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Hepatitis C Virus and Autophagy

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Abstract

Autophagy is a catabolic process by which cells remove protein aggregates and damaged organelles for recycling. It can also be used by cells to remove intracellular microbial pathogens including viruses in a process known as xenophagy. However, many viruses have developed mechanisms to subvert this intracellular antiviral response and even use this pathway to support their own replications. Hepatitis C virus (HCV) is one such virus. HCV is an important human pathogen that can cause severe liver diseases. Recent studies indicated that HCV could activate the autophagic pathway to support its replication. This review summarizes the current knowledge on the interplay between HCV and autophagy and how this interplay affects HCV replication and host innate immune responses.

Keywords

autolysosomes; autophagosomes; autophagy; hepatitis C virus; innate immunity; unfolded protein response

Introduction

Autophagy is a catabolic process that is important for maintaining cellular homeostasis. It removes long-lived proteins and damaged organelles in cells and can also be induced during nutrient starvation. This process begins by the formation of membrane crescents termed phagophores or isolation membranes in the cytosol (Figure 1). The edges of these phagophores will subsequently extend to sequester part of the cytoplasm, leading to the formation of enclosed double-membrane vesicles, known as autophagosomes. Autophagosomes mature by fusing with lysosomes to form autolysosomes, in which the cargos of autophagosomes are digested by lysosomal enzymes for recycling (Levine and Kroemer, 2008).

Many protein factors that are important for autophagy have been identified. Class III phosphatidylinositol-3-kinase (PI3KC3) is one of these factors. It catalyzes the formation of phosphatidylinositol-3-phosphate (PI3P) and is important for the initiation of autophagy (Simonsen and Tooze, 2009) (Figure 1). PI3KC3 consists of three core components, hVps34, p150 and Beclin-1 (Li et al., 2005; Simonsen and Tooze, 2009). Atg5 and Atg12,

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which are covalent linked, and their association with Atg16 are important for the formation of phagophores. Another factor that is important for autophagy is the microtubule-associated protein light-chain 3 (LC3). LC3 is a cytosolic protein. During autophagy, it is covalently linked by Atg3, Atg4 and Atg7 to phosphatidylethanolamine (PE), a phospholipid. This lipidation allows LC3 to localize to autophagosomal membranes. LC3 is de-lipidated by Atg4 after the maturation of autophagosomes and released back into the cytosol. It can also be degraded by lysosomal enzymes, if it is localized to the inner membrane of autophagosomes (Levine and Kroemer, 2008). The lipidation of LC3 and its localization to autophagosomes are often used as markers for autophagy.

Autophagy can be used by cells to remove intracellular microbial pathogens in a process known as xenophagy (Gomes and Dikic, 2014). However, many microbial pathogens including viruses have developed mechanisms to subvert this intracellular anti-microbial pathway and even use it to support their own replications. In recent years, many reports have been published to show that hepatitis C virus (HCV) could induce autophagy to support its own replication. In this review, we will summarize and discuss these recent findings.

Hepatitis C virus

Hepatitis C virus (HCV) is a human pathogen that causes severe liver diseases including liver cirrhosis and hepatocellular carcinoma (HCC). Globally, there are 170 million people that are chronically infected by this virus. Many of the HCV carriers will require liver transplantation for survival within two decades of infection (Shepard et al., 2005).

HCV belongs to the *Flaviviridae* family. It is an enveloped virus with a positive-stranded RNA genome of about 9.6 *Kb* in size. Based on the nucleotide sequence of its genome, HCV has been grouped into six major genotypes and many more subtypes. The HCV genome encodes a polyprotein, which is translated by a cap-independent manner via an internal ribosomal entry site (IRES) located near its 5’-end (Moradpour et al., 2007). The HCV polyprotein is cleaved by cellular and viral proteases to generate ten mature viral gene products, arranged in the order of Core-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B (Tellinghuisen et al., 2007), with structural proteins located at the N-terminus and nonstructural proteins that are required for viral RNA replication located at the C-terminus (Bartenschlager and Lohmann, 2000).

