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L'IMPLICATION DE L'AUTOPHAGIE DANS L'INFECTION PAR LE VIRUS DE L'HÉPATITE C

Par

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«Look deep into nature, and then you will understand everything better»

Albert_Einstein

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RÉSUMÉ EN FRANÇAIS

L'infection par le virus de l'hépatite C (VHC) est un problème majeur de santé publique avec plus de deux cent millions d'individus chroniquement infectés dans le monde. Malgré le développement d'antiviraux très puissants à action directe (AAD) pour le traitement de l'infection chronique par le VHC, il reste encore de nombreux cas de personnes infectées. Ce nombre devrait augmenter dans les prochaines années en raison du coût élevé de ces médicaments antiviraux qui en limite l'accès et le grand nombre d'infections occultes. Bien que de nombreuses avancées majeures aient été réalisées dans la compréhension de la pathogénèse et de la réplication virale, les liens étroits entre le VHC et la cellule hôte sont encore mal compris.

L'autophagie, un mécanisme cellulaire de la cellule hôte, s'est révélée être modulée par le VHC. L'autophagie est un mécanisme de dégradation cellulaire qui vise à recycler les protéines ainsi que les organites qui ne sont plus nécessaires mais également à protéger la cellule d'éventuels agents pathogènes. Nous avons montré que le VHC exploite l'autophagie pour se répliquer. Le mécanisme par lequel le VHC détourne à son avantage l'autophagie et/ou les composantes autophagiques est encore débattu. Dans une étude récente au sein de notre laboratoire, nous avons montré que l'ARN-polymérase dépendante de l'ARN du VHC (NS5B) interagit avec la protéine autophagique ATG5. De plus, dans des cellules arborant un réplicon du VHC, la protéine ATG5 colocalise avec un marqueur des usines de réplication membranaire du VHC, la protéine NS4B. Finalement, le « knock-down » d'ATG5 a permis de diminuer l'ARN du virus ainsi que les niveaux d'expressions des protéines virales.

Puisque ATG5 est une composante du complexe d'élongation de l'autophagie (ATG5-12/16L1), l'objectif principal de ce projet de recherche est d'approfondir le rôle de ATG5-12/16L1 dans le cycle de réplication du VHC.

Dans le premier article, nous avons évalué la localisation du complexe ATG5-12/16L1 dans une infection chronique *in vitro*. En effet, nous avons montré que les composants du complexe ATG5-12/16L1 colocalisent avec les composantes de la réplicase du VHC (NS3, NS4B, NS5A et NS5B).

De plus, en utilisant la méthode de ligation de proximité « Proximity Ligation Assay» (PLA), nous avons montré, *in situ,* que ATG5-12 interagit avec les composantes de la réplicase du VHC. Bien que ATG16L1 colocalise avec la réplicase de VHC, aucune interaction entre ATG16L1 et la réplicase du HCV n'a été observée.

Ces résultats suggèrent que l'interaction de ATG5-12/16L1 avec la réplicase virale se produit via ATG5-12 et non pas avec ATG16L1.

Contrairement à nos attentes, la protéine LC3, un marqueur autophagique, n'a pas été recrutée avec le complexe d'élongation au site de réplication du VHC et aucune colocalisation des protéines virales avec sa forme active (LC3II) n'a été observée.

En utilisant l'expression de dominants négatifs des protéines ATG, nous avons démontré que le conjugué ATG5-12 est nécessaire pour la réplication virale du VHC. Par ailleurs, LC3II ne semble pas impliquée dans la réplication virale.

Dans le deuxième article, nous avons étendu nos travaux afin de déterminer à quelle étape du cycle de réplication du VHC, le complexe ATG5-12/16L1 est nécessaire.

En utilisant la méthode de «knock-down», nous avons montré que ATG5-12/16L1 est nécessaire pour la réplication de l'ARN viral du VHC. Cependant, ATG5-12/16L1 n'est pas nécessaire pour l'entrée du virus ou pour la formation de particules virales infectieuses. En contraste, LC3 semble requise pour la traduction du génome viral au début de l'infection.

En purifiant les usines de réplication du VHC, nous avons détecté les composants du complexe d'élongation par immuno-buvardage de type western. Par contre, tel qu'anticipé, la protéine LC3 n'a pas été détecté dans ces échantillons.

Nous avons également montré que la diminution de l'expression d'ATG12 ou ATG7 est associée à l'agrégation subcellulaire des protéines de la réplicase du VHC.

L'analyse en microscopie électronique a révélé une diminution drastique du nombre et de la taille des vésicules à double membranes qui sont des composantes majeures des usines de réplication membranaire du VHC. Finalement, nous avons démontré une disparition complète des vésicules multi-membranaires dans ces mêmes usines de réplication en contexte de « knock-down » d'ATG12.

En conclusion, l'ensemble de ces résultats ont mis en évidence un nouveau rôle pour le complexe ATG5-12/16L1 dans la réplication du génome et dans la formation des usines de réplication membranaire du VHC.

ENGLISH SUMMARY

Hepatitis C virus (HCV) infection is a major health problem that accounts for around 200 million chronic infections worldwide. Albeit the development and approval of highly potent direct acting antivirals (DAAs) for the treatment of HCV infection, the burden of HCV infection remains and is expected to increase in the coming years due to the high cost of these drugs, the limited accessibility to the treatment, and the large number of occult infections. Even though, major breakthroughs have been achieved in the understanding of viral pathogenesis and replication, the tight link between HCV and host cell is still poorly understood.

One host mechanism that has been shown to be modulated by HCV is autophagy. Autophagy is a cellular degradation mechanism that functions in the recycling of unwanted cellular proteins and organelles and to protect the cell from invading pathogens. Uniquely, HCV was shown to exploit autophagy for the purpose of viral replication. The mechanism by which HCV usurps autophagy and/or autophagic components is widely argued. In a previous report, our lab revealed that HCV RNA-dependent RNA polymerase (NS5B) interacts with the autophagy protein ATG5. Furthermore, ATG5 colocalized with the HCV NS4B, a viral nonstructural protein that is associated with the HCV-induced membranous web (MW), in HCV replicon cells. Silencing of ATG5 has been shown to attenuate HCV RNA and proteins level. Since ATG5 is one component of what is called the autophagy elongation complex (ATG5-12/16L1), the main objective of this research project was to further investigate the role of ATG5-12/16L1 in HCV replication cycle.

In the first article, we assessed the localization of ATG5-12/16L1 complex in chronically infected cells. Clearly, ATG5-12/16L1 components colocalized with HCV viral replicase (NS3, NS4B, NS5A, and NS5B). In addition, we showed that ATG5-12 interacts *in situ* with HCV replicase components by using proximity ligation assay (PLA). Although ATG16L1 colocalized with HCV replicase, no interaction between ATG16L1 and the HCV replicase was observed suggesting that the interaction of ATG5-12/16L1 with viral replicase occurs via ATG5-12 but not ATG16L1. Surprisingly, LC3 was not recruited along with the elongation complex to the site of HCV replication and no colocalization of LC3-II with HCV proteins was observed. By using dominant negative forms of ATG proteins, we

demonstrated that ATG5-12 conjugate, but not LC3-II formation, was vital for HCV replication.

In the second article, we extended our research to investigate at which stage of HCV replication cycle the ATG5-12/16L1 was required. By using siRNA approach, we showed that ATG5-12/16L1 was required for HCV RNA replication but not HCV entry or infectious virus particle formation. In contrast, LC3 was required only for the onset of HCV genome translation. By isolating HCV-induced membranous web, we were able to detect the elongation complex component by using western blotting whereas LC3 was undetectable in the isolated membranes. Interestingly, we revealed that silencing of ATG12 or ATG7, but not LC3, has led to subcellular aggregation of the HCV replicase proteins. Electron microscopy analysis has revealed a dramatic decrease in number and size of double membrane vesicles, a major component of HCV MW, and a complete disappearance of multimembrane vesicles from the MW composition upon ATG12 silencing.

In conclusion, the results represented in this thesis highlights a novel role of the ATG5-12/16L1 in HCV genome replication and the formation of HCV MW.

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LIST OF ABBREVIATIONS

aa: Amino acid

AH1: Alpha helix 1

AH2: Alpha helix 2

apo: Apolipoprotein

ATF6: Activating transcription factor 6

ATG: Autophagy-related gene

ATG12-DN2: ATG12 dominant negative

ATG4BDN2: ATG4B dominant negative

ATG5-DN2: ATG5 dominant negative

CDK: Cyclin-dependent kinase

CEs: Cholesteryl esters

CKII: casein kinase II

CLDN1: Claudin-1

cLDs: Cytoplasmic lipids droplets

CMA: Chaperon-mediated autophagy

coV: Coronaviruses

CypA: cyclophilin A

D1: Core N-terminal hydrophilic domain

D2: Core C-terminal hydrophobic domain

DAAs: Direct Acting Antivirals

DENV: Dengue virus

DFCP1: Double-FYVE-containing protein 1

DGAT1: Diacylglycerol acyltransferase-1

EGFR: Epidermal growth factor receptor

EIA: Enzyme immunoassay

EM: Electron microscopy

EphA2: Ephrin receptor A2

ER: Endoplasmic reticulum

ERAD: ER-associated degradation

gHV68: Murine gamma-herpesvirus 68

GSK3: Glycogen synthase kinase 3

HAV: Hepatitis A virus

HBV: Hepatitis B virus

HCC: Hepatocellular carcinoma

HCMV: Human cytomegalovirus

HCV: Hepatitis C virus

HCVcc: Cell culture grown HCV

HCVpp: HCV pseudoparticles

HDLs: High-density lipoproteins

hPLIC1: Human homolog 1 of protein linking integrin-associated protein and cytoskeleton

HSPG: Heparan sulfate proteoglycan

HSV1: Herpes Simplex Virus 1

HVRs: Hyper variable regions

IAV: Influenza A virus

igVR: Intergenotypic variable region

IRE1: Inositol-requiring enzyme 1

IRES: Internal ribosomal entry site

IRGM: Immunity-associated GTPase family M

KSHV: Kaposi's sarcoma-associated herpesvirus

LCS: Low complexity sequences

LDLR: LDL receptor

LDLs: Low-density lipoproteins

LDs: Lipid droplets

LVP: Lipo-viro-particle

MAVS: Mitochondrial antiviral signaling protein

MDA-5: Melanoma differentiation-associated protein 5

MEFs: Mouse embryonic fibroblasts

MHV: Mouse hepatitis virus

MKNK1: MAPK interacting serine/threonine kinase 1

MNV: Murine norovirus

mTORC1: Mechanistic/mammalian target of rapamycin

MW: Membranous web

NANBH: Non-A, Non-B hepatitis

NBM: Nucleotide-binding motif

NPC1L1: Niemann-Pick C1-like 1 cholesterol absorption receptor

NS: Nonstructural protein

nt: Nucleotide

NTR: Untranslated region

OCLN: Occludin

ORF: Open reading frame

PAS: Phagophore assembly site

PERK: PKR-like ER kinase

PLA: Proximity ligation assay

PLEKHM1: Pleckstrin homology domain containing protein family member 1

pRb: Retinoblastoma tumor suppressor protein

RdRp: RNA-dependent RNA polymerase

RLRs: RIG-I-like receptors

RTK: Receptor tyrosine kinases

SARS: Severe Acute Respiratory Syndrome

SLI: Stem-loop I

SMVs: Single-membrane vesicles

SNARE: Soluble N-ethylmaleimide-sensitive factor attachment protein receptor

SPP: Signal peptide peptidase

SR-BI: Scavenger receptor class B type I

STING: STimulator of INterferon Genes

TC-PTP: T-cell protein tyrosine phosphatase

TFA: Tubule forming agent

TfR1: Transferrin receptor 1

TM: Transmembrane

TMD: Transmembrane domain

TRIF: Toll/IL-1 receptor homology domain-containing adaptor inducing IFN-β

Ub: Ubiquitin

UBDs: Ub-binding domains

UPR: Unfolded protein response

UVRAG: UV radiation resistance-associated gene product

VCP: Valosin-containing protein

VLDLs: Very low density lipoproteins

CHAPTER 1: INTRODUCTION

1 HEPATITIS C VIRUS

1.1 Hepatitis C history and discovery

Hepatitis C virus (HCV) is a pathogen with globally high prevalence and is a leading cause of liver cirrhosis, hepatocellular carcinoma (HCC), and death. The initiation of the field of viral hepatitis in the 1940s and 1960s was by the recognition of infectious serum hepatitis (Krugman *et al.*, 1962). Later on, it was identified as hepatitis A virus (HAV) infection by Dr. Feinstone and colleagues (Feinstone *et al.*, 1973) and hepatitis B virus (HBV) infection by Dr. Blumberg and Dr. Prince (Bayer *et al.*, 1968). Following the development of serological tests to detect HAV and HBV infections in the 1970s, the majority of parentally transmitted hepatitis were surprisingly not due to either virus (Feinstone *et al.*, 1975). Thus, it was termed Non-A, Non-B hepatitis (NANBH). Initial studies on viral hepatitis used chimpanzees as a reliable model for passaging infection from human materials (Alter *et al.*, 1978, Hollinger *et al.*, 1978). The use of chimpanzees as a model provided evidences about the existence of NANBH agents. These agents have been shown to cause the formation of tubule-like structures within the cytoplasm of the chimpanzee hepatocytes (Shimizu *et al.*, 1979). The tubule forming agent (TFA) has appeared to be a lipid-enveloped agent as it could be inactivated upon organic solvents treatment and it could be filtered through a 80 nM pore-size filter (Bradley, 1985).

In the 1980s, it was suggested that it could be a novel enveloped virus that is related to either the flaviviridae, togaviridae or hepatitis delta (Bradley, 1985). Simultaneously, it has been reported that with gradual progression of NANBH disease about 20% of infected patients slowly develop liver cirrhosis over many years (Dienstag *et al.*, 1986). During this period, several trials to propagate the NANBH agents in culture were performed. However, no successful cell culturing of NANBH was observed during this period.

In 1989, Dr. Michael Houghton and colleagues in Chiron company were able for the first time to isolate and identify the NANBH agent, named as HCV, by immunoscreening bacterial expression cDNA libraries from NANBH patient sera (Figure 1) (Choo *et al.*, 1989). Later on, the same group developed the first enzyme immunoassays (EIA) tests

specific for NANBH-specific antibodies, which showed that HCV was the main cause of parenterally-transmitted NANBH around the world (Kuo *et al.*, 1989).

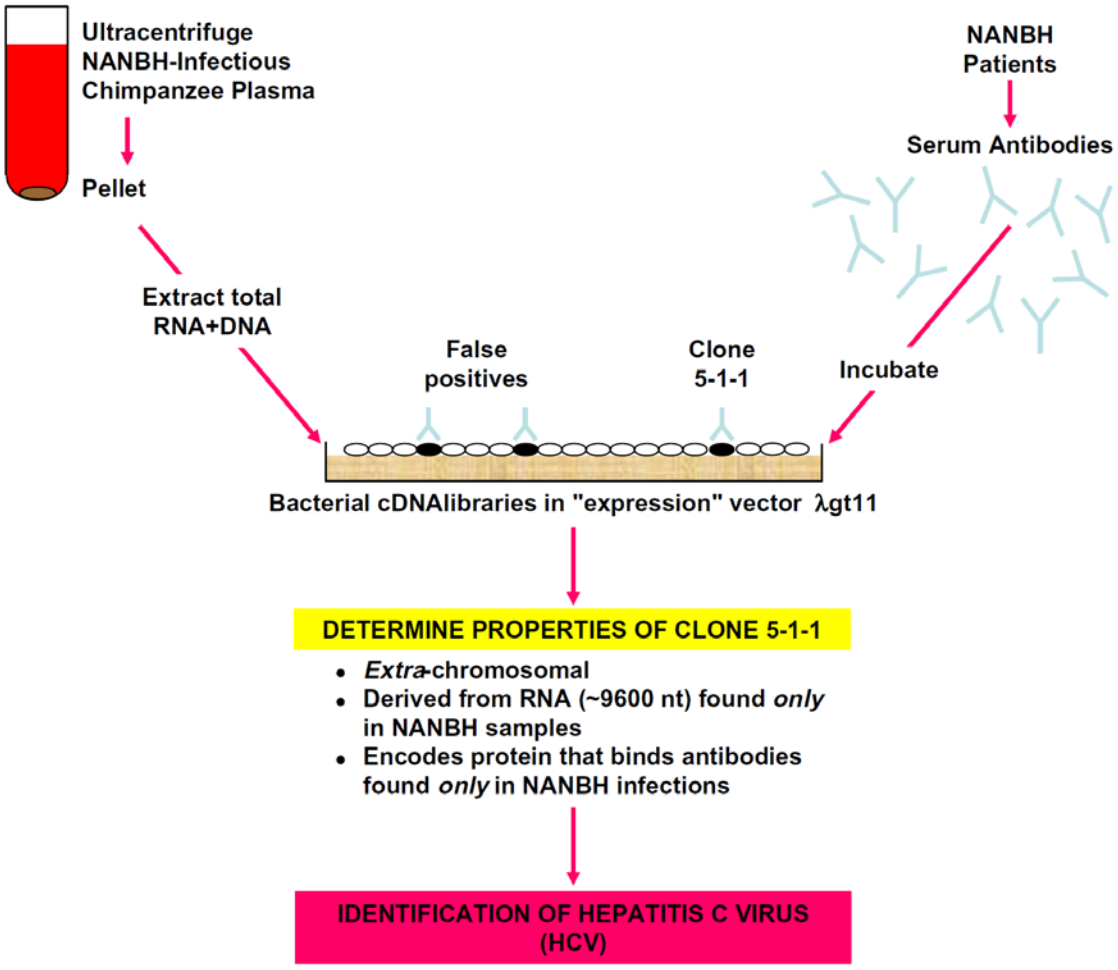


Figure 1. Schematic diagram for the first successful identification of HCV. A lambda gt11 cDNA library from both RNA and DNA present in the ultra-centrifuged pellet NANBH chimpanzee plasma was generated using random primers and screened with serum obtained from a NANBH patient with high serum ALT levels. One small clone of 150 bp, named clone 5-1-1, was found to be derived from HCV genome using the criteria in the figure (Houghton, 2009) .

1.2 HCV prevalence and genotypes distribution

The most recent estimation of HCV global burden showed an increased seroprevalence over the last 15 years to more than 185 million people worldwide. The global distribution of HCV infection is markedly variable (Figure 2). The highest prevalence is reported in Africa and Middle East with lower prevalence in Europe, Australia and North and South America (Hajarizadeh *et al.*, 2013). Based on phylogenetic and sequence analysis, HCV strains are classified into seven genotypes (1-7). Different genotypes vary in 30-35% of nucleotide content. Genotypes are further classified into 67 confirmed subtypes, 20 provisional and 21 unassigned ones. Strains that belong to the same subtype differ in less than 15% of nucleotide content (Smith *et al.*, 2014).

Globally, genotype 1 accounts for the highest number of HCV cases (46% of cases) compared to other genotypes with over one third of its cases located in East Asia. Genotype 3 is the second most abundant and accounts for 30.1% of cases, nearly three-quarters of which occur in South Asia. The majority of the remaining cases worldwide are due to genotype 2,4, and 6 (9.1%, 8.3%and 5.4% of the cases respectively). Genotype 2 and 6 are mainly located in East Asia while genotype 4 is located mainly in North Africa and the Middle East. Genotype 5 was estimated to account for the fewest HCV cases in the world, less than 1% of all HCV cases, most of which occur in Southern and Eastern sub-Saharan Africa (Messina *et al.*, 2015). Until now, only three genotype 7 subjects have been reported. These subjects were all Central African immigrants located in Canada (Murphy *et al.*, 2015).

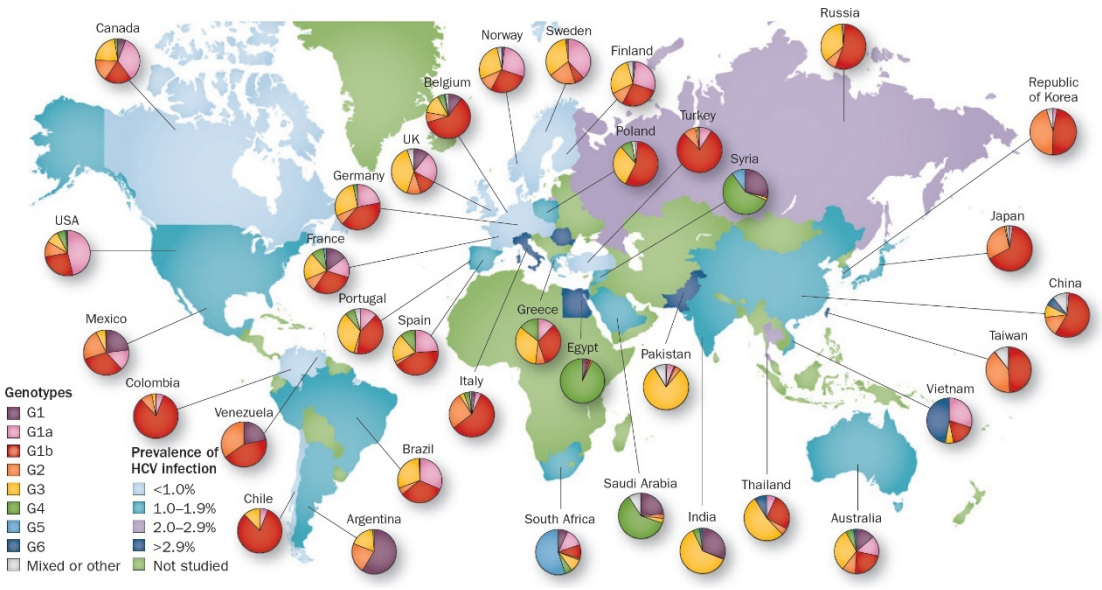


Figure 2. World HCV prevalence and genotypes distribution (Hajarizadeh *et al.*, 2013)

1.3 HCV genome organization

HCV in an enveloped positive-sense single-stranded RNA virus and is grouped with the genus Hepacivirus within the Flaviviridae family (Lindenbach *et al.*, 2013).HCV genomic RNA is 9,6 kb in length that is composed of a single open reading frame (ORF) of nearly 3040 codons flanked by two untranslated regions (NTR) the 5' and 3' NTRs (Figure 3). The viral genome is directly translated by a mechanism mediated by the internal ribosomal entry site (IRES) to a single polyprotein that is localized to the endoplasmic reticulum (ER). This polyprotein is cleaved co- and post-translationally by the action of viral and cellular proteases into 10 mature structural and nonstructural proteins (NS). The one third amino-terminal region of the polyprotein encodes the structural proteins E1, E2 and Core, which are incorporated, into viral particles. The remaining two thirds of the carboxy-terminal region encodes the NS proteins which includes P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Figure 3). These NS proteins are not incorporated in viral particles. Instead, they function to orchestrate different activities required for HCV RNA replication, forming what is called the replication complex (Moradpour *et al.*,

2013). The expression of the NS protein has been shown to induce a massive membrane remodeling within the cytoplasm of the infected cell forming the replication factories, termed as the membranous web (MW) (Egger *et al.*, 2002).With the help of host factors and viral structural proteins, the NS proteins have also been shown to participate in virion maturation (Lindenbach, 2013). In addition, several NS proteins modulate host immune defense and play a role in establishing chronicity (Lindenbach, 2013, Morikawa *et al.*, 2011).

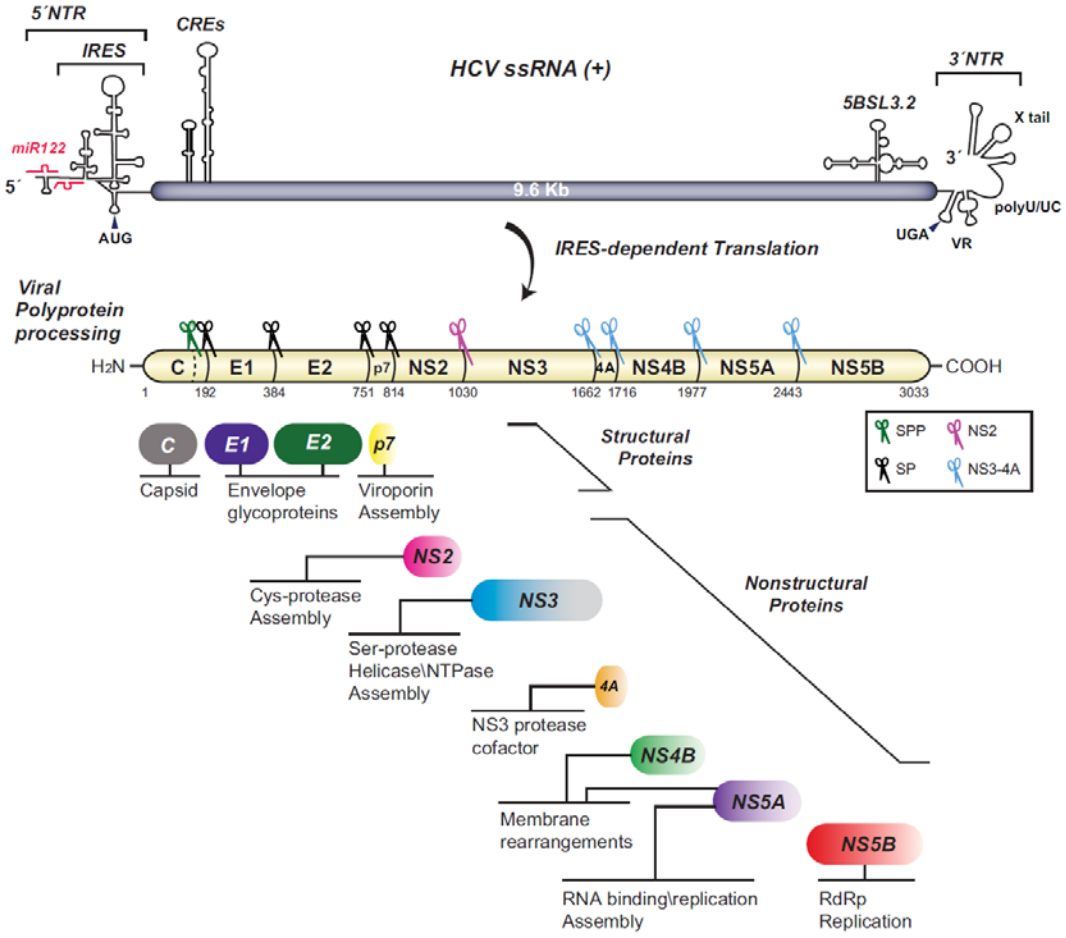


Figure 3. HCV genome organization and polyprotein processing. The HCV RNA single strand (SS) with positive polarity is shown on the top. The RNA Secondary structures include cis-acting RNA elements (CREs) in the untranslated regions (NTRs) are also shown on the top. miR-122 binding sites in the 5′ NTR containing the internal ribosome entry site (IRES) are shown. The proteins resulting from the single polyprotein cleavage and their functions are indicated. Scissors represent proteases that cleaves the virus polyprotein. SP, signal peptidase; SPP, signal peptide peptidase. **(Paul *et al.*, 2014).**

1.3.1 IRES-mediated translation initiation

HCV protein translation initiation is directed by a group of regulatory structural RNA elements at the 5' NTR that define IRES. The HCV IRES spans a region of ~341 nucleotides (nt) and is composed of three distinctly structured domains II, III, and IV. The boundaries of the IRES have been carefully mapped to be between 25 and 46 nt of the 5′NTR, stretching up to about 24-40 nt within the coding region. The first domain of the IRES, stem-loop I (SLI), is not required for translation, but is important for replication (Figure 4a). SLII is comprised of a stem having several internal loops (Figure 4a, boxed in yellow, left side). The larger domain SLIII consists of branching hairpin stem–loops (IIIabcdef) organized in 3- and 4-way junctions and a pseudoknot near the base of the stem–loop. SLIV is a small structure that resembles a hairpin and contains the start codon AUG at position 342. After its binding with the 40S ribosomal subunit, the central IRES core domain with the double pseudoknot positions the start codon on the 40S into the mRNA binding cleft (Berry *et al.*, 2011). To form preinitiation 48S ribosomal complexes, the HCV IRES requires just three initiation factors, eukaryotic initiation factor (eIF) 2, eIF3, and eIF5. The eIF3 binds to the apical region of SLIII (Sizova *et al.*, 1998) (Figure 4a, boxed in magenta) and associates with the ribosomal 40S subunit (Jackson *et al.*, 2010). The eIF2 associates with the initiator tRNA and GTP to form the ternary complex eIF2-GTP-Met-tRNAi^{Met} which brings the Met-tRNAi^{Met} to the 40S subunit (Pestova *et al.*, 1998). Consequently, eIF5 promotes start codon recognition by the ternary complex eIF2-GTP-Met-tRNAi^{Met} and acts as a GTPase-activator protein for eIF2 (Pestova *et al.*, 1998). This is sufficient to allow the bound 40S subunit to lock onto the HCV initiation codon and associate with the large ribosomal 60S subunit to form translation-competent 80S (Pestova *et al.*, 1998, Terenin *et al.*, 2008) (Figure 4b).

In addition to these canonical translation initiation factors, several host factors have been identified to interact with HCV IRES and/or 3'NTR and modulate viral RNA translation (Table 1). Since the IRES-mediated translation is a critical step in HCV life cycle, the IRES-mediated translation became an important target for developing therapeutic strategies to eradicate HCV.

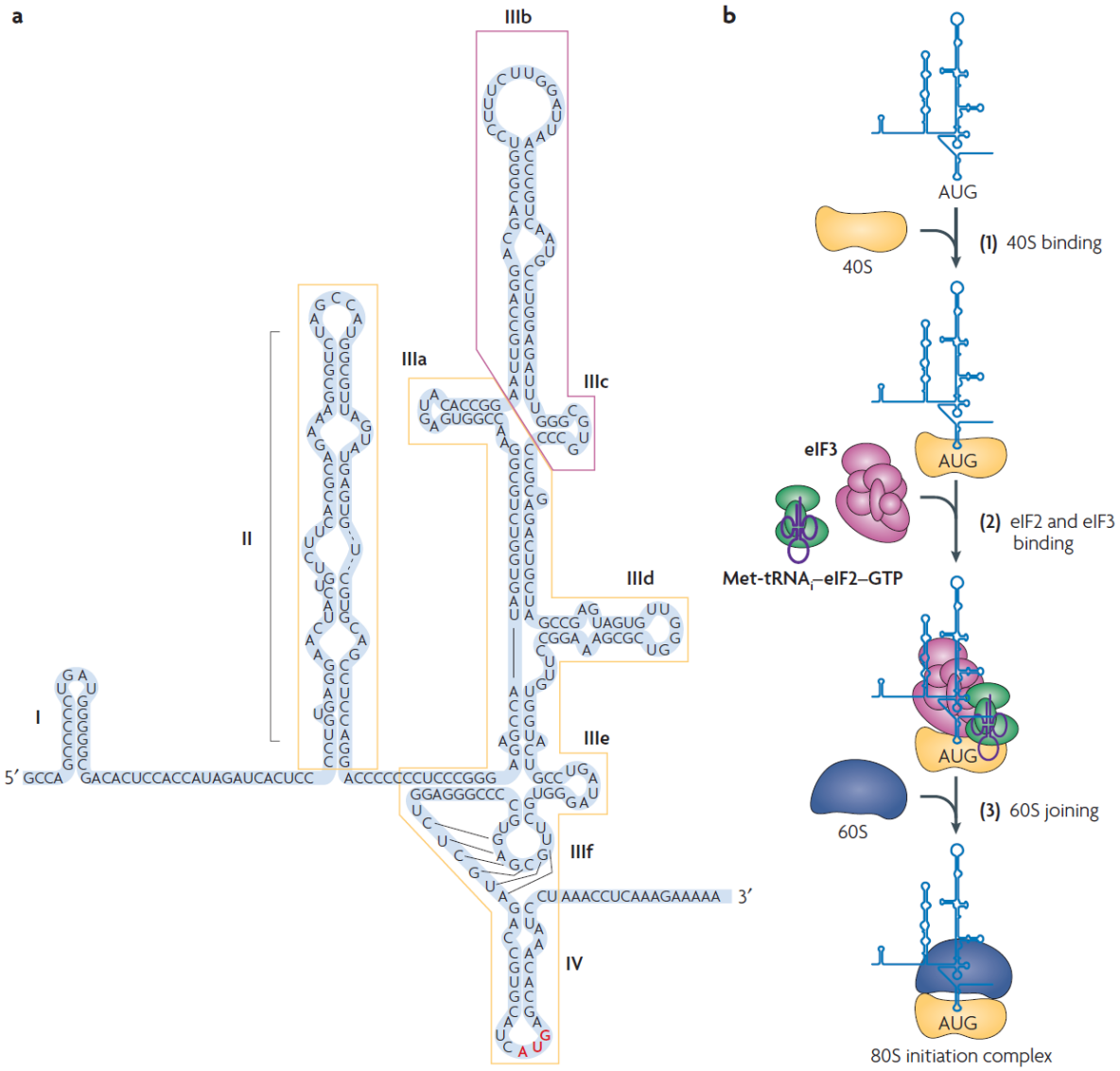


Figure 4. HCV IRES structure and model for 80s complex formation. (a) The secondary structure of the HCV 5'NTR consisting of 4 different domains (I, II, III, IV). The domains for 40S binding are boxed in yellow while the domain for eIF3 binding is boxed in magenta. The translation start codon is in red color (b) a model for HCV IRES mediated initiation of translation. [modified from (Fraser et al., 2007)]

Table 1. Host factors involved in HCV RNA translation [adopted from (Hoffman *et al.*, 2011)]

Cellular factors	Effect on HCV translation	Proposed mechanisms	Cellular functions	References
Gemin5	-	Binds to IRES and forms complex with eIF3a, b, c. Addition of purified Gemin5 resulted in down-regulation of HCV IRES activity, while shRNA knockdown of Germin5 resulted in increase of HCV IRES activity	snRNA binding protein of SMN complex, which is involved in biogenesis of snRNPs, a component of mRNA slicing machinery. Also, involved in down-regulation of cellular translation in which a complex containing eIF4E is formed	(Pacheco *et al.*, 2009)
HMGB1	+	Facilitates viral replication by interaction with the HCV RNA	A nuclear protein involved in a group of diseases, including infectious diseases, metabolic and immune disorders, and cancer.	(R. Yu *et al.*, 2015)
hnRNP D (AUF1)	+	Interacts with SLII of HCV IRES. Knockdown inhibits translation but increases replication suggesting a role in balancing viral translation and replication	Involved in mRNA decay, telomere maintenance and translation initiation	(Paek *et al.*, 2008)
hnRNP L	+	Binds to 30 -end of HCV IRES. hnRNP L-specific RNA aptamers inhibited IRES function in dose dependent manner	Role in mRNA processing including alternative splicing, mRNA export and mRNA stability	(Hahm *et al.*, 1998, B. Hwang *et al.*, 2009)
Hu antigen R (HuR)	+	Binds to polyU/UC region of 30 -UTR. Overexpression enhances HCV IRES activity while knockdown down-regulates HCV IRES activity	Selectively binds and stabilizes AU-rich element containing mRNAs	(Rivas-Aravena *et al.*, 2009, Spangberg *et al.*, 2000)
IGF2BP1 (IMP-2)	+	Binds to 50 - and 30 -UTRs. siRNA knockdown down-regulates HCV IRES activity. May stimulate translation by promoting RNA circularization and/or recruitment of eIF3	Binds to and regulates the translation of certain mRNA such as insulin-like growth factor 2 and b-actin	(Weinlich *et al.*, 2009)
La autoantigen	+	Binds near the initiation AUG codon of IRES. Thought to alter the conformation which facilitates formation of the initiation complex and stimulate internal initiation of translation	Multifunctional protein with roles in RNA biogenesis. Binds to the 30 termini of many newly synthesized RNAs, particularly those made by RNA pol III, protecting the 30 ends from exonucleases. Autoantigen in systemic autoimmune diseases	(Ali *et al.*, 1997, Pudi *et al.*, 2004)
LSm1-7	+	LSm1-7 ring binds to 50 -UTR dependent on SLIII. Also binds to poly(U/UC) tract of 30 -UTR. Silencing of LSm1 selectively down-regulates HCV translation	Component of P-bodies. Involved in mRNA turnover. Binds to short oligo (A) tracts at 30 -end of de-adenylated mRNA and inhibits 30 degradation while promoting decapping and 50 –30 degradation	(Scheller *et al.*, 2009)
miR-122	+	Binding to S1 and S2 sites in 50 -UTR upstream of the IRES enhances translation. Thought to enhance the association of 40S ribosomal subunit and HCV RNA. May protect 5'-end of viral genome from degradation	Well-conserved, highly abundant, liver specific microRNA. Binding to 30 -UTR of cellular mRNA encoding cationic amino acid transporter CAT-1 results in down-regulation of CAT-1 protein levels	(Henke *et al.*, 2008, Jangra *et al.*, 2010, Jopling *et al.*, 2005)
miR-199a	?(-)	Target sequence in domain II of the HCV IRES. Over-expression results in down-regulation of viral replication	Implicated in the post-transcriptional regulation of gene expression for various genes such as Ceruloplasmin (CP)	(Murakami *et al.*, 2009)
miR-196	-	Potential target sequence present in HCV NS5A coding region. Over-expression results in down-regulation of HCV protein expression and replication	Implicated in the post-transcriptional regulation of gene expression for various genes such as Bach1	(Hou *et al.*, 2010)
Nucleolin	?(+)	No data using HCV reporter. Identified by mass spectrometry as a component of HCV IRES bound 40S ribosomal subunit. Bound by yeast inhibitor RNA (IRNA). Stimulates poliovirus IRES	Implicated in a variety of processes including rRNA maturation, and ribosome assembly	(Izumi *et al.*, 2001)
NSAP1 (SYNCRIP, hnRNP Q)	+	Binds to adenosine rich region downstream of AUG start codon in core coding sequence. Over-expression enhances IRES activity while knockdown down-regulates IRES activity	A member of hnRNP family of proteins implicated in mRNA processing mechanisms. Component of the spliceosome	(Ali *et al.*, 1995)
PTB	?	Interacts with both 50 - and 30 -UTRs of HCV RNA. Role in HCV protein translation is unclear with contradictory reports suggesting stimulation, inhibition, or no effects on HCV protein translation	Implicated in the regulation of pre-mRNA splicing	(Ali *et al.*, 1995, Tischendorf *et al.*, 2004)

PSMA7	+	Knockdown by siRNA or ribozymes results in significant inhibition of HCV translation	Human 20S proteasome a-subunit type 7 is a component of 20S core structure of proteasome	(Kruger *et al.*, 2001)
Staufen 1	+	Knockdown by siRNA decreased virus secretion	a dsRNA-binding protein involved in the regulation of translation, trafficking, and degradation of cellular RNAs	(Blackham *et al.*, 2013)

1.4 Structural proteins

1.4.1 Core Protein

The first protein to be translated from the ORF is the core protein. The presence of an internal signal sequence between core and E1 directs the nascent polyprotein to the ER where cleavage of the signal sequence takes place by the action of signal peptidase (SP) (Figure 3). This cleavage yields immature core protein with 191 amino acid (aa). Further processing of the C-terminus by signal peptide peptidase (SPP), an intramembrane cleaving protease, yielding the mature 21-kDa core protein, with ~177 amino acid (aa) (Figure 3) (Oehler *et al.*, 2012, Santolini *et al.*, 1994). Mature core is a homodimeric membrane protein stabilized by the formation of disulfide bond at Cys 128 (Kushima *et al.*, 2010). HCV core has two main domains, the N-terminal hydrophilic domain (D1) that is rich in basic aa residues with highly flexible intrinsic disordered structure that allows an expansion of interaction with viral and host molecules (Uversky, 2011). It also harbors critical residues required for Core function. Indeed, core D1 can bind HCV RNA promoting RNA encapsidation. As other nucleocapsid proteins, D1 has RNA chaperone activity required for structural remodeling and packaging of the RNA during virion formation (Cristofari *et al.*, 2004). In addition, core D1 can regulate translation of viral proteins (Boni *et al.*, 2005, Lourenco *et al.*, 2008). Moreover, the C-terminus of D1 harbors motifs for BH3 interaction capable of binding cellular proteins to regulate apoptosis (Mohd-Ismail *et al.*, 2009). The other core protein domain is the C-terminal hydrophobic domain (D2), which mediates the binding with lipid droplets (LDs). This association with LDs impedes core nuclear localization exerted by a nuclear localization signal found in the N-terminal RNA binding domain (Suzuki *et al.*, 1995). Core D2 contains a central hydrophobic

loop that connects two amphipathic α-helices and interacts with phospholipids interface on LDs (Boulant *et al.*, 2007).

It has been shown that the association of core with LD and its interaction with NS5A plays a crucial role in virion assembly process (Miyanari *et al.*, 2007, Shavinskaya *et al.*, 2007).

1.4.2 E1 and E2 glycoproteins

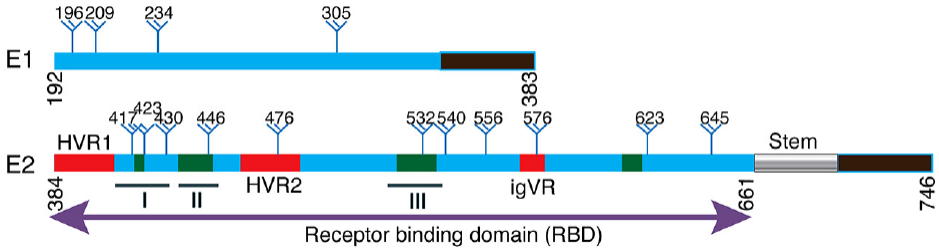
The envelope glycoproteins E1 and E2 are type 1 transmembrane with an ectodomain at the N terminus (~160 aa and ~360 aa for E1 and E2 respectively) facing ER lumen and a short transmembrane domain (TMD) of ~30 aa at C terminus (Moradpour *et al.*, 2013) (Figure 5a). During their biogenesis, the ectodomains of E1 and E2 translocate into the ER lumen and inserts their TMDs (shown in black, Figure 5a) into the ER membrane. The TMDs are strongly implicated in the functions of E1 and E2, including membrane anchoring and ER retention. In addition, they participate in the formation of E1–E2 noncovalent heterodimer, which is supposed to represent the building units for HCV envelope. The E1 and E2 embedded in the virus envelope were shown to form large covalent complexes stabilized by disulfide bonds (Vieyres *et al.*, 2010). The folding of these two proteins, that occurs in the ER, is a complex process that requires ER chaperones, disulfide bridging, and glycosylation. Indeed, E1 and E2 contain up to 6 and 11 glycosylation sites (trees in Figure 5a), respectively. Despite the high genetic variability, this extensive glycosylation is quite conserved across all genotypes (Brown *et al.*, 2010, Goffard *et al.*, 2005).

The genes that encode E1 and E2 are uniquely variable with several hyper variable regions (HVRs) that have been identified within E2 (shown in red, Figure 5a). These HVRs differs by up to 80% across the different genotypes. The HVR1 consists of the first 27 aa of E2 and is responsible for eliciting type-specific neutralizing antibodies (Figure 5a) (Penin *et al.*, 2001). HVR2, from aa 91 to 97 of E2, has up to 100% sequence diversity across genotypes (Figure 5a). The intergenotypic variable region (igVR), aa 187 to 197, is widely variable between different genotypes while it contains a single conserved N-linked sugar moiety

(Figure 5a). HVR2 and igVR are essential for the structural integrity and hence for the function of HCV glycoproteins (McCaffrey *et al.*, 2011). However, in contrast to HVR1, they are not targets for humoral immune response.

Recently, two independent crystal structures have been provided for the core ectodomain of E2 protein (Khan *et al.*, 2014, Kong *et al.*, 2013) (Figure 5b and c, respectively) and amino-terminal domain of E1 (El Omari *et al.*, 2014) (Figure 5d). The structures of E1 and E2 lack the hallmarks of viral membrane fusion proteins suggesting a possible new entry mechanism for HCV.

a



b c d

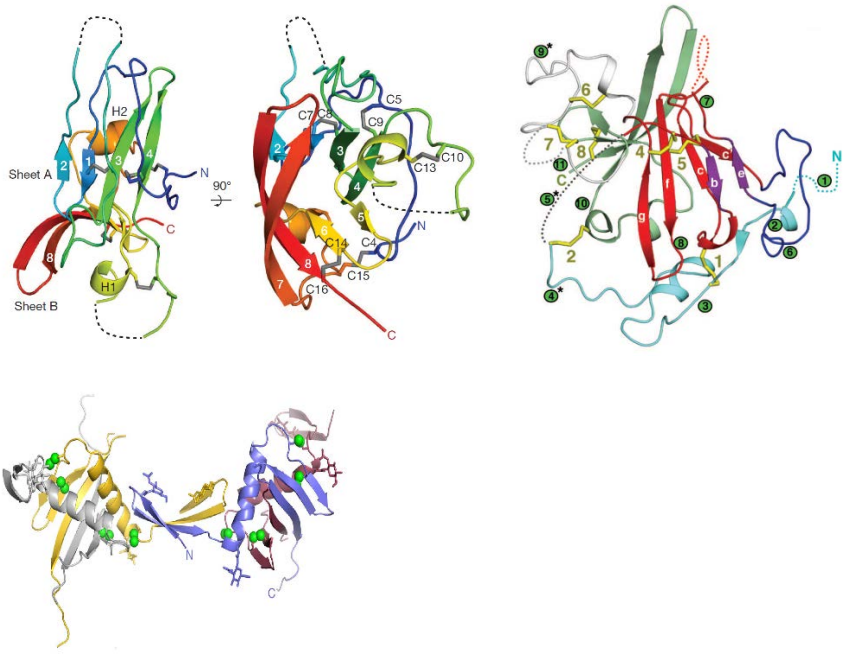


Figure 5. HCV E1 and E2 glycoproteins. (a) Schematic of the HCV E1 and E2 that shows the conserved glycosylation sites (trees). (b) and (C) Two structures of the core ectodomain of E2. (D) Represents the structure of the amino terminal domain of E2.

1.5 Nonstructural proteins

1.5.1 P7

P7 is a small, 63 aa, integral membrane protein located at the junction between structural and nonstructural proteins (Lin *et al.*, 1994).It is classified as viroporin, as influenza virus M2 protein, based on its ability to alter membrane permeability (Nieva *et al.*, 2012). It comprises two transmembrane α-helices with an N and C termini oriented towards ER lumen connected by a cytosolic loop (Carrere-Kremer *et al.*, 2002, Vieyres *et al.*, 2013). P7 forms hexameric or heptameric structures with cation channel activity to facilitate the formation of infectious viral particles (Chandler *et al.*, 2012). It was shown that trafficking of viral and cellular glycoproteins is delayed by the action of P7. Furthermore, P7 could act to prevent acidification in intracellular compartment to promote infectious viral particles production (Wozniak *et al.*, 2010). Recently, it was shown that, in addition to its function as viroporin, P7 could act as lipid raft adhesion factor for HCV budding process (G. Y. Lee *et al.*, 2016). Although P7 is required for virus assembly but not replication, the exact function of P7 has not been elucidated yet (Scull *et al.*, 2015).

1.5.2 NS2

NS2 is a 217 aa integral membrane protein. It binds to intracellular membranes via its hydrophobic N-terminal domain that contains 3 transmembrane segments (Jirasko *et al.*, 2008). NS2 gene encodes for a cysteine protease responsible for its autocleavage from the HCV polyprotein precursor at NS2/NS3 junction. The activity of this cysteine protease has been shown to be greatly improved by one third N-terminal of the NS3 protein (Schregel *et al.*, 2009). The catalytic activity resides at the C-terminal domain. The crystal structure of this domain revealed a dimer with two active sites (Lorenz *et al.*, 2006). Despite the fact that NS2 is dispensable for RNA replication, NS2 plays a crucial organizing role in the assembly of infectious viral particles. This action was shown to be independent of its protease activity and may involve interaction network with other structural and

nonstructural proteins (Boson *et al.*, 2011, Stapleford *et al.*, 2011). Recently, it was proposed that NS2 protease activation in modulated by a conserved NS3 surface patch (Isken *et al.*, 2015).

1.5.3 NS3-4A complex

NS3 and its co-factor, NS4A, form a non-covalent complex (J. L. Kim *et al.*, 1996). NS3 is a 70 kDa multifunctional protein with a serine protease located in the N-terminal (aa 1-180) and an NTPase/RNA helicase in the C-terminal (aa 181-631). The activity of both enzymes is well characterized and their crystal structures have been resolved (Morikawa *et al.*, 2011, Raney *et al.*, 2010, Yao *et al.*, 1999). NS3-4A protease activity is required for cleavage at four junctions in the HCV polyprotein, NS3/NS4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B (Bartenschlager *et al.*, 1993, Grakoui *et al.*, 1993). In addition, NS3-4A protease was shown to target, so far, three cellular factors for cleavage. These include T-cell protein tyrosine phosphatase (TC-PTP), mitochondrial antiviral signaling protein (MAVS), and toll/IL-1 receptor homology domain-containing adaptor inducing IFN-β (TRIF), which may be implicated in the development of chronic infection and HCC (K. Li *et al.*, 2005, Meylan *et al.*, 2005). Accordingly, NS3-4A protease plays essential roles not only in the replication but also in the persistence and pathogenesis of HCV (Morikawa *et al.*, 2011). This made the NS3-4A protease a primary target for DAAs. The binding of NS4A to NS3 was shown to stabilize the NS3 structure and hence enhances its catalytic activity and directs it to cellular membrane localization via the hydrophobicity of the N-terminal transmembrane α-helix of NS4A (Abian *et al.*, 2010). Furthermore, the C-terminal acidic portion of NS4A is involved in the regulation of HCV RNA replication and virus assembly by interacting with other viral nonstructural proteins forming what is called the replication complex (Morikawa *et al.*, 2011). NS4A was also shown to regulate HCV replication through its role in NS5A hyperphosphorylation (Lindenbach *et al.*, 2007). The NS3 NTPase/RNA helicase belongs to the superfamily 2 DExH/Dbox helicases (Raney *et al.*, 2010). It combines ATP hydrolysis activity to its capability

to unwind either double-stranded RNA or single-stranded RNA regions with extensive secondary structure. Although the NS3 helicase is essential for HCV RNA replication and also plays a role in viral particle assembly, its precise role (s) in HCV life cycle remain(s) still not determined.

1.5.4 NS4B

NS4B is a hydrophobic 27 kDa protein that is poorly characterized (Hugle *et al.*, 2001). It is an integral protein consists of a N-terminal part (aa 1-69), a central portion with at least four predicted transmembrane (TM) domains (aa 70-190), and a C-terminal part (aa 191-261) (Gouttenoire *et al.*, 2009b, Hugle *et al.*, 2001, Lundin *et al.*, 2003). The N-terminal part comprises two amphipathic α-helices, AH1 (aa 3-35) and AH2 (aa 42-66). The AH2 is conserved across different genotypes and is crucial for HCV replication (Elazar *et al.*, 2004, Gouttenoire *et al.*, 2014). This segment plays an important role in the assembly of a functional replication complex. The C-terminus comprises a highly conserved amphipathic α-helix, H1, (aa 201–213) and a membrane-associated amphipathic α-helix, H2, (aa 229–253), and two other palmitoylation sites (Gouttenoire *et al.*, 2009b, G. Y. Yu *et al.*, 2006). Upon NS4B dimerization/ multimerization, the N-terminus can translocate its AH1 part into the ER lumen promoting recruitment of the replication complex via its role in the formation of membranous web (MW), a specific membrane alteration consisting of confined membranous vesicles that serves as a scaffold for the HCV replication complex, thus allowing HCV RNA replication (Egger *et al.*, 2002, Gosert *et al.*, 2003, Gouttenoire *et al.*, 2009a, Romero-Brey *et al.*, 2012). In addition, NS4B interacts with other viral nonstructural proteins and can bind viral RNA (Einav *et al.*, 2008). The central portion of NS4B harbors a nucleotide-binding motif (NBM) Walker A located between TM2 and TM3 domains with NTPase activity. This NBM walker A exists in all HCV genotypes and shows importance in HCV life cycle (Einav *et al.*, 2004, Jones *et al.*, 2009). Altogether, NS4B is considered as a master organizer of HCV replication complex formation.

Therefore, NS4B is also considered as a potential target for antivirals (Cannalire *et al.*, 2016, Rai *et al.*, 2011).

1.5.5 NS5A

NS5A is a 447-aa membrane-associated phosphoprotein that plays an important role in modulating HCV RNA replication and particle formation. It can be found in basally phosphorylated (56 kDa) and hyperphosphorylated (58 kDa) forms. NS5A has N-terminal membrane anchoring domain in addition to three other domains, D1, D2 and D3 separated by two low complexity sequences (LCS). D1 (aa 36-213) and D2 (aa 250-342) are mainly involved in HCV RNA replication while D3 (aa 356-447) is implicated in viral assembly (Appel *et al.*, 2008, S. Kim *et al.*, 2011, Tellinghuisen *et al.*, 2008, Tellinghuisen *et al.*, 2004). Moreover, D1 was shown to be involved in lipid droplets (LDs) binding (Miyanari *et al.*, 2007) whereas D2 is involved in the interaction with core protein (Masaki *et al.*, 2008). The basal phosphorylation of NS5A protein is a conserved feature within flaviviruses (Reed *et al.*, 1998). In the case of HCV NS5A, it occurs at the C-terminal and the central portion of the protein while hyperphosphorylation of the protein requires the serine residues 225, 229, and 232 in the first LCS. Indeed, cell culture adaptive changes often affect these centrally located serine residues suggesting that the phosphorylation state of NS5A modulates the efficiency of HCV RNA replication (Appel *et al.*, 2005, Evans *et al.*, 2004, Neddermann *et al.*, 2004). Cellular protein kinases in the CMGC kinase family, named by the initials of some members of the family, mediate NS5A phosphorylation. These include cyclin-dependent kinase (CDK), mitogen activated protein kinase (MAPK), glycogen synthase kinase 3 (GSK3) and casein kinase II (CKII) (Asabe *et al.*, 1997, Koch *et al.*, 1999, Lindenbach *et al.*, 2007, Macdonald *et al.*, 2004, Tellinghuisen *et al.*, 2008). The subcellular distribution of both forms of NS5A seems to be similar. Thus, the phosphorylation level of NS5A does not alter its localization to the ER membrane (Tanji *et al.*, 1995). However, the phosphorylation of NS5A is shown to enhance its degradation and decreases the protein half-life (Y. Huang *et al.*, 2007b). NS5A

is also involved in the production of infectious virions through its interaction with core protein, which needs a basal phosphorylation of NS5A (Masaki *et al.*, 2008). The role of NS5A in HCV RNA replication and virion formation made it an attractive target for DAA (Adler *et al.*, 2014)

1.5.6 NS5B

NS5B is a conserved 68 kDa protein with RNA-dependent RNA polymerase (RdRp) activity. It is the key enzyme responsible for the initiation of complementary negative-strand RNA synthesis and subsequent synthesis of genomic positive-strand RNA using this negative-strand RNA as template. Due to the lack of proofreading of RdRp, HCV replication is error-prone. This 591 aa viral enzyme has been widely studied and characterized (Behrens *et al.*, 1996, Lesburg *et al.*, 1999, Lohmann *et al.*, 1997, Simister *et al.*, 2009). The NS5B catalytic domain is located at the N-terminal 530 aa and contains motifs that are common in all RdRps. These include the hallmark GDD sequence within motif C, and the classical fingers, palm, and thumb subdomain organization of a right hand. The catalytic domain and the C-terminal membrane anchor are separated by a 40-aa linker between (aa 570-591) that occludes the active site. Within the palm and thumb domains, there are four allosteric sites which serve as potential targets for DAA development (Beaulieu, 2009, Court *et al.*, 2016, Pierra Rouviere *et al.*, 2016). NS5B can interact with other viral proteins such as NS3, NS4A and NS5A (Ishido *et al.*, 1998). In addition, it can interact with cellular proteins like hVAP-33, which facilitates the formation of the viral RNA replication complex (Tu *et al.*, 1999). Recently, it was shown to interact with Valosin-containing protein (VCP), which is a AAA+ATPase that can modulate viral replication (Yi *et al.*, 2016). It can also form a complex with the retinoblastoma tumor suppressor protein (pRb) and promote pRb degradation in a ubiquitin-dependent manner, thus contributing to HCC development (Munakata *et al.*, 2007). Moreover, NS5B was found to interact with ubiquitin-like protein hPLIC1 (human homolog 1 of protein linking integrin-associated protein and cytoskeleton) and causes NS5B ubiquitination. The

ubiquitination modification of NS5B through hPLIC1 binding promotes ubiquitin-dependent proteasome degradation, resulting in reduction of NS5B level (Gao *et al.*, 2003). As NS5B primarily functions in RNA replication, decrease in NS5B through proteasome degradation leads to decrease in HCV RNA replication. Thus up-regulation of NS5B ubiquitination could be a potential target for anti-viral development.

1.6 HCV virion structure

Despite the significant progress in cell culture systems which enables the production of viral particles as well as several biochemical and morphological studies, the structure of the HCV particle remains elusive.

Viral particles purified from patients' sera or cell culture media by filtration and visualized by electron microscopy (EM) revealed that, HCV has a spherical morphology of different sizes (range 40-80 nm diameter) (Catanese *et al.*, 2013, Kaito *et al.*, 1994, X. Li *et al.*, 1995). The HCV genome interacts with the core protein to form the nucleocapsid that is surrounded by a lipid membrane, called the viral envelope, in which are anchored two envelope glycoproteins, E1 and E2. These two glycoproteins assemble as a E1E2 heterodimer that is stabilized by disulfide bonds on the viral particle where they represent the major viral determinants of HCV entry (Figure 6).

One unique feature of HCV virion is its association with apolipoproteins (apo) such as apoE, apoB, apoA1, apoC1, apoC2 and apoC3 (Andre *et al.*, 2002, Catanese *et al.*, 2013, Chang *et al.*, 2007, Meunier *et al.*, 2008, Nielsen *et al.*, 2006). Furthermore, analysis of cell culture-produced viral particles by mass spectrometry indicates that their lipid composition resembles the one of the very low-density lipoproteins (VLDLs) and low-density lipoproteins (LDLs) with cholesteryl esters (CEs) accounting for almost half of the total HCV lipids (Merz *et al.*, 2011). Purified infectious virions showed a pleomorphic nature of HCV virions and show viral particles with a rather smooth and even surface (Merz *et al.*, 2011).

The type of the interactions occurring between HCV virion components and the lipoprotein remains elusive. One proposed model suggested that HCV particles form a lipo-viro-particle (LVP), a combination of a virion moiety and a lipoprotein moiety (see figure 6A) (Andre *et al.*, 2002, Bartenschlager *et al.*, 2011). However, alternative models have also been suggested, with lipoproteins peripherally associated with canonical viral particles via interaction between apolipoproteins and HCV envelope lipids or proteins (Lindenbach, 2013). In both models, the interaction of virus particles with lipoprotein may contribute to the protection of virus particles from circulating neutralizing antibodies (Andre *et al.*, 2002, Catanese *et al.*, 2013, Dao Thi *et al.*, 2012). Importantly, apolipoprotein(s) associated with HCV particles play a critical role in HCV entry. The difficulty to investigate natural HCV, or LVP, and its different lipoprotein or immunoglobulin-associated forms is due to low infectivity of serum-derived HCV either in primary hepatocytes or hepatoma cell lines (Fournier *et al.*, 1998, Rumin *et al.*, 1999). In addition, the mechanism(s) by which LVPs mediate cell entry, leading to release of HCV genetic material and RNA replication, have not been fully characterized.

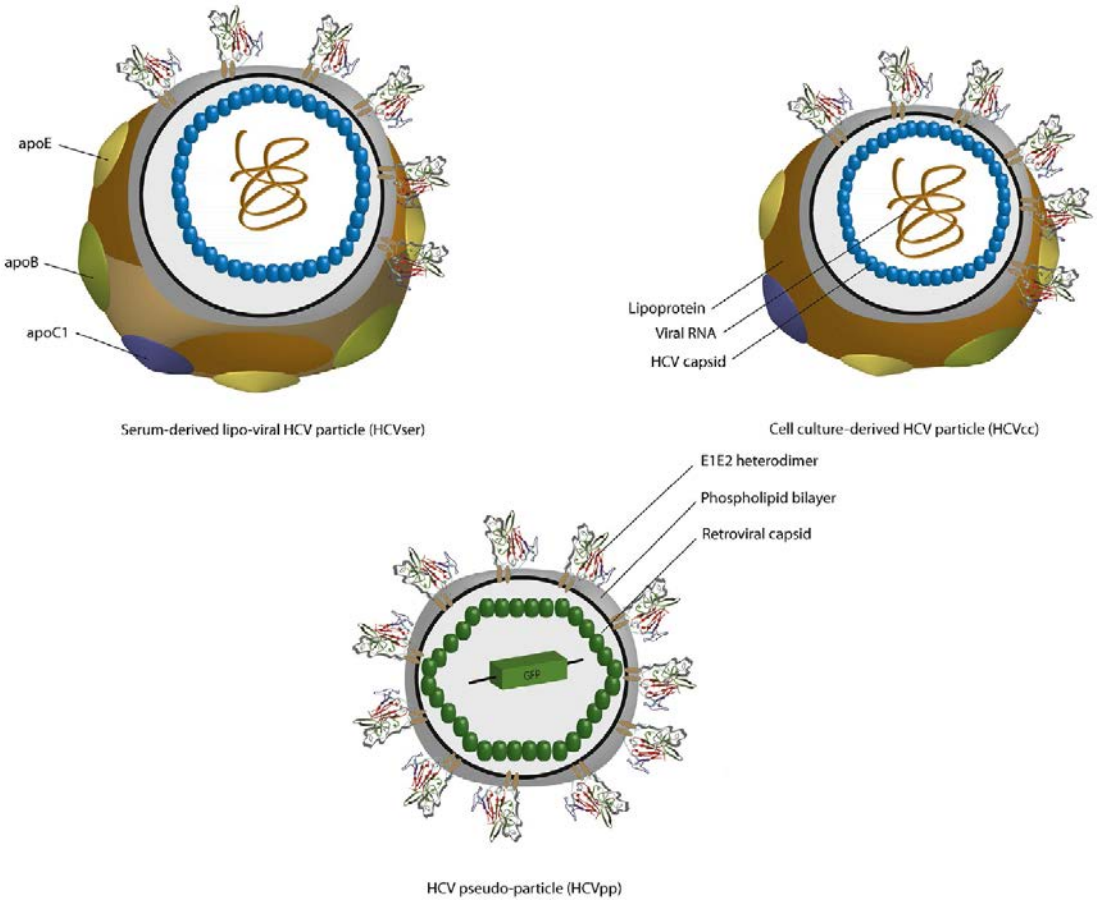


Figure 6. The components of each HCV particle type. The apolipoprotein composition of each type of viral particle is indicated in the figure. [modified from (Douam *et al.*, 2015)]

The development of several surrogate in vitro assays to overcome these limitations has been successfully achieved. Among them, two main assays consist of cell culture grown HCV (HCVcc) derived from a fulminant hepatitis C JFH-1 and JFH-1-derived recombinant genomes (Lindenbach *et al.*, 2005, Wakita *et al.*, 2005) and of HCV pseudoparticles (HCVpp) harboring authentic E1E2 glycoproteins which is particularly beneficial to study viral entry (see figure 6C) (Bartosch *et al.*, 2003a, Drummer *et al.*, 2003). HCVcc exists as a mixture of infectious and noninfectious particles and displays a broad density profile similar to HCV derived from patients' sera. Higher specific infectivity was shown to be associated to low-density fractions (Gastaminza *et al.*, 2006, Lindenbach *et al.*, 2006, Podevin *et al.*, 2010). The lipid composition of HCVcc particles was determined and found similar to that of VLDL and LDL (Merz *et al.*, 2011). Highly purified HCVcc particles were found to contain

several apoE molecules on their surface as well as apoC-I and, to some extent, apoB (see figure 6B). This comes in line with the finding that HCV formation and secretion rely on VLDL assembly and secretion pathway (Chang *et al.*, 2007, Gastaminza *et al.*, 2008, H. Huang *et al.*, 2007a, Jiang *et al.*, 2009, Meunier *et al.*, 2008). While HCVcc, which are produced in human hepatoma cells typically Huh7 and Huh-7.5 cells, further permit investigation of the late steps of infection, HCVpp provide a flexible platform to study the structure/ function relationship of HCV glycoproteins *in vitro*. It is noteworthy that, since HCVpp are produced from 293T kidney cells, they are not associated to lipoproteins (Flint *et al.*, 2004), which, in turn, allows to investigate the cell entry events that are specifically linked to the functions of the E1E2 glycoproteins (Bartosch *et al.*, 2005). In addition, this provides a suitable tool to reconstitute some of the interactions of HCV with lipoproteins or apolipoproteins (Dreux *et al.*, 2007, Dreux *et al.*, 2006, Meunier *et al.*, 2005). Altogether, utilizing HCVcc and HCVpp allows the dissection of the cellular and viral factors involved in HCV infection.

1.7 HCV attachement

Hepatocytes are the main target cells for HCV infection. However, infection of dendritic cells, B cells, and other cell types have also been reported. Viral entry into the host cell involves a complex multistep series of interactions including attachment, entry and fusion. The initial capture of HCV virion by its cell surface receptor/co-receptors may involve HVR1 in HCV E2 (Flint *et al.*, 2000). Depending on virion density, this step may be mediated by heparan sulfate proteoglycan (HSPG) syndecan-1 and/or syndecan-4 (Lefevre *et al.*, 2014, Shi *et al.*, 2013) or by the scavenger receptor class B type I (SR-BI) (Figure 7) (Dao Thi *et al.*, 2012). Although, the initial binding of virions to HSPG or SR-BI was thought to be through HCV glycoproteins (Barth *et al.*, 2003) (Scarselli *et al.*, 2002), a more recent data showed that apoE, rather than HCV glycoproteins, could be implicated in this binding (Dao Thi *et al.*, 2012, Jiang *et al.*, 2013). As HCV particle interacts with

lipoproteins, the LDL receptor (LDLR) has also been suggested to be involved in the early phase of HCV entry (Agnello *et al.*, 1999). However, this interaction may result in non-productive entry pathway and can potentially lead to viral particle degradation (Albecka *et al.*, 2012). After initial attachment step, and in addition to HSPG, SR-BI, and LDLR, several entry factors for HCV have been identified, including CD81, tight junction proteins claudin-1 (CLDN1) and occludin (OCLN), the receptor tyrosine kinases (RTK) epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2), Niemann-Pick C1-like 1 cholesterol absorption receptor (NPC1L1) (Evans *et al.*, 2007, Liu *et al.*, 2009, Lupberger *et al.*, 2011, Pileri *et al.*, 1998, Ploss *et al.*, 2009, Sainz *et al.*, 2012), and the two recently identified entry factors transferrin receptor 1 and E-cadherin (Q. Li *et al.*, 2016, Martin *et al.*, 2013).

1.7.1 Scavenger receptor class B type I

The SR-BI is a 509 aa cell surface glycoprotein receptor that is present on many cell types with higher expression within the liver (Meredith *et al.*, 2012). It has been identified as HCV E2 binding partner along with CD81(Pileri *et al.*, 1998, Scarselli *et al.*, 2002). SR-BI is involved in the lipid metabolism and is known to be a major receptor for high-density lipoproteins (HDLs) promoting its selective uptake into hepatocytes (Acton *et al.*, 1996). It can also bind other lipoprotein classes such as the VLDL and LDL (Van Eck *et al.*, 2008). A role of SRB1 in HCV entry was first suggested through its binding of HCV E2 HVR1 (Bartosch *et al.*, 2003b, Catanese *et al.*, 2007, Scarselli *et al.*, 2002). The lipid transfer capability of SRB1 may be required for HCV cell entry as HCV entry is enhanced by HDL and could be inhibited by oxidized LDL (Dreux *et al.*, 2009a, Meuleman *et al.*, 2012). It was shown that SR-BI mediates primary attachment of HCV particles that have intermediate density to cell surface and that these interactions require apolipoproteins, such as apoE (Dao Thi *et al.*, 2012). Recently it was shown that, introducing three specific mutations (Y507L, V514A, and V515A) located within E2 neutralizing epitope modulated HCV dependence on the viral receptor SRB1

(Lavie *et al.*, 2014). SRB1 antagonist was shown to inhibit HCV replication with additive to synergistic potency when used in combination with other antiviral therapeutics (Zhu *et al.*, 2012).

1.7.2 CD81

Human CD81 is a tetraspanin adaptor cell surface molecule that is broadly expressed in many cell types (van Spriel *et al.*, 2010). It is involved in many cellular functions including adhesion, morphology, proliferation and differentiation. It harbors four transmembrane domains, two short intracellular domains and two extracellular loops, named SEL and LEL (Meredith *et al.*, 2012). The involvement of CD81 in HCV entry is likely to be after the very early attachment phase (i.e. after SRB1), promoting a conformational change in the HCV E1/E2 glycoproteins. This conformational change facilitates low pH-dependent fusion and viral endocytosis (Sharma *et al.*, 2011). HCV, through its envelope glycoprotein E2, binds CD81 large extracellular loop to facilitate HCV entry (Petracca *et al.*, 2000, Pileri *et al.*, 1998). The CD81 large extracellular loop sequence is conserved between humans and chimpanzees. However, CD81 from other species, including species that are not permissive to HCV infection, can support HCV entry *in vitro* suggesting that CD81 sequence variability is not sufficient to define HCV susceptibility (Flint *et al.*, 2006).

1.7.3 Claudin 1 and Occludin

Claudin 1 and Occludin are two tight junction proteins that were identified after screening of a cDNA library of potential cellular factors implicated in HCV entry utilizing HCVpp (Evans *et al.*, 2007, Ploss *et al.*, 2009). Neither CLDN1 nor OCLN directly interact with HCV envelope. Although, CLDN1 may interact with CD81 as a part of the HCV receptor complex (Harris *et al.*, 2010, Harris *et al.*, 2008). It was suggested that CLDN1 and OCLN are involved in a later phase of HCV entry, after SRB1 and CD81, although their exact roles are still elusive. It was also shown that

HCV envelope glycoproteins promote co-endocytosis of CD81 and CLDN1 and fusion with early endosome (Farquhar *et al.*, 2012). It was shown that anti-CLDN1 monoclonal antibodies can inhibit HCV infection in primary hepatocytes *in vitro* as well as in cell lines by neutralizing the interactions between HCV E2 and CLDN1 (Fofana *et al.*, 2010, Krieger *et al.*, 2010). Synchronized infection assays showed that HCV utilizes CD81, Claudin-1, and Occludin in this order during the attachment and entry. In addition, downregulation of Occludin reduces both HCV entry and glycoprotein-mediated cell fusion (Sourisseau *et al.*, 2013).

1.7.4 Receptor tyrosine kinases

The two RTKs including the EGFR and EphA2 were identified as HCV entry co-factors using a functional RNAi kinase screen (Lupberger *et al.*, 2011). EGFR is known to regulate several key processes including cell proliferation, survival, and differentiation during development, tissue homeostasis and tumorigenesis (Schneider *et al.*, 2009). While EphA2 is involved in cell positioning, cell morphology, polarity and motility (Lackmann *et al.*, 2008). These RTKs appear to participate in HCV entry after the initial engagement step, by regulating CD81 and CLDN1 co-receptor interactions and viral E2-mediated membrane fusion by EGFR-dependent signaling pathways (Lupberger *et al.*, 2011). Indeed, the small GTPase HRas (Harvey Rat Sarcoma oncogene homologue) was identified as a host signal transducer that represents the physical link between the HCV–CD81–CLDN1 complex and the EGFR/Shc1/Grb2/HRas signaling pathway (Zona *et al.*, 2013). Stimulation of EGFR activates the Ras/MEK/ERK pathway which promotes MAPK interacting serine/threonine kinase 1 (MKNK1) activation. Later on, MKNK1 was identified as a host factor in HCV entry, which possibly act to facilitate the steps that are downstream of the EGFR (S. Kim *et al.*, 2013a).

1.7.5 Niemann-Pick C1-like 1 cholesterol absorption receptor

Niemann-Pick C1-like 1 cholesterol absorption receptor (NPC1L1) is a 13 transmembrane-domain cell surface cholesterol-sensing receptor expressed on the apical surface of intestinal enterocytes and human hepatocytes. NPC1L1 is responsible for cellular cholesterol absorption and whole-body cholesterol homeostasis (Castro-Torres *et al.*, 2014, L. Yu, 2008). HCV entry was shown to be inhibited by the available NPC1L1 antagonist ezetimibe which is FDA-approved to treat hypercholesterolemia (Sainz *et al.*, 2012). Based on an experiment comparing the susceptibility of HCVpp and HCVcc entry to ezetimibe, it was demonstrated that the cholesterol content of viral particles correlates with NPC1L1-mediated infection (Sainz *et al.*, 2012).

1.7.6 Transferrin receptor 1

Transferrin receptor 1 (TfR1) is the main receptor for cellular iron uptake into cells and is ubiquitously expressed in all tissues. After TfR1 binds to its extracellular ligand, iron-bound transferrin, the TfR1-trafficking protein (TTP) facilitates its internalization via clathrin-mediated endocytosis. Once iron is released in the cytoplasm, TfR1 will be recycled back to the cell surface (Tosoni *et al.*, 2005). It has been shown that HCV infection can alter the expression of TfR1. In addition, blocking of the cell surface TfR1 inhibits HCVcc and HCVpp infection, demonstrating that TfR1 acts at the level of HCV glycoprotein-dependent entry. The role of TfR1 in HCV entry was suggested to be beyond CD81 and might be specifically involved in HCV particle internalization (Martin *et al.*, 2013).

1.7.7 E-cadherin

E-cadherin, encoded by the CDH1 gene, is a major classical adherent junction protein. It is a transmembrane glycoprotein that plays an important role in maintaining cell-cell adhesion. In addition, it performs a vital role in establishing

epithelial architecture and maintaining cell polarity and differentiation (Stemmler, 2008, van Roy *et al.*, 2008). Recently, E-cadherin was identified as key modulator of HCV entry (Q. Li *et al.*, 2016). Depletion of E-cadherin has been shown to drastically diminishes the cell-surface distribution of the CLDN1 and OCLN in various hepatic cell lines. Thus, suggesting that E-cadherin plays an important regulatory role in CLDN1/OCLN localization on the cell surface (Q. Li *et al.*, 2016).

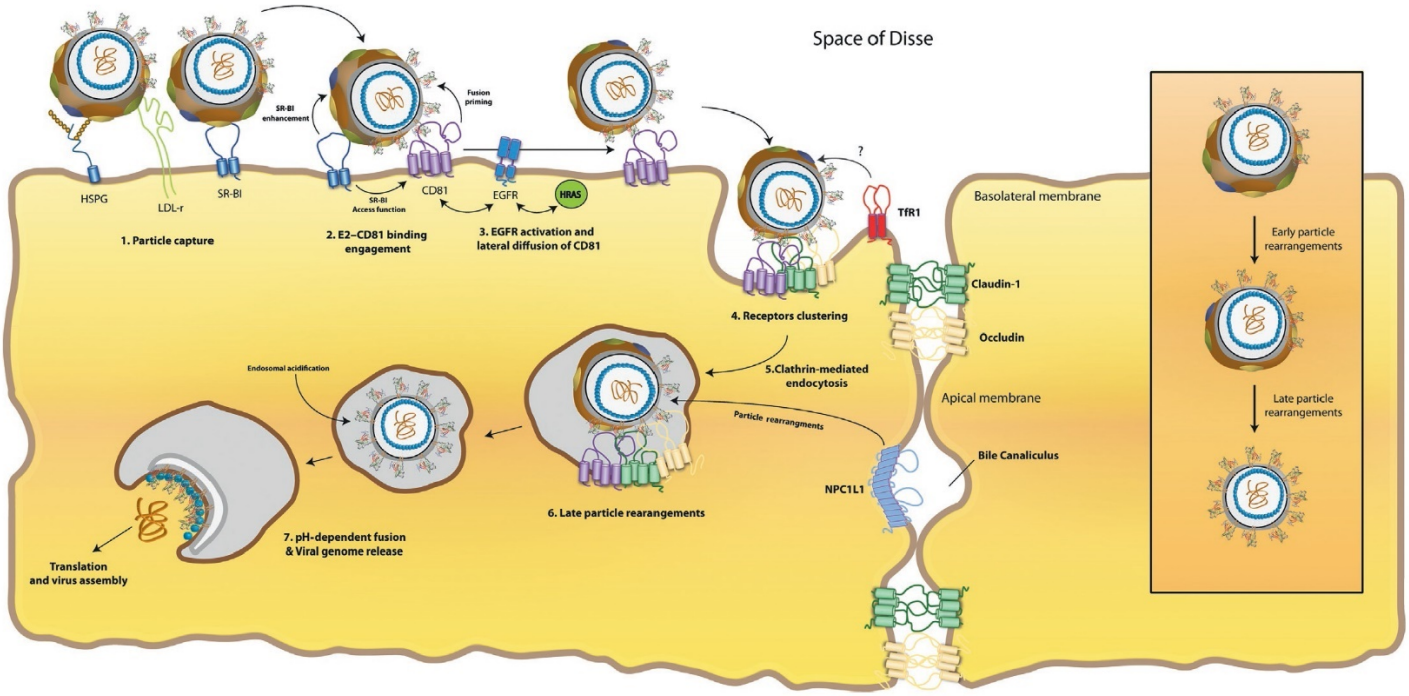


Figure 7. Model of HCV entry. The main HCV entry receptors and cofactors are indicated. The entry is composed of 7 main steps as indicated. (Douam *et al.*, 2015)

HCV entry to the cell occurs by clathrin-mediated endocytosis, a dynamin-dependent pinocytosis endocytic mechanism (Figure 7) (E. Blanchard *et al.*, 2006b). During this process, particle attachment induces the inward budding of the plasma membrane and the formation of a clathrin pit that internalizes the particle–receptor complex. Down regulation of the clathrin heavy chain was shown to inhibit

HCVpp and HCVcc infection (A. A. Blanchard *et al.*, 2006a). Moreover, internalization of CD81–Claudin-1 complexes through clathrin-dependent endocytosis has been observed during HCV infection. This was shown to be consistent with the fact that CD81–Claudin-1 association is critical for virus internalization and that virus internalization occurs prior to the fusion step (Farquhar *et al.*, 2012, Harris *et al.*, 2010, Harris *et al.*, 2008, Krieger *et al.*, 2010). Following endocytosis, HCV-receptor complexes migrate to the RAB5A-positive endosomal compartments. Fusion is thought to occur at these compartments to allow the release of HCV RNA (Coller *et al.*, 2012, Meertens *et al.*, 2006). The release of viral RNA is believed to be through membrane fusion which is mediated by endosomal acidification. This is based on the observation that the use of bafilomycin A1 which prevents the endosomal re-acidification, has been shown to block HCV infection (Meertens *et al.*, 2006, Sharma *et al.*, 2011). By using transdominant-negative mutants in an HCVpp experiment, it has been shown that HCV membrane fusion occurs in an early endosomal compartment that was not positive for RAB7 (Meertens *et al.*, 2006). Few evidences suggested that the interaction of HCV with CD81 may trigger a conformational change permitting low pH-induced membrane fusion (Sharma *et al.*, 2011). Altogether, the mechanisms behind the activation of HCV for low pH-induced fusion, the fusion step, and the identity of the fusion peptide(s) are still unknown.

1.8 HCV replication and membranous web formation

A hallmark of all positive-strand RNA viruses is the induction of extensive cytoplasmic membrane proliferation and remodeling (Romero-Brey *et al.*, 2014). Depending on the virus type, the induced membrane structures derived from either ER, Golgi apparatus, mitochondria or even lysosomes provide suitable microenvironments for viral replication. The exact role of membranes in viral RNA synthesis is not understood. Evidences suggest that they may serve to increase local concentration of host and viral factors required for efficient RNA replication,

to facilitate spatial coordination of different steps of the viral replication cycle, and to protect viral RNA and proteins from degradation and immune defense (Paul *et al.*, 2013, Paul *et al.*, 2014, Romero-Brey *et al.*, 2015). The induced membrane rearrangement by positive-strand RNA viruses can be confined to two main morphological subclasses: the invaginated vesicle/spherule type within the ER and the double-membrane vesicle (DMV) type (Chatel-Chaix *et al.*, 2014, Romero-Brey *et al.*, 2014). Unlike other related flaviviruses, HCV is known uniquely to induce a matrix of DMVs, designated membranous web (MW) (Figure 8). EM analysis of HCV polyprotein expressing cells in recent studies revealed that the diameter of these DMVs is with an average of 150 nm. and contain active replicase complexes, supporting the model that they form to support viral RNA replication (Paul *et al.*, 2013, Romero-Brey *et al.*, 2012). The outer membranes of ~50% of DMVs connect to the ER membrane via a neck-like structure. Biochemical analyses of purified vesicles revealed the presence of ER calnexin and calreticulin proteins, confirming the ER as a major source of membranes for MW formation (Ferraris *et al.*, 2010, Miyanari *et al.*, 2003)

For a period of time, it was believed that viral NS4B protein was responsible for the induction of MW formation during HCV infection since its sole expression can induce massive membrane rearrangements (Egger *et al.*, 2002). Moreover, mutations in NS4B inhibiting self-interaction affect DMVs morphology and block HCV RNA replication (Paul *et al.*, 2011). However, by using a replication-independent polyprotein expression approach, it has been found that NS5A was the only protein capable of inducing DMVs, while only single-membrane vesicles (SMVs) were observed in NS3/4A-, NS4B-, and NS5B-expressing cells (Reiss *et al.*, 2011, Romero-Brey *et al.*, 2012). The contribution of SMVs to HCV replication is unknown, but one model postulates that local, HCV-induced exvaginations form SMVs and, while the vesicles remain attached to the ER, a secondary invagination produces DMVs (Romero-Brey *et al.*, 2012). Since none of the HCV proteins expressed solely was capable of inducing a MW, this suggests that MW formation most likely requires a concerted action of the replicase proteins.

Several host cell factors are also involved in this membrane remodeling. Among these critical players is the prolyl-peptidyl *cis-trans* isomerase cyclophilin A (CypA) that binds to the D2 domain of NS5A. CypA has been shown to be critical for RNA replication and its pharmacological inhibition has been shown to block *de novo* formation of DMVs (Madan *et al.*, 2014). In addition to CypA, NS5A also associates with phosphatidyl-inositol-4-kinase-IIIα (PI4KIIIα) and induces the accumulation of PI4P within the MW (Figure 8). Knockdown of PI4KIIIα expression or NS5A mutations abrogates this interaction cause size reduction and aggregation of DMVs, strongly correlating with serious defects in RNA replication (Reiss *et al.*, 2013).

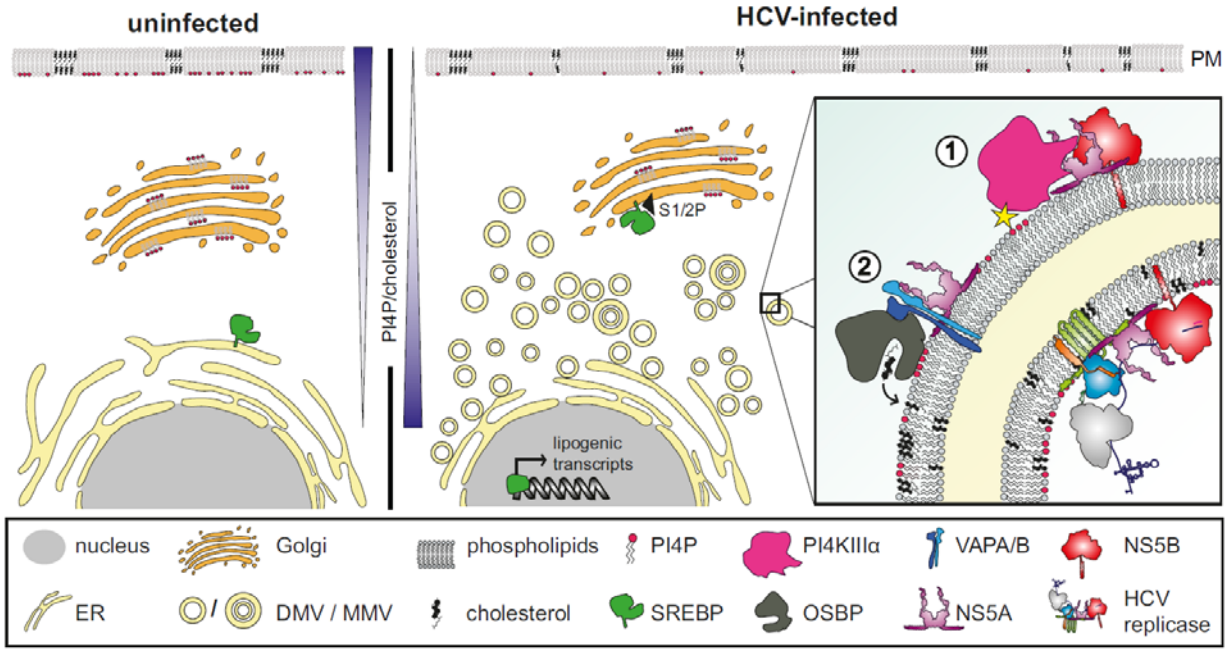


Figure 8. HCV induced membranous web formation. HCV induces a massive remodeling of intracellular membranes, the majority of which are double-membrane vesicles (DMVs) while multimembrane vesicles (MMVs) are less frequent. (1) The interaction of PI4KIIIα with NS5A and NS5B induces high levels of PI4P. (2) The oxysterol-binding protein (OSBP) traffics cholesterol to HCV-induced MW which is facilitated by the increase of PI4P levels and the interactions with VAPs and NS5A. (Paul *et al.*, 2014)

1.9 HCV assembly and release

HCV assembly is the complex process of packaging viral genome into nucleocapsid and their subsequent envelopment. This process is dependent on

cytoplasmic lipids droplets (cLDs), which are organelles responsible for neutral lipid storage and involved in many cellular processes (McLauchlan, 2009). cLDs are composed of a neutral lipid core consisting of triglycerides and cholesterol esters, surrounded by a phospholipid monolayer likely derived from the ER outer leaflet. Within the MW in a position juxtaposed to the ER as revealed by EM, cLDs are regarded as platforms for HCV virion assembly (Miyanari *et al.*, 2007).

Two HCV proteins, the core and NS5A, associate with cLDs during virion assembly. On one hand, the core recruits other viral proteins and host cell factors involved in regulation of infectious particle production to the periphery of cLDs. On the other hand, NS5A has an intrinsic cLD targeting sequence and may act to traffic viral RNA out of DMVs to the cLD surface where the encapsidation by core is initiated (Appel *et al.*, 2008, Filipe *et al.*, 2015). Interestingly, the transfer of core and NS5A to cLDs is linked via common host factors (e.g., diacylglycerol acyltransferase-1 (DGAT1), Rab18 and apolipoprotein J) (Filipe *et al.*, 2015). The process of infectious HCV particles assembly appears to be orchestrated by NS2 that interacts on one hand with p7 and the envelope glycoproteins (E1 and E2) and on the other hand with NS3, which is part of the viral replicase (Lindenbach, 2013). Thus, NS2 might link the RNA-containing replicase with the machinery required for envelopment of HCV particles. Moreover, p7 appears to be involved in capsid envelopment and may protect virus particles against acidic pH during passage through the secretory pathway.

In addition to cLDs, HCV assembly also relies on host cell machineries, such as components of the low-density lipoprotein pathway (H. Huang *et al.*, 2007a). This is consistent with the nature of HCV particles circulating in the blood as the LVP (Andre *et al.*, 2002).

During egress, HCV particles depend on p7 to neutralize acidic compartments within the secretory pathway. The virus particles might undergo post-synthetic lipidation, similar to the VLDL assembly pathway, since the intracellular HCVcc particles have a higher buoyant density than extracellular particles and acquire their low buoyant density in a post-ER compartment (Gastaminza *et al.*, 2008). Recently, it has been shown that, instead of being packaged into nucleocapsid,

HCV RNA can be released within exosomes and transferred between cells (Bukong *et al.*, 2014). This suggests that exosomes mediate viral transmission of HCV to cells in a receptor-independent manner.

2 AUTOPHAGY

2.1 The autophagy machinery

Autophagy (literally “self-eating”) is an intracellular degradation process that mammalian cell performs in order to survive under stress conditions related to nutrition starvation or infection (Deretic *et al.*, 2009). The canonical autophagy process can be divided in six sequential steps: (1) induction, (2) membrane nucleation, (3) membrane expansion and autophagosome formation, (4) fusion with the lysosome, and (5) degradation (Figure 9). In the initial step of autophagy induction (initiation and vesicle nucleation), the ULK/Atg1 complex activates the class III phosphatidylinositol 3 kinase complex, which recruits a series of autophagy-related genes (ATG) proteins to the phagophore (isolation membrane) which originates either via *de novo* lipid synthesis or from preexisting organelles (Green *et al.*, 2014, Simonsen *et al.*, 2009). Subsequently, the edges of these phagophores elongate to sequester part of the cytoplasm, leading to the formation of double-membrane vesicles, known as autophagosomes.

During phagophore elongation and vesicle completion, two ubiquitin-like conjugation systems (Atg12-conjugation and microtubule-associated protein 1A/1B light chain 3 (LC3)-conjugation system) control the covalent conjugation of Atg5 to Atg12 and the conversion of LC3 (LC3-I) to its phosphatidylethanolamine-conjugated form (LC3-II) (Ohsumi, 2001). LC3-II is generated by a process resembling ubiquitination that involves E1, E2, and E3 ligases. The LC3-I is generated by the action of a protease, ATG4, which cleaves LC3 to form LC3-I. This is bound by the E1, ATG7, and transferred to the E2, ATG3 which facilitate the lipidation of LC3-I forming the membrane bound form LC3-II with the help of the E3 ligase. The E3 ligase is a complex composed of ATG16L1 and ATG5-12 (named as the elongation complex); the latter is produced by another reaction in

which ATG12 is bound by the E1, ATG7, transferred to a different E2, ATG10, and from there to ATG5. The process by which ATG12-5 and LC3-II are formed is referred to as the conjugation reaction and is required for the formation of the autophagosome.

Once the autophagosome is formed, the outer membrane of the mature autophagosome fuses with the lysosome to form an autolysosome allowing the degradation of the sequestered cytoplasmic material by the action of lysosomal enzymes (Levine *et al.*, 2008). The precise mechanism that mediates this fusion event has been recently identified, and involves soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes, including STX17, SNAP29, and VAMP8 (Itakura *et al.*, 2012b). The fusion between autophagosomes and lysosomes is regulated by the adaptor protein SNAP29 through two-way interactions, with SNAP29-STX17 located on the autophagosome membrane and SNAP29-VAMP8 located on the lysosome membrane. In addition, Rab7, a small GTPase belonging to the Ras-like GTPase superfamily, has been shown to modulate autophagosome-lysosome fusion (Gutierrez *et al.*, 2004, Jager *et al.*, 2004). Recently, Pleckstrin homology domain containing protein family member 1 (PLEKHM1), a multivalent endocytic adaptor protein that links LC3 to HOPS and Rab7, and ATG14 were found to be critical for the maturation of autophagosomes (Diao *et al.*, 2015, McEwan *et al.*, 2015). Interestingly, in a recent study it has been shown that the mechanistic target of rapamycin (mTORC1), a known negative regulator of early autophagy events, regulates autophagosome maturation by phosphorylating UV radiation resistance-associated gene product (UVRAG) protein (Y. M. Kim *et al.*, 2015)

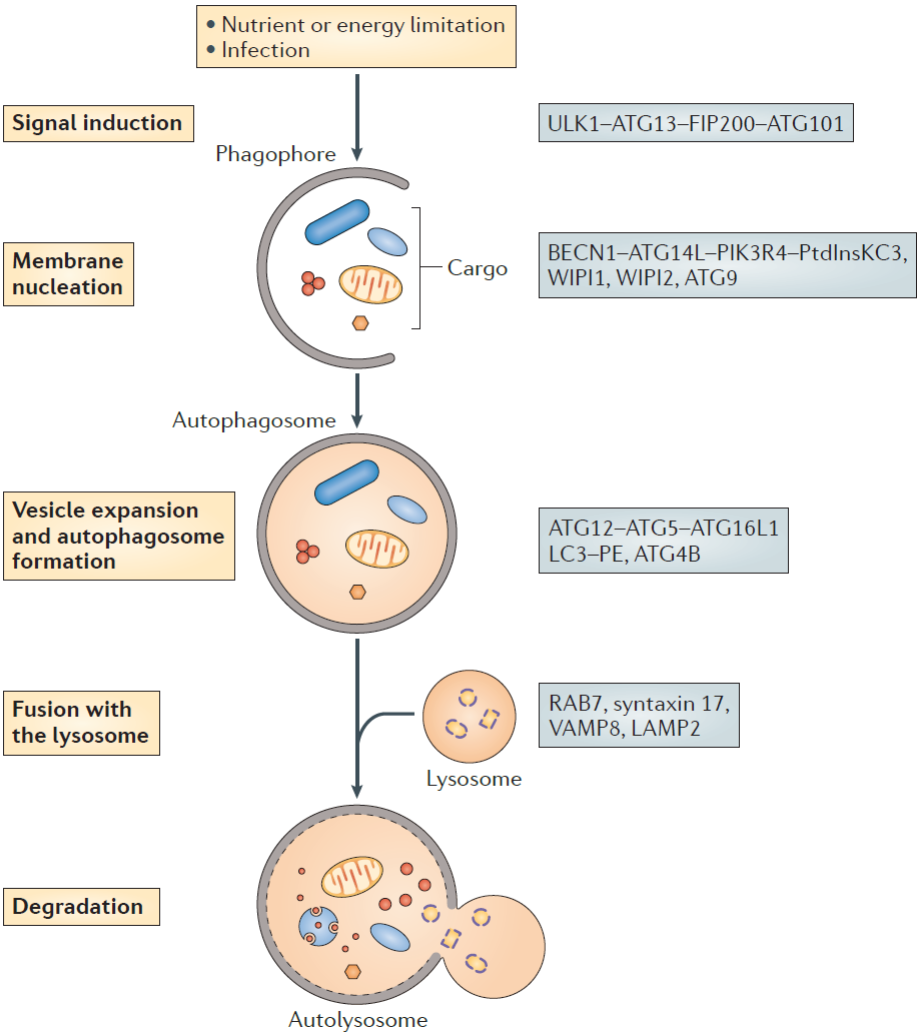


Figure 9. Scheme of autophagy mechanism. Autophagy is initiated with the formation of limiting membrane, phagophore. Cargo sequestration occurs either in bulk or selective manner. After engulfment, the sealed autophagosome fuses with lysosome to deliver the cargo where its degradation takes place by lysosomal hydrolases.(J. Huang *et al.*, 2014)

2.2 The non-canonical autophagy

Canonical autophagy involves the sequential activity of defined molecular complexes, whose roles and importance have been described earlier. However, it has been revealed that the formation of functional autophagosomes can bypass certain steps, eliminating the use of particular molecular autophagy complexes (Figure 10) (Codogno *et al.*, 2011). For example, LC3 lipidation still occurs in *ulk1/(atg1) and ulk2* knockout mouse embryonic fibroblasts (MEFs), suggesting

that the ULK1 complex is not essential for activation of the LC3 conjugation machinery and that the autophagy events can be initiated in an ULK1-independent manner (Cheong *et al.*, 2011, Wong *et al.*, 2013). In addition, Atg5- and Atg7-independent autophagy has also been described, which does not involve LC3 conversion, and might specifically involve the formation of autophagosomes from late endosomes and the *trans*-Golgi network (Figure 10) (Nishida *et al.*, 2009). Recently, Atg5- and Atg7-independent autophagy has been shown to be required for clearance of mitochondria in reticulocytes (Honda *et al.*, 2014)

The discovery of alternative, autophagy pathways suggests that autophagic processes are more diverse than initially anticipated. Concomitantly, depending on the stimuli and cellular condition, only a subset of autophagy-related complexes/proteins may be activated at one time to degrade and recycle material.

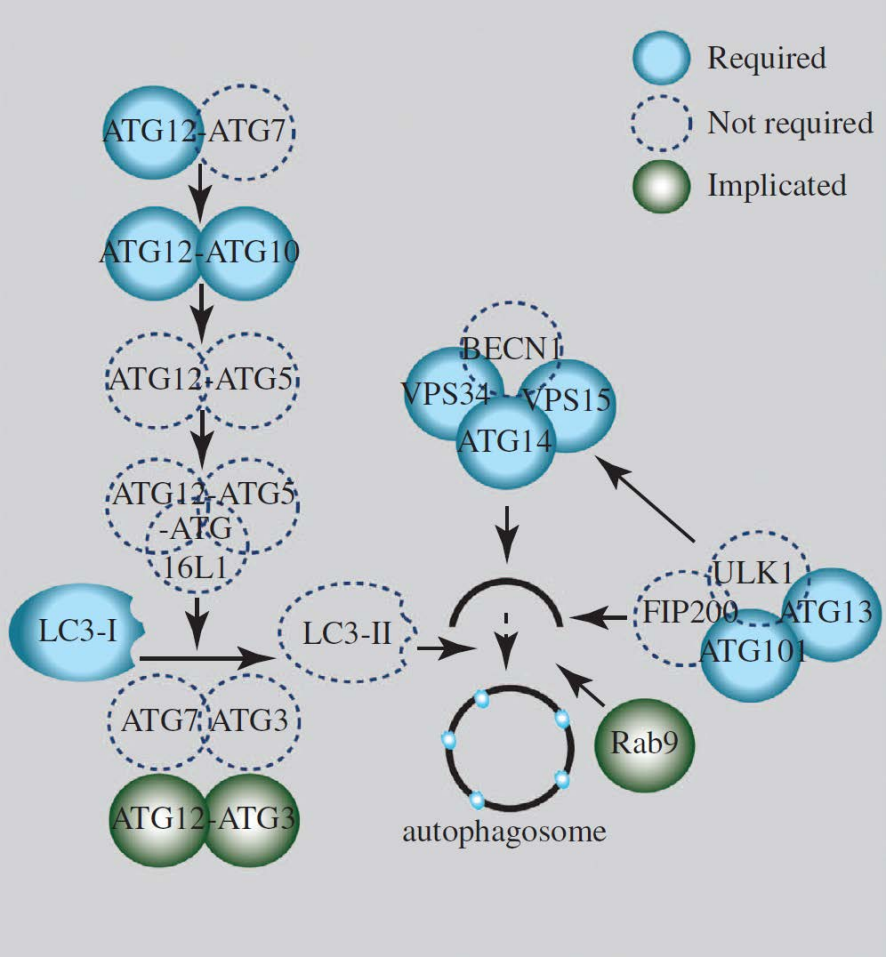


Figure 10. Non-canonical autophagy. The autophagic proteins that has been proposed to participate in non-canonical autophagy are in blue, while proteins that are not required are transparent. Proteins that are required for certain forms of non-canonical autophagy are in green (Lindqvist *et al.*, 2015).

2.3 Selective autophagy

In contrast to the canonical autophagy, which is characterized by random sequestration of cytosolic contents, selective autophagy operates under nutrient-rich conditions and is marked by the presence of specialized autophagosomes confining substrates such as damaged or superfluous organelles, including mitochondria and peroxisomes, as well as invasive microbes in a selective manner (Yang *et al.*, 2010). Each specific process involves a core set of machinery, as well

as specific components, and accordingly is identified with a unique name. For example, selective degradation of mitochondria by autophagy is termed mitophagy, xenophagy for pathogens, pexophagy for peroxisomes, aggrephagy for aggregated proteins, *etc.* (Figure 11) (Deffieu *et al.*, 2009, Dunn *et al.*, 2005). The selectivity relies on autophagy receptors which link the cargo, through their degradation signals, to the autophagosomal membrane, leading to the engulfment of cargo by the autophagosome (Johansen *et al.*, 2011). In yeast, five receptors have been described so far to mediate cargo selection: Atg19 and Atg34 (Cvt pathway), Atg32 (mitophagy), Atg36 and Atg30 (pexophagy), some of which have homologues in higher eukaryotes (Meijer *et al.*, 2007). In mammalian cells, more than 20 autophagy receptors were identified by the yeast two-hybrid system and proteomic approaches (Behrends *et al.*, 2010, Kirkin *et al.*, 2009). The most prevalent autophagy targeting signal in mammals is the modification of cargos with ubiquitin (Ub), a small conserved protein that when originally added to a protein signals its degradation via proteasomes (Kirkin *et al.*, 2007). The efficiency of ubiquitylation, as a cargo signal, is both critical and sufficient to induce their autophagic clearance through binding to Ub-binding domains (UBDs) that are currently identified in most of autophagy receptors (Wild *et al.*, 2014). In contrary, selective autophagy receptors in yeast do not use Ub modifications as a degradation signal which is similar to few mammalian autophagy receptors like NIX or BNIP3, which are located at the outer mitochondrial membrane (J. Zhang *et al.*, 2009).

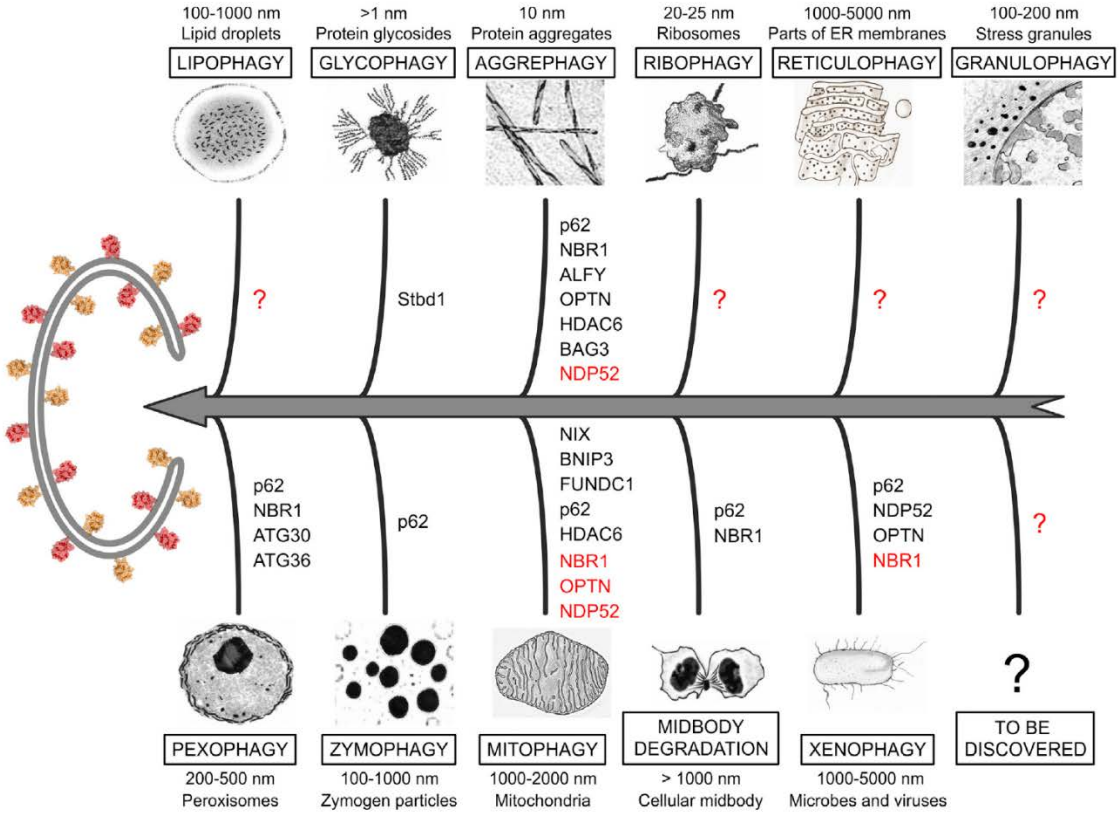


Figure 11. Different types of mammalian selective autophagy and their respective receptors. Receptors in black are well established selective autophagy receptors, while receptors in red are putative selective autophagy receptors (Rogov *et al.*, 2014).

3 AUTOPHAGY AND VIRUSES

Autophagosomes were first observed during viral infection in 1965 when Dales and colleagues observed the presence of vesicles with two lipid bilayers late in poliovirus infection using EM (Dales *et al.*, 1965). They noticed the existence of virions near and often within, these vesicles. The authors suggested that these vesicles, termed at that time autolytic vesicles, appear as a secondary response to infection. Similar vesicles, named membrane-vesicle complexes, were later observed by EM of mouse pancreas infected with Coxsackie virus, suggesting that these structures, autophagosomes, were present during infection of multiple picornaviruses (Burch *et al.*, 1979).

3.1 Autophagy as anti-viral response

The hypothesis that autophagy would engulf and destroy pathogens has proven to be true for several pathogens and the process has been termed xenophagy (Paulus *et al.*, 2015). In the case of viruses, it has been shown that binding partner of the anti-apoptotic regulatory protein Bcl-2, Beclin 1, a homologue of the yeast ATG6, when expressed from a recombinant Sindbis Virus, reduces virus load and protects mice from fatal encephalitis (X. H. Liang *et al.*, 1999, X. H. Liang *et al.*, 1998). In addition, several viruses encode a Beclin 1-binding protein, such as the Herpes Simplex Virus 1 (HSV1) protein, ICP34.5, (Orvedahl *et al.*, 2007). Indeed, ICP34.5 binding to Beclin 1 inhibits the formation of autophagosomes in neurons, suggesting that the virus has evolved to actively inhibit autophagy. Other viral proteins inhibiting autophagy through Beclin 1 binding such as the Kaposi's sarcoma-associated herpesvirus (KSHV) open-reading-frame 16 protein and the murine gammaherpesvirus-68 M11 protein have been reported (Ku *et al.*, 2008, M. Su *et al.*, 2014). In addition to ICP34.5's Beclin 1 binding, HSV US11 protein inhibits autophagy through direct interaction with the PKR kinase, suggesting that HSV encodes at least two proteins capable of inhibiting autophagy, which may indicate the antiviral role of autophagy against this particular virus (Lussignol *et al.*, 2013).

In xenophagy, the STimulator of INterferon Genes (STING), a transmembrane protein that act as xenophagy effector, senses dsDNA viruses and directs them for autophagic degradation (Barber, 2014). In addition, STING induces type I IFN, which suggests a role for autophagy in cell-to-cell immune signaling. Degradation of viral antigens via xenophagy can feed into the MHC Class II presentation pathway as well, indicating that xenophagy can play a role in both cell clearance of pathogens and prolonged presentation of antigen from those pathogens (Figure 12) (Paludan *et al.*, 2005).

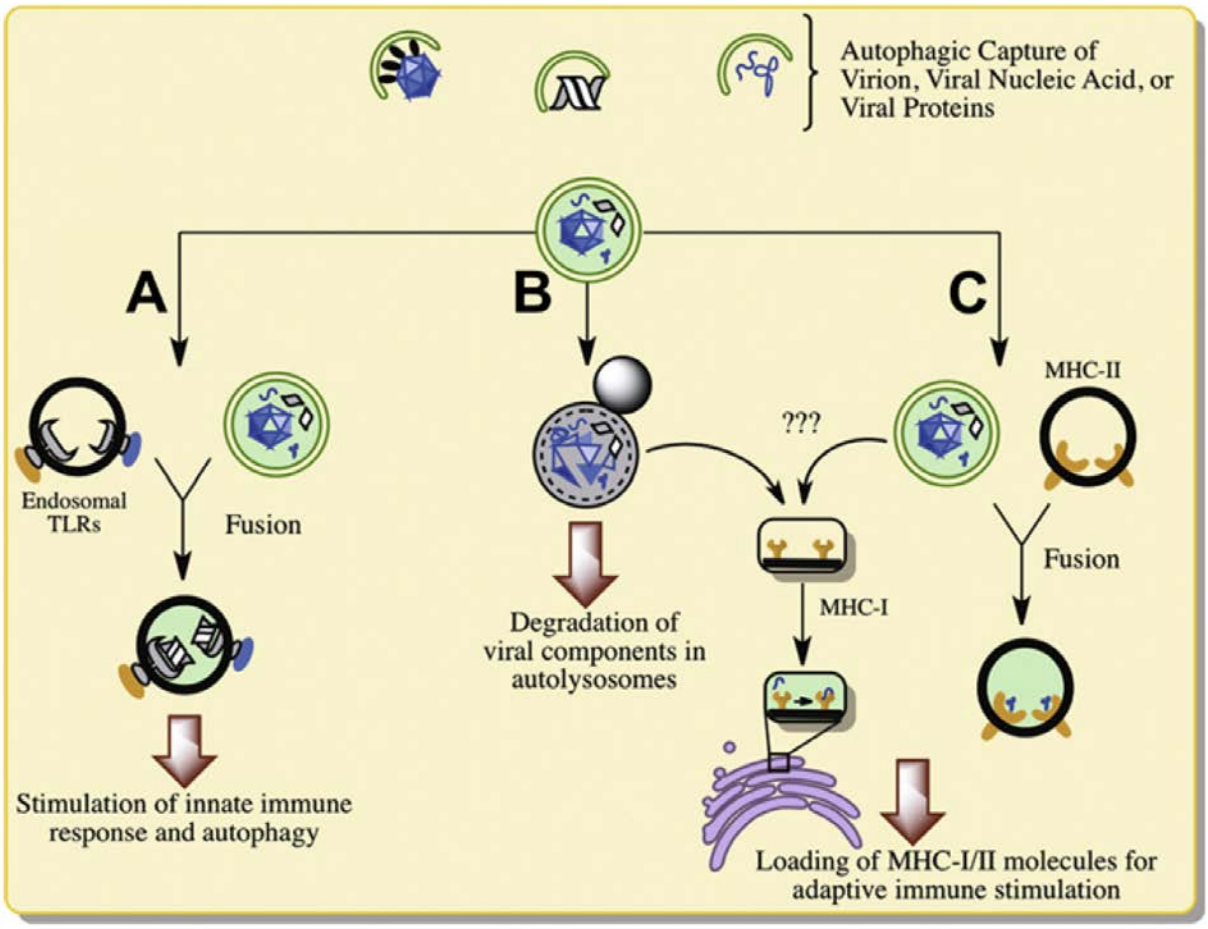


Figure 12. The antiviral role of autophagy. Autophagy can limit viral replication and spread by the direct capture of virions, viral genome, or viral proteins, and the subsequent delivery of these to their respective compartments. A) Viral genome will be delivered to endosomal TLRs to induce innate immune sensors which, in turn, can also stimulate autophagy. B) Fusion of autophagosome, containing captured virions, with the lysosome which will lead to direct virion degradation. C) The autophagosome can transport viral antigens to MHC-I and -II complexes to stimulate antigen presentation (Jordan *et al.*, 2012).

3.2 Autophagy in viral immune response

The anti-viral capacity of autophagy in systemic immunity varies depending on the overall environment of the host and type of viral infection. Toll-like receptors (TLRs) are involved in detecting virus components, such as single- and double-stranded viral RNAs (Zeng *et al.*, 2012). Intracellular sensors such as retinoic acid-inducible gene-1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA-5), all named RIG-I-like receptors (RLRs), can also detect viral nucleic acids in the

cytoplasm (Loo *et al.*, 2011) . Several TLRs are known to activate innate responses during viral infections, including TLR2, TLR3, TLR4, TLR7 and TLR8. TLR-dependent autophagy has been shown to be required for an antiviral response against Rift Valley fever virus, Vesicular Stomatitis virus, and other viruses (Figure 12) (Moy *et al.*, 2014).

The autophagy machinery has also been shown to be required for a successful IFNγ response against murine norovirus (MNV). Interestingly, this effect does not involve autophagic degradation (S. Hwang *et al.*, 2012). In addition, evidence suggests that placental trophoblasts protect the fetus from viruses by secreting exosomes that contain C19MC miRNAs, the largest known miRNA cluster, which are capable of inducing autophagy in neighboring cells to confer anti-viral resistance (Delorme-Axford *et al.*, 2013).Surprisingly, in this specific case, autophagy is anti-viral even for viruses which have been shown to be resistant to the anti-viral effects of autophagy, suggesting that a cell tropism governs the nature of the relationship between viruses and the autophagy.

3.3 Virus subversion of autophagy

Similar to evasion of the innate immune response, some viruses have developed strategies to defeat the antiviral effects of autophagy. Viruses have been reported to interfere with autophagy mechanism at two checkpoints. Either early during autophagosome formation or at the step of autophagosome fusion with late endosomes or lysosomes. HSV-1 ICP34.5 represents an example of viruses that inhibit early checkpoint of the mechanism by blocking the induction of autophagy by binding Beclin 1, thereby contributing to HSV-1 neurovirulence (Leib *et al.*, 2009, Orvedahl *et al.*, 2007). In addition, ICP34.5 blocks PKR/eIF2a signaling via dephosphorylation of eIF2a, thus employing a second mechanism to inhibit autophagosome formation, which is induced by PKR/eIF2a signaling (Talloczy *et al.*, 2002, Talloczy *et al.*, 2006). Other viruses including murine gamma-herpesvirus 68 (gHV68) and Kaposi's Sarcoma-associated herpesvirus (KSHV)

also encode a Beclin-1-interacting protein, v-Bcl2, that prevents autophagy at the step of autophagosome formation (Ku *et al.*, 2008, Pattingre *et al.*, 2005, Sinha *et al.*, 2008). On the other hand, HSV encodes for another protein, US11, that inhibits autophagy without interacting with Beclin 1.(Lussignol *et al.*, 2013). Moreover, KSHV also inhibits autophagy in a BECN1-independent manner by the action of its K7 protein, which blocks autophagosome maturation and fusion with lysosomes (Q. Liang *et al.*, 2013). A beta-herpesvirus, the human cytomegalovirus (HCMV) also inhibits autophagosome formation in primary human fibroblasts, possibly by stimulating the mTOR signaling pathway in infected cells.

Another example for viruses that subvert autophagy is influenza A virus (IAV). This subversion has been found to be dependent on the viral Matrix 2 (M2) ion-channel protein, which blocks fusion of autophagosome with lysosome (Gannage *et al.*, 2009).

3.4 Autophagy as a proviral mechanism

In contrast to DNA viruses, most of which inhibit autophagosome formation, RNA viruses often induce the accumulation of autophagic vesicles. As an intracellular membranous structure, autophagy has been proposed to act as a scaffold for replication factories of RNA viruses that are known to replicate and assemble in the cytoplasm or to support viral particle release (Figure 13). It has been shown that viral RNA replication/transcription complexes of nidoviruses, such as equine arteritis virus and coronaviruses (coV), are found anchored on DMVs which have two membranous layers and a size similar to autophagosomes (Figure 13).(Goldsmith *et al.*, 2004, Gosert *et al.*, 2002, Pedersen *et al.*, 1999, Posthuma *et al.*, 2008, Snijder *et al.*, 2001). Although these observations remain controversial, DMVs in cells infected with corona viruses like mouse hepatitis virus (MHV) or Severe Acute Respiratory Syndrome (SARS)-CoV have been shown to contain viral components and to be positive for the autophagic marker, LC3II (Prentice *et al.*, 2004a, Prentice *et al.*, 2004b, Snijder *et al.*, 2006). In rota virus

infection, NSP4, a viral protein involved in virus replication and in budding of immature double- layered particles into the lumen of ER, co-localizes with LC3 and NSP5, a marker of rota virus RNA replication site (Berkova *et al.*, 2006). It is noteworthy that, in certain cases, the autophagy machinery is not necessarily involved in the formation of DMVs. As an example, the autophagy regulators Beclin1 and Atg5 are not involved in the formation of DMVs that contain immature vaccinia virions (H. Zhang *et al.*, 2006). Additionally, it has been reported that Atg5 is not required for replication and release of MHV, and that the morphological analysis did not show any differences in the structure of the intracellular membranes regardless of the presence or absence of Atg5 in MHV-infected primary macrophages and low passage of primary MEFs, despite of the controversy in this regard (Stertz *et al.*, 2007, Zhao *et al.*, 2007). Nevertheless, it is still possible that MHV induces DMVs formation by hijacking only part of the autophagy machinery.

Dengue virus (DENV), a single-stranded RNA virus, stimulates autolysosome formation during infection (Y. R. Lee *et al.*, 2008, Panyasrivanit *et al.*, 2009). Induction or inhibition of autophagy by using pharmacological drugs modulate DENV infection suggesting that the autophagy machinery is required for viral replication (Y. R. Lee *et al.*, 2008). In cell infected with DENV, LC3II colocalizes with the DENV non-structural protein NS1 and with double-stranded RNA, suggesting the existence of replication complexes in autophagic vesicles (Panyasrivanit *et al.*, 2009). It has been also shown that DENV induction of autophagy regulates lipid metabolism by processing LDs and triglycerides to release free fatty acids and production of ATP (Figure 13) (Heaton *et al.*, 2010). A recent study has showed that autophagy-associated DENV vesicles can be released from infected cells and are capable of infecting new cells (Wu *et al.*, 2016). These vesicles contained viral proteins E, NS1, prM/M, and viral RNA, as well as host LDs and LC3-II. The authors suggested that the virus uses this vesicle-mediated transmission to evade the neutralizing antibodies (Wu *et al.*, 2016). It is important to note that, while inhibition of autophagic and lysosomal membrane fusion increased DENV serotype 2 yield, it resulted in decreased production for

DENV serotype 3 (Khakpoor *et al.*, 2009, Panyasrivanit *et al.*, 2009). This sheds light on the complex relation between the autophagy machinery and DENV infection that could be determined, at least in part, by viral factors whose roles vary between DENV serotypes.

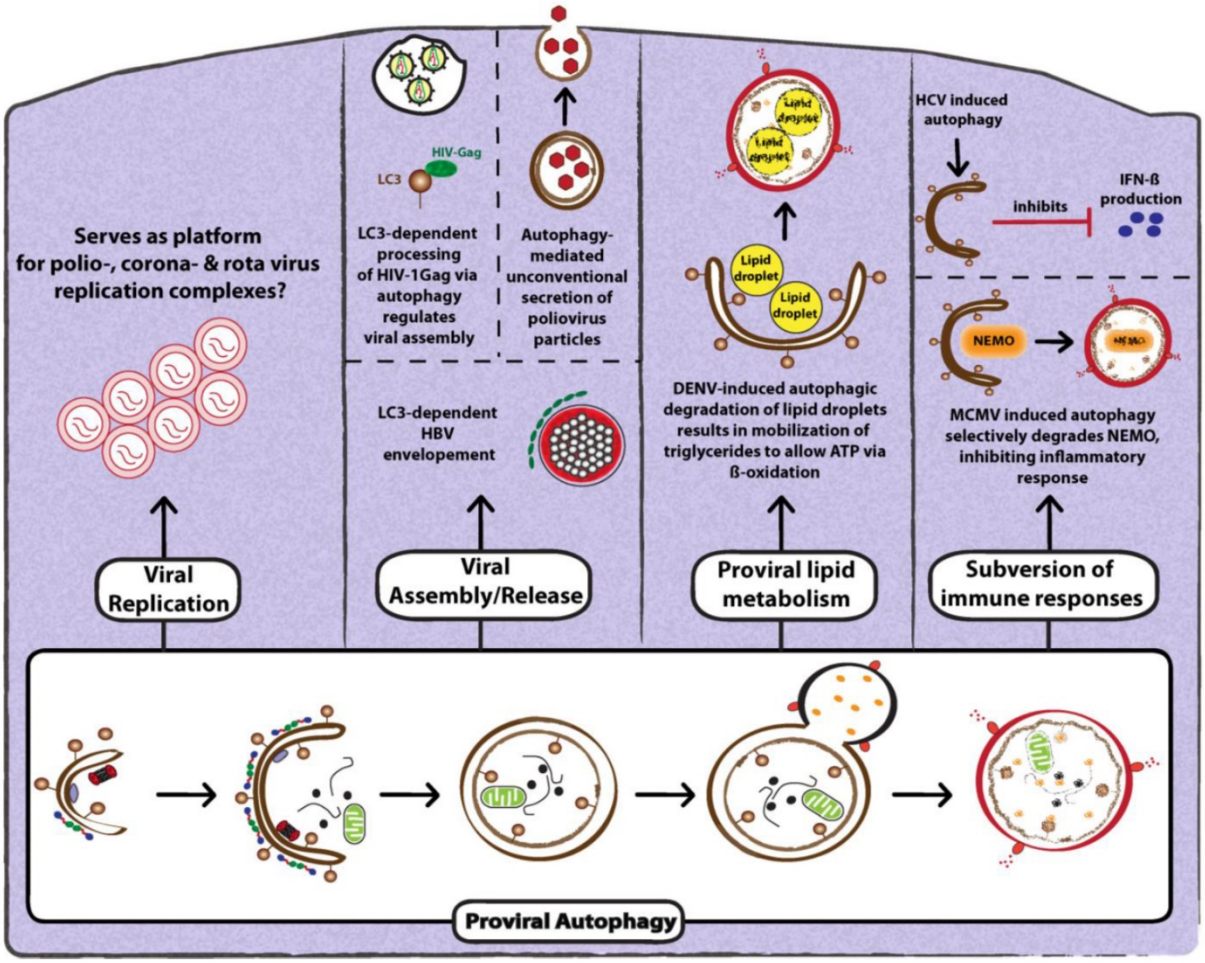


Figure 13. The proviral activity of autophagy. By harnessing autophagosomes as membrane platform for viral replication complexes or to mediate virus assembly and release. In addition, viruses can trigger selective autophagy to degrade either lipids (LDs) for energy production during viral replication or to subvert immune responses via selectively degrading specific regulatory molecules (Chiramel *et al.*, 2013)

3.5 Autophagy and HCV

In the past few years, many research articles have been published to show that HCV could induce autophagy to support its own replication (mostly summarized in table 2). It could induce LC3 lipidation and the accumulation of autophagic vesicles

in immortalized primary human hepatocytes, Huh7 hepatoma cells and the derivatives of Huh7 cells. This induction has been shown to be independent of HCV genotypes (Ait-Goughoulte *et al.*, 2008, Dreux *et al.*, 2009b, Sir *et al.*, 2008, Tanida *et al.*, 2009). The induction of autophagic vesicles was observed in the hepatocytes derived from patients chronically infected by HCV (Rautou *et al.*, 2011, Vescovo *et al.*, 2012). Moreover, this induction was shown in cells either transfected by the HCV RNA or infected by HCV, and also in cells harboring the replicating HCV subgenomic RNA replicon (Ke *et al.*, 2011, Mizui *et al.*, 2010, Shrivastava *et al.*, 2012, Sir *et al.*, 2012, Taguwa *et al.*, 2011, L. Wang *et al.*, 2015). Sir *et al*. reported that HCV JFH1, a genotype 2a strain, induced the accumulation of autophagosomes in Huh7 cells. In addition, they observed that the fusion between autophagosomes and lysosomes was perturbed. This has raised the question regarding whether HCV is able to induce a complete autophagic flux (Sir *et al.*, 2008). However, it was subsequently shown that HCV could efficiently induce the fusion between autophagosomes and lysosomes and allow a complete autophagic flux (H. Huang *et al.*, 2013, Ke *et al.*, 2011). In a more recent report the authors proposed an explanation to why HCV induced incomplete autophagy in some studies but complete autophagy in others. They noticed that the maturation of autophagosomes in HCV-infected cells was temporally regulated (L. Wang *et al.*, 2015). In this study, the maturation of autophagosomes was inefficient early in HCV infection while it was efficient in the late stage of infection. The author attributed this temporal regulation to the differential induction of Rubicon and UVRAG by HCV, which negatively and positively regulate the maturation of autophagosomes, respectively (C. Liang *et al.*, 2008, Matsunaga *et al.*, 2009, Sun *et al.*, 2010). They showed that the induction of Rubicon by HCV infection preceded the induction of UVRAG. This has led to an inhibition of the fusion between autophagosomes and lysosomes at the beginning and subsequently accumulation of the autophagosomes. This inhibition was reversed in the later stage of infection when the expression of UVRAG exceeded that of Rubicon (L. Wang *et al.*, 2015). It is noteworthy that different HCV genotypes had also been

shown to act differently on the maturation of autophagosomes (Taguwa et *al.*, 2011).

HCV had also been shown to induce selective removal of mitochondria by mitophagy. Indeed, Siddiqui and colleagues have shown that HCV could induce the expression of PINK1 and Parkin and cause the perinuclear clustering of mitochondria and the translocation of Parkin to mitochondria to initiate mitophagy (S. J. Kim *et al.*, 2013b). They suggested that the fission of mitochondria enhances HCV replication and attenuates cellular apoptosis (S. J. Kim *et al.*, 2014, S. J. Kim *et al.*, 2013b). Kurt and colleagues have reported that HCV could also induce Chaperon-mediated autophagy (CMA) by promoting the expression of LAMP2A, a CMA receptor, and HSC70, a cytosolic chaperone required for CMA. Silencing of CMA machinery enhanced innate antiviral response and restored degradation of interferon-alpha receptor-1 expression (Kurt *et al.*, 2015).

Table 2. Summary of HCV-autophagy interactions modified from (Ke *et al.*, 2014)

HCV genotype	Expression	Model	Analysis of autophagy activation	Physiological significance	Ref.
HCV-77 (1a)	Transfection of viral RNA	IHH cells	1 Detection of GFP-LC3 punctate structure formation 2 Accumulated autophagosome in TEM analysis 3 Upregulation of Beclin expression and ATG5-ATG12 conjugate	Promotion of viral RNA replication	(Ait-Goughoulte *et al.*, 2008)
HCV-JFH1 (2a)	Transfection of viral RNA	Huh7.5 cells	1 Upregulated expression of LC3-II 2 No overlapping signal of GFP-LC3 punctate with lysosome 3 Autophagic activation by UPR 4 An incomplete autophagic process lacking enhanced autophagic degradation of long-lived proteins and p62	Promotion of viral RNA replication	(Sir *et al.*, 2008)
HCV-JFH1 (2a)	HCVcc infection	Huh7 cells	1 Upregulation of LC3-II 2 Accumulation of GFP-LC3 dot-like vesicles 3 No colocalization of autophagic vacuoles with viral proteins	Enhanced translation of the incoming viral RNA	(Dreux *et al.*, 2009b)
HCV-JFH1 (2a)	HCVcc infection	Huh7.5-1 cells	1 Increase of GFP-LC3 dot-like structures 2 No colocalization of autophagic vacuoles with viral proteins	Promotion of virion assembly	(Tanida *et al.*, 2009)
HCV-JFH1 (2a)	HCVcc infection	Huh7 cells	1 Transient interaction of ATG5 with NS5B and NS4B	Promotion of viral RNA replication	(Guevin *et al.*, 2010)
HCV-JFH1 (2a) infection	HCVcc infection	Huh7 cells	1 Detection of early- and late-stage autophagic vacuoles by TEM analysis 2 Colocalization of autophagic vacuoles with lysosome 3 Complete autophagic process by HCV infection	Promotion of viral RNA replication by suppressing antiviral immunity	(Ke *et al.*, 2011)
HCV-H77 (1a); HCV-JFH1 (2a)	HCVcc infection	IHH	1 Activated IFN response in the HCV-infected cells by silencing of Beclin and ATG7 2 Increased caspase-dependent apoptosis by knockdown of Beclin and ATG7 in the HCV-infection cells	Promotion of viral RNA replication by suppressing antiviral immunity	(Shrivastava *et al.*, 2011)

HCV-Con1 (1b) and JFH1 (2a)	Replicon viral RNA transfection	Huh7 cells; Huh7.5-1 cells; Liver biopsy	1 An inverse correlation between hepatic steatosis and activation of autophagy in liver biopsy samples of infected patients 2 Colocalization of autophagic vacuoles with LDs	Promotion of catabolism of LDs	(Vescovo *et al.*, 2012)
HCV-JFH1 (2a)	Replicon viral RNA transfection	Huh7 cells; HCV-transgenic mice	1 Enhanced ROS level in mitochondria in HCV viral RNA-transfected cells 2 Activated autophagy by expression of HCV NS proteins 3 Alteration of antioxidant response by upregulation of antioxidant enzymes in HCV NS protein-expressing cells	Regulation of oxidative response in mitochondria	(Chu *et al.*, 2011)
HCV-JFH1 (2a)	HCVcc infection	Huh7 cells; Huh7.5-1 cells	1 Accumulation of mito autolysosome in HCV-infected cells 2 Stimulation of Parkin and Pink 1 expression in HCV-infected cells 3 Activation of mitophagy *via* a Parkin-dependent pathway	Elimination of damaged mitochondria and promotion of viral RNA replication	(S. J. Kim *et al.*, 2013b)
HCV-JFH1 (2a) HCV-N (1b)	HCVcc infection ; Replicon viral RNA transfectio	Huh7 cells	1 Activation of autophagy through AKT1-TSC-mTORC1 signaling 2 Activation of autophagy *via* UPR	Promotion of viral RNA replication	(H. Huang *et al.*, 2013)
HCV-JC1 (2a) infection ; HCV NS4B	HCVcc infection ; Ectopic overexpression	Huh7.5 cells	1 Activation of autophagy by HCV NS4B amino acid 1-190 2 Requirement of Rab5 and PI3K for autophagic activation	Organization of virus replication site	(W. C. Su *et al.*, 2011)
HCV-JFH1 (2a)	HCVcc infection	IHH cells	1 Transcriptional activation of Beclin gene expression 2 Autophagy activation in a Bcl2-Beclin dissociation- and mTOR inhibition-independent manner	Promotion of viral RNA replication	(Shrivastava *et al.*, 2012)
HCV-JFH1 (2a)	HCVcc infection	Huh7.5 cells	1 Activation of autophagy through IRGM 2 Interaction of HCV NS3 with IRGM	Promotion of viral RNA replication;	(Gregoire *et al.*, 2012)

3.5.1 ER-stress in HCV-induced autophagy

The accumulation of unfolded or misfolded protein in the endoplasmic reticulum will induce ER-stress. This induction of ER-stress 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 which are collectively known as the unfolded protein response (UPR) (Figure 14) (Hetz, 2012). The UPR activation will mitigate ER stress by reducing protein synthesis, promoting the expression of ER chaperon proteins to facilitate protein folding, and enhancing protein degradation through autophagy and the ER-associated degradation (ERAD) pathway. The failure of UPR to alleviate ER stress will lead to the induction of apoptosis (M. Wang *et al.*, 2014b).

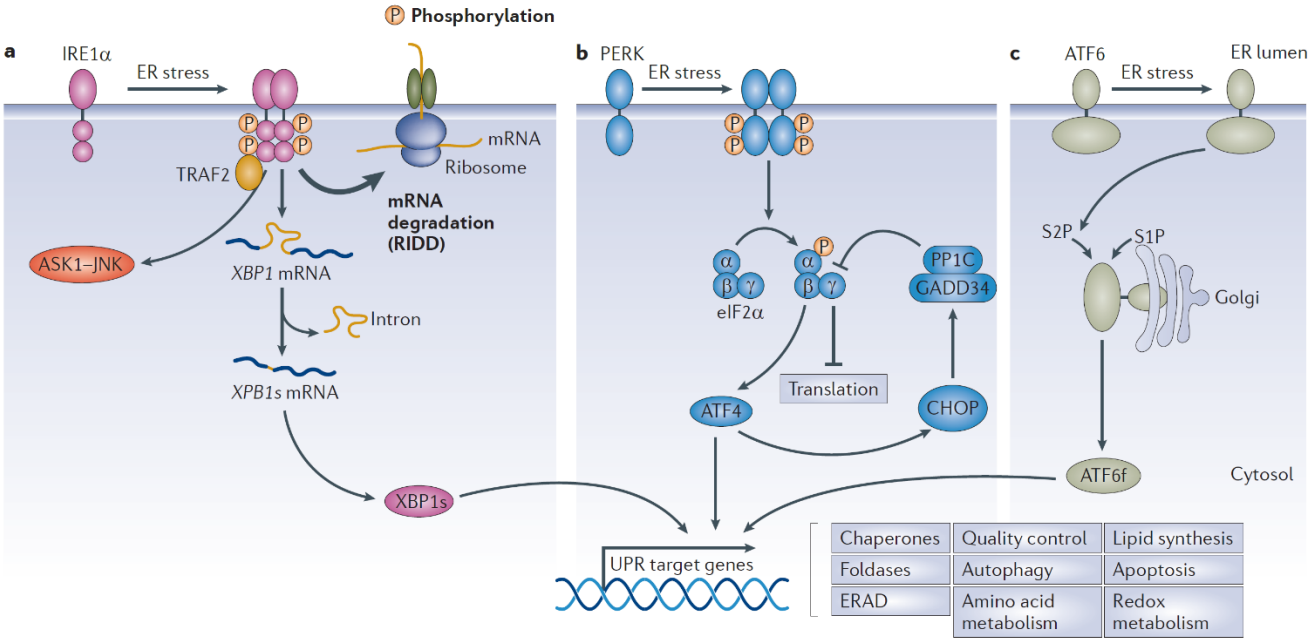


Figure 14. The mechanism of unfolded protein response. ER stress induces the UPR.as an adaptive response. Three main stress sensors control UPR-dependent responses: IRE1α, PERK and activating transcription factor 6 (ATF6). These proteins transduce signals to the cytosol and nucleus to restore the capacity of protein folding via various pathways. (a) IRE1α RNase activity processes the mRNA encoding the transcription factor X-box binding protein 1 (XBP1). This results in the expression of an active XBP1s that upregulates a group of UPR target genes, ER-associated protein degradation (ERAD), protein quality control, and organelle biogenesis. IRE1α also capable of degrading selected mRNAs via regulated IRE1-dependent decay (RIDD). Furthermore, IRE1α activates the JUN N-terminal kinase (JNK)– apoptosis signal-regulating kinase 1 (ASK1) pathway through its binding to adaptor proteins, such as tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2). (b) Activation of PERK decreases protein synthesis via phosphorylation of the eIF2α. EIF2α phosphorylation permits the selective translation of the *ATF4* mRNA, which encodes a transcription factor that induces the expression of genes involved in different processes including autophagy. ATF4 controls the expression of the pro-apoptotic components GADD34 and CHOP. GADD34 can bind protein phosphatase 1C (PP1C) to dephosphorylate eIF2α. c Under ER stress, ATF6 translocates from the ER to Golgi apparatus where it is processed by a site 1 protease (S1P) and site 2 protease (S2P) releasing its cytosolic domain (ATF6f). ATF6f mediates the upregulation of specific UPR target genes. (Hetz *et al.*, 2013)

HCV infection has been shown to induce ER stress and activate the UPR (Joyce *et al.*, 2009, Tardif *et al.*, 2005). This induction of UPR has been reported to be necessary for the induction of autophagy by HCV (Ke *et al.*, 2011, Shinohara *et al.*, 2013, Sir *et al.*, 2008, J. Wang *et al.*, 2014a). Obviously, the silencing of three UPR sensors, PERK, ATF6 or IRE1, by using of siRNAs or the inhibition of the ER stress using pharmacological inhibitors reduced the ability of HCV to induce

autophagy (Ke *et al.*, 2011, Sir *et al.*, 2008, J. Wang *et al.*, 2014a). In contrary, a separate report has challenged the importance of the UPR for the induction of autophagy by HCV. This challenge was based on three main observations. The first was that the accumulation of LC3II in HCV-infected cells has preceded the detection of the UPR induction; second, the HCV SGR, which did not induce UPR, could still induce autophagy; and third, the silencing of IRE1 did not inhibit HCV-induced autophagy (Mohl *et al.*, 2012). However, the observations that the HCV SGR could not induce the UPR and that the silencing of IRE1 could not inhibit autophagy were not consistent with the previous reports (Sir *et al.*, 2008, Tardif *et al.*, 2002). The explanation of this discrepancy is still unknown and further investigations are needed to resolve this apparent discrepancy.

3.5.2 Autophagy modulation by HCV proteins

Both HCV structural and non-structural proteins have been shown to modulate autophagy. The HCV core protein has been shown to induce the ER stress and activate PERK and ATF6 signaling pathways, but not the IRE1 pathway, of the UPR (J. Wang *et al.*, 2014a). The same group have shown that the activation of PERK induced the expression of the transcription factor ATF4 and its downstream effector CHOP, which, in turn, upregulated the expression of LC3 and ATG12 to induce autophagy (J. Wang *et al.*, 2014a). Even though HCV p7 was shown to bind Beclin-1, the over-expression of p7 did not affect autophagy (Aweya *et al.*, 2013). On the other hand, the expression of HCV nonstructural polyprotein NS3-NS5B has been shown to be sufficient to induce DMVs that are similar to autophagosomes (Chatterji *et al.*, 2015).

It is worth mentioning that, HCV NS3/4A can bind to mitochondria-associated immunity-associated GTPase family M (IRGM), a member of the interferon-inducible GTPase family (Gregoire *et al.*, 2011). IRGM can interact with multiple autophagy proteins, including ATG5 and ATG10, to regulate autophagy. The knockdown of IRGM expression impaired the lipidation of LC3 and the induction of autophagy 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 still elusive.

The HCV NS4B protein has also been shown to induce LC3II and autophagosomes accumulation (W. C. Su *et al.*, 2011, L. Wang *et al.*, 2015). However, NS4B has been shown to block autophagosome maturation by inducing the expression of Rubicon (L. Wang *et al.*, 2015). NS4B could also bind to Beclin-1, Rab5, and hVps34 (Stone *et al.*, 2007, W. C. Su *et al.*, 2011). Rab5 is an early endosomal GTPase that regulates membrane trafficking while hVps34 is the catalytic subunit of the PI3KC3 complex that positively regulates autophagy. Either knocking down Rab5 or inhibiting hVps34 has diminished the ability of NS4B to induce autophagosome accumulation (W. C. Su *et al.*, 2011). The sole expression of NS4B has been reported to be sufficient for the activation of the UPR which could be the mechanism by which NS4B induces autophagy (S. Li *et al.*, 2009, L. Wang *et al.*, 2015, Zheng *et al.*, 2005).

HCV NS5A protein has also been shown to upregulate the expression of Beclin-1 via NS5ATP9, a protein involved in the regulation of diverse pathways including DNA repair, cellular signaling, cell cycle control and cell growth, and could induce autophagy (Emanuele *et al.*, 2011, Quan *et al.*, 2014, Shrivastava *et al.*, 2012).

In a previous report by our group, we showed that HCV NS5B can bind ATG5 in yeast two-hybrid and co-immunoprecipitation experiments and colocalize with ATG5 in the early time points after HCV infection (Guevin *et al.*, 2010). ATG5 also colocalized with HCV NS4B in HCV SGR cells. The silencing of ATG5 inhibited HCV RNA replication, suggesting a role of autophagy and/or the interaction between ATG5 and NS5B in HCV RNA replication (Guevin *et al.*, 2010).

3.5.3 Autophagy in HCV replication

Several groups, including our lab, have demonstrated that the inhibition of autophagy would suppress HCV replication, indicating the participation of autophagy in HCV replication cycle (Ait-Goughoulte *et al.*, 2008, Dreux *et al.*, 2009b, Guevin *et al.*, 2010, Ke *et al.*, 2011, Mohl *et al.*, 2012, Shrivastava *et al.*,

2012, Sir *et al.*, 2008, W. C. Su *et al.*, 2011, Taguwa *et al.*, 2011). These reports have investigated the role of autophagy in HCV replication utilizing different HCV infection models including cells infected by HCVcc, cells transfected by the HCV genomic RNA or SGRs, or cells that stably express HCV SGR. Although all of them agreed on that autophagy positively regulates HCV replication cycle, there were controversies regarding the stage of HCV replication cycle at which autophagy is involved.

Dreux *et al.* reported that autophagy was important for the initiation of HCV RNA translation upon infection but not important for the later stage of HCV replication cycle (Dreux *et al.*, 2009b). However, the role of autophagy in the onset of HCV RNA translation has been challenged by Ke *et al.* who suggested that autophagy was required for the efficient production of infectious HCV particles (Ke *et al.*, 2011). While several groups, including ours, showed consensus on a crucial role of autophagy in HCV RNA replication (Ferraris *et al.*, 2010, Guevin *et al.*, 2010, Ke *et al.*, 2011, Sir *et al.*, 2008, Sir *et al.*, 2012).

By using iodexanol fractionation assay, Ferraris et al. have shown that membranous fractions containing LC3 were positive for components of HCV replication complex (Ferraris *et al.*, 2010). By conducting electron microscopy and immunogold staining, they also demonstrated the presence of HCV double-stranded RNA (i.e., replicative intermediates) on double-membrane vesicles that resembled autophagosomes (Ferraris *et al.*, 2010). Their results were also consistent with a more recent study by Sir et al. who showed that NS5A and NS5B proteins, two important components of the HCV replication complex, along with HCV RNA colocalized with LC3 positive vesicles, likely autophagosomes, in HCV SGR cells by using immunofluorescence (Sir *et al.*, 2012). They further confirmed these results by co-immunoprecipitation and immunoelectron microscopy studies (Sir *et al.*, 2012). Interestingly, they failed to reproduce similar results in HCV infected cells (Sir *et al.*, 2012). Moreover, other reports were unable to mark colocalization between HCV replication complex and LC3II suggesting a differential involvement of autophagy in different HCV replication systems (Dreux *et al.*, 2009b). Recently, a study by Mohl *et al.* has shown that the double-FYVE-

containing protein 1 (DFCP1) is required for HCV RNA replication, in both SGR cells and virus infection, however, it did not modulate virus entry or initial translation (Mohl *et al.*, 2016). DFCP1 is a PI3P binding protein that generates cup-shaped protrusions from the ER membrane, termed omegasomes, which afford a platform for the formation of autophagosomes (Axe *et al.*, 2008). They also showed that, early during HCV infection, the viral replication complexes transiently colocalize with omegasomes (Mohl *et al.*, 2016). These findings highlighted the possibility that, early autophagosomal membranes could be utilized by HCV to generate DMVs which facilitate viral replication.

Hypothesis and objectives

We based our research project on our lab'sprevious reports which showed a potential involvement of the autophagy protein ATG5 in HCV replication. As ATG5 represents one component of the autophagy elongation complex (ATG5-1216L1), our hypothesis was that the ATG5-12/16L1 complex plays a role in HCV replication. This has led us to the following research objectives:

1) Assessment of the subcellular localization of the ATG5-12/16L1 complex and its possible interactions with HCV replicase.
2) Identification of the HCV replication cycle step at which ATG5-12/16L1 complex is involved.

CHAPTER 2: PUBLICATIONS

Publication No.1

(Submitted manuscript)

The autophagy elongation complex (ATG5-12/16L1) is recruited at HCV replication site and is required for viral replication

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Running title: Autophagy elongation complex is involved in HCV replication.

Contribution of the authors:

Ahmed M. Fahmy: Designed the experiments, performed experiments for all the manuscript figures, and wrote the manuscript

Matthieu Blanchet: Performed the qRT-PCR for Figure 6 and revised and corrected the manuscript

Christian Duguay: Optimized the Iodexanol gradient floatation assay, participated in parts of Figure 5 and 6.

Patrick Labonté: Designed the experiments, revised and corrected the manuscript and figures.

Abstract

Hepatitis C virus (HCV) infection is known to induce autophagosome accumulation as observed by the typical punctate cytoplasmic distribution of LC3-II in infected cells. Previously, we showed that viral RNA-dependent RNA polymerase (NS5B) interacts with ATG5, a major component of the autophagy elongation complex and is involved in the formation of double-membrane vesicles (DMV). In this study, we evaluate the involvement of the autophagy elongation complex (ATG5-12/16L1) in HCV replication. We demonstrate that the elongation complex is recruited at the site of viral replication and act as a proviral factor. Indeed, ATG5-12 as well as ATG16L colocalized with the viral replicase in infected cells. Using *in situ* proximity ligation assay, we show that ATG5 interacts with several replicase components. Interestingly, LC3 is not recruited along with the elongation complex to the site of viral replication and no sign of colocalization of LC3-II with viral proteins was observed. Finally, using dominant negative forms of ATG proteins, we demonstrate that ATG5-12 conjugate, but not LC3-II formation, is critical for viral replication. Altogether, these findings suggest that HCV recruits the autophagy elongation complex to the site of viral replication where it promotes replication.

Introduction

Hepatitis C virus (HCV) infection leads to a wide spectrum of diseases ranging from asymptomatic to end-stage liver diseases including cirrhosis and hepatocellular carcinoma (1). HCV is an enveloped, positive-strand RNA virus that belongs to the *Flaviviridae* family. The HCV genome is approximately 9.6 kb in length and consists of a single ORF flanked by two non-coding regions (NCRs). The translated polyprotein is processed by cellular and viral proteases into the structural proteins (core, E1, and E2) and the nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B)(2). HCV replication is marked by the formation of a membrane-associated replication complex with a unique membrane alteration referred to as the membranous web (3). Double membrane vesicles (DMVs) have been observed inside the membranous web suggesting that autophagy is involved in the establishment of the HCV replication scaffold (4-6).

Autophagy is an intracellular catabolic process essential to maintain cell homeostasis which is particularly noticeable under nutrient-deprivation conditions such as starvation (7). In addition, autophagy provides a cell-autonomous defense system against microbial infections and intracellular pathogens via the autophagosome/lysosome pathway (8, 9). Autophagy is initiated by the formation of the isolation membrane, the phagophore, which extends to form a closed DMV known as the autophagosome. This structure then, fuses with a lysosome to form an autolysosome. The fusion allows the degradation of the autophagosomal cargo by lysosomal enzymes. So far, more than 30 autophagy-related gene (ATG) products have been identified (10).

Although autophagy has antiviral capability, several viruses and especially positive-strand RNA viruses can use the autophagy machinery for their own benefit (11-15). Among them, HCV is known to induce accumulation of LC3-II punctate structures (16, 17). Furthermore, it was shown that at least a part of the autophagy process is absolutely required for the HCV life cycle *in vitro* (18, 19). It has been proposed that HCV may induce autophagosome formation through the unfolded protein response (UPR)(20, 21); however recent data have suggested that autophagy is triggered independently of the UPR in HCV-infected cells (22). Apart from other HCV proteins, NS4B expression has been shown to be sufficient to induce the accumulation of autophagosomes as seen by the redistribution of diffused LC3 (LC3-I) to punctate structures (LC3-II) in NS4B-transfected cells (23). It has been demonstrated that induction of autophagy by HCV is important for the suppression of the antiviral interferon response (21, 24). In addition to this indirect action of autophagy that favors the establishment and the maintenance of HCV, it has been suggested that autophagic proteins promote HCV replication by either facilitating protein translation (17) or virus maturation (19). It was also shown that upon HCV infection, NS5A transcriptionally upregulates Beclin1, enhances phospho-mTOR expression, and thus, activates mTOR signaling pathway (25). On the other hand, a more recent study proposed that HCV-induced autophagy occurs through inhibition of AKT-TSC-mTOR via ER stress (26). In a previous study, we have shown that HCV RNA-dependent RNA polymerase (RdRp), the NS5B, colocalizes and interacts with ATG5, a component of the autophagy elongation complex and a key factor for the formation of autophagosomes (27). Therefore, we have proposed that autophagy may provide assistance in the formation of membranous structures used by the virus for its replication. In this study, we confirm that the autophagy elongation

complex (ATG5-12/16L1) is recruited at the HCV replication site, where it acts to promote HCV replication.

Materials and methods

Cell culture and reagents

Huh7 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) (Gibco), supplemented with 10% v/v fetal bovine serum (FBS) (Multicell), 100 U/ml penicillin, 100µg/ml streptomycin, 2 mM L-glutamine (Gibco) at 37ºC, 5% CO2, in a humidified incubator.

Plasmids and antibodies

hATG5 and hATG16L1 sequences were cloned into peGFP-C1 plasmid (Clontech) to form pGFP-ATG5 and pGFP-ATG16L1, respectively. The peGFP-LC3 construct was kindly provided by Dr. Tamotsu Yoshimori (Japan) (28). The pmStrawberry-ATG4BC74A (ATG4B-DN), pcDNA3-mRuby2 and pCI-neo-hApg5-K130R-HA (ATG5-DN) constructs were purchased from Addgene (Cambridge, USA). The Flag-tagged ATG12 (pATG12) and its dominant-negative derivative pATG12ΔG140 (ATG12-DN) constructs were kindly provided by Dr. Adi Kimchi (Israel) (29). Rabbit polyclonal anti-LC3, rabbit polyclonal anti-ATG5 (used for western blot), mouse monoclonal anti-Flag, and mouse monoclonal anti-β-actin antibodies were purchased from Sigma Aldrich (USA). Mouse monoclonal anti-ATG5 (used for immunofluorescence) and anti-P62 antibodies were purchased from Abnova (Taiwan). Rabbit polyclonal anti-ATG12 was purchased from Cell Signaling (USA). Rabbit polyclonal anti-ATG16L1 antibody was purchased from MBL (USA). Mouse monoclonal anti-dsRNA was purchased from English & Scientific

Consulting (Hungary). Mouse monoclonal anti-HA was purchased from Roche (USA). Mouse monoclonal anti-Core was purchased from Virostat (USA). Mouse monoclonal anti-NS3 and anti-NS5A antibodies were purchased from BioFront (USA). Rabbit polyclonal anti-NS3 and NS5A were obtained from Dr. Olivier Nicolas. Rabbit polyclonal anti-NS4B and anti-NS5B antibodies were kindly provided by Drs. Kouacou Konan (USA) and Takaji Wakita (Japan), respectively. Mouse monoclonal anti-GAPDH was purchased from Santa Cruz (USA). Rabbit polyclonal anti-calnexin was purchased from Enzo.Life Sciences (USA).

Preparation of viral stock and infections

The cell culture-derived HCV (HCVcc) JFH1 virus was generated in Huh7 cells by transfection of *in vitro*-transcribed full-length JFH1 RNA (MEGAscript, Ambion) and viral stocks were produced by infection of Huh7 cells at a multiplicity of infection (MOI) of 0.01, as described previously (30). To reach 90% infected cells, huh7 cells were infected at MOI of 0.01 and passaged for 7 days then analyzed by immunofluorescence using anti-NS5A antibody.

Western blot analysis

Cells were lysed in 300 µl of lysis buffer [25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP40, Complete™ protease inhibitor (Roche)]. Lysates were normalized for total protein content using the BCA protein assay kit (Pierce). Proteins were then resolved by SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad), blocked for 30 min at room temperature (RT) with PBS-5% milk, and then incubated overnight at 4°C with primary antibody in PBS-5% milk. After washing with 0.1% Tween 20 in PBS (PBST), membranes were incubated 1 h at RT with a goat-anti-rabbit or goat-

anti-mouse IgG conjugated to horseradish peroxidase in PBS-5% milk. Protein bands were visualized with either the Super Signal West-Pico or -Femto chemiluminescence substrates (Pierce).

Indirect immunofluorescence

Huh7 cells infected at greater than 90% were transfected with different plasmids as indicated in figure legends. At 24 h post-transfection, cells were trypsinized and grown on glass coverslips for another 24 h. The coverslips were then fixed with 4% formaldehyde in PBS for 10 min, washed in PBS and incubated in blocking buffer (PBS, 3% bovine serum albumin, 10% FBS, 0.1% Triton X-100) for 30 min at RT. In case of GFP-LC3 cells were permeabilized with 0.05% saponin to remove dispersed LC3 (26). After washing with PBS, the coverslips were incubated with primary antibody in blocking buffer for 1 h at RT. Coverslips were then washed in PBS and incubated with either Alexa fluor™-(488 or 568) goat anti-mouse IgG or Alexa fluor™-(488 or 568) goat anti-rabbit IgG (Invitrogen) for 1h at RT. After washing, coverslips were mounted on glass slides with Prolong Antifade (Invitrogen) and examined with either a laser scanning confocal BioRad Radiance 2000 or a Zeiss LSM 780. The Manders'coefficient of colocalization was obtained using ImageJ software (NIH) in randomly selected regions that were positive for the targeted proteins from different cells. Manders'coefficient values over 0.4 were considered as strong colocalization.

In situ proximity ligation assay (PLA)

Huh7 cells infected at greater than 90% were grown on glass coverslips for 24 h prior to fixation with 4% formaldehyde in PBS for 10 min. They were then washed in PBS and incubated in blocking buffer (PBS, 3% bovine serum albumin, 10% FBS, 0.1% Triton X-

100) for 30 min at RT. Coverslips were incubated with primary antibodies for 1 h at RT then washed three times with 1x wash buffer A (Duolink) and incubated with PLA probes (anti-rabbit plus and anti-mouse minus) diluted with the provided buffer in a humidity chamber for 1 h at 37°C. Coverslips were then washed three times with 1x wash buffer A and incubated with ligase-ligation solution in a humidity chamber for 30 min at 37°C. Amplification and mounting steps were performed according to manufacturer's instructions. Mounted coverslips were examined with laser scanning confocal a Zeiss LSM 780. Each detected signal represents an interaction event. The analysis of PLA signal frequency was done using Duolink Image Tool.

Denisty gradient fractionation

Huh7 cells were infected with HCVcc JFH1 at a MOI of 0.01. Three weeks post-infection cells were trypsinized, washed with PBS, resuspended in 500 µl of 0.25M sucrose in PBS and left on ice for 20 min. Cells were lysed using a Dounce homogenizer. Cell debris and nuclei were then removed by centrifugation at 1000 xg for 10 min at 4◦C. Supernatant was mixed with iodixanol to make a solution of 40 % W/V. This solution was then overlaid with 420 µl of 25%, 1.68 ml of 20% and 630 µl of 15% iodixanol. This gradient solution was centrifuged at 100,000–150,000 xg for 16 h at 4°C before collecting 13 fractions and subject them to Western Blotting as described earlier.

Quantification of HCV RNA in iodixanol fractions by qRT-PCR

RNA isolated from equal volumes of all fractions was reverse transcribed with M-MLV (Invitrogen). Generated cDNA was used for qPCR as described earlier (31). Results were analyzed using the comparative ΔCt method. Strand specific detection of HCV RNA was conducted as follow: Total RNA was extracted as described above. Reverse transcriptions

for the specific detection of HCV minus- and plus-strand were conducted using forward primer (5'-TCTGCGGAAACCGGTGAGT-3') and reverse primer (5'-GAGTGGGTTTATCCAAGAAAG-3'), respectively. SuperScript® III Reverse Transcriptase (Life technologies) was used for the reverse transcription according to the manufacturer's instruction with the exception of a polymerization at 60°C for 8 min to increase reaction specificity. The resulting cDNA were then quantified as described above. For the detection of GAPDH mRNA, total RNA was reverse transcribed using the iScript reverse transcription Kit (Biorad) according the manufacturer's instructions. qPCR amplification was carried out using the Ssofast Evagreen Supermix (Biorad) with the following primers: F-5'-GTGAACCATGAGAAGTATGAC-3'; R-5'-ATGAGTCCTTCCACGATAC-3'. Cycling was conducted as described above.

Flow cytometry

Huh7 cells infected at greater than 90% were transfected with either mock (pcDNA3-mRuby2) or ATG4B-DN. At day 2 cells were trypsinized and washed twice with PBS. Dead cells were labeled using FVD780 (eBioscience). Cells were fixed with 4% formaldehyde for 10 min at RT, incubated with blocking buffer (PBS, 2% BSA, 0.2% Saponin) for 20 min at 4°C and incubated with primary antibody diluted in blocking buffer for 30 min at 4°C. After washing with PBS, cells were incubated with Alexa fluor™-488 goat anti-rabbit IgG (Invitrogen) for 30 min at 4°C. Cells were analyzed using BD LSRFortessa cell analyzer (BD Biosciences) and Cyflogic software (CyFlo Ltd).

Statistical analyses

Results shown represent the mean of at least three independent experiments. ANOVA analysis was performed to identify statistically significant differences in viral NS3 protein

expression and LC3-I/LC3-II ratios. P values below 0.05 were considered statistically significant.

Results

Formation of the ATG5-12 conjugate and the autophagy elongation complex ATG5-12/16L1 in Huh7 cells

We have shown in a previous study that HCV polymerase interacts with ATG5, a protein that participates in early events during induction of autophagy (27). Since ATG5 is normally conjugated to ATG12, we first compared the conjugation status of ATG5 in Huh7 infected and uninfected cells. The results showed that the unconjugated form of ATG5 (32 kDa) was undetectable in both infected and uninfected cells. Indeed, ATG5 was exclusively detected as ATG5-12-conjugated form (55 kDa) (Fig. 1A). The absence of detection of the unconjugated ATG5 suggests that most of the ATG5 is readily conjugated to ATG12 in Huh7 cells as previously reported for others cell types (32). Additionally, HCV infection did not hamper this conjugation. Moreover, results show that infection induces the accumulation of LC3-II, a hallmark of induced autophagy (Fig. 1A).

Once ATG5 is conjugated to ATG12, it can form a multimeric complex by association with ATG16L1 (33) . To confirm that this interaction occurs within the cells at endogenous level of the proteins, we investigated the presence of ATG5-12/16L1 complex by *in situ* proximity ligation assay (PLA). This novel technique has been used in several studies to detect specific *in situ* interactions (34-37). Clearly, endogenous ATG5-12/16L1 complexes were detected in both infected and uninfected cells (Fig. 1B and C). It is noteworthy that the count of interaction signals was slightly lower in infected cells. Although, the

significance of this reduction is not ascertained at this moment, our results suggest that HCV infection still allows the formation of the high molecular weight complex ATG5-12/16L1 that normally occurs spontaneously *in vitro* (38, 39).

The ATG5-12 conjugate co-localizes and interacts with viral nonstructural proteins

We then assessed the colocalization between the endogenous ATG5-12 conjugate and the components of the viral replicase in JFH1-infected Huh7 cells using an anti-ATG5 antibody. The results presented in Figure 2 (A and B) show distinct membrane-like structures that are positive for the ATG5-12 conjugate as well as for HCV NS3, NS4B, NS5A, and NS5B. The distribution of ATG5-12 in uninfected cells is shown in Figure 2 (C). We also confirmed the colocalization of ATG5-12 conjugate with the viral nonstructural proteins using an ATG12-Flag protein (Fig. 3). Because NS3, NS5A, and NS5B are the main constituents of the viral replicase and since NS4B is localized on vesicles of the membranous web where the replicase is located, these results raise the hypothesis of an action of the ATG5-12 conjugate as a proviral factor at either the viral translation and/or replication site.

To evaluate putative weak and/or transient *in situ* interaction between the ATG5-12 conjugate and components of the viral replicase, we first analyzed the known ATG5/NS5B interaction in infected cells by PLA. The result showed that on average, around 380 ATG5-12/NS5B interactions were detected in infected cells (Fig. 4E). We then sought to screen

for all other possible interactions between ATG5-12 conjugate and HCV non-structural proteins for which colocalization was observed. As we showed earlier, NS3, NS4B and NS5A colocalize with ATG5-12 (Fig. 2 and 3). Indeed, PLA experiments showed that ATG5 interacts with these viral proteins *in situ* (Fig. 4B-D). In contrast, no interaction was observed between endogenous ATG5 and the viral core protein (Fig. 4A). Since no colocalization in infected cells was detected between these two proteins (data not shown), this PLA experiment was used as a specificity control of the assay. These results confirm the previous colocalization findings from figure 2 and 3 and strengthen the putative involvement of ATG5-12 conjugate in HCV replication.

The autophagy elongation complex (ATG5-12/ATG16L1) is found at the HCV replication site

In order to complete its normal function, the ATG5-12 conjugate associated with ATG16L1 to form the autophagy elongation complex that allows the expansion of the autophagosomal membrane (33, 40). Therefore, we sought to determine if the elongation complex, and not only ATG5-12 conjugate, is recruited to the site of viral replication. The subcellular localizations of either the endogenous ATG16L1 or a GFP-tagged human ATG16L1 protein were monitored in infected Huh7 cells. The GFP-ATG16L1 was used only when detection of endogenous ATG16L1 was not readily possible due to conflict in antibody species. Results show a marked colocalizations between ATG16L1 and several HCV nonstructural proteins that constitute the viral replicase as well as the NS4B protein (Fig. 5). Despite the obvious colocalization of ATG16L1 with NS3 and NS5A, we were

unable to detect *in situ* interaction of endogenous ATG16L1 and these proteins in infected cells using PLA (Fig. S1A and B). These results suggest that the interaction of ATG5-12/16L1 with viral NS3 and NS5A occurs through ATG5-12 rather than ATG16L1.

We next hypothesized that the elongation complex ATG5-12/16L1 resides with the viral replicase at the replication site. To test this hypothesis, we performed an iodixanol density gradient to segregate the replicase from the endoplasmic reticulum (ER). In this gradient, fractions where collected from bottom to top; higher to lower density. The chaperone protein calnexin was used to identify the fraction corresponding to the ER (Fig. 6A lower panel). Most of the calnexin protein was located in fraction 4 to 7 while lower amount of the protein was detected in lighter fractions (11-13) which most likely represent ER-derived HCV-induced membranous web (6). While the majority of ATG5-12 conjugate fractionated at higher density fractions where the soluble protein GAPDH is located, a proportion of ATG5-12 co-fractionated with ATG16L1 and the viral replicase proteins NS3 and NS5B at lower density fractions (Fig. 6A lower panel, lanes 12-13). Next, we examined all fractions for viral RNA and found that it segregated into two distinct areas, one overlapping the ER marker (Fig. 6A upper and lower panels lanes 4-7), and the other fractionating with the autophagy elongation complex (Fig. 6A upper and lower panels, lanes 11-13). From these results, we deduced that HCV RNA translation site was within fractions 5-6 whereas viral replication machinery was located in fractions 11-13 (Fig. 6A). To verify this, we performed strand-specific HCV RNA amplification by RT-qPCR. We found that HCV negative-strand RNA was located at the gradient top fractions (12 and 13) whereas the HCV positive-strand RNA resides at both the top as well as with the ER marker (Fig. 6A lower panel and B). These results are in line with the presence of the replication

complex in fraction 12 and 13. As control, the mRNA of GAPDH was amplified and used to mark the translation site (fractions 4-6). Since the segregation of the proteins in different fractions is based on density and might not reflect their subcellular localization. We confirmed the recruitment of the autophagy elongation complex by analyzing its colocalization with dsRNA (Fig.7). In infected cells, most of the dsRNA is expected to represent the HCV replication intermediate and thus, the replication site. As a positive control, the dsRNA-NS3 colocalization was assessed and as expected, HCV dsRNA markedly colocalized with the viral NS3 protein which harbors helicase activity and is known to be a constituent of the replicase. The specificity of dsRNA antibody was confirmed in uninfected Huh7 cells (Fig. 7C). Results showed that HCV dsRNA colocalizes with both ATG5-12 conjugate and ATG16L1 confirming that ATG5-12 and ATG16L1 are present at the HCV replication site. However, no *in situ* interaction was observed between ATG16L1 and HCV dsRNA (Fig. S1C).

LC3 lipidation is not essential for HCV replication in vitro

Following the recruitment of ATG5-12/16L1 complex to the isolation membrane, LC3-I protein is activated by conjugation to phosphatidylethanolamine (PE) to form the LC3-II membrane-bound protein. This action is made with the help of ATG4B, a cysteine protease that prepares LC3-I for conjugation through a proteolytic process (41). The active LC3-II protein, in turn, decorates the inner and the outer side of the isolation membrane during autophagosome formation. Thus, as a consequence of elongation complex recruitment, LC3-II was expected to be found at the HCV replication site (42). However, no

colocalization was observed between GFP-LC3 and NS3, NS5A, or core protein in infected cells (Fig. S2) suggesting that the presence of the elongation complex (ATG5-12/16L1) at the HCV replication site did not result in LC3 lipidation at this site. To determine the importance of LC3 lipidation for HCV replication, we utilized a dominant negative form of ATG4B (ATG4B-DN). Overexpression of this ATG4B-DN has been shown to inhibit LC3 lipidation and its downstream-dependent events such as P62 degradation (43). Indeed, upon overexpression of ATG4B-DN in Huh7 cells, we observed a marked decrease in LC3-II formation and accumulation of P62 (Fig. 8A). Surprisingly, overexpression of ATG4B-DN had no obvious inhibitory effect on HCV polyprotein expression in cells already infected, as indicated by the levels of NS3 and core protein (Fig. 8B). Since these results were obtained upon transient expression using transfection with efficiency around 40-50%, we decided to perform a FACS analysis gating specifically on Huh7 cells expressing ATG4B-DN and monitoring the level of NS3 expression in this targeted population. The result confirmed that expression of the dominant-negative form of ATG4B has no adverse effect on viral protein expression (Fig. 8C). Together, these results suggest that LC3 lipidation is not mandatory for viral replication in established infection.

ATG12 conjugation to ATG5 is required for HCV replication in Huh7 cells

We next investigated the effect of decoupling the ATG5-12 conjugate on HCV replication by overexpressing the dominant-negative form of ATG12 (ATG12-DN) lacking the C-terminal glycine that is essential for conjugation with ATG5 in HCV-infected Huh7 cells (29). As expected, the overexpression of this dominant negative form of the protein

hampered the conjugation of ATG12 to ATG5 as well as its subsequent events, the formation of ATG5-12/16L1 complex, and LC3 lipidation (Fig. 8D). Interestingly, this conjugation-defective mutant, when compared to a wild-type ATG12, displays an adverse effect on HCV lifecycle, as indicated by a decrease in the NS3 and core protein (Fig. 8B). To confirm this result, we overexpressed a dominant negative form of ATG5 (ATG5-DN) that blocks its conjugation to ATG12 (Fig. 8D) and showed that overexpression of ATG5-DN also affected HCV lifecycle (Fig. 8B). Together, these results suggest that the ATG5-12 conjugated form, rather than the individual ATG5 and ATG12 proteins, act as HCV proviral factor.

Discussion

In a previous study, we showed that HCV RdRp colocalizes and interacts with ATG5, a member of the elongation complex (27). Here we show that the ATG5-12 conjugate colocalizes clearly on structures that harbor several HCV nonstructural proteins such as NS3, NS4B, NS5A, and NS5B (Fig. 2 and 3). We then performed *in situ* PLA to identify interaction between members of the viral replicase and that of the elongation complex. The results indicate that several HCV nonstructural proteins are in very close proximity to ATG5 in infected cells (Fig. 4). Since ATG5-12 forms a high molecular weight multimeric complex with ATG16L1 that is absolutely required for autophagosome formation (40), we then analyzed the recruitment of ATG16L1 at the site of HCV replication (Fig. 5). Several membranous structures were positive for both ATG16L1 and viral nonstructural proteins. Using cell fractionation, we showed that proteins of the autophagy elongation complex were present in the fractions harboring the HCV replication complex. Indeed, HCV RNA (positive and negative-strand) as well as the viral helicase and polymerase were found at higher concentrations in those fractions (Fig. 6A, lanes 12-13 and Fig. 6B). Furthermore, by labeling dsRNA, we were able to show that the replicating HCV RNA colocalizes with the autophagy elongation complex (Fig. 7). Together, these results confirm that ATG16L1 is recruited at the site of HCV replication where the elongation complex is formed.

Although the mechanism by which the ATG5-12/16 complex leads to autophagosome formation is mostly uncharacterized, it is believed that direct interaction of this complex with Rab33b is required and precedes LC3-I lipidation (42, 44). Therefore, LC3-II was expected to colocalize with NS4B, NS3, or NS5B. Interestingly we, as well as several other groups (16, 17, 19), were unable to observe colocalization of LC3 with HCV proteins (Fig.

S2). This was very intriguing since HCV has ben shown to trigger the appearance of LC3-II throughout the infected cell (16).

As LC3 was not recruited at the site where viral replicase is localized, we decided to analyze the contribution of LC3-II formation in HCV replication. For this purpose, we used a dominant-negative form of ATG4B (ATG4B-DN) that blocks LC3-II formation. The results demonstrate that although LC3-II formation was indeed severely affected by the ATG4B-DN expression, no inhibitory effect on HCV replication was observed. The results presented in figure 8 also confirm that the ATG5-12 conjugate is truly important for HCV replication. Indeed, transfection of Huh7 with either ATG5-DN or ATG12-DN, led to a significant decrease in HCV replication. This result not only demonstrates the importance of both proteins but also shows that their conjugation is required for HCV replication.

How exactly the ATG5-12/16L1 complex modulates HCV replication is still unknown. We postulated that HCV infection might trigger *de novo* synthesis of DMV through activation of autophagy. Recently, it was shown that DFCP-1, a protein that generates omegasomes, is required for HCV RNA replication. Viral NS5A transiently colocalizes with DFCP-1 on ER protrusions suggesting that omegasomes may provide vesicles on which HCV can replicate (45). Since the autophagy elongation complex could be recruited at the nascent omegasome for its elongation, it might participate in the creation of the HCV- induced membranous web. Alternatively, the autophagy elongation complex could facilitate membranous web formation through its known capability of enhancing membrane tethering and vesicles aggregation *in vitro* (46).

In our previous study, using overexpression of GFP-ATG5, we showed that ATG5-NS5B colocalization was disappearing at day 5 post-infection. Here, all colocalizations were performed using endogenous ATG5 and the colocalization remained obvious in infected cells for a longer period. The discrepancy may arise from the unconjugation of ATG5 when overexpressed as exogenous GFP-ATG5. Indeed, endogenous ATG5 can only be seen as an ATG5-12 conjugated form (Fig. 1A) and this conjugation is likely important for HCV replication (Fig. 8).

In summary, recruitment of the autophagy elongation complex, which is normally involved in DMV formation, to the HCV replication site, promotes viral replication. However, our results do not exclude the possible involvement of autophagy proteins in the translation of the viral RNA as it has been suggested previously (17). Interestingly, the recruitment of the elongation complex is not accompanied by LC3 lipidation at this site. Therefore, we believe that HCV infection cycle is more dependent on ATG5-12 conjugation than on LC3 lipidation. Thus, the link between the recruitment of the elongation complex and the formation of DMV through an autophagy-like mechanism needs further confirmation. The recent identification of DMVs within the membranous web (4, 5) may suggest that autophagy proteins act by promoting membrane formation used for viral replication by a LC3-independent mechanism.

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Figure legends

Figure 1. Formation of the autophagy elongation complex in Huh7 cells.

A. Detection of the ATG5-12 conjugate by Western blot in mock (UI) and JFH1 infected Huh7 cells at more than 90% using an anti-ATG5 antibody. HCV infection and autophagosome accumulation were detected using anti-NS3 and anti-LC3 antibodies, respectively. β-actin represents loading control.

B. *In situ* ATG5-12/16L1 complex formation was analyzed using PLA in JFH1-infected at more than 90% or uninfected cells. Cells were labeled for ATG5-12 and ATG16L1 using anti-ATG5 and anti-ATG16L1 respectively. CTL represents negative control lacking anti-ATG5 antibody. Nuclei were counterstained with DAPI (blue).

C. The frequency of PLA signals were significantly higher in both JFH1-infected and uninfected cells compared to control cells (CTL) (based on the count in 40 cells for each condition) ($P<0.0001$, 1way ANOVA).

Figure 2. Components of the HCV replicase colocalize with ATG5-12 conjugate in Huh7 cells.

A. JFH1-infected Huh7 cells at more than 90% were probed for endogenous ATG5-12 conjugate using mouse anti-ATG5 antibody and HCV nonstructural proteins (NS3, NS4B, NS5A, and NS5B) using rabbit specific antibodies as described in the materials and methods section. The nuclei were stained with DRAQ5 (blue). Confocal microscopy images displaying subcellular localization of endogenous ATG5-12 conjugate and viral NS3, NS4A, NS5A, and NS5B in merged image panels are shown. Marked colocalization

between endogenous ATG5-12 conjugate and components of the viral replicase (NS3, NS5A, and NS5B) or the membranous web (NS4B) was observed.

B. The average of colocalized pixels of ATG5-12 and HCV nonstructural proteins ($n \geq 5$ cells) was determined. The values of overlapping fluorescence signal with HCV nonstructural proteins were calculated using Manders' colocalization coefficient.

C. Localization of ATG5-12 in uninfected cells. Uninfected Huh7 cells were immunostained for ATG5-12 using mouse anti-ATG5 antibody antibody.

Figure 3. HCV viral nonstructural proteins colocalize with ATG12 protein in Huh7 cells.

A. Huh7 cells were infected with HCVcc and then transfected with recombinant ATG12. Confocal microscopy images displaying subcellular localization of ATG12 (green) and viral NS3, NS4B and NS5B (red) in merged images are shown. Colocalization between ATG12 and components of the viral replicase (NS3, and NS5B) or the membranous web (NS4B) was observed.

B. The average colocalization of ATG12 and HCV nonstructural proteins ($n \geq 5$ cells) was calculated using Manders' colocalization coefficient.

Figure 4. Assessment of ATG5-12 interactions with viral proteins in infected cells as observed by proximity-ligation assay (PLA).

JFH1-infected at more than 90% or uninfected cells were fixed and processed for detection of ATG5-12-Core, ATG5-12-NS3, ATG5-12-NS4B, ATG5-12-NS5A or ATG5-12-NS5B complexes by PLA using appropriate antibodies. Nuclei were stained with DAPI (blue).

Each PLA signal (red dot) indicates one interaction and were calculated as described in experimental procedures. In A, no significant difference in the frequency of PLA signals between JFH1-infected (n=50) cells compared to uninfected controls (n=50) indicating undetectable interaction between ATG5-12 conjugate and core. However, the incidence of PLA signals in B-D were significantly higher in JFH1-infected (n ≥23) cells compared to uninfected negative controls (N=50) (P<0.0001, Student's t-test) indicating complexes formation between ATG5-12 and NS5B, NS5A, NS4B and NS4A.

Figure 5. HCV nonstructural proteins colocalize with ATG16L1 in Huh7 cells.

A. Huh7 cells infected with JFH1 at more than 90% and then transfected with pGFP-ATG16L1 or immunostained for endogenous ATG16L1 using rabbit specific antibody. Confocal microscopy images displaying subcellular localization of GFP-ATG16L1 or endogenous ATG16L1 and viral NS3 (mouse), NS4B (rabbit), NS5A (mouse), and NS5B (rabbit) in merged images are shown.

B. The values of overlapping fluorescence signal of ATG16L1 and GFP-ATG16L1 with HCV nonstructural proteins were calculated in ≥ 5 cells using Manders' colocalization coefficient.

C. Distribution of endogenous ATG16L1 using rabbit specific antibody and ATG16L1-GFP in uninfected cells. Uninfected Huh7 cells were stained for ATG16L1 or transfected with ATG16L1-GFP and examined using confocal microscopy.

Figure 6. Autophagy elongation complex (ATG5-12/16L1) is present at the HCV replication site.

(A) Analysis of HCV-infected cell homogenates separated on iodixanol gradient. The iodixanol step gradients were separated into 13 fractions and subjected to Western blotting. All fractions were analyzed for calnexin (ER marker), HCV replicase components (NS3, NS5A and NS5B), and the ATG5-12/16L1 complex using specific antibodies. RT-qPCR was used to quantify HCV RNA in the same fractions (A, upper panel). (B) Strand-specific RT-qPCR was used to quantify HCV RNA with positive or negative polarity in different fractions of iodixanol step gradients (see materials and methods). GAPDH mRNA quantification was used as a marker for cellular RNA fractionating with the translational machinery.

Figure 7. The autophagy elongation complex colocalizes with HCV replicative intermediate dsRNA.

A. JFH1-infected Huh7 cells at more than 90% were immunostained for dsRNA and NS3 or ATG16L1. Alternatively, infected cells were transfected with pGFP-ATG5 and analyzed by confocal microscopy for dsRNA and GFP-ATG5.

B. The average of percent colocalization of NS3, GFP-ATG5 or ATG16L1 with dsRNA ($n \geq 5$ cells) was determined. The values of overlapping fluorescence signal with dsRNA were calculated using Manders' colocalization coefficient.

C. Uninfected Huh7 cells immunostained for dsRNA. Negative staining shows specificity of dsRNA antibody utilized in this experiment.

Figure 8. ATG5-12 conjugate disruption affects HCV lifecycle.

A. Huh7 cells were either transfected with an empty plasmid (mock) or a plasmid encoding an enzymatically inactive dominant negative form of ATG4B (ATG4B-DN). Cell lysates were analyzed by Western blot at 72 h post-transfection for LC3-I to LC3-II conversion and P62 accumulation.

B. JFH1-infected Huh7 cells (>90% infected) were transfected with plasmids encoding the dominant negative forms of ATG5, ATG12, or ATG4B. Cell lysates of transfected cells were analyzed 72 h post-transfection for HCV core and helicase expression using anti-NS3 by western blot. β-actin was used for normalization.

C. Infected Huh7 cells (>90% infected) were either mock-transfected (empty plasmid) or transfected with a plasmid encoding ATG4B-DN. Transfected cells were stained for NS3 as described in materials and methods then analyzed by flow cytometry at 72 h post-transfection for NS3 expression getting on ATG4B-DN positive cells.

D. Huh7 cells were either transfected with an empty plasmid (mock) or a plasmid encoding a conjugation-defective dominant negative form of ATG5 (ATG5-DN) or ATG12 (ATG12-DN) tagged with HA or Flag respectively. Cell lysates were analyzed by Western blot at 72 h post-transfection for elongation complex formation (ATG5-12/16L1), ATG5-12 conjugation and LC3-I to LC3-II conversion.

Figure S1. PLA of ATG16L1 and viral replicase components.

No detectable interactions of ATG16L1 with NS3, NS5A or dsRNA (A, B and C respectively) as indicated by the frequency of PLA signals between JFH1-infected (n=50) cells compared to uninfected controls (n=50).

Figure S2. HCV viral proteins does not colocalize with LC3 protein.

A. Huh7 cells were infected with JFH1 and then transfected with GFP-LC3. Confocal microscopy images displaying subcellular localization of GFP-LC3 and viral core, NS3, NS4B, NS5A and NS5B.

B. The values of overlapping fluorescence signal of GFP-LC3 with HCV nonstructural proteins were calculated in ≥ 5 cells using Manders' colocalization coefficient.

Figures

Figure 1

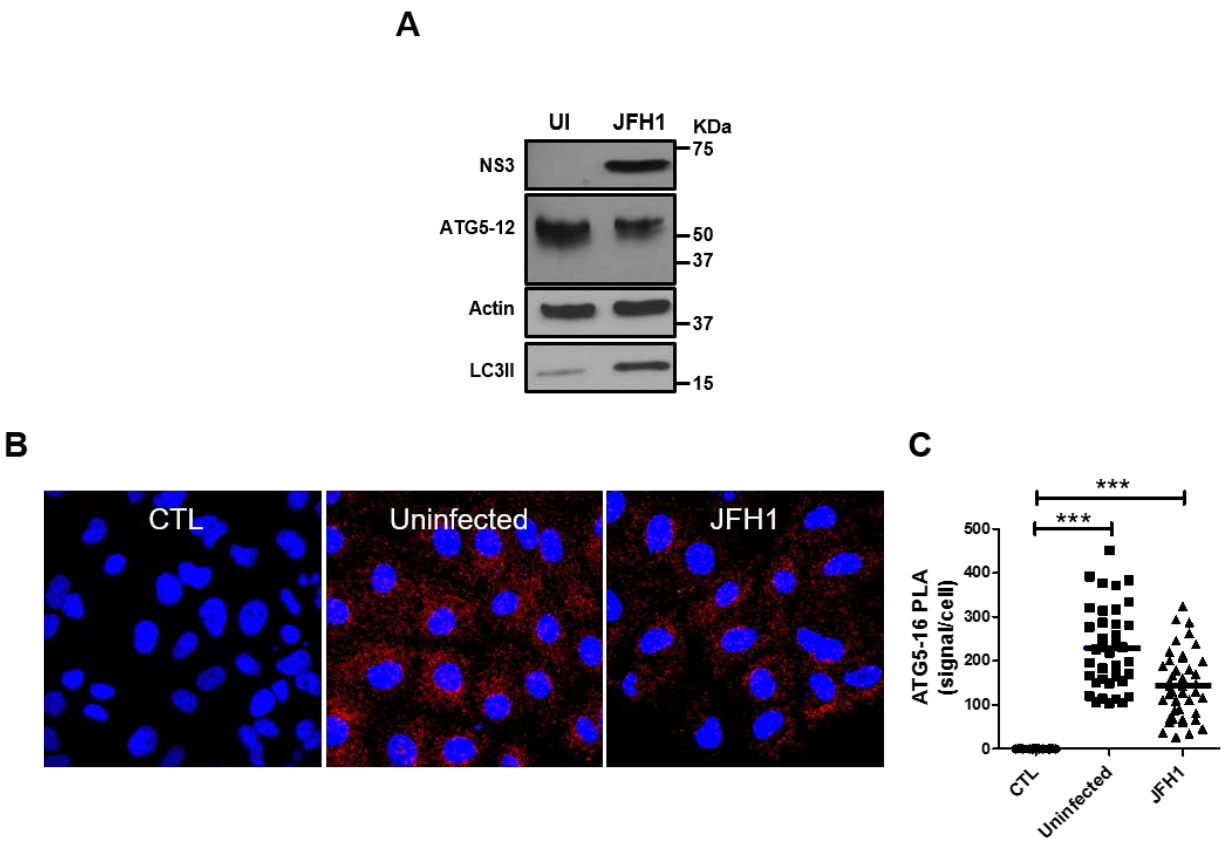

A
UI
JFH1
KDa
NS3
ATG5-12
Actin
LC3II
75
50
37
37
15
B
CTL
Uninfected
JFH1
C
***
***
ATG5-16 PLA (signal/cell)
500
400
300
200
100
0
CTL
Uninfected
JFH1

Figure 2

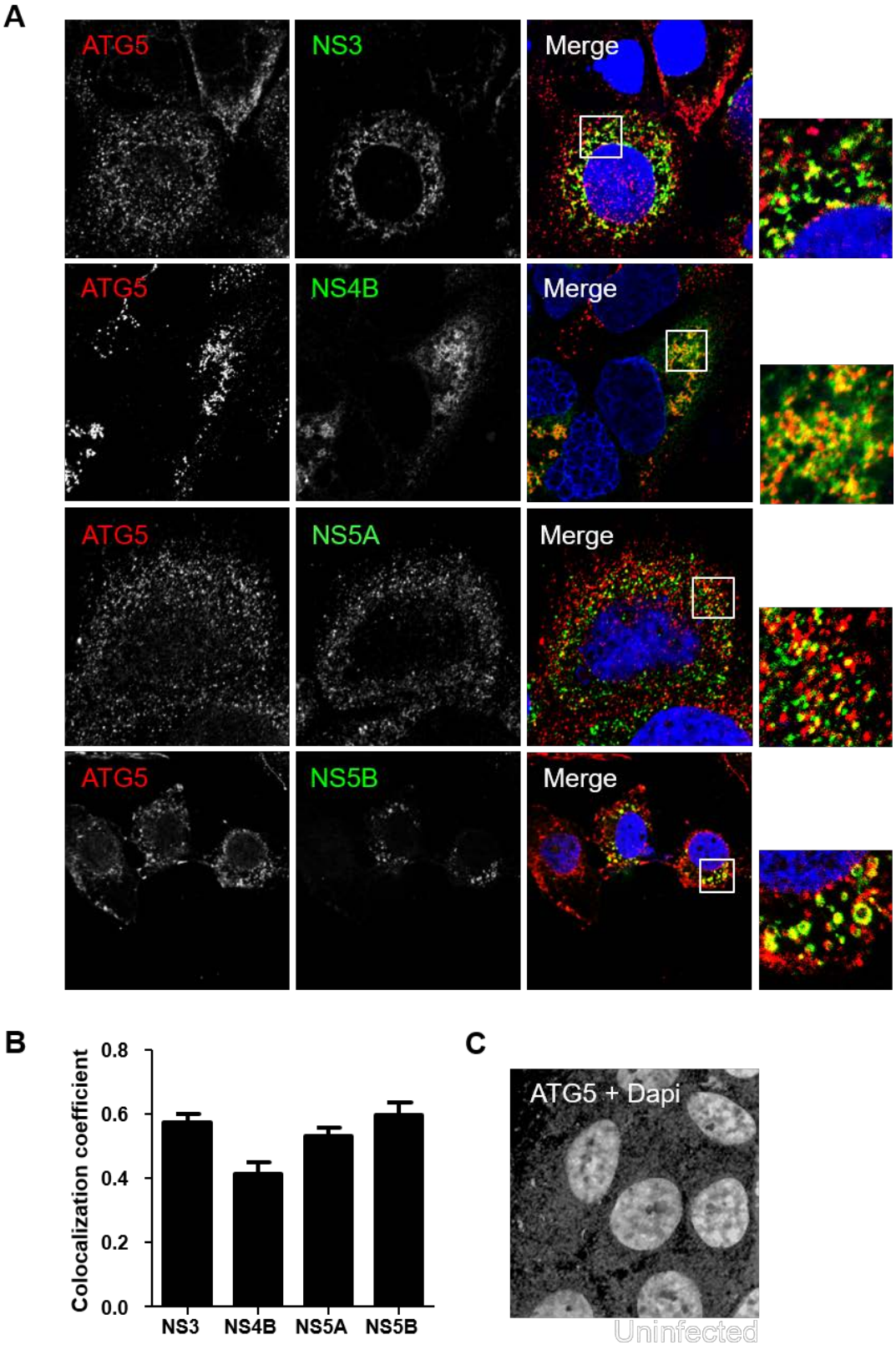

A
ATG5
NS3
Merge
ATG5
NS4B
Merge
ATG5
NS5A
Merge
ATG5
NS5B
Merge
B
Colocalization coefficient
0.8
0.6
0.4
0.2
0.0
NS3
NS4B
NS5A
NS5B
C
ATG5 + Dapi
Uninfected

Figure 3

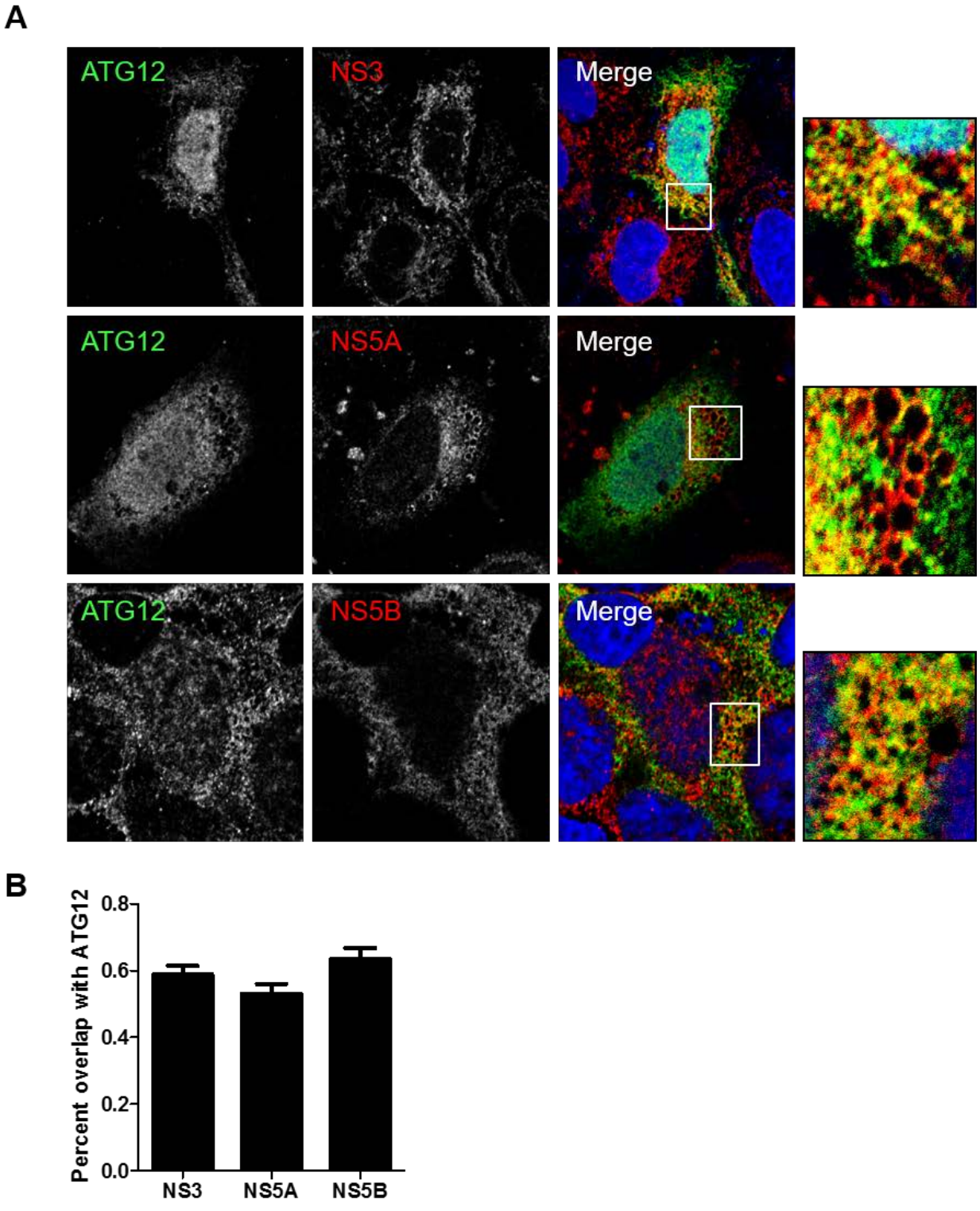

A
ATG12
NS3
Merge
ATG12
NS5A
Merge
ATG12
NS5B
Merge
B
Percent overlap with ATG12
0.0
0.2
0.4
0.6
0.8
NS3
NS5A
NS5B

Figure 4

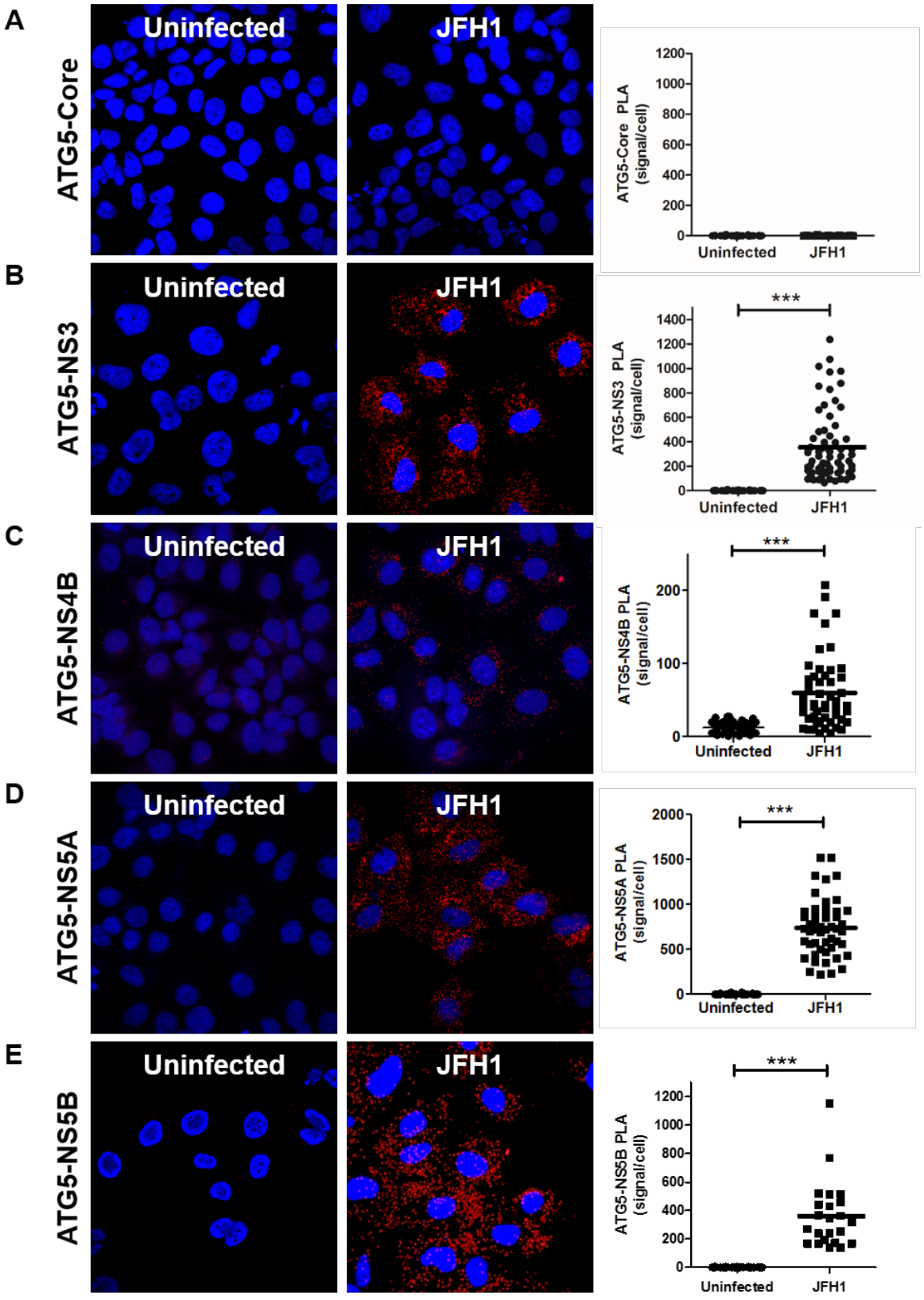

A
ATG5-Core
Uninfected
JFH1
ATG5-Core PLA (signal/cell)
1200
1000
800
600
400
200
0
Uninfected
JFH1
B
ATG5-NS3
Uninfected
JFH1
***
ATG5-NS3 PLA (signal/cell)
1400
1200
1000
800
600
400
200
0
Uninfected
JFH1
C
ATG5-NS4B
Uninfected
JFH1
***
ATG5-NS4B PLA (signal/cell)
200
100
0
Uninfected
JFH1
D
ATG5-NS5A
Uninfected
JFH1
***
ATG5-NS5A PLA (signal/cell)
2000
1500
1000
500
0
Uninfected
JFH1
E
ATG5-NS5B
Uninfected
JFH1
***
ATG5-NS5B PLA (signal/cell)
1200
1000
800
600
400
200
0
Uninfected
JFH1

Figure 5

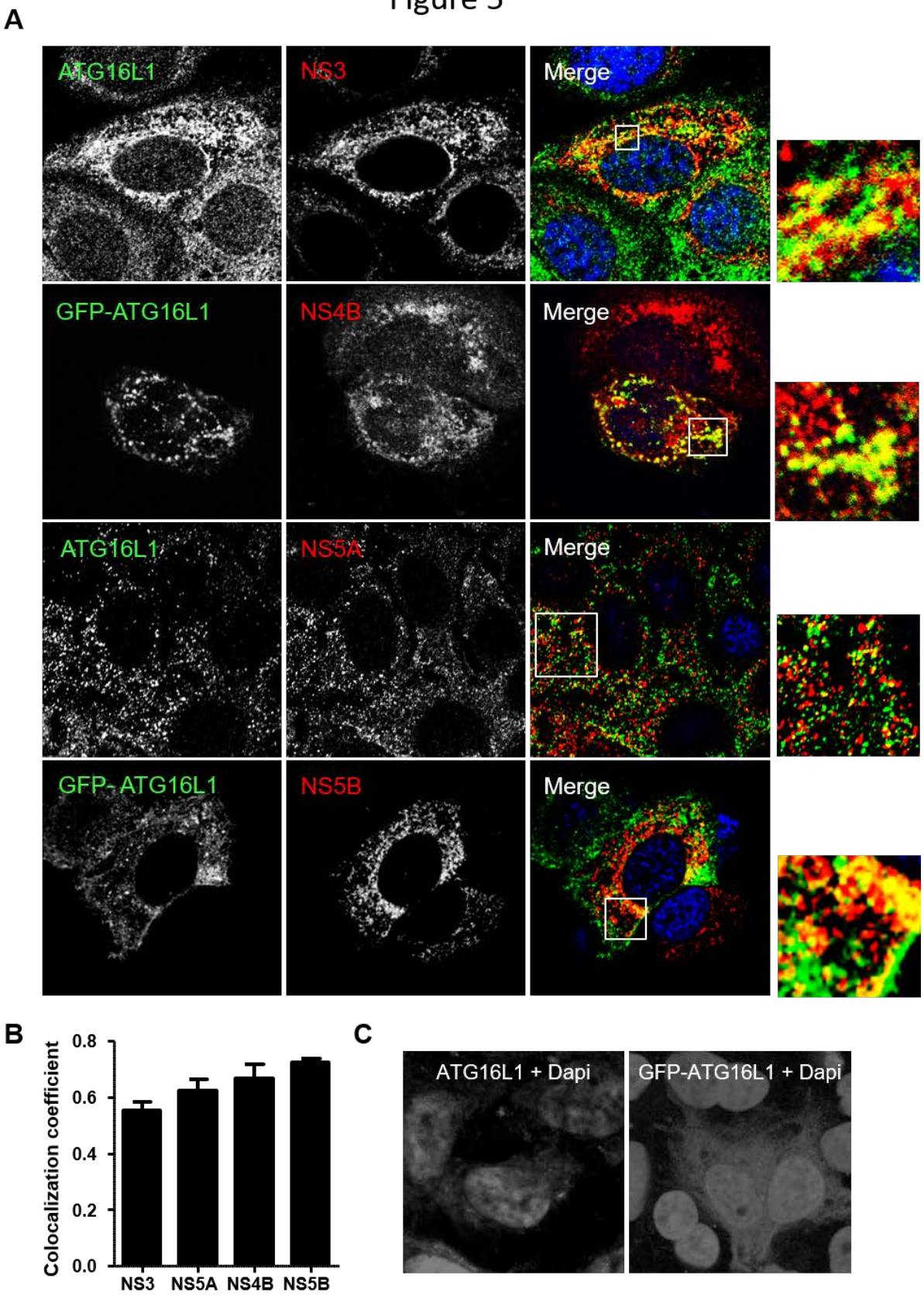
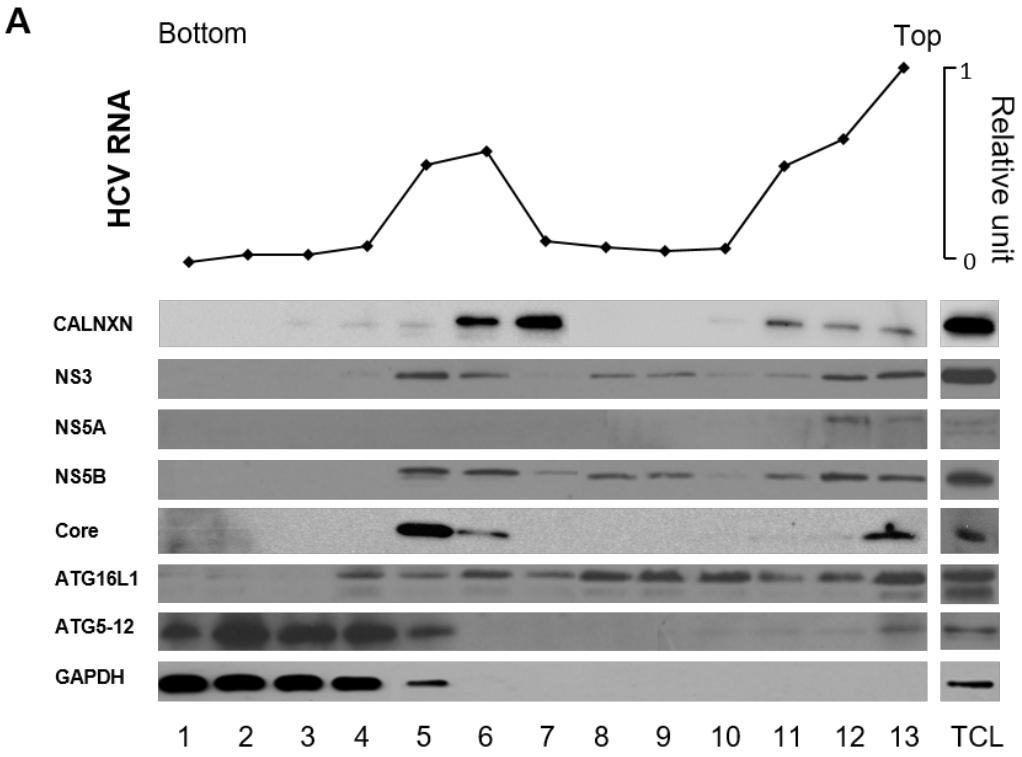

A
ATG16L1
NS3
Merge
GFP-ATG16L1
NS4B
Merge
ATG16L1
NS5A
Merge
GFP- ATG16L1
NS5B
Merge
B
Colocalization coefficient
0.8
0.6
0.4
0.2
0.0
NS3
NS5A
NS4B
NS5B
C
ATG16L1 + Dapi
GFP-ATG16L1 + Dapi

Figure 6


A
Bottom
Top
HCV RNA
1
0
Relative unit
CALNXN
NS3
NS5A
NS5B
Core
ATG16L1
ATG5-12
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13 TCL

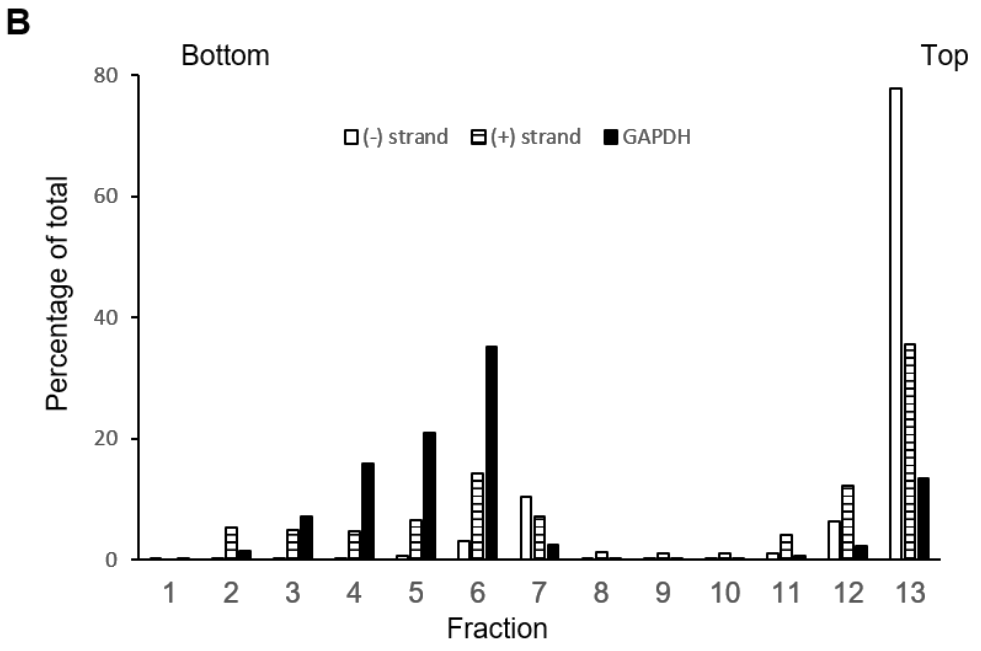

B
Bottom
Top
(-) strand
(+) strand
GAPDH
Percentage of total
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13
Fraction

Figure 7

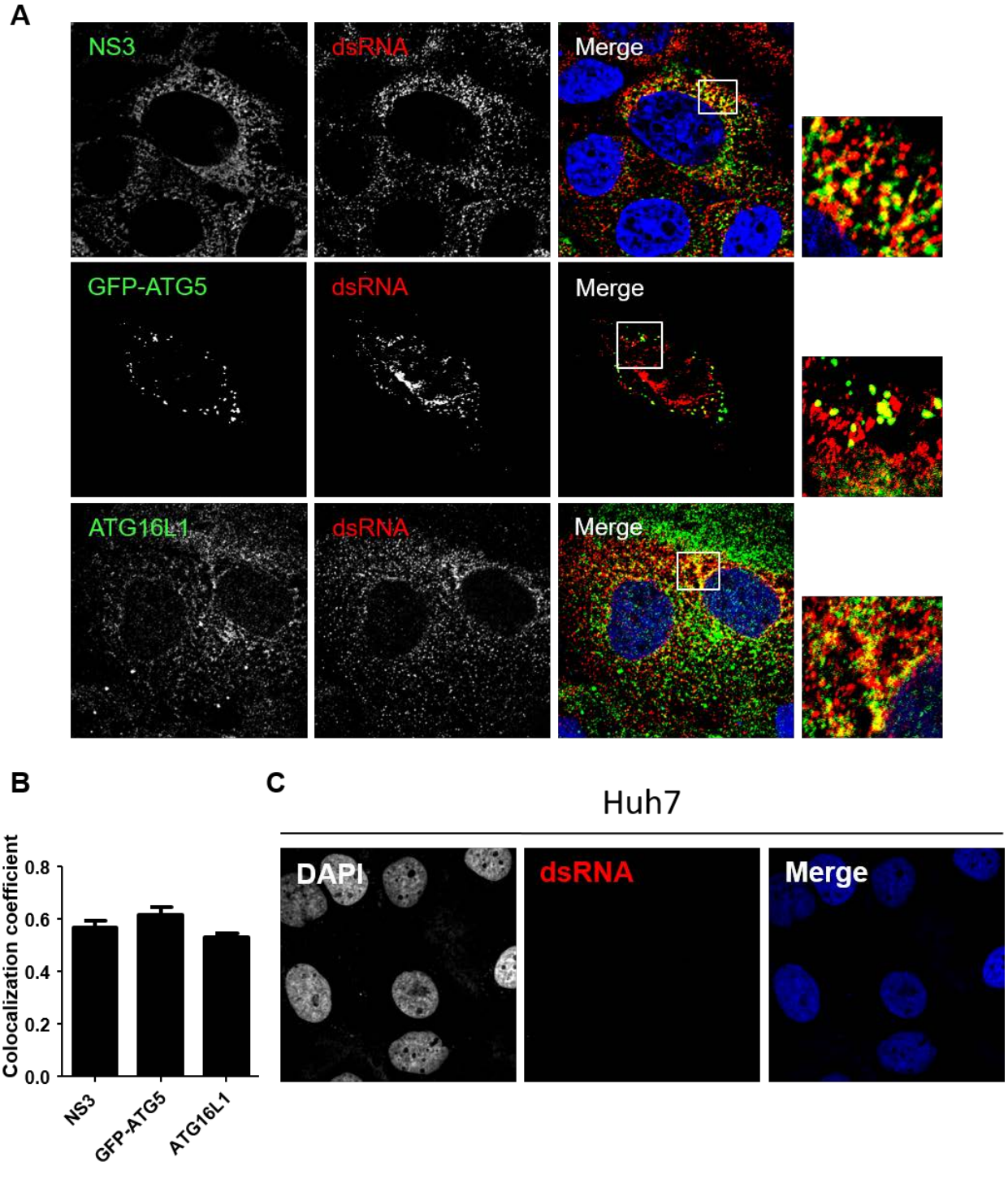
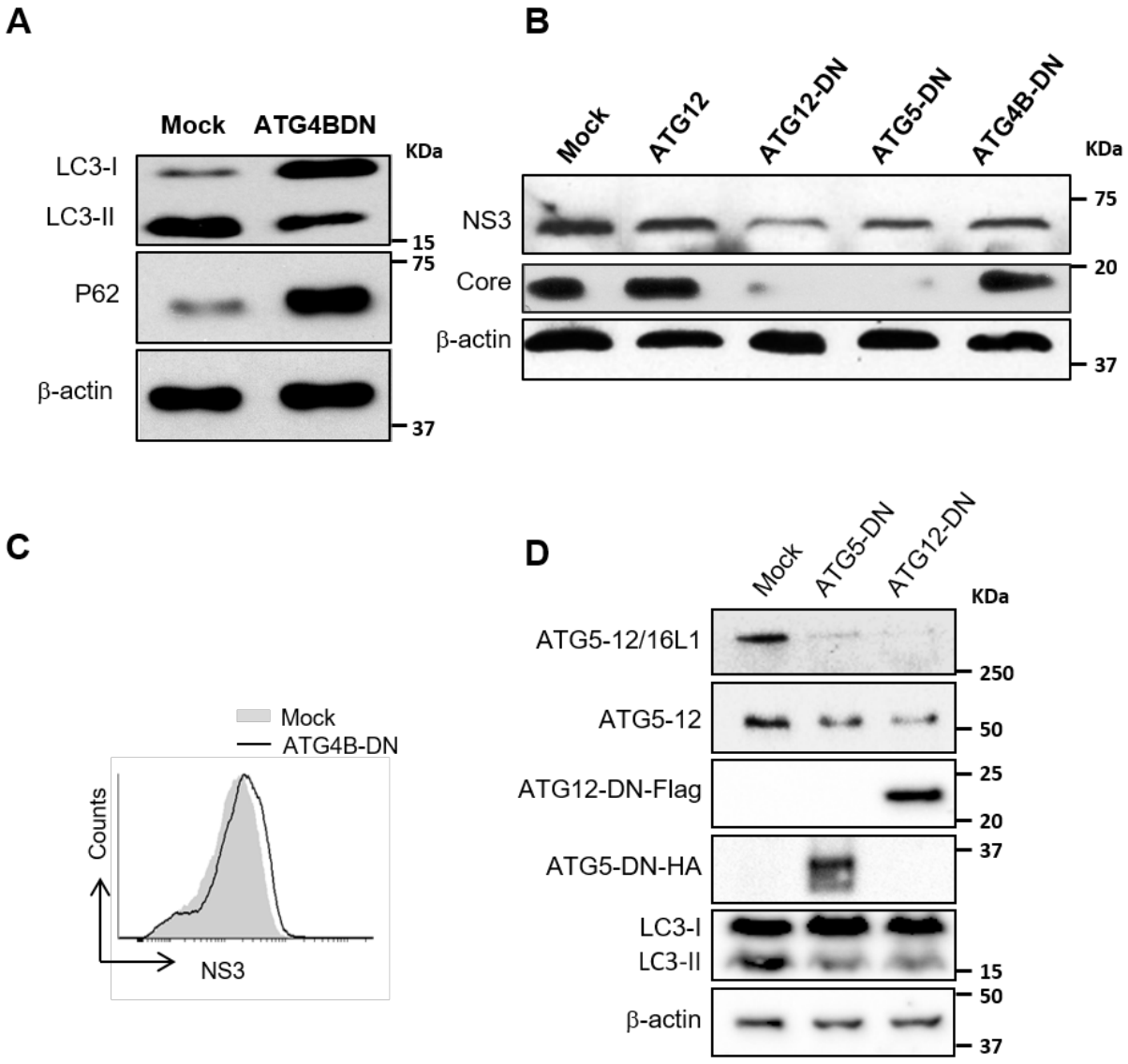
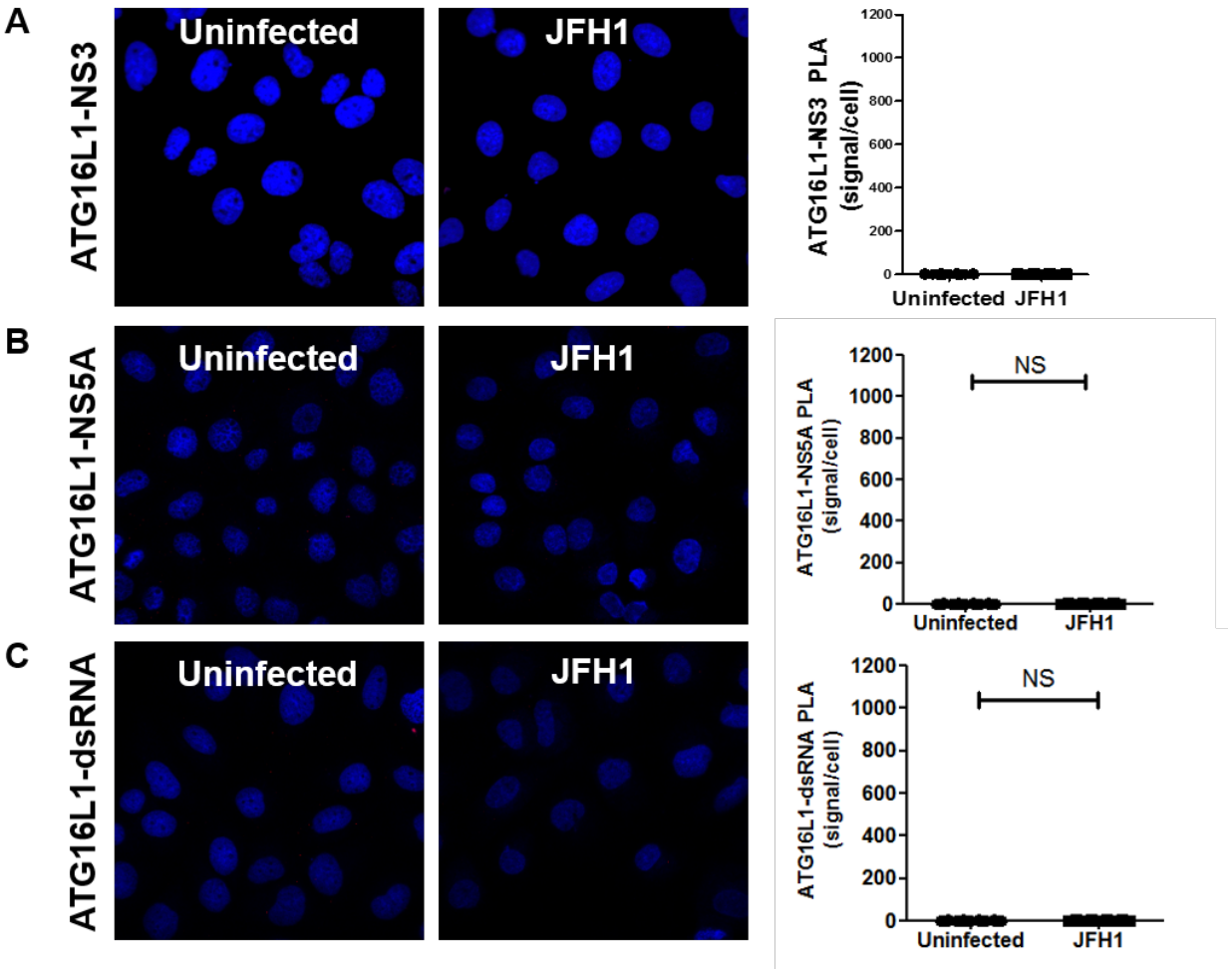

A
NS3
dsRNA
Merge
GFP-ATG5
dsRNA
Merge
ATG16L1
dsRNA
Merge
B
Colocalization coefficient
0.0
0.2
0.4
0.6
0.8
NS3
GFP-ATG5
ATG16L1
C
Huh7
DAPI
dsRNA
Merge

Figure 8

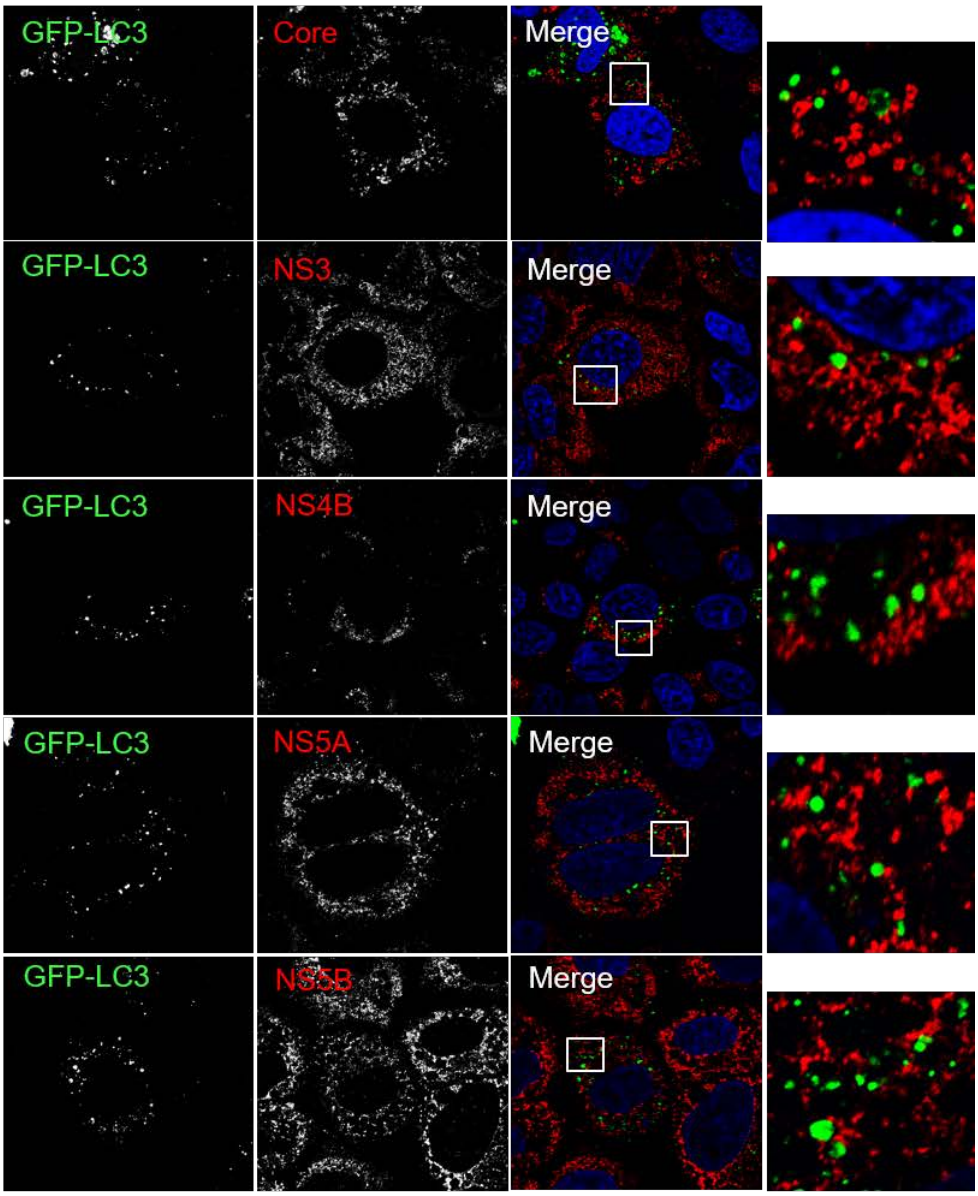

A
Mock
ATG4BDN
KDa
LC3-I
LC3-II
P62
β-actin
15
75
37
B
Mock
ATG12
ATG12-DN
ATG5-DN
ATG4B-DN
KDa
NS3
Core
β-actin
75
20
37
C
Mock
ATG4B-DN
Counts
NS3
D
Mock
ATG5-DN
ATG12-DN
KDa
ATG5-12/16L1
ATG5-12
ATG12-DN-Flag
ATG5-DN-HA
LC3-I
LC3-II
β-actin
250
50
25
20
37
15
50
37

Supplementary 1

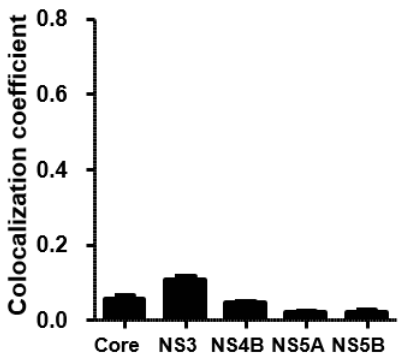


Supplementary 2

A



B



Publication No.2

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The autophagy elongation complex (ATG5-12/16L1) positively regulates HCV replication and is required for wild-type membranous web formation

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Abstract: 184 words

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Running title: The autophagy elongation complex is involved in HCV membranous web formation.

Contribution of the authors:

Ahmed M. Fahmy: Designed and performed the experiments, and wrote the manuscript

Patrick Labonté : Designed the experiments, and revised the manuscript.

Abstract

Hepatitis C virus (HCV) infection induces intracellular membrane rearrangements, thus forming a membranous web (MW) in which HCV replication and assembly occur. The HCV-induced MW is primarily composed of double membrane vesicles (DMVs) transfused by multi-membrane vesicles. The autophagy machinery has been proposed to participate in the formation of such vesicles. However, no clear evidence has been found linking autophagy to the formation of these DMVs. In this study, we evaluated the role of the autophagy elongation complex (ATG5-12/16L1) in HCV replication and MW formation. Using a dominant negative form of ATG12 and an siRNA approach, we demonstrated that the ATG5-12 conjugate, but not LC3-II formation, is crucial for efficient viral replication. Furthermore, purification of HCV MW revealed the presence of ATG5-12 and ATG16L1 along with HCV nonstructural proteins. Interestingly, LC3 was not recruited along with the elongation complex to the site of viral replication. Finally, inhibition of the elongation complex, but not LC3, greatly impaired the formation of the wild-type MW phenotype. To our knowledge, this study provides the first evidence of the involvement of autophagy proteins in the formation of wild-type MWs.

Introduction

Hepatitis C virus (HCV) infection is a leading cause of liver diseases, including cirrhosis and hepatocellular carcinoma. HCV, a member of the *Flaviviridae* family, is a *Hepacivirus* with a positive-strand RNA genome[1]. The virus replicates exclusively in the cytoplasm of the host cell. After cell entry, the 9.6 kb HCV genome is released and translated at the rough endoplasmic reticulum (rER) into a single polyprotein. This translated polyprotein is then proteolytically processed by cellular and viral proteases into 10 distinct proteins consisting of structural (core, E1, and E2) and nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins[2]. The expression of HCV proteins results in the induction of a major rearrangement of host cell membranes, thus leading to the formation of a complex membranous compartment termed the membranous web (MW), which favors viral RNA replication and assembly[3,4]. This massive remodeling of the host cell membrane network is associated with all positive-strand RNA viruses and is typically characterized by the generation of either convoluted membranes or double membrane vesicles (DMVs)[5-8]. Importantly, the HCV-induced MW is primarily composed of DMVs thus suggesting that autophagy plays a role in the construction of the HCV replication scaffold[7,9].

Macroautophagy, referred to hereafter as autophagy, is a catabolic pathway that degrades proteins and organelles, thereby maintaining cell homeostasis and directing cell fate. During cellular stress such as amino acid starvation, autophagy is triggered, thereby forming an organelle called the autophagosome. The *de novo* formation of the autophagosome begins by initiation of the growth of a double-membraned phagophore that, by closing, sequesters cytoplasmic contents. The autophagosome then fuses with the lysosome, thus allowing the degradation of the intra-autophagosomal cargo by the action of lysosomal enzymes and the release of free amino acids and other products. This process is orchestrated by more than 30 autophagy-related gene (ATG) proteins and other autophagy-linked proteins[10].

During the early steps of autophagosome biogenesis, ATG5, ATG12, and ATG16L1 form a stoichiometric complex known as the autophagy elongation complex (ATG5-12/16L1).

The elongation complex has been shown to determine the site of LC3 lipidation[11], a process required for the association of LC3 with the autophagosomal membrane. The membrane-associated LC3 allows the completion of autophagosome formation[12].

Although autophagy can act as an anti-viral mechanism, many reports have shown that positive-strand RNA viruses, including HCV, can hijack the autophagy machinery for virion morphogenesis and viral replication[13-17]. Several studies have shown that HCV induces autophagosome formation *in vitro*[9,18-21].This finding has also been confirmed in liver biopsies obtained from patients chronically infected with HCV[22]. In addition, HCV infection has been shown to induce mitophagy, the selective removal of mitochondria, through the induction of phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) and Parkin on the outer surface of the mitochondria. It has been proposed that degradation of mitochondria enhances HCV replication and suppresses cellular apoptosis[23]. Although several studies have shown that HCV infection induces autophagy by ER-stress via induction of the unfolded protein response (UPR)[20,24-27], other studies have shown that HCV induces autophagy in a manner independent of ER-stress, through the direct interaction of viral proteins with autophagy proteins[28,29]. Moreover, HCV-induced autophagy has been found to play a crucial role in evading host microbial defense mechanisms at the level of innate and adaptive immune responses[30]. The induction of autophagy promotes HCV replication either by enhancing protein translation[31] or via viral maturation[32]. Owing to the morphological similarities between HCV-induced DMVs and the double-membrane nature of autophagosomes, it has been proposed that autophagy plays a role in the biogenesis of viral replication compartments[7]. Importantly, Atg5 induces the formation of DMVs in embryonic stem cells[33]. In addition, a previous study conducted by our lab has shown that ATG5 interacts with the viral polymerase (NS5B) and co-localizes with NS4B, a MW-associated viral protein[34]. Another group has shown that ATG12, Beclin 1, and ATG4B are required for the establishment of viral replication[31]. However, studies on the involvement of autophagy proteins in the assembly of the MW remain elusive. In this study, we investigated the putative role of the ATG5-12/16L1 complex in the HCV replication-cycle. We found that the autophagy elongation complex (ATG5-12/16L1) is recruited at the MW, where it promotes HCV replication in an LC3-

independent manner. Surprisingly, knock-down of one component of the elongation complex, ATG12, led to an aberrant MW phenotype, thus suggesting a novel role of autophagy proteins in the formation of the MW.

Results

HCV does not perturb formation of the ATG5-12/16L1 complex.

ATG5 forms a conjugate with ATG12, but the monomeric forms of these two proteins have been shown to be nearly undetectable under normal conditions[35]. We first tested whether this conjugation occurs in HCV-infected cells. The assessment of the ATG5 protein by western blotting showed that monomeric ATG5 (32 kDa) was undetectable in both infected and uninfected cells. ATG5 was detected only in the ATG5-12-conjugated form (55 kDa) (Fig. 1A). Infection with HCV JFH1 strain was confirmed by detecting HCV NS3 protein using anti-NS3 antibody (Fig. 1A). The difficulty in detecting unconjugated ATG5 suggested that the majority of the ATG5 is readily conjugated to ATG12 in Huh7 cells, as has previously been reported for other cell types[36]. In addition, HCV infection did not inhibit this conjugation. Furthermore, HCV infection induced LC3-II accumulation (Fig. 1A)This result confirms the capability of HCV to modulate autophagy, as has previously been reported by several groups[9,18,19].

After ATG5 is conjugated to ATG12, it forms a multimeric complex by associating with ATG16L1. To test whether this complex forms in HCV-infected cells, we overexpressed ATG12-Flag in infected and uninfected cells. Using co-immunoprecipitation with an anti-flag monoclonal antibody, we detected the ATG5-12/16L1 complex under both conditions (Fig. 1B). Whereas ATG5-12 associates spontaneously with ATG16L1[37,38], our results indicated that HCV infection did not disturb the formation of the ATG5-12/16L1 complex.

Conjugation of ATG5-12 is required for HCV replication in infected cells.

To test whether the decoupling of the ATG5-12 conjugate influences HCV replication, we overexpressed the dominant-negative form of ATG12 (ATG12DN) in HCV-infected Huh7 cells. This mutated form of the ATG12 protein lacks the C-terminal glycine, which is crucial for conjugation with ATG5[39]. Interestingly, the overexpression of this conjugation-defective mutant had an adverse effect on HCV lifecycle, as indicated by a decrease in the NS3 protein (Fig. 1C), as well as the viral RNA level (Fig. 1D), as compared with the levels in mock-treated cells. The specificity of the effect of ATG12DN overexpression was assessed by trans-complementation with wild-type ATG12. As expected, trans-complementation with ATG12 restored the normal level of replication (Fig. 1C and D). Altogether, these results suggest that the ATG5-12 conjugate, rather than the monomeric form of the ATG5 and ATG12 proteins, acts as an HCV proviral factor.

The ATG5-12 conjugate is involved in the HCV lifecycle at a post-translational step.

The conjugation of ATG12 to lysine 130 of ATG5 is mediated by ATG7, an E1-like enzyme, thus allowing the formation of the ATG5-12/16L1 complex. A fraction of the ATG5-12/16L1 complex localizes to the isolation membrane, where it facilitates LC3 lipidation. Another role of ATG7, along with ATG3, an E2-like enzyme, is to activate LC3 after its processing at the C-terminus by ATG4B, thus allowing its conjugation to the amino group of phosphatidylethanolamine (PE) and formation of the membrane-associated LC3-II, which assists in the expansion and closure of the autophagosome[40,41]. Therefore, silencing of ATG7 allows the inhibition of LC3-II and ATG5-12 conjugation (Fig. 2B). Thus, by silencing LC3, ATG7 or ATG12, we were able to analyze the independent contributions of the two autophagy conjugation systems in the HCV lifecycle. For this purpose, we first determined the efficiency of the selected siRNA to knock down their respective targets (Fig. 2A, B and C). We then analyzed the effects of the silencing of ATG12, LC3 or ATG7 on HCV entry, viral RNA translation/replication and virion

maturation and secretion. As shown in Figure 2D, Huh7 infection by HCVpp was not altered in cells treated with siRNA against LC3, ATG7 or ATG12, thus suggesting that neither LC3 nor the ATG5-12 conjugate is involved in viral entry.

Previously, Dreux and colleagues have reported that HCV RNA translation is affected by inhibition of LC3 conjugation using siRNA against ATG4B. In that report, the authors followed the luciferase activity expressed from a replication-defective subgenomic replicon RLuc/SGR harboring an inactivation mutation (GDD to GND) at the active site of the HCV polymerase NS5B (RLuc/SGR-GND)[31]. Using a similar approach, we evaluated the effects of LC3, ATG7 and ATG12 silencing on viral RNA translation and/or replication. We used full-length HCV JFH1 RNA with a firefly luciferase reporter containing the GND mutation in the active site of NS5B (JFH1/Fluc-GND) and then analyzed the Fluc activity expressed from the HCV internal ribosomal entry site (IRES). A significant decrease in luciferase activity (>80%) was observed in JFH1/Fluc-GND-transfected cells pretreated with siLC3 (Fig. 2E). Silencing of ATG7, which inhibits LC3-II formation as well as ATG5-12 conjugation, decreased viral translation by 50%. This effect probably occurred through the inhibition of LC3-II conjugation, because silencing of ATG12 expression was much less efficient than LC3 silencing at inhibiting viral RNA translation (Fig. 2E). In contrast, silencing of ATG12 severely affected the luciferase activity of a replication competent JFH1/Fluc wild-type virus. The effect on replication was not due to the toxicity of siRNA treatment (Fig. S1), thus suggesting that the ATG5-12 conjugate is involved in an HCV lifecycle step(s) beyond entry and RNA translation (Fig. 2E), whereas LC3 expression and/or conjugation is primarily important for viral translation, as previously suggested[31].

The ATG5-12 conjugate positively regulates HCV RNA replication in an LC3-independent manner.

To determine whether LC3 or the ATG5-12 conjugate modulates HCV RNA replication, we analyzed the effects of silencing these autophagy genes on viral RNA replication in Huh7 cells stably expressing the JFH1 subgenomic replicon (SGR). Using these specific cells, which are capable of only intracellular HCV RNA replication and lacked the capacity

to produce infectious viral particles, allowed us to study HCV RNA replication independently of viral entry and egress. Again, silencing of ATG12 but not LC3 efficiently inhibited RNA replication, thus supporting the role of the ATG5-12 conjugate in viral replication and ruling out the possibility that LC3 participates in viral replication (Fig. 3A and B). Because silencing of LC3 expression led to a clear inhibition of HCV RNA translation after electroporation of the viral RNA but did not significantly affect replication in JFH1-SGR cells, we sought to compare the effect of siRNA treatment before and after infection with HCVcc JFH1 (Fig. 3C). The results clearly demonstrated that siLC3 inhibited HCV only when it was transfected before infection, whereas siATG7 was effective when it was transfected before or after infection. These results suggest that LC3 is important early in infection and primarily for initial HCV RNA translation, as has previously been reported[31]. Finally, we evaluated the effects of siRNA treatment on intracellular and extracellular HCV infectious particle production in JFH1-infected cells (Fig. 3D). These results suggested that HCV maturation and secretion was not significantly affected by siRNA treatment. Although silencing ATG12 led to a significant decrease in HCV particle formation, this effect was attributed to a severe reduction in viral replication. Collectively, ATG12 silencing and to a lesser extent ATG7 but not LC3, impaired viral replication.

ATG5-12 and ATG16L1 are associated with purified MW extract.

In a previous study from our lab, we have shown that ATG5 interacts with the HCV polymerase (NS5B) and co-localizes with the MW associated protein NS4B[34]. The major limitation in our ability to investigate the composition of the MW has recently been resolved by Dr. Ralf Bartenschlager's group, which has developed a method to purify the HCV MW by using HCV replicon cells harboring an HA-tag NS4B (NS4B-HA)[42]. This method allowed us to evaluate the presence of the autophagy elongation complex proteins in purified MW extract. Through this protocol, after membrane enrichment from a discontinuous sucrose gradient via ultracentrifugation, we pooled fractions that were rich in viral nonstructural proteins (fraction 3 to 7) but mostly devoid of soluble proteins

(GAPDH or LC3I, fractions 8-10) (Fig. 4A). The MW vesicles were then pulled down from pooled fractions by using a specific antibody against the HA-tag of the HCV NS4B protein. Subsequently, the MW-enriched extract was used for either western blot analysis or vesicle visualization by transmission electron microscopy (TEM). As expected, HCV NS4B^{HA}, NS3 and NS5A were readily detectable in the purified extract from NS4B^{HA} replicon cells but not that from control untagged NS4B replicon cells (Fig. 4B). The autophagy elongation complex proteins (ATG5-12 and ATG16L1) were also detected in the purified MW from NS4B^{HA} replicon cells, but not in the control extract, thus indicating that the elongation complex is indeed present at the HCV replication site. In contrast, we were unable to detect LC3II in the purified MW (Fig. 4B), thereby suggesting that LC3 is not recruited with the autophagy elongation complex to the MW. We then examined the morphology of purified membranes and compared them with ER membranes purified from a cell line expressing HA-tagged Calnexin, as previously described[42]. The results showed that almost 90% of the NS4B^{HA} purified membranes were spherical vesicles as compared with CLNXN^{HA} purified material, in which the majority of membranes were composed of partially collapsed large membranes (Fig. 5A and B). Our results are in agreement with those of Paul and colleagues, who have demonstrated that most of the purified ER membranes are composed of elongated structures, as opposed to the spherical vesicles found in MW extracts[42]. Finally, the specificity of the pull-down using HA-beads was confirmed by using extracts from untagged SGR cells (Fig. 5C). Altogether, these results indicate that at least a fraction of the autophagy elongation complex is localized in the virus-induced MW compartments.

Silencing of ATG12 or ATG7, but not LC3, alters the phenotype of the MW.

To evaluate the putative role of host cell proteins in MW formation, Reiss and colleagues have established a T7-polymerase-based HCV RNA synthesis system in which continuous production of HCV polyproteins persists even when HCV replication is abrogated[43]. This system is particularly useful to evaluate the formation of the MW while targeting host cell proteins in the absence of HCV RNA replication. With this system, it has been shown that

alterations in MW formation result in a clustered phenotype of HCV nonstructural proteins[43,44]. Thus, using the same system (obtained from Dr. Volker Lohmann), we analyzed MW formation indirectly by monitoring viral protein localization after treatment with siLC3, siATG7 and siATG12. Under normal conditions, the NS3 and NS5A cellular distribution appeared as small punctate structures that appeared to be membrane associated (Fig. 6A and B). Treatment with siLC3 did not alter the cellular distribution of the viral proteins, as observed by confocal microscopy (Fig. 6A, B and C). Strikingly, silencing of ATG7 or ATG12 resulted in the formation of larger protein clusters in most of the transfected cells (Fig. 6A, B and C). This effect was not due to decreased HCV protein expression level (Fig. 6D), thus suggesting that the ATG5-12 conjugate, but not LC3, is required to obtain a wild-type MW phenotype.

Silencing of ATG12 or ATG7, but not LC3, modifies MW morphology.

Next, using TEM, we analyzed MW morphology after treatment with siLC3, siATG7 or siATG12. Expression of pTM-NS3-5B in cells treated with siCTL induced heterogeneous membrane alterations composed of DMVs of an average size of 200 nm interspersed by multi-membrane vesicles (MMVs) that were distributed throughout the cytoplasm (Fig. 7A and B) and that were not seen in negative cells (Fig. 7E). Silencing of ATG7 resulted in more homogenous DMVs with a markedly decreased average size (90 nm) and led to the disappearance of MMVs (Fig. 7A, B and D). Silencing of ATG12 led to a similar effect but with a much lower abundance of DMVs (Fig. 7C). However, silencing of LC3 had no effect on the DMV size (average diameter 200 nm) (Fig. 7B) or on the vesicle types in which both DMVs and MMVs coexist (Fig. 7C and D). Thus, a similar morphology of membrane alterations was observed in siCTL-treated cells (Fig. 7A and B). These results strongly suggest that the ATG5-12 conjugate is crucial for the formation of a typical HCV-induced MW architecture.

Discussion

In the present study, we demonstrated the requirement of the ATG5-12/16L1 complex for the completion of the HCV lifecycle. HCV infection does not hamper ATG12 conjugation to ATG5 or the formation of the multimeric complex ATG5-12/16L1 (Fig. 1A and B). In contrast, the conjugation of ATG12 to ATG5 is crucial for the HCV lifecycle. More specifically, our study suggests a role of the ATG5-12/16L1 complex in HCV genome replication and the formation of the MW. The involvement of the autophagy elongation complex in the HCV replication step was investigated by using siRNA targeting of ATG7, ATG12 or LC3. Because the silencing of ATG7 is known to inhibit the conjugation of both LC3 and ATG5, we were able to address the importance of these two conjugation systems in HCV replication. Indeed, the ATG5-12 conjugate acted as a proviral factor at a step beyond entry and RNA translation but before virion maturation and secretion, as depicted in Figures 2 and 3. We also observed that silencing of LC3 interfered with HCV RNA translation after electroporation of replication-defective replicon (Fig. 2E). These results are consistent with those of Dreux and colleagues, who have found a defect in viral RNA translation after silencing of Beclin-1 or ATG4B, thus leading to inhibition of LC3-II formation[31]. Silencing of ATG12 had little effect on replication-deficient virus but was detrimental to the replication of the JFH1/Fluc virus, thus indicating that its primary target is beyond the translation step (Fig. 2E). This result was further confirmed in cells stably expressing the JFH1 subgenomic replicon, in which silencing of ATG7 or ATG12, but not LC3, significantly inhibited HCV replication (Fig. 3A and B). Silencing of LC3 impeded HCV only when performed before infection, thus suggesting that the ATG5-12 conjugate, but not LC3, is important in viral replication after the establishment of infection (Fig. 3C).

In addition, the co-purification of the elongation complex proteins with the MW suggested that the ATG5-NS5B interaction previously described by our laboratory[34] might actually participate in targeting of the elongation complex to the MW and/or in supplying of autophagic isolation membranes for the formation of the virus-induced vesicles. In canonical autophagy, the ATG5-12/16L1 complex is recruited to the isolation membrane prior to LC3 and is released just before the completion of autophagosomes. The absence

of LC3 in the purified MW suggests that HCV either hijacks ATG5-12/16L1-positive LC3-negative isolation membranes or initiates the *de novo* formation of the isolation membrane at the MW rather than utilizing LC3-positive autophagosomes for the formation of DMVs within the MW (Fig. 4). Interestingly, the recruitment of the elongation complex to the MW was not accompanied by LC3 lipidation or its relocation at that site. Recently, it has been demonstrated that the ATG5-12/16L1 complex has a membrane-tethering activity that is independent of LC3[45,46]. This finding highlights the possibility that in HCV infected cells the major role of the elongation complex is to tether vesicles during MW formation. Concomitantly, it has been reported that some ATG proteins, including ATG16L1, can traffic in LC3-free vesicle-like structures to the site where they probably act to generate *de novo* isolation membranes[47]. This finding also raises the possibility that HCV may recruit similar structures that aid in the formation of the MW.

Recently, Reiss and colleagues have developed a system to evaluate the importance of host factors in membranous web formation[43]. Using this system, we demonstrated that ATG7 as well as ATG12 expression, but not LC3, are important to obtain a wild-type MW phenotype, as observed using confocal microscopy (Fig. 6). Furthermore, the morphology of the HCV-induced vesicles was severely altered after silencing of ATG7 or ATG12, but not LC3. Notably, knocking down ATG12 decreased the size and the number of DMVs, whereas silencing of ATG7 mainly affected their size (Fig. 7). At the moment, it remains unknown whether the altered MW is HCV-replication competent. However, the importance of the ATG5-12 conjugate in HCV RNA replication suggests that the autophagy elongation complex inhibits HCV replication through destabilization of the viral replication factories present within the MW.

In summary, recruitment of the autophagy elongation complex to the MW, which is normally involved in DMV formation, promotes viral replication and maintains proper formation of the wild type MW.

Methods

Cell culture and reagents. Huh7 and Huh7-Lunet cells stably expressing Calnexin or NS4B-HA replicon were obtained from Dr Ralf Bartenschlager. Huh7-Lunet cells stably expressing the T7 polymerase (Huh7-Lunet-T7) was obtained from Dr. Volker Lohmann, and the Huh7.5 cell line was obtained from Dr. Charles Rice. All Huh7-derived cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% v/v fetal bovine serum (FBS) (Multicell), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Gibco) at 37°C, 5% CO_2, in a humidified incubator. Cell lines harboring the wild-type replicon or $NS4B^{HA}$ were maintained in medium supplemented with G418 (Gibco) at a final concentration of 500 µg/ml. Huh7-Lunet-T7 and Huh7-Lunet cells stably expressing calnexin were cultured in the presence of 10 µg/ml blasticidin (InvivoGen).

Plasmids and antibodies. The hATG5 and hATG16L1 sequences were cloned into the peGFP-C1 plasmid (Clontech), thus forming pGFP-ATG5 and pGFP-ATG16L1, respectively. The Flag-tagged ATG12 (pATG12) and its dominant-negative derivative pATG12ΔG140 (ATG12DN) constructs were kindly provided by Dr. Adi Kimchi[39]. The PTM vector for the expression of HCV nonstructural proteins NS3 to 5B (pTM-NS3-5B) was kindly provided by Dr. Volker Lohmann. Rabbit polyclonal anti-LC3, rabbit polyclonal anti-ATG5, mouse monoclonal anti-Flag, and mouse monoclonal anti-β-actin antibodies were purchased from Sigma Aldrich. Rabbit polyclonal anti-ATG12 and anti-ATG7 were purchased from Cell Signaling. Rabbit polyclonal anti-ATG16L1 antibody was purchased from MBL. Mouse monoclonal anti-HA was purchased from Roche. Mouse monoclonal anti-NS3 and anti-NS5A antibodies were purchased from BioFront. Rabbit polyclonal anti-NS3 and NS5A were obtained from Dr. Olivier Nicolas. Rabbit polyclonal anti-NS4B and anti-NS5B antibodies were kindly provided by Drs. Kouacou Konan and Takaji Wakita, respectively. Mouse monoclonal anti-GAPDH was purchased from Santa Cruz.

Preparation of viral stock and infections. The cell culture-derived HCV (HCVcc) JFH1 virus was generated in Huh7 cells by transfection of *in vitro*-transcribed full-length JFH1 RNA (MEGAscript, Ambion). Viral stocks were produced by infection of Huh7 cells at a multiplicity of infection (MOI) of 0.01, as described previously[48]. A replicative bicistronic JFH1-based full-genome construct expressing Firefly luciferase (pJFH1/Fluc) and a clone with a mutation in the viral polymerase (GDD-to-GND) (pJFH1/Fluc-GND) were generated as previously described[49]. To reach 90% infected cells, Huh7 cells were infected at an MOI of 0.01, passaged for 7 days and then analyzed by immunofluorescence using an anti-NS5A antibody.

Western blot analysis. Cells were lysed in 300 µl of lysis buffer [25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP40, Complete protease inhibitor (Roche)]. The lysates were normalized for total protein content using the BCA protein assay kit (Pierce). The proteins were then resolved by SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad), blocked for 30 min at room temperature (RT) with PBS-5% milk, and then incubated overnight at 4°C with primary antibody in PBS-2% BSA. After being washed with 0.4% Tween 20 in PBS (PBST), the membranes were incubated for 1 h at RT with a goat-anti-rabbit or goat-anti-mouse IgG conjugated to horseradish peroxidase in PBS-5% milk. Protein bands were visualized with either the Clarity western ECL (Bio-Rad) or Femto chemiluminescence substrates (Pierce).

Immunoprecipitation. HCVcc-infected and uninfected Huh7 cells were transfected with a plasmid encoding ATG12-Flag. At day 2 post-transfection, the cells were placed on ice and washed with PBS containing 1 mM Na_3VO_4. The cells were scraped in the presence of 300 µl of lysis buffer [1% NP-40, 20 mM Tris-HCl (pH 7.5), 135 mM NaCl supplemented with 50 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1.5 mM EGTA and Complete™ protease inhibitor (Roche)]. ATG12-Flag was immunoprecipitated from the total lysate by using anti-Flag antibody.

Purification of HCV-induced MW. HCV-remodeled membrane purification was performed using a method adopted from a previously described protocol[42]. Briefly, 7.5 x 10^7 Huh7-Lunet cells harboring either wild-type or HA-tagged NS4B replicons or control cells stably overexpressing $CANX^{HA}$ were washed, scraped and then resuspended in 500 µl of hypotonic buffer and incubated on ice for 30 min. The cells were lysed with 50 strokes with a Dounce homogenizer. The lysates were centrifuged at 800 x g for 10 min at 4°C. Supernatants were collected and layered on top of a discontinuous sucrose gradient (70% to 30%) and centrifuged at 130,000 x g for 4 h at 4°C using an SW60i rotor (Beckman Coulter). Ten fractions were collected from the bottom (300 µl each) and analyzed for protein content. For HA affinity capture, fractions 3 to 7 were pooled, and then an equal amount of protein contained in pooled fractions was equilibrated to 150 mM NaCl. Incubation with HA-agarose beads (Sigma-Aldrich) was performed as previously described[42].

Membrane visualization by transmission electron microscopy. To examine purified membranes, 50 µl of eluted material was centrifuged at 10 p.s.i. on a copper grid for 5 min at RT in an Airfuge (Beckman). Structures were negatively stained using 2% aqueous uranyl acetate for 30 sec and examined with an H-7100 (Hitachi) transmission electron microscope.

Quantification of HCV RNA by RT-qPCR. Isolated RNA was reverse transcribed with M-MLV (Invitrogen). The generated cDNA was used for qPCR using Taqman probes, as previously described[50]. Results were analyzed using the comparative ΔCt method.

Small interfering RNA (siRNA) transfection. Huh7 cells were reverse transfected in a 24-well plate with siRNA (25 nM final concentration) using Lipofectamine RNAiMAX reagent (Life Technologies) according to the manufacturer's protocol. Huh7 cells were transfected with siRNA to GFP, LC3B siRNA (UACCUGUAUACGUUAGUGAAAUU)

or with an ON-TARGETplus human ATG7 siRNA-SMART pool (catalog no. L-020112-00-0005). To study the onset of replication, Huh7.5 cells were reverse transfected with siRNA, as described above, in 6-well plates. Forty-eight hours later, the cells were trypsinized, washed twice with cold PBS, resuspended in 100 µl of cold Ingenio electroporation solution (Mirus) and then electroporated with 5 µg of ***in vitro***-transcribed viral RNA (JFH1/Fluc or JFH1/Fluc-GND) in 2 mm gap electroporation cuvettes by using a BTX Harvard Apparatus with the following settings: 820 V, 99 µS, 4 pulses, 1.1 s interval. The cells were then resuspended in DMEM-10% FBS and seeded in 96-well plates and further cultured for 24 h. The cells were then lysed in 20 µl luciferase lysis buffer (RLB) and stored at -80°C until measurement of luciferase activity. For the determination of intra- and extracellular virus titers, JFH1-infected Huh7 cells were reverse transfected with siRNA in 6-well plates. Two days later, the cells were washed three times with PBS and supplemented with fresh DMEM. After 24 h, cells and supernatants were harvested. The cells were washed twice with PBS, trypsinized, resuspended in 1 ml culture medium and subjected to 3 rapid freeze-thaw cycles in a dry ice/ethanol bath and 37°C water bath, respectively. Cell debris was removed by centrifugation at 10,000 rpm for 3 minutes. Samples were analyzed using a limiting dilution assay.

Production of HCVpp and cell entry assay. Viral pseudotyped particles harboring the HCV glycoproteins (HCVpp) were produced by transfection in Hek-293T cells of vectors encoding viral glycoproteins, packaging proteins and a Luciferase marker. After 48 h, viral pseudoparticle supernatants were harvested and filtered through 45-µM filters to remove the cell debris. For the entry assay, Huh7.5 cells were reverse transfected with siRNA in 96-well plates. After 48 h, the cells were infected with 50 µl HCVpp containing supernatant. Forty-eight hours post-infection, the cells were washed three times with PBS, lysed in 20 µl luciferase lysis buffer (RLB) and stored at -80°C until measurement of luciferase activity.

Luciferase assay. Cell lysates were prepared with Reporter Lysis Buffer (RLB) (Promega), and luciferase activity was measured with a luciferase assay system (Promega), per the manufacturer's protocol.

Assessment of the MW phenotype. For the immunofluorescence experiment, Huh7-Lunet-T7 cells were reverse transfected with siRNA as previously described[43]. Forty-eight hours later, a second round of transfection with siRNA was performed. After 48 h, cells were transfected with pTM-NS3-5B using Lipofectamine 3000 (Invitrogen). The coverslips were then fixed with 4% formaldehyde in PBS for 10 min, washed in PBS and incubated in blocking buffer (PBS, 3% bovine serum albumin, 10% FBS, 0.1% Triton X-100) for 30 min at RT. After being washed three times with PBS, the coverslips were incubated with primary antibody in blocking buffer for 1 h at RT. Then, the coverslips were washed with PBS and incubated with either Alexa Fluor™-(488 or 568) goat anti-mouse IgG or Alexa Fluor™-(488 or 568) goat anti-rabbit IgG (Invitrogen) for 1 h at RT. After being washed, the coverslips were mounted on glass slides with Prolong™ Antifade (Invitrogen) and examined with a laser scanning confocal Zeiss LSM 780.

For TEM analysis, a similar setup was used, except that after transfection with pTM-NS3-5B, the cells were trypsinized and seeded into lab-tek chamber slides (Thermo Fisher). After 24 h, the monolayer of cells was washed with PBS, fixed with 2.5% glutaraldehyde (Electron Microscopy Science) and incubated overnight at 4°C. The cells were then washed in 0.1 M cacodylate (Electron Microscopy Science) and incubated in 1% osmium tetroxide (Mecalab) for 1 h at 4°C. The cells were dehydrated in a graded series of ethanol/deionized water solutions (from 50% to 100%). The cells were then infiltrated with a 1:1 and 3:1 Epon 812 for 1 h for embedding and polymerized overnight in an oven at 60°C. The polymerized blocks were trimmed, and 100 nm ultrathin sections cut with an UltraCut E ultramicrotome (Reichert Jung) and transferred onto 200-mesh copper grids (Electron Microscopy Science) with formvar support film. The sections were stained with 4% uranyl acetate (Electron Microscopy Science) for 8 min, then with lead citrate for 5 min (Fisher scientific). The cells were imaged with an FEI Tecnai 12 transmission electron microscope

(FEI company) operating at an accelerating voltage of 120 kV and equipped with an AMT XR80C CCD camera. Vesicle size was measured using Image J (NIH).

Cell viability assay. Cells were reverse transfected with different siRNAs used in this study in a 96-well plate for 48 h. Cell viability was then assayed using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay reagent (Promega).

Statistical analyses. The results shown represent the mean of at least three independent experiments. Student's-t-test and one-way ANOVA with Dunnett's post-test (as indicated in the figure legends) were performed using GraphPad Prism 5. P-values below 0.05 were considered statistically significant**.**

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Author contributions

AF and PL designed the study. AF performed the experiments. AF and PL analyzed the data and wrote the manuscript.

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FIGURE LEGENDS

Figure 1. HCV does not alter the formation of the autophagy elongation complex in Huh7 cell.

(**A**) ATG5-12 conjugation was assessed by western blotting in mock (UI) or Huh7 cells infected at more than 90% with JFH1 using specific anti-ATG5 antibody. HCV infection and LC3 lipidation were detected using anti-NS3 and anti-LC3 antibodies, respectively. β-actin represents loading control. (**B**) Uninfected or JFH1-infected Huh7 cells at more than 90% were transiently transfected with a plasmid encoding for Flag-ATG12 protein. Two days later, cells were lysed and ATG12 was immunoprecipitated using anti-Flag antibody or IgG as a control followed by western blot analysis using anti-Flag and anti-ATG16L1. (**C**) Huh7 cells were infected with JFH1 (>90% infected) before being transfected with control (mock), Flag-ATG12, GFP-ATG5, Flag-ATG12DN or Flag-ATG12 and Flag-ATG12DN encoding plasmids. Cell lysates were analyzed for HCV NS3, ATG5 and ATG12 protein expressions at 72 h post-transfection by western blotting. (**D**) JFH1-infected cells at more than 90% were transfected with control plasmid (mock), Flag-ATG12DN or Flag-ATG12 and Flag-ATG12DN. Two days later, intracellular HCV RNA was quantified by RT-qPCR. Data were collected from three independent experiments (n=3). (***P*<0.005, NS, None-significant. Statistical analysis was performed by using One-way ANOVA).

Figure 2. Silencing of ATG7 or ATG12 inhibits HCV lifecycle.

(**A**), (**B**) and (**C**) Huh7 cells were transfected with siRNA against a non-specific target (siCTL), LC3 (siLC3), ATG7 (siATG7) or ATG12 (siATG12) siRNA, respectively. The cells were lysed 48 h post-transfection and extracts from each treatment were analyzed by western blotting for the corresponding proteins as indicated in the figure. β-actin was used as loading control. (**D**) Huh7.5 cells were transfected with siCTL, siLC3, siATG7 or siATG12. The cells were infected with HCVpp 48 h later and the luciferase activity was assessed 24 h post-infection. Data were derived from two independent experiments (n=3). (**E**) Huh7.5 cells were transfected with siCTL, siLC3, siATG7 or siATG12 for 48 h and

then electroporated with 5 μg of *in vitro*-transcribed viral RNA (JFH1/Fluc or JFH1/Fluc-GND). The cells were lysed 24 h later and the luciferase activity was determined from three independent experiments (n=3). (***P<0.001, Statistical analysis was performed by using Student's-t-test). RLU represents relative luciferase units.

Figure 3. Silencing of ATG7 or ATG12 but not LC3 inhibits HCV replication.

(**A**) Huh7 cells stably expressing wild-type JFH1 SGR were transfected with siCTL, siLC3, siATG7 or siATG12 each for 48 h and then cell lysates were analyzed for NS3 by western blotting using specific anti-NS3 antibody. β-actin served as loading control. (**B**) Huh7 cells stably expressing wild-type JFH1 SGR were transfected as in (**A**) and analyzed for SGR RNA by using RT-qPCR. Data are derived from three independent experiments (n=2). (*P<0.05, NS, none-significant. Statistical analysis was performed by using One-way ANOVA). (**C**) Huh7 cells were transfected with siCTL, siLC3 or siATG7 prior to or 7 days post-infection with JFH1 (MOI=0.01). Transfected cells were cultured for 72 h, lysed and subjected to RT-qPCR for intracellular viral RNA quantification. Data are derived from three independent experiments (n=3) (***P<0.001, Student's-t-test). RU represents relative units. (**D**) JFH1-infected Huh7 cells were transfected with siCTL, siLC3, siATG7 or siATG12. Extracellular and intracellular infectivity were determined 48 h post-transfection and expressed as percentage of control. Data shown are from three independent experiments (n=2)

Figure 4. Purified MW extracts harbor the autophagy elongation complex proteins.

(**A**) Huh7-Lunet cells harboring HA-tagged NS4B replicons were subjected to sucrose gradient fractionation as described in materials and methods section. The fractions were analyzed for their protein content by western blotting. Specific antibodies were used to detect NS3, $NS4B^{HA}$, NS5A, LC3 and GAPDH as indicated on the left. Fractions containing membrane-associated proteins (boxed in red) were pooled for affinity capture immunoprecipitation. The density of the different fractions is shown in the lower panel. TCL, total cell lysate. (**B**) HA-specific affinity-captured protein content from pooled fraction in (**A**) was analyzed by western blotting to detect viral NS3, NS4B, NS5A and autophagy elongation complex ATG5-12/16L1 by using specific antibodies. Pooled fractions from SGR cell lysate was used to demonstrate pull-down specificity.

Figure 5. Analysis of the purified MW membranes morphology and number.

(**A**) Morphological analysis of HA-captured membranes. HA-captured material from $NS4B^{HA}$ or $CLNXN^{HA}$ were negatively stained and examined using TEM. Scale bars represents 100 nm. (**B**) The purified membranes were categorized into elongated or spherical structures and were represented as relative values. (n >100). (**C**) Number of membrane structures per area of one hexagon from 10 randomly chosen grid hexagons. (***P<0.0001, Statistical analysis was performed by using Student's-t-test).

Figure 6. The impact of LC3, ATG7 or ATG12 silencing on membranous web phenotype.

(**A**) and (**B**) Huh7-Lunet-T7 cells were transfected with siCTL, siLC3, siATG7 or siATG12. After 48 h, the cells were transfected with pTM-NS3-5B and were stained 24 h later for NS3 and NS5A using specific antibodies (red). Nuclei were stained with DAPI (blue). (**C**) The percentage of wild-type and clustered phenotype was determined in 100 NS5A-positive cells per condition. (**D**) Huh7-Lunet-T7 cells were transfected with the different siRNAs as in (**A**) and then transfected with pTM-NS3-5B. After 24 h, cells were lysed and examined for HCV NS3 proteins expression by using western blotting. GAPDH was used as loading control.

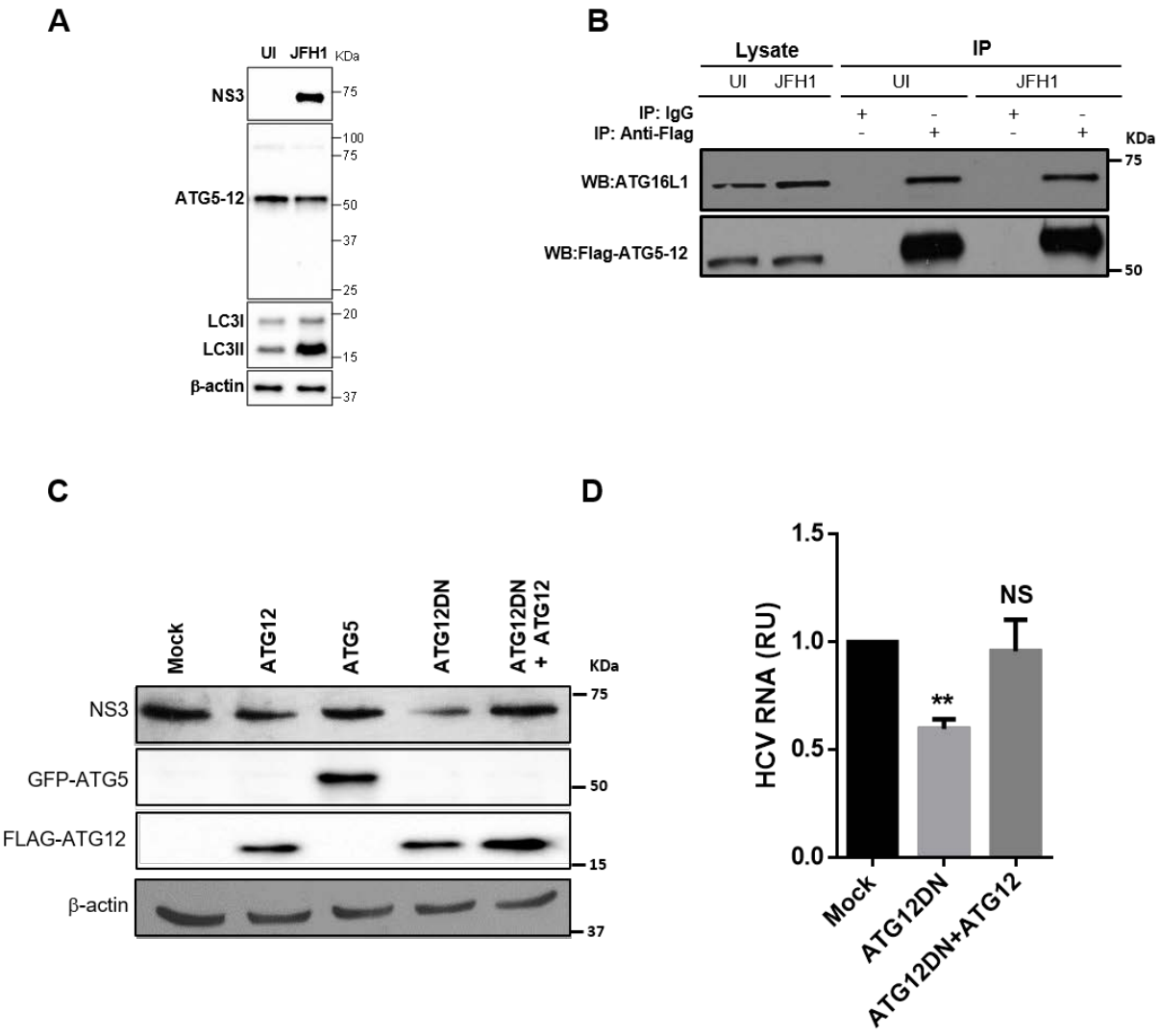
Figure 7. The impact of LC3, ATG7 or ATG12 silencing on membranous web ultrastructure.

(**A**) Huh7-Lunet-T7 cells knocked down for LC3, ATG7 or ATG12 were incubated for 48 h prior to transfection with pTM-NS3-5B. After 24 h, cells were fixed and processed for TEM analysis. Lower magnification images and shown on the left with their respective enlargement depicted on the right. Red arrows refer to DMVs. Arrowheads refer to MMVs. N is assigned for nucleus, LD for lipid droplet; M for mitochondria; G for Golgi apparatus. Scale bars are shown in the lower right corner of each panel. (**B**) The average diameter of DMVs assessed in each condition in at least 5 different cells (n=50 DMVs/cell) (****P*<0.005, Statistical analysis was performed by using One-way ANOVA). (**C and D**) The average number of DMVs and MMVs per μM^2 as calculated from 40 randomly-selected areas per cell (n=3 cells). (****P*<0.005, Statistical analysis was performed by using One-way ANOVA). (**E**) TEM analysis of naïve Huh7-Lunet-T7 cells.

Figure S1. Cell viability of Huh7 cells treated with different siRNAs.

Naïve Huh7 cells were transfected with siCTL, siLC3, siATG7 or siATG12. After 48 h, cells were incubated with CellTiter 96® AQueous reagent for 2 h and the optical density was measured according to the manufacturer's protocol. (n=4).

Figure 1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NS3
ATG5-12
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LC3II
β-actin
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100
75
50
37
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20
15
37
B
Lysate
IP
UI
JFH1
UI
JFH1
IP: IgG
IP: Anti-Flag
KDa
WB:ATG16L1
WB:Flag-ATG5-12
75
50
C
Mock
ATG12
ATG5
ATG12DN
ATG12DN + ATG12
KDa
NS3
GFP-ATG5
FLAG-ATG12
β-actin
75
50
15
37
D
HCV RNA (RU)
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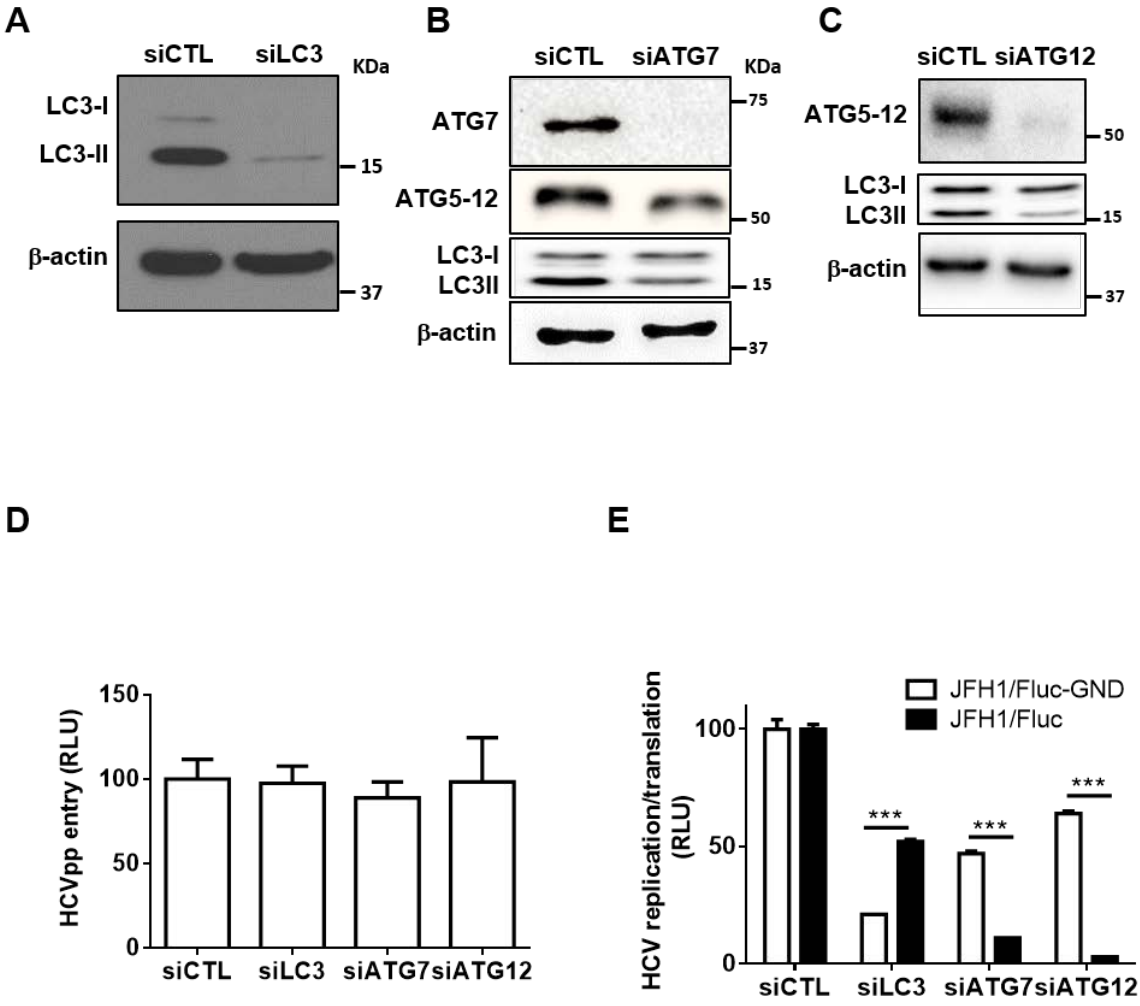
1.0
0.5
0.0
NS
**
Mock
ATG12DN
ATG12DN+ATG12

Figure 2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KDa
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ATG5-12
50
LC3-I
LC3II
15
β-actin
37
C
siCTL siATG12
ATG5-12
50
LC3-I
LC3II
15
β-actin
37
D
HCVