle morphogenesis occurs on ER membranes in close juxta-position to the lipid droplets.[11,12] Both HBV and HCV affect a variety of cellular functions and cause structural changes in the subcellular organelles in the infected cells.[13–15] HCV infection induces ultra-structural changes which include, ER ballooning, formation of ER membranous web, mitochondrial swelling, induction of lipid droplets, and Golgi fragmentation.[11,12,16,17] Effect on cellular functions manifest as an ER response, altered calcium signaling, mitochondrial dynamics and modification of secretory pathways.[12,18–20] In many instances, some of the viral proteins associate with organelles directly affecting some of these processes.[20]

Following translation of HCV RNA genomes on the ER-associated ribosomes, viral proteins are associated with the ER either as transmembrane proteins or tethered to the ER.[11] These activities cause an ER stress leading to unfolded protein response (UPR) and calcium release.[19] Released calcium is taken up by mitochondria where calcium induces an oxidative stress state thus causing damage to mitochondria. In HCV infections, UPR, ER stress and autophagy processes are linked with each other.[21–23] ER stress response can potentially induce autophagy.[24] HCV infection is associated with ER stress response that can trigger the activation of initial steps of autophagy in infected cells.[19,21,25,26] Both HBV and HCV gene expression induce bulk autophagy.[22,27,28] Several reports have highlighted roles of autophagic machinery in various steps of HBV and HCV life cycle (such as viral replication, translation, and propagation).[22,27,28] Although a few reported findings relating the autophagy with HBV or HCV are controversial and remain to be resolved, but overall the results consistently support the notion that HBV and HCV, can subvert autophagy to benefit

the infectious process. Although many reports have described the involvement of bulk autophagy in various steps of HBV and HCV lifecycle, our understanding of the biological significance and the precise role of autophagy in the viral lifecycle is still incomplete.

1.2. Autophagy

The seminal observation that portion(s) of cytoplasmic material can be engulfed by lysosomes was first described by De Duve and Wattiaux.[29] With this observation, the events of autophagy were first proposed 50 years ago. Then, in the late 1990s, autophagy-related genes (*ATG*) were identified in yeast genetic screen that further provided the insights into the molecular mechanism of autophagy in higher eukaryotes.[30,31] Several aspects of autophagy have been uncovered since and were implicated in various biological processes and pathological conditions.[32] The picture that emerges from a number of reports to-date confirmed that autophagy is a complex and multistep process.[32] In general, autophagy can be broadly classified as macroautophagy, microautophagy and chaperone-mediated autophagy (CMA).[33–36] All the three classes of autophagy share a common mode of degradation via lysosome but occur by different mechanisms and pathways (Fig. 1). Briefly, in microautophagy, the targeted cargo is directly engulfed by the lysosome. A selected portion of the lysosomal membrane undergoes structural remodeling throughout the dynamic process of invagination, protrusion and/or septation of limited lysosomal membrane eventually leading the engulfment of the target cargo by the lysosome for subsequent degradation. Unlike microautophagy, macroautophagy is a multi-step process, in which the cargo is first wrapped within a double-layered membrane and then sequestered at the lysosome for degradation.[33] A complete process includes membrane initiation, nucleation, elongation, maturation, and fusion. CMA is another type of autophagic process, which recognizes the KFERQ motifs in the cargo protein to target them to the lysosomes.[34] Lysosomal membrane receptor, lysosome-associated membrane protein type 2A (LAMP-2A) is a critical component of CMA.[34] The targeted substrate protein, containing the KFERQ-like motif, binds with the cellular chaperone heat shock cognate protein 70 (HSC70) (or heat shock 70 kDa protein) and this complex is then transported to the outer surface of the lysosome.[37] LAMP-2A at the surface of lysosome binds with this complex and substrate protein gets unfolded and translocated to the lysosome.[37] Autophagy can be selective or non-selective.[34,38] Non-selective autophagy plays important role in energy balance by mass degradation of subcellular organelles and protein aggregates thereby recycling them back in terms of energy supply to the nutritionally deprived cellular environment.[39] On the other hand, selective autophagy uses specific adapter proteins, such as p62, neighbor of BRCA1 gene 1 (*NBR1*), B cell leukemia 2 (BCL2) and adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) and NIP3-like protein X (NLX) that recognize a selective subset of autophagy substrates and exclusively target them to lysosomal degradation.[38] For example, NBR1 can selectively mediate peroxisomal autophagy or pexophagy.[40] Parkin and phosphatase and tensin homolog deleted on chromosome ten (PTEN)-induced putative kinase 1 (PINK1) can induce mitochondria-selective autophagy or mitophagy.[41–43] In addition to the cell organelles, previous studies have shown that the autophagy can also selectively eliminate intracellular pathogens such as *Mycobacterium tuberculosis* and Sindbis virus.[44–46] Autophagic removal of pathogens is termed as

"Xenophagy". Growing evidence has extended the functions of autophagy to immune response (innate or adaptive).[47]

1.3. Autophagy and viral infection

The crosstalk between viruses and autophagy is very complex. Viruses can either induce or inhibit autophagy. Providing a detailed picture of virus-autophagy interaction of all viruses is beyond the scope of this review. This review intends to discuss the correlations between HBV/HCV-induced autophagy/mitophagy and liver disease pathogenesis.

2. HBV and autophagy

2.1. Crosstalk between HBV and autophagy

HBV induces autophagy in cultured cells, during natural infection and in transgenic mice (expressing full length HBV genome).[28,48,49] All the studies conducted so far, have unanimously established that autophagy machinery is influenced by HBV infection and required for HBV pathogenesis. However, differences in the proposed mechanisms remain to be addressed. Although multiple investigations have implicated that HBV induced changes in the initial, the middle and the late stages of autophagy, the underlying mechanisms are not fully elucidated. For example, a few studies have implicated HBx as a trigger for autophagy induction while others have identified small hepatitis B surface antigen (SHBs) as autophagy inducer.[28,48] Autophagy induction was observed in transgenic mice expressing the full length HBV genome.[50] However, it was not observed in mice expressing HBV mutant (*HBVΔX*) that was defective in producing HBx.[28,50] Moreover, ectopic expression of HBx alone could induce autophagy in hepatocytes.[13,28] SHBs mediated induction of ER stress and UPR is thought to be responsible for autophagy induction.[48] Similar to *HBVΔX*, HBV mutant, incapable of producing SHBs was found unable to induce autophagy.[48] Overall, the evidence for HBx (not SHBs) to be the better trigger for autophagy induction is relatively more convincing because: (i) HBV mutant with a deletion in its PreS2 region can still induce ER stress;[51–53] (ii) the reduced expression of SHBs was found to be a trigger for ER stress.[54,55] When SHBs level is reduced, more large HBV surface antigen (LHBs) accumulates in the ER-lumen and causes the ER stress.[56,57] Further investigation is warranted to resolve the issue whether it is HBx or SHBs that induces autophagy.

In addition to the early stage of autophagy, the mid-stage of autophagy in HBV infection appeared to have partly differing hypothesis. HBx was shown to upregulate beclin-1 expression via activating its promoter.[58] Elevated level of beclin-1 then activates phosphatidylinositol 3-kinase catalytic subunit type 3 (PI3KC3).[58] In general, PI3KC3 activation induced production of phosphatidylinositol-3-phosphate (PI3P), a lipid that is required for membrane biogenesis.[59] Higher level of PI3P increases the step of nucleation and as a result, autophagic vacuoles and autophagosomes surrounded by double layer membrane are generated within cytoplasm.[59] However, Sir *et al.*[28] observed that beclin-1 is not induced in HBV or HBx expressing cells. They observed that instead of acting via beclin-1, HBx can directly bind and activate the PI3KC3 complex.[28] Despite the differences in the proposed upstream events, both studies are in agreement that the PI3KC3 activation is important for HBV or HBx mediated autophagy induction.

Last step, in which the autophagosomes fuse with the lysosome and execute the final step of cargo degradation is also apparently complex. The increased biogenesis of autophagic vacuoles by HBx does not degrade the autophagic proteins to the same extent.[28] This observation suggested that the HBx-induced augmentation of autophagic vacuoles does not increase the sequestration of cellular proteins or organelles for degradation. In other words, the process of autophagy appeared to be incomplete where the matured autophagosome is unable to fully degrade the targeted protein. It was concluded that HBV induces incomplete autophagy. However, Kim *et al.*[13] focusing on selective autophagy of mitochondria (mitophagy), clearly showed that HBV infection can specifically target damaged mitochondria for autophagic degradation. Whole HBV genome expression or HBx alone was able to induce mitophagy, while *HBVΔX* genome failed to do so.[13] It is possible that in HBV infected cells, events of bulk and selective autophagy are differentially regulated.

In summary, HBV infection induces autophagy in hepatocytes, which is crucial for the HBV pathogenesis. Variations in the proposed models and hypothesis could be due to different cell types, tissue or virus genotype used. Genotype variability seems to affect autophagy differently. It was observed that HBV-C (genotype C) was more potent inducer of autophagy than HBV-B (genotype B). HBV-C can cause more severe liver disease outcome than HBV-B but it is not confirmed whether the higher virulence of HBV-C is because of its higher efficiency to induce autophagy.[49,60]

2.2. Mechanisms of HBV-induced autophagy

Possible mechanism(s) of HBV-induced autophagy is a combined action executed by multiple cellular events summarized in Fig. 2. Although these pathways are intricately linked, for the sake of clarity, each of these pathways is discussed separately. It should be noted that these pathways are often found to co-exist and operate together.

2.2.1. ER stress and UPR pathways—ER is an important cell organelle in which protein synthesis and folding occur. Accumulation of unfolded protein aggregates in the ER causes an ER stress response. As a result, a complex signaling cascade is initiated within the cells that work to neutralize the stress. ER stress can be relieved by UPR that stops protein synthesis, promotes protein degradation via ER-associated degradation (ERAD) pathways and induces the involvement of chaperone proteins that assist in proper folding of proteins.[61] Similar to other stimuli of ER stress, HBV infection also can activate ER stress signaling in infected cells.[62] The HBV surface protein (HBsAg), that translocates to ER, is a principal inducer of ER stress and modulates autophagy pathways.[48] HBV expression can also induce BiP (alias glucose-regulated protein 78) expression, which helps autophagosome biogenesis via C/EBP homologous protein (CHOP).[63,64] In general, ER stress turns on UPR signaling, which is mainly constituted with 3 major signaling arms: protein kinase RNA-like ER kinase (PERK), inositol-requiring protein-1α (IRE1) and activating transcription factor 6 (ATF6).[65] Activation of PERK pathways is mainly for controlled translation. It was observed that HBx or HBs can activate all the three pathways *i.e.* PERK, IRE1 and ATF6.[52] Interestingly, if any of these three pathways is blocked, lipidation of LC3 in HBs expressing cells is also inhibited.[48] This observation confirmed that all the three arms of UPR pathway are

important to induce autophagy in HBV expressing cells. In summary, published reports pointed out a strong link between HBV induced autophagy and UPR signaling.

2.2.2. HBx-induced pathways—HBx-induced autophagy follows three major routes, which include beclin-1 upregulation, activation of PI3KC3 enzyme activity and activation of death associated protein kinase (DAPK).[28,58,66] HBx activates beclin-1 gene promoter at site −277/179 and then enhanced beclin-1 localizes with PI3KC3 and UV radiation associated gene (*UVRAG*) to promote pre-autophagosome formation sites.[58] Moreover, HBx-mediated increase in enzymatic activity of PI3KC3 helps accumulation of PI3P, which helps autophagosome formation.[28] Recently, HBx mediated autophagy was also found to be DAPK dependent.[66] In general, DAPK phosphorylates beclin-1.[67] HBx first activates DAPK by dephosphorylation and then, active form of DAPK phosphorylates beclin-1 at Bcl2-homology (BH3) domain. Phosphorylated beclin-1 gets dissociated from Bcl-XL and induces autophagy.[66] In summary, the involvement of HBx in autophagy has been confirmed by multiple reports.

2.2.3. Mitogen activated protein kinase (MAPK) and reactive oxygen species (ROS) induced pathways—HBV expression can stimulate MAPK.[68] In vertebrates, three pathways of MAPK have been characterized. These pathways are, extracellular signal regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK).[69] Previous studies have suggested that MAPK pathways are involved in the progression of autophagy.[70] Interestingly HBx was reported to activate all the three pathways of MAPK.[71] If JNK signaling is blocked, HBx mediated phosphorylation of Bcl2 is abolished, which targets the dissociation of beclin-1 from Bcl2, a step fundamental for autophagy induction.[72] In addition, HBx can induce autophagy via ROS generation.[72] If ROS level is neutralized, both JNK signaling and autophagosome formation are inhibited.[72] Moreover, Sirtuin 1 (SIRT1) is a known modulator of autophagy and interestingly HBx can interact with SIRT1 and modulate the acetylation status of key proteins required for autophagy.[73–75]

2.2.4. AMP-activated protein kinase (AMPK) pathways—AMPK is a sensor for the cellular energy state.[76] If the cellular adenosine triphosphate (ATP) level is low, AMPK is activated and initiates downstream signaling to establish energy homeostasis.[76] Several reports have suggested that ROS mediated activation of AMPK is also a reason for induction of autophagy which benefits HBV replication.[72] However, another report claimed that the increased autophagic degradation can restrict the HBV production.[77] Therefore the involvement of AMPK in HBV replication appeared very complex until the recent report reconciled this complexity and added that HBx, when it activates AMPK signaling, it also turns on mammalian target of rapamycin complex-1 (mTORC1) and can acts as a rheostat that simultaneously can stimulate pathways to activate or repress HBV replication.[78]

2.2.5. PINK1 and Parkin mediated mitophagy—Autophagy was previously considered a non-specific process. However, the recent advancement in the field has clearly indicated that the selective-autophagy also exists.[44] It is now well-accepted that the damaged cell organelles are selectively sequestered to the lysosomes and degraded.[13,15,40–43] For instance, autophagic removal of peroxisomes (pexophagy), mitochondria (mitophagy) and

ER (reticulophagy) are selectively operated by specific proteins.[13,15,40–43] Mitophagy is a process of selective autophagy of mitochondria. In mammalian cells, mitophagy is mainly governed by the two key protein, Parkin (an E3 ubiquitin ligase) and PINK1.[42,43] Molecular mechanism of Pink1-Parkin mediated mitophagy has been characterized in much detail and multiple hypotheses have been proposed to explain the mitochondrial translocation of PINK1-Parkin. PINK1 contains a mitochondrial targeting sequence and hence is translocated to mitochondria regardless of polarization status.[43] From outer mitochondrial membrane (OMM), translocase enzymes import PINK1 to the inner mitochondrial membrane (IMM).[43] At IMM, PINK1 is then processed by mitochondrial processing protease (MPP) and as result, mitochondrial targeting sequence is removed.[43] Processed PINK1 is subsequently cleaved by presenilin associated rhomboid-like protease (PARL) to generate 52 kDa form and then subsequently degraded by proteasomal pathways.[43] In short, if the mitochondria are not impaired, PINK1 will be quickly imported to IMM and degraded by combined action of MPP, PARL and proteasomal machinery. However, in impaired mitochondria, altered mitochondrial potential dampens the PINK1 translocation to the IMM and its subsequent degradation.[42,43] As a result, PINK1 is displayed at OMM and initiates the Parkin recruitment and phosphorylates ubiquitin.[79] Phospho-ubiquitin is then transferred onto Parkin and activates Parkin.[79] Mitochondrial Parkin then ubiquitinates mitochondrial surface proteins to recruit p62 and other key molecules required for the initiation of mitophagy.[41] Kim *et al.*[13] reported that the HBV infection induces mitophagy. They found that the HBV perturbs mitochondrial dynamics and induces mitochondrial fission.[13] Full length HBV genome or HBx expression alone is sufficient to activate dynamin-related protein (Drp1).[13] HBV stimulates the Ser616 phosphorylation of Drp1 and induces its translocation of mitochondria.[13] Mitochondrial Drp1 forms a ring shape structure around the mitochondria and via its GTPase activity, it cuts a fully elongated mitochondria into smaller fragments.[20] It has been shown that the fragmented mitochondria are easy target for lysosomal degradation.[20] In addition to Drp1, Kim *et al.*[13] also found that HBV upregulates the Parkin, PINK1 and LC3B expression. HBV or HBx alone cause mitochondrial translocation of Parkin. Mitochondrially localized Parkin not only initiates mitophagy but also regulates mitochondrial fission by inducing proteasomal degradation of mitofusin 2 (Mfn2), a mediator of mitochondrial fusion. The PINK1/Parkin mediated mitophagy was found to be critical for sustained cell viability and support chronic infection. Inhibition of mitophagy by silencing Parkin or Drp1 in HBV infected cells results in significant cell death, indicating that mitophagy contributes to the establishment of persistent viral infectious process.[13]

2.2.6. Involvement of long non-coding (lnc) RNA—Many lncRNAs are reportedly dysregulated in HBV-related HCC and HBV/HBx expressing cells.[80,81] Most of the studies only focused on the dysregulation of lncRNA and a few of these have actually focused on the molecular mechanism. In HBV related HCC, two autophagy-related lncRNAs, HOTAIR and HULC were studied in detail.[82,83] HOTAIR holds multimeric complex consists of polycomb repressive complex 2 (PRC2), and LSD1/Co-REST/HDAC1, together.[82] It has been shown that the regulation of HOTAIR expression by HBV/HBx is associated with transcriptional repression.[82] In addition, HBV/HBx mediated regulation of HULC modulates cAMP responsive element binding protein (CREB) signaling.[83] Interestingly, increased

expression of HOTAIR or HULC activates, while reduced expression of HOTAIR or MALAT1 diminishes autophagy in liver cells.[84–86] Although in HBV expressing cells, the direct relation of these lncRNAs with autophagy is yet to be established but indirect correlation strongly points to the lncRNA-mediated signaling could be one of the mechanisms that regulates HBV induced autophagy in infected hepatocytes.

3. HCV and autophagy

3.1. Crosstalk between HCV and autophagy

Several studies have shown that HCV infection induces autophagy.[22,87,88] HCV infected cells and hepatocytes derived from infected individual show significant lipidation of LC3, a marker for the initial onset of autophagic response. Sir and colleagues, reported that HCV induces autophagic vacuole formation in hepatoma cells. However, they showed that the process of autophagosomes fusion with the lysosome is not fully executed and remains incomplete. Subsequent investigations reported that HCV does enhance autophagic flux and induces complete autophagy.[22] Huang *et al.*[89] observed that process of autophagosome maturation in HCV infected cells is temporally regulated. Later Wang *et al.*[90] also observed that during early stage of HCV infection, the process of autophagosome maturation is less efficient and as the infection progresses, it becomes efficient at late stage of HCV infection. Differential induction of Rubicon and UVRAG proteins contribute to the temporal regulation of the autophagosome maturation.[91] Rubicon is a negative regulator of autophagy while the UVRAG positively regulates autophagosome maturation. During HCV infection, Rubicon is upregulated at early stage of infection, inhibits UVRAG to support autophagosome maturation and the process of autophagy remains incomplete.[91] However, at late stage of infection, UVRAG is upregulated significantly and this enhanced level of UVRAG is sufficient to overcome the inhibitory effect exerted by Rubicon. As a result, at late stage of infection UVRAG efficiently support the process of autophagosome maturation and process of autophagy becomes complete.[91] This observation is nicely reconciled with the observation made by Kim *et al.*[15] demonstrating that the effects of mitophagy are seen at late stage of infection. Similar to HBV, different genotypes of HCV have also been shown to exert different effects on autophagy process.[92]

3.2. Mechanisms of HCV-induced autophagy

HCV-induced autophagy is also accompanied by multiple arms of cellular signaling pathways.[90] Broadly, the events of autophagic induction are categorized as direct or indirect consequences of HCV replication. These events are discussed below and summarized in Fig. 3. It should be noted that all these upstream events induced by HCV are highly interlinked and often cooperate with each other.

3.2.1. ER stress-associated mechanisms—In general, accumulation of unfolded or misfolded proteins induces ER stress which activates three arms of UPR signaling *i.e.* ATF6, IRE1 and PERK.[61] UPR acts to alleviate the ER stress and failure of UPR to alleviate ER stress results in apoptosis.[61] HCV infection has been shown to induce ER stress and activate UPR signaling.[19,25,26] In an earlier investigation, it was observed that HCV mediated autophagy induction is UPR-dependent. The HCV core protein can alone induce UPR

signaling via activating PERK and ATF6 but not IRE1.[23] However, small interfering RNA (siRNA)-mediated silencing of IRE1, ATF6 and PERK respectively in HCV infected cells, resulted in significant inhibition of autophagy.[23] Interestingly, it was also reported that UPR may not be necessary for HCV induced autophagy.[93] Mohl *et al.*[93] showed that the autophagy induction precedes the initiation of UPR. Moreover, they showed that hepatoma cell line harboring various subgenomic replicon of HCV induces autophagy but not UPR and silencing of IRE1 has no effect on autophagy induction.[93] This report did show that HCV infection is associated with activation of autophagy and UPR but rejected any correlation between the two. The reason for this discrepancy is not clear yet but the contradiction pertaining to the LC3 lipidation precedes UPR can be reconciled by the fact that at early stage, minute activation of UPR that could not be detected, might be sufficient to initiate LC3 lipidation. Additionally, the UPR activation during immediate early stage of HCV infection possibly is transient in nature but sufficient to induce autophagy, and eventually gets robust only at later stage of infection. Nonetheless, further studies are required to reconcile this discrepancy with experimental evidence.

3.2.2. Mitochondria-associated mechanisms—HCV core protein is known to be in close association with mitochondria, which causes significant increase in ROS (reactive oxygen species) and RNS (reactive nitrogen species).[25,26] Elevated level of ROS activate autophagy via NF-E2 related factor 2 (Nrf2) mediated pathway.[94] In a recent investigation, it has been reported that HCV abrogates the activation of Nrf2 and antioxidant response element (ARE) target genes.[95] HCV NS3 protein can directly bind with the Nrf2 and holds it in the cytoplasm and prevents its nuclear translocation.[95] Impairment of this signaling leads to an imbalance in the cellular oxidative status and as a result, ROS level is increased, which causes phosphorylation of p62, a crucial protein for autophagy machinery.[95] Moreover, reduction in GSH and NADPH levels, decrease in mitochondrial complex I activities and imbalance of mitochondrial Ca^{+2} uptake, eventually disrupt the mitochondrial membrane integrity and lead to mitochondrial dysfunction. Cytosolic imbalance in calcium causes activation of nuclear factor-kappa B (NF-κB) and STAT3. Moreover, HCV NS5a is also a potent modulator of calcium signaling and induces oxidative stress.[19] Use of anti-oxidants or calcium chelators eliminates the HCV NS5a mediated alterations in hepatoma cells. Interestingly, NF-κB and STAT3 can regulate the cellular autophagy.[19] In summary, oxidative stress, calcium burst and activation of NF-κB/STAT3 are the mitochondria-centric pathways that can eventually induce cellular autophagy in HCV infected cells.

3.2.3. Golgi-associated mechanisms—Recently Golgi-situated immunity related GTPase M (IRGM) protein has been shown to regulate autophagy in HCV infected hepatoma cells.[17] It was observed that the IRGM protein regulates the unc-51 like autophagy activating kinase 1 (ULK1) signaling and induce autophagy. ULK1 and AMPK are the key proteins in autophagy signaling cascade.[17] ULK1 is a target of AMPK, which interacts with, phosphorylates and activate ULK1 to induce autophagy.[96] In HCV infected cells, IRGM was found to positively regulate both, AMPK and ULK1 by separate mechanisms.[17] Moreover this event of IRGM mediated activation of autophagy was found to be co-operated with the event of Golgi fragmentation.[17] The event of Golgi-fragmentation during HCV infection was previously reported by Siddiqui[16] laboratory and later Hansen *et al.*[17] further revealed

detailed mechanism demonstrating that the IRGM is required for HCV-induced autophagy and thereby HCV replication. Interestingly, they further showed that IRGM is critical for Golgi fragmentation. Fragmented Golgi was proposed to play important role in the formation of membranous web (MW) and virus replication complex (VRC). However the precise mechanism pertaining to how Golgi fragmentation can produce MW and VRC is still unknown. Even before this report by Hansen *et al.* another study had shown that HCV-NS3 can directly interact with IRGM and this interaction is sufficient to induce autophagy in IRGM dependent manner.[97]

3.2.4. Direct effects of HCV proteins—In addition to the multiple indirect effects exerted during HCV replication, HCV encoded proteins can also modulate autophagy in a direct manner. Both the types of HCV proteins (structural and nonstructural) have ability to modulate autophagy. For example p7 ion channel protein of HCV can directly bind with the beclin-1, which is an important component of PI3KC3 complex.[90] However, the overall significance of this observation remains elusive as the ectopic expression of p7 alone cannot induce autophagy.[90] In addition, when NS3-NS5B is expressed in cells, significant appearance of double layered vesicles was observed and pharmacological inhibition also suggested that cyclophillin-A inhibitor, but not phosphatidylinositol-4 kinase IIIα (PI4KIIIα) inhibitors, stop NS3-NS5B induced formation of double layered vesicles.[98] HCV NS3 is also a potent modulator of autophagy. It is a direct interacting partner of IRGM which is a member of GTPase family. Interestingly, IRGM can directly interact with many crucial proteins of autophagy network, such as ATG5 and ATG10.[97] With this correlation, it can be assumed that IRGM could act as an adapter to establish interaction between ATG5/ATG10 and HCV-NS3. HCV NS4B is shown to modulate autophagy via inducing Rubicon expression and affecting autophagosome maturation.[91] HCV NS4B can interact with beclin-1, hVsp34 and Rab5 and if the expression of any of these three molecules are suppressed, the autophagy induced by HCV-NS4B, is inhibited.[90] In HCV infected cells, NS4B colocalized with the ATG5 suggesting that this protein can directly interfere in the autophagy signaling.[99]

4. Roles of autophagy in HBV/HCV pathogenesis

As discussed above, the implication of autophagy in HBV/HCV infection are not confined only to the viral replication. Growing body of evidence establishes that autophagy rather exerts multiple effects on the overall pathogenesis of these two hepatotropic viruses.[90] A series of events influenced by autophagy in cells infected with HBV or HCV are depicted in Fig. 4, and discussed below.

4.1. Innate immunity

During viral infection, autophagy induction has been linked with the stimulation of toll like receptors (TLRs) and antigen presentation by major histocompatibility complexes (MHCs).[47] In general autophagy has been found to be a critical regulator of host cells anti-viral signaling.[100] The interplay between pathogen associated molecular pattern (PAMP) and pattern recognition receptor (PRR) during HBV and HCV infection has been characterized.[101–103] For additional details, the readers are advised to read published articles.[101–106] This

section mainly covers the innate immune signaling pertaining to the HBV and HCV infections. In case of HCV infection, autophagy has been shown to disrupt innate immune singling.[107] In HCV genomic RNA, UC rich sequence located in the 5' UTR (untranslated region) is recognized by cellular PRR, retinoic acid-inducible gene I (*RIG-I*).[107] It was previously shown that the inhibition of lysosomal acidification with chloroquine or suppression of *ATG*5 can induce RIG-I mediated interferon signaling.[108] Interestingly, ATG5-ATG12 complex can interact with mitochondria antiviral signaling protein (MAVS).[108] In support of this, beclin-1 and ATG7 were also implicated in autophagy mediated modulation of innate immune signaling in HCV infected cells.[109] However, the study conducted on transgenic mice offered a slightly different view on this aspect. It was reported that in the presence of interferons (IFNs), autophagy could regulate HCV expression negatively.[90] Mice with liver specific expression of HCV proteins showed that the HCV core and NS¾A protein are degraded by autophagy.[110] It was also observed that IFN-beta but not IFN-alpha stimulates this degradation.[110] Recently, Pan *et al.*[111] reported that mycophenolic acid (MPA), which is a potent inhibitor of inosine monophosphate dehydrogenase (IMPDH), can inhibit HCV replication via inducing the expression of ISGs in HCV infected cells. This finding was further supported by Fang *et al.*[112] who also observed that MPA can inhibit HCV replication by autophagy dependent mechanism. In addition, HCV infection has been shown to down-regulate tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) expression. TRAF6 mediates toll-like receptor (TLR) signaling cascade and by autophagic degradation of TRAF6, HCV ensures to suppress the NF-κB and inflammatory signaling in infected hepatocytes.[113] HCV induced reprogramming of host cell antiviral signaling appeared to be a multilayered event. HCV also cripples innate immunity by cleaving MAVS via its NS¾a protease activity.[105,106]

While a direct correlation between autophagy and innate immunity has not been reported so far in HBV infections, HBV-induced mitophagy has been studied in greater details. As discussed above, HBV induces mitochondrial translocation of Parkin.[13] How Parkin regulates cellular events other than mitophagy is a potential area investigation. Similar to HCV, HBV and/or HBx expression can recruit Parkin to mitochondria during mitophagy. HBx, Parkin and MAVS form a ternary complex which results in inactivation of MAVS downstream signaling.[114] In addition, Parkin also recruits a cytoplasmic linear ubiquitin assembly complex (LUBAC) onto mitochondria.[114] Parkin, together with LUBAC which causes linear ubiquitination on MAVS signalosome, abrogate IFN synthesis.[114] Thus, it is clear that the key proteins of mitophagy pathway play critical and pivotal roles in modulating innate immunity.

4.2. Viral replication

Autophagy is a positive regulator of HCV-or HBV-associated liver disease pathogenesis.[90] Inhibition of autophagy reduces the HBV replication in hepatoma cells, which proves that the autophagy is a positive regulator of HBV infection.[13,28,48] It was observed that the depletion of PI3KC3 or ATG7 by pharmacological or genetic approaches results in reduction in HBV DNA while the HBV transcription, translation and pgRNA packaging is slightly affected.[28] ATG5 is essential for the formation of autophagosomes and liver-specific knock-out of ATG5 in HBV transgenic mice inhibited autophagy and resulted decreased HBV viral

load in the blood.[50] Similar to the observations made by Tian *et al*.[50] mice model also showed slight effects of autophagy inhibition on RNA transcription and HBV-translation. It is not fully clear how HBV DNA replication is supported by autophagy. However, Sir *et al*.[28] claimed that inhibiting autophagy, had little or no effect on HBV RNA synthesis. Moreover, a report also claimed that suppression of beclin-1 or ATG5 by RNA interference, does not affect HBV DNA replication, but disrupts the release of HBV.[48] Li *et al*.[48] observed that the SHBs co-localizes with autophagosomes and LC3 and concluded that HBV usurps autophagy for viral envelopment. Further investigation is required to address whether autophagy regulates HBV replication or envelopment. Notably, similar to the Li and Sir *et al*.[28,48] also observed co-localization of HBV core/ precore with the autophagosome. However there is a possibility that the association of these HBV proteins with the autophagosome might be the events related to the autophagic removal of the excess proteins and not the requirement for HBV envelopment as Li *et al*.[48] reported.

Dreux *et al*.[27] concluded that the early events (RNA translation) but not late events of HCV infection are supported by autophagy. They observed that the key molecules of autophagic machinery are required only for the newly coming HCV RNA to establish successful translation.[27] However, these autophagy molecules are not required if the HCV infection is already established.[27] This proposed effect of autophagy on HCV translation was challenged in other subsequent reports. Multiple reports had claimed that autophagy is actually required for efficient HCV particle production.[22,87,90] These reports claimed that autophagy is essential for HCV RNA replication and the evidence presented to support this claim were convincingly strong. Ultrastructural analysis by electron or confocal microscopy revealed that the nascent HCV genome is co-localized with the autophagosomes.[115] Moreover, cells harboring HCV subgenomic and expressing GFP-LC3 showed that the purified autophagosome could mediate HCV replication *in vitro*.[116]

4.3. Establishment of chronic infection

Kim *et al*.[14,15] showed that HCV disrupts mitochondrial dynamics, an event that helps infected cells to escape from apoptosis and innate immunity to sustain persistent viral infection. HBV infection also affects the events of mitochondrial dynamics and mitophagy similar to HCV.[13] Both HBV and HCV upregulate Drp1-mediated mitochondrial fission and fragmented mitochondria are degraded by Parkin dependent mitophagy.[13–15] In case of HCV, silencing of Drp1 and Parkin lead to the suppression of HCV secretion, reduced glycolysis and ATP generation, and increase in apoptosis in HCV infected cells.[14,15] In case of HBV infection, Parkin translocates to mitochondria and degrade mitofusin 2 protein.[13] Perturbation in HBV induced mitochondrial dynamics and mitophagy is detrimental of cell survival and if these events are blocked, HBV infected cells cannot survive.[13] These observation suggested that HBV induced mitochondrial dynamics and mitophagy promote cell survival and thereby viral persistence.[13–15] These reports also implicated the functional relevance of altered mitochondrial dynamics and mitophagy in the pathogenesis of chronic liver disease associated with HBV and HCV infection. In summary, mitochondria-mediated pathways regulating bulk autophagy and mitophagy aid in the establishment of chronic infection.

4.4. HBV or HCV related hepatocellular carcinoma

HBV and HCV infections cause chronic hepatitis. Severe liver complications, such as fibrosis, cirrhosis, and development of HCC are associated with HBV and HCV. Growing body of evidence suggests that severity of virus-associated HCC, is often determined by the autophagy.[117] Previously it has been hypothesized that the *beclin-1* is a haplo-insufficient tumor suppressor gene and mice with heterozygous disruption of beclin-1 showed increased frequency of spontaneous malignancies and accelerated development of HBV induced premalignant tumors.[118] Lan *et al*.[119] showed that in the tumors of HBx-transgenic mice reduction of autophagy is associated with HBV-associated HCC. They further concluded that autophagy downregulation is inversely correlated with *miR-224* expression in HBV-associated HCC patient specimens.[119] *In vitro* studies demonstrate that oncogenic role played by miR-224 is critical for the regulation of cell migration and tumorigenesis of hepatoma cells.[120] Moreover, in HCC patients, significant correlation was observed between expression of low *ATG5*, high-*miR-224*, with overall poor survival rate in HBV patients.[120] Many pharmaceutical inhibitors that block different steps of the autophagy are also the inhibitors of HCC growth. These inhibitors include bafilomycin A1, 3 MA, azithromycin, spautin-1, wortmannin, vorinostat, chloroquine (CQ), thapsigargin, hydroxychloroquine (HCQ), lucanthone, matrine, xanthohumol, monensin, and concanamycin A99. In addition, autophagy inducers such as amiodarone has also been proposed to be used as a potential therapy for various HCC with different etiology.[121] Thus, these studies suggest that both autophagy inducers and inhibitors, are potentially effective in anti-HCC therapy. The exact effect of each reagent requires careful evaluation before they can be used to treat HCC patients.

5. Conclusion and perspectives

HBV and HCV are among the major human pathogens associated with development of severe diseases including HCC. The molecular details of how these viruses lead to high rates of chronic infection are still elusive. However multiple reports strongly established the fact that HBV and HCV have evolved mechanisms to subvert the cellular autophagy to benefit their multiplication, and associated pathogenesis. In the past years, multiple reports have demonstrated that HBV and HCV directly or indirectly utilize the autophagic machinery. Recent advancements in the field of selective autophagy and the in-depth characterization of the key proteins such as Parkin, PINK1 and LUBAC have provided a solid platform to further investigate and identify new therapeutic targets. For instance, inhibition of PINK1/Parkin mediated mitophagy in HBV or HCV infected cells could induces apoptosis.[13–15] Similarly, the involvement of multifunctional protein, LUBAC has been shown to modulate the antiviral and inflammatory signaling in HBV-and HCV-infected cells.[114,122] It is now established that the LUBAC, Parkin and mitophagy are tightly related with each other and it is possible that by synthesizing M-1 ubiquitin chains, LUBAC helps these viruses to hijack the cellular machinery to ensure cell survival and reduced IFN synthesis.[114,122] Such observations suggest that M-1 ubiquitin specific proteases (USPs) or de-ubiquitinases (DUBs) are promising tools that should be investigated further. It is of interest that a few DUBs such as USP-15, are specific to cleave M-1 ubiquitin chains and USP30 is the only mitochondria localized DUB, which has a potential to modulate mitophagy. Therefore, the

abrogation of mitophagy or LUBAC signaling by pharmacological inhibition or genetic ablation of USPs/DUBs, could be the potential means for elimination of HBV-or HCV-infected hepatocytes. Role of autophagy/mitophagy in HBV/HCV-associated liver disease pathogenesis, and its potential association with USPs and DUBs open fresh avenues for the development of novel therapeutic targets.

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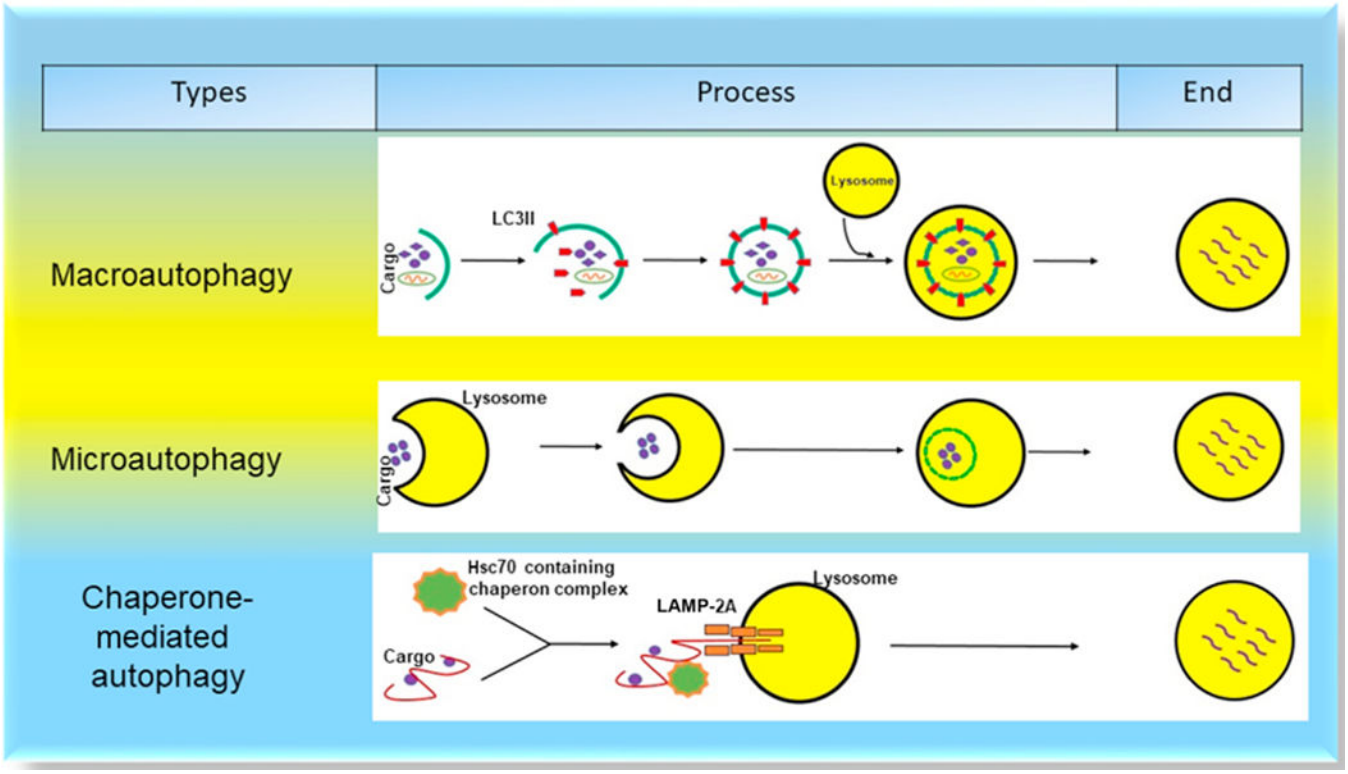

Fig. 1. Schematic for different types of autophagy.

Macroautophagy: a small portion of the cytoplasm and cellular organelles are isolated by a membrane (called phagophore) to form an autophagosome. Then lysosomes fuse with the outer membrane of autophagosome to degrade the internal material in autolysosome structures. Microautophagy: the lysosomal or late endosomal membrane directly engulfs the small cargo of cytoplasm by inward invagination. Chaperone-mediated autophagy: KFERQ-like motif-containing targeted proteins are recognized by cellular chaperone HSC70 and then they are translocated into lysosome after binding with LAMP-2A. Abbreviations: HSC70, heat shock cognate protein 70; LAMP-2A, lysosome-associated membrane protein type 2A.

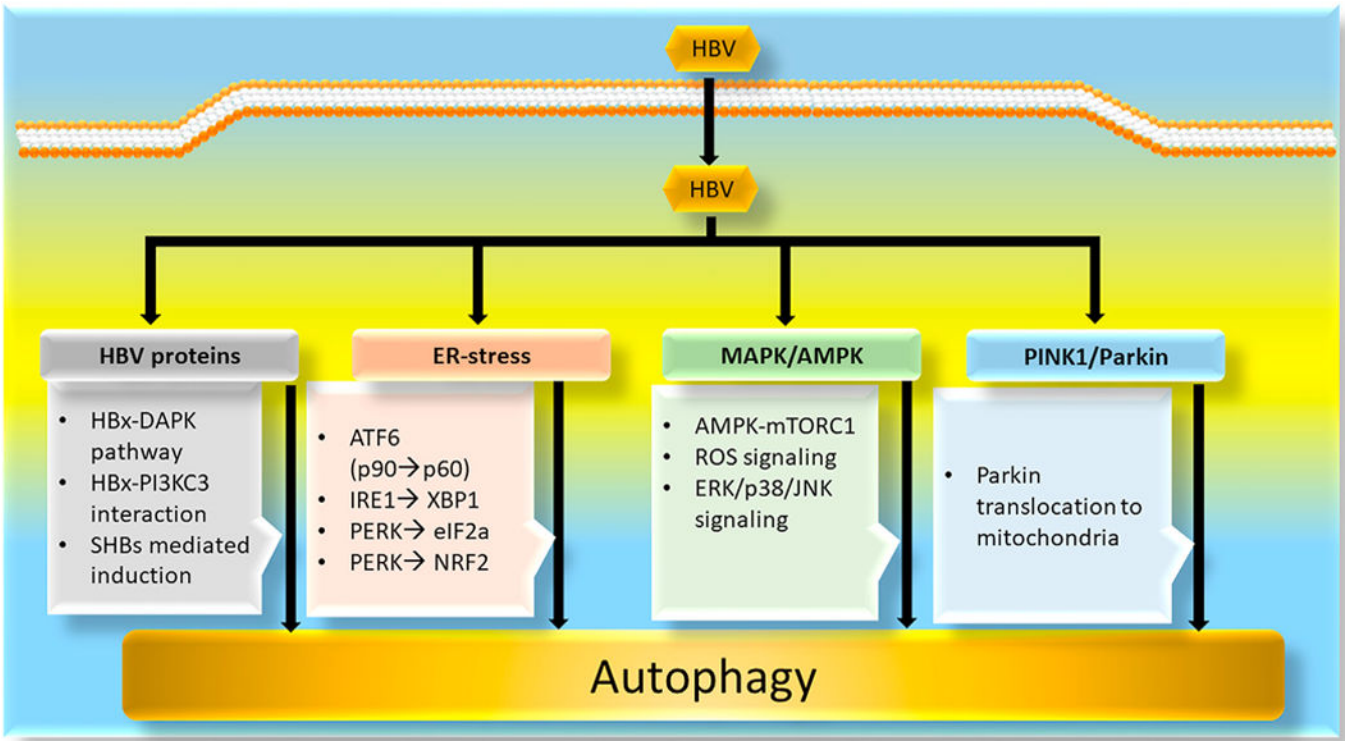

Fig. 2. Mechanisms of HBV-mediated autophagy.

HBV proteins: the HBx protein induced autophagy by upregulation of beclin-1 and activation of DAPK and PI3KC3 enzyme activity. The HBV surface antigen protein (HBs) also mediates autophagy induction. ER stress: expression of HBs can induce ER stress mediated autophagy by switching on UPR signaling via PERK, IRE1 and ATF6. MAPK/AMPK: three pathways of MAPK (ERK, p38 MAPK and JNK) signaling, ROS signaling and AMPK are involved in HBV mediated autophagy. PINK/Parkin: PINK1 and Parkin proteins are associated with HBV induced mitophagy. Abbreviations: HBV, hepatitis B virus; ER, endoplasmic reticulum; DAPK, death associated protein kinase; PI3KC3, phosphatidylinositol 3-kinase catalytic subunit type 3; UPR, unfolded protein response; PERK, protein kinase RNA-like ER kinase; IRE1, inositol-requiring protein-1α; ATF6, activating transcription factor 6; MAPK, mitogen activated protein kinase; AMPK, AMP-activated protein kinase; ERK, extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase; ROS, reactive oxygen species; PINK, phosphatase and tensin homolog deleted on chromosome ten (PTEN)-induced putative kinase 1;SHBs, small hepatitis B surface antigen; XBP1, X-box binding protein 1; NRF2, NF-E2 related factor 2; mTORC1, mammalian target of rapamycin complex-1.

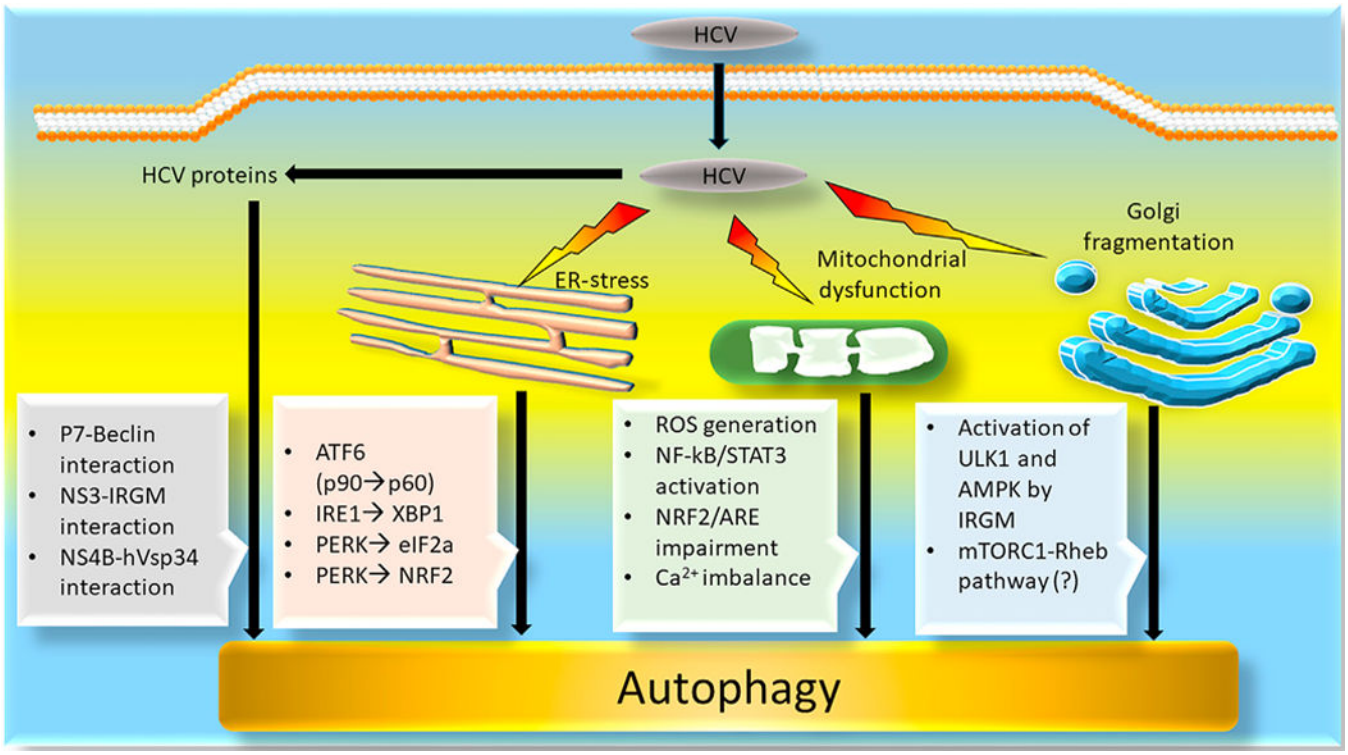

Fig. 3. Mechanism of HCV-mediated autophagy.

HCV proteins: direct interaction of p7 protein with beclin-1; NS3 protein with IRGM; and NS4B protein with beclin-1, hVps34 and Rab5 can subvert autophagy. ER-centric mechanism: HCV can induced ER stress mediated autophagy by turning on the three arms of UPR signaling *i.e.* PERK, IRE1 and ATF6. Mitochondria-centric mechanisms: HCV can induce mitochondrial dysfunction mediated autophagy by increasing ROS, NRF2/ARE impairment, NF- κB/STAT3 activation and mitochondrial Ca^{2+} imbalance. Golgi-centric mechanisms: HCV can induce autophagy through Golgi fragmentation by the activation of ULK1 and AMPK by IRGM. Abbreviations: HCV, hepatitis C virus; ER, endoplasmic reticulum; NF-κB, nuclear factor-kappa B; IRGM, immunity related GTPase M; UPR, unfolded protein response; PERK, protein kinase RNA-like ER kinase; IRE, inositol-requiring protein-1α; ATF6, activating transcription factor 6; ROS, reactive oxygen species; NRF2, NF-E2 related factor 2; ARE, antioxidant response element; ULK1, Unc-51 like autophagy activating kinase 1; AMPK, AMP-activated protein kinase; XBP1, X-box binding protein 1; mTORC1, mammalian target of rapamycin complex-1.

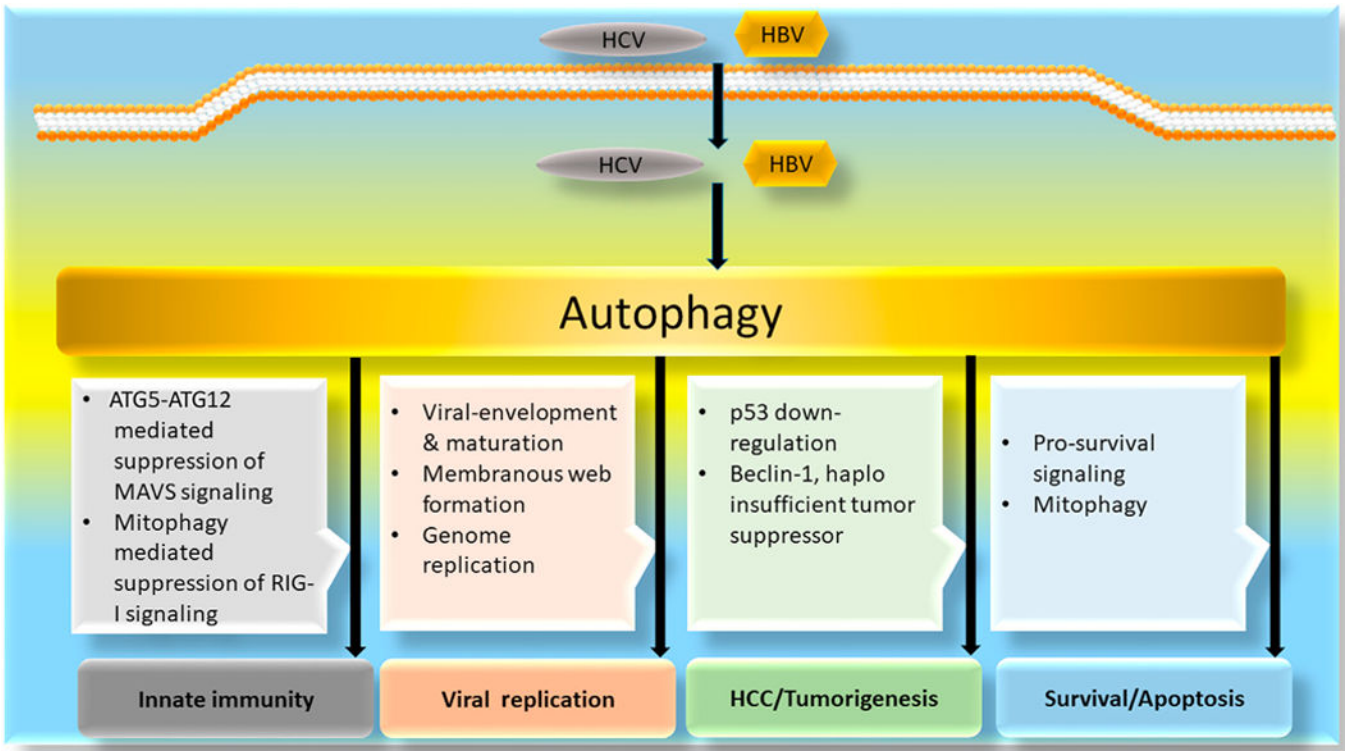

Fig. 4. Roles of autophagy in HBV/HCV pathogenesis.

Innate immunity: HCV infection mediated autophagy interrupts innate immune IFN signaling through ATG5-ATG12 mediated suppression of MAVS signaling and mitophagy-mediated suppression of RIG-I signaling. Viral replication: autophagy can act as a positive regulator for HCV or HBV replication through viral envelopment and maturation, membranous web formation and genome replication. HCC/Tumorigenesis: autophagy is involved in the HBV-associated HCC through p53 downregulation and heterozygous disruption of *beclin-1* gene. Survival/Apoptosis: HCV induced autophagy is associated with apoptosis through various pro-survival signaling and mitophagy. Abbreviations: HCV, hepatitis C virus; HBV, hepatitis B virus; IFN, interferon; MAVS, mitochondria antiviral signaling protein; ATG, autophagy-related genes; HCC, hepatocellular carcinoma.