HCV and the autophagic response

HCV has been to shown to induce the autophagic response by many different laboratories. It could induce the lipidation of LC3 and the accumulation of autophagic vacuoles in immortalized primary human hepatocytes, Huh7 hepatoma cells and the derivatives of Huh7 cells, and this induction is independent of HCV genotypes (Ait-Goughoulte et al., 2008; Dreux et al., 2009; Sir et al., 2008; Tanida et al., 2009). The induction of autophagic vacuoles was observed in cells either transfected by the HCV genomic RNA or infected by HCV, and in cells harboring the replicating HCV subgenomic RNA replicon (Ke and Chen, 2011; Mizui et al., 2010; Shrivastava et al., 2012; Sir et al., 2012; Taguwa et al., 2011; Wang et al., 2015). It is also observed in the hepatocytes of patients chronically infected by HCV (Rautou et al., 2011; Vescovo et al., 2012).

Sir et al. reported that HCV JFH1 (genotype 2a) induced the accumulation of autophagosomes in Huh7 hepatoma cells. They also observed that the fusion between autophagosomes and lysosomes was inefficient, raising the question regarding whether HCV was able to induce a complete autophagic response (Sir et al., 2008). However, it was subsequently shown that HCV could efficiently induce the fusion between autophagosomes and lysosomes and enhance the autophagic flux (Huang et al., 2013; Ke and Chen, 2011). Our recent results offered an explanation to why HCV induced incomplete autophagy in some studies but complete autophagy in others. We found that the maturation of autophagosomes in HCV-infected cells was temporally regulated (Wang et al., 2015). The maturation of autophagosomes was inefficient in the early stage of HCV infection whereas it was efficient in the late stage (Wang et al., 2015). This temporal regulation was also observed by Huang et al. (Huang et al., 2013), and was due to the differential induction of Rubicon and UVRAG by HCV (Wang et al., 2015), which negatively and positively regulate the maturation of autophagosomes, respectively (Liang et al., 2008; Matsunaga et al., 2009; Sun et al., 2010). The induction of Rubicon by HCV preceded the induction of UVRAG, which led to the initial inhibition of the fusion between autophagosomes and lysosomes and the accumulation of the former (Figure 2). This inhibition was overcome in the later stage of infection when the fold induction of UVRAG by HCV exceeded that of Rubicon (Wang et al., 2015). It is noteworthy that different HCV genotypes had also been shown to have different effects on the maturation of autophagosomes (Taguwa et al., 2011).

Autophagy can remove damaged organelles including mitochondria. The selective removal of mitochondria by autophagy is termed mitophagy (Youle and Narendra, 2011). HCV had also been shown to induce mitophagy. Siddiqui and colleagues reported that HCV could induce the expression of PINK1 and Parkin and cause the perinuclear clustering of mitochondria and the translocation of Parkin to mitochondria (Kim et al., 2013). PINK1 is a serine/threonine kinase. Its localization to the outer mitochondrial membrane will recruit its substrate Parkin, an E3 ubiquitin ligase, to mitochondria to initiate mitophagy. They subsequently demonstrated that mitophagy induced by HCV could promote mitochondrial fission and attenuate HCV-induced apoptosis (Kim et al., 2014). Mitophagy apparently plays a very important role in HCV replication and persistence, as its inhibition suppressed HCV replication and enhanced cellular apoptosis (Kim et al., 2014; Kim et al., 2013).

The unfolded protein response and HCV-induced autophagy

The accumulation of unfolded or misfolded protein in the endoplasmic reticulum (ER) will induce ER stress, which will lead to the activation of the activating transcription factor 6 (ATF6), the inositol-requiring enzyme 1 (IRE1), and the double-stranded RNA-activated protein kinase-like ER kinase (PERK) to trigger downstream signaling events collectively known as the unfolded protein response (UPR) (Hetz, 2012). The UPR will alleviate ER stress by attenuating protein synthesis, inducing the expression of ER chaperon proteins to facilitate protein folding, and enhancing protein degradation via the ER-associated degradation (ERAD) pathway and autophagy. If the UPR fails to alleviate ER stress, it will induce apoptosis (Wang and Kaufman, 2014). HCV infection can induce ER stress and activate the UPR (Joyce et al., 2009; Tardif et al., 2005), and this induction of UPR has also been shown to be important for the induction of autophagy by HCV (Ke and Chen, 2011;

Shinohara et al., 2013; Sir et al., 2008; Wang et al., 2014), as the silencing of ATF6, IRE1 or PERK with RNAi or the alleviation of the ER stress using phenyl butyric acid, a chemical chaperon, diminished the ability of HCV to induce autophagy (Ke and Chen, 2011; Sir et al., 2008; Wang et al., 2014). In a separate report, whether the UPR is important for HCV to induce autophagy was challenged. This challenge was made based on the observations that first, the accumulation of lipidated LC3 in HCV-infected cells preceded the detection of the UPR; second, the HCV subgenomic RNA replicon, which did not express viral envelope proteins and induce UPR, could nevertheless induce autophagy; and third, the silencing of IRE1 did not abolish autophagy induced by HCV (Mohl et al., 2012). The findings that the HCV subgenomic RNA replicon could not induce the UPR and the silencing of IRE1 could not abolish autophagy were not consistent with the previous reports (Sir et al., 2008; Tardif et al., 2002). The reason for this discrepancy is unclear and further studies will be required to resolve this discrepancy. The observation that the level of lipidated LC3 preceded that of the UPR may be explained by the low level of activation of UPR, which could not be detected but nevertheless was sufficient to induce the LC3 lipidation, or alternatively due to the induction of Rubicon by HCV in the early time points of infection, which could suppress the maturation of autophagosomes to lead to the accumulation lipidated LC3 and autophagosomes in HCV-infected cells (Wang et al., 2015) (Figure 2).

Role of HCV proteins in the induction of autophagy

Both HCV structural and non-structural proteins have been reported to regulate the autophagic pathway. It has been demonstrated that the HCV core protein could induce the ER stress and activate PERK and ATF6 signaling pathways, but curiously not the IRE1 pathway, of the UPR (Wang et al., 2014) (Figure 3). The activation of PERK induced the expression of the transcription factor ATF4 and its downstream effector CHOP, which then upregulated the expression of LC3B and ATG12 to stimulate autophagy (Wang et al., 2014). The HCV p7 ion channel protein had also been found to bind to Beclin-1, a core component of the PI3KC3 complex. However, the biological significance of this finding is unclear, as the over-expression of p7 did not affect autophagy (Aweya et al., 2013). The expression of HCV nonstructural polyprotein NS3-NS5B had also been shown to be sufficient to induce double-membrane vesicles that resembled autophagosomes (Chatterji et al., 2015). The HCV NS3/4A could bind to mitochondria-associated immunity-associated GTPase family M (IRGM) (Gregoire et al., 2011) (Figure 3), which is a member of the interferon-inducible GTPase family and can interact with multiple autophagy-associated proteins including ATG5 and ATG10 to regulate autophagy. The inhibition of IRGM expression prevented the lipidation of LC3 and the induction of autophagosomes by HCV (Gregoire et al., 2011). Although IRGM is clearly important for the induction of autophagy by HCV, the possible role of its interaction with HCV NS3/4A in this induction is less unclear. The HCV NS4B protein had also been shown to induce the lipidation of LC3 and the accumulation of autophagosomes (Su et al., 2011; Wang et al., 2015). NS4B could induce the expression of Rubicon, which, as mentioned above, inhibits the maturation of autophagosomes (Wang et al., 2015). It could also form a complex with Rab5, hVps34 and Beclin-1 (Stone et al., 2007; Su et al., 2011) (Figure 3). Rab5 is a small GTPase that regulates membrane trafficking, and hVps34 is the catalytic subunit of the PI3KC3 complex. The suppression of Rab5 or hVps34 both impaired the ability of NS4B to induce autophagosome (Su et al., 2011). HCV NS4B

by itself was sufficient to activate the UPR and may also induce autophagy via this pathway (Li et al., 2009; Wang et al., 2015; Zheng et al., 2005). HCV NS5A had also been reported to upregulate the expression of Beclin-1 via NS5ATP9, a multifunctional protein involved in DNA damage response, cellular signaling, cell cycle control and cell death, and could induce autophagy (Chatterji et al., 2015; Quan et al., 2014; Shrivastava et al., 2012) (Figure 3). HCV NS5B had also been found to bind ATG5 in yeast two-hybrid and co-immunoprecipitation studies and colocalize with ATG5 in the early time points after HCV infection but not in the later time points (Guevin et al., 2010). ATG5 also colocalized with HCV NS4B in HCV-infected cells. The silencing of ATG5 inhibited HCV RNA replication, supporting a role of autophagy and/or the interaction between ATG5 and NS5B in HCV RNA replication (Guevin et al., 2010). It remains to be determined, however, regarding whether the interaction between ATG5 and NS5B plays any role in the progression of autophagy in HCV-infected cells and whether ATG5 positions HCV RNA replication complexes to autophagosomes.

Autophagy and HCV replication

Many laboratories have demonstrated that the suppression of autophagy would suppress HCV replication, indicating a positive role of autophagy in HCV replication (Ait-Goughoulte et al., 2008; Dreux et al., 2009; Gregoire et al., 2011; Guevin et al., 2010; Ke and Chen, 2011; Mohl et al., 2012; Shrivastava et al., 2012; Sir et al., 2008; Sir et al., 2012; Su et al., 2011; Taguwa et al., 2011). These studies involved the analysis of cells infected by HCV, cells transfected by the HCV genomic RNA or subgenomic RNA replicon, or stable cells containing HCV subgenomic RNA replicon. While there is a consensus that autophagy is important for HCV replication, there are controversies regarding how autophagy may help HCV replication. Dreux et al. reported that autophagy was important for the translation of HCV RNA upon infection but not important for the later stage of HCV infection (Dreux et al., 2009). The possible effect of autophagy on HCV RNA translation has been disputed (Ke and Chen, 2011). In addition, a separate study suggested that autophagy was required for the efficient production of HCV particles (Tanida et al., 2009). Several groups, however, reported that autophagy played important roles in HCV RNA replication (Ferraris et al., 2010; Guevin et al., 2010; Ke and Chen, 2011; Sir et al., 2008; Sir et al., 2012). By conducting confocal microscopy and immunogold analysis, Sir et al. demonstrated that nascent HCV RNA labeled with bromouridine triphosphate colocalized with autophagosomes (Sir et al., 2012). They further established stable HCV subgenomic RNA replicon cells that expressed the GFP-LC3 fusion protein and showed that autophagosomes purified with the anti-GFP antibody from these cells could mediate HCV RNA replication *in vitro* (Sir et al., 2012). Ferraris et al. used lipidated LC3 as the marker to isolate membrane vesicles from a sucrose gradient (Ferraris et al., 2010). By conducting electron microscopy and immunogold staining, they demonstrated the presence of HCV double-stranded RNA (i.e., replicative intermediates) on double-membrane vesicles that resembled autophagosomes. Their results were also consistent with the replication of HCV RNA on autophagosomal membranes (Ferraris et al., 2010). As mentioned above, Guevin et al. also found the colocalization of HCV NS4B and NS5B with ATG5 and that ATG5 was important for HCV RNA replication. All these results support the notion that HCV RNA replication takes place on autophagosomal membranes. Note that HCV RNA replication

may require only a small fraction of HCV nonstructural proteins produced by the virus (Romero-Brey et al., 2012). This could explain why HCV nonstructural proteins were not always found associated with autophagosomes (Mohl et al., 2012).

By conducting immunofluorescence staining and electron microscopy, Bartenschlager and colleagues studied the membranous web induced by HCV and found both single-membrane and double-membrane vesicles (DMVs) in HCV-infected cells (Romero-Brey et al., 2012). The DMVs were originated from the ER and had an average diameter of ~150 nm, which is smaller than that of autophagosomes (~500 nm). These DMVs could be induced by HCV NS5A alone and were largely devoid of LC3, the autophagosomal marker. The subsequent studies of Bartenschlager and colleagues indicated the association of HCV RNA replication complex with these DMVs (Paul et al., 2013). Whether DMVs and autophagosomes are related to each other and whether one is the derivative of the other require further studies. However, it is clear that both autophagosomes and DMVs could support HCV RNA replication.

As HCV induces the accumulation of autophagosomes in the early time points after infection and these autophagosomes mature efficiently in the later time points (Wang et al., 2015), it is possible that autophagosomes as well as autophagy-associated proteins may only be involved in HCV RNA replication in the early time points. This possibility will also be consistent with the previous observation that the colocalization of HCV NS5B and ATG5 occurred only in the early time points, but not in the later time points of HCV infection (Guevin et al., 2010). Note that in stable cell lines containing HCV subgenomic RNA replicon, autophagosomes mature inefficiently and could support HCV RNA replication in these cells (Sir et al., 2012; Wang et al., 2015).

Autophagy and interferon response in HCV-infected cells

The autophagy induced by HCV had also been shown to suppress the interferon (IFN) innate immune response. Ke and Chen showed that the knockdown of ATG5 or the treatment of HCV-infected cells with chloroquine, which inhibits the acidification of lysosomes, could induce the expression of type I IFNs and activate the IFN signaling pathway via RIG-I, a cytosolic pattern recognition receptor that can be activated by the UC-rich sequence located at the 3’-untranslated region of the HCV genomic RNA (Ke and Chen, 2011). Their finding was reminiscent of the previous report by Jounai et al. who found that the ATG5-ATG12 conjugate, which is essential for autophagy, could directly interact with RIG-I and the mitochondrial antiviral signaling protein (MAVS), a downstream effector of RIG-I, through the caspase recruitment domains (CARDs) to inhibit signal transduction and the induction of type I IFNs (Jounai et al., 2007) (Figure 4). The inhibition of the RIG-I signaling pathway by autophagy likely is only important in the early stage of HCV infection, as the HCV NS3/4A protease can cleave MAVS to disrupt the RIG-I signaling pathway and prevent the induction of interferons in the later stage of infection when the protease level is high (Foy et al., 2005; Li et al., 2011; Li et al., 2005). Shrivastava et al. also found that the knockdown of Beclin-1 or ATG7 in HCV-infected cells could induce the expression of type I IFNs and IFN-stimulated genes (ISGs) (Shrivastava et al., 2011). In contrast, the studies by Desai et al. offered a different view regarding the relationship among HCV, autophagy and innate

immunity. By studying transgenic mice that expressed HCV proteins in the liver, they found that IFN-β, but not IFN-α, could stimulate the autophagic degradation of HCV core and NS3/4A proteins (Desai et al., 2011). Their results indicated that autophagy could also negatively regulate HCV replication in the presence of IFN-β.

Conclusion and perspectives

The studies in the past few years provided strong evidence that HCV could induce autophagy to support its replication. The mechanism of this induction of autophagy by HCV likely involves many factors that include the combined effects of multiple HCV gene products and the activation of UPR. HCV can clearly use the autophagic pathway to enhance its replication, as the inhibition of autophagy leads to the suppression of HCV replication. HCV may use autophagic vacuoles to support its replication and the autophagic pathway to suppress the IFN response. However, interferon-β can also induce the autophagic degradation of HCV NS3/4A and core proteins and turn autophagy against HCV. Although the relationship between HCV and autophagy is becoming increasingly clear, many questions remain to be answered. For examples, it is unclear how IRGM participates in the induction of autophagy in HCV-infected cells and how HCV NS4B induces the expression of Rubicon to suppress the maturation of autophagosomes. It is also unclear how autophagy attenuates RIG-I signaling to suppress the IFN response. Further work will also be necessary to identify the membrane sources of autophagosomes in HCV-infected cells and the relationship, if any, between autophagosomes and DMVs.

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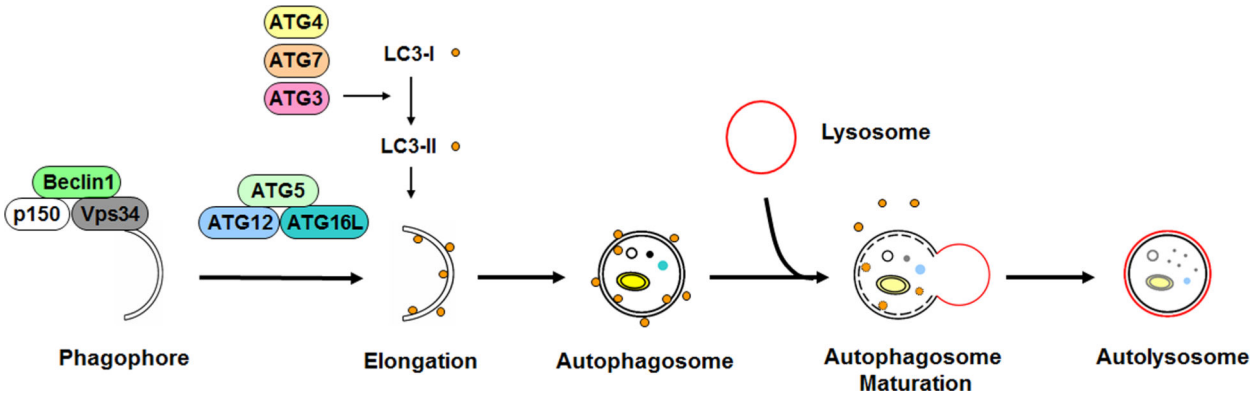

Figure 1. Illustration of the autophagic pathway

Beclin-1, p150 and hVps34, which are the three core components of PI3KC3, is important for the initiation of autophagy. ATG5, ATG12 and ATG16L, which form a complex, are important for the elongation of phagophores and the formation of autophagosomes. LC3-I and LC3-II are the non-lipidated and lipidated LC3, respectively. The lipidation of LC3, which enables LC3 to localize to autophagosomes, is mediated by ATG4, ATG7 and ATG3. Autophagosomes mature by fusing with lysosomes to form autolysosomes, in which the cargos of autophagosomes are digested for recycling.

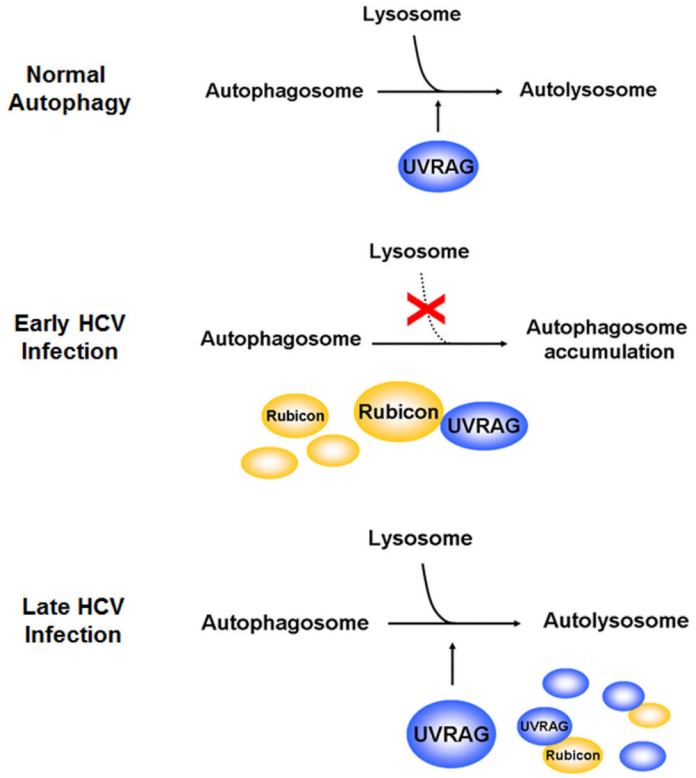

Figure 2. Roles of Rubicon and UVRAG in the maturation of autophagosomes in HCV-infected cells

In the normal autophagic pathway, UVRAG facilitates the fusion between autophagosomes and lysosomes to form autolysosomes. The induction of Rubicon by HCV in the early stage of infection inhibits the UVRAG activity and the fusion between autophagosomes and lysosomes. This leads to the accumulation of autophagosomes. The induction of UVRAG in the late stage of HCV infection overcomes the inhibitory effect of Rubicon and results in the maturation of autophagosomes.

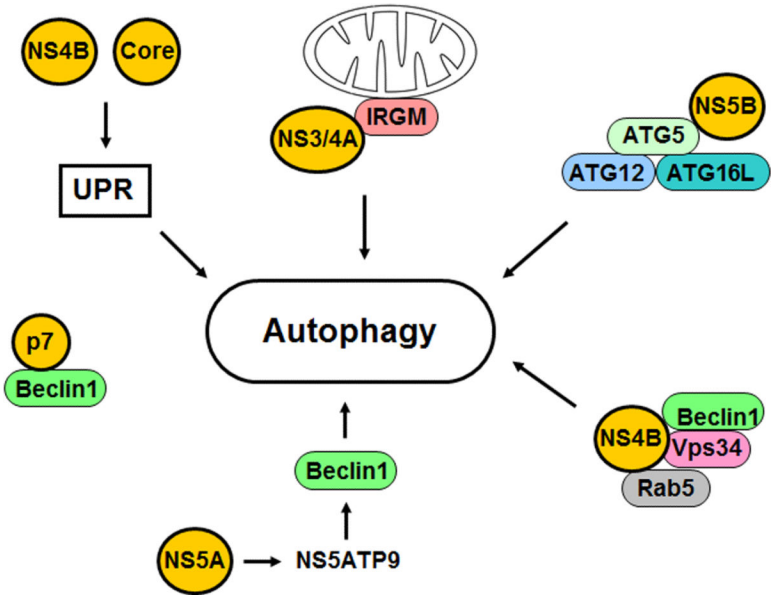

Figure 3. HCV proteins and the autophagic pathway

Both HCV core and NS4B proteins could induce the ER stress and activate the UPR, which has been known to induce autophagy. The HCV p7 protein could bind to Beclin-1, although it is unclear whether this binding plays any role in HCV-induced autophagy. The HCV NS3/4A protease could bind to mitochondria-associated IRGM, which plays an important role in HCV-induced autophagy. HCV NS4B could form a complex with Rab5, hVps34 and Beclin-1, which are important regulatory factors for autophagy. HCV NS5A could upregulate the expression of Beclin-1 via NS5ATP9. HCV NS5B could bind to ATG5, which, as illustrated in Figure 1, is conjugated to ATG12 and associated with ATG16L and important for the elongation of phagophores and the formation of autophagosomes.

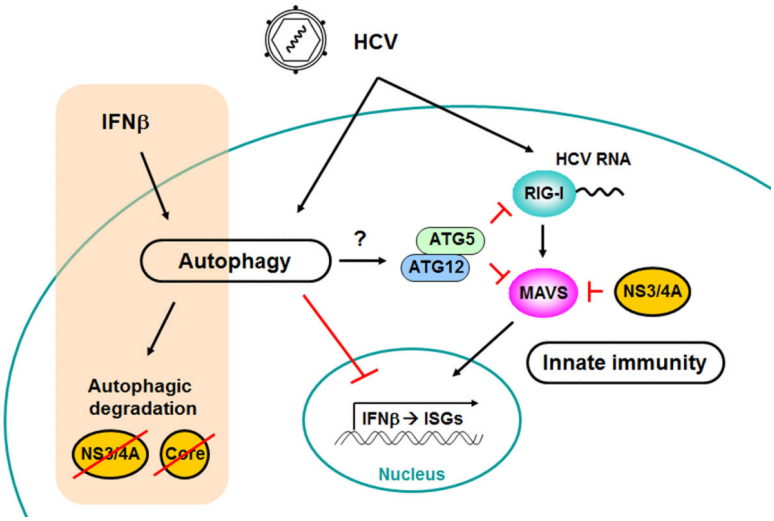

Figure 4. Autophagy and the IFN response to HCV infection

HCV viral RNA is recognized by RIG-I, which activates MAVS to induce the expression of type I IFNs. The HCV NS3/4A protease cleaves MAVS to disrupt the RIG-I signaling pathway and blocks the induction of IFN-β and ISGs. The ATG5-ATG12 conjugate can bind to RIG-I and MAVS to inhibit their activities. HCV-induced autophagy also inhibits the RIG-I signaling pathway, although it is unclear whether this inhibition is mediated by the ATG5-ATG12 conjugate. In the presence of IFN-β, autophagy can mediate the degradation of HCV core and NS3/4A proteins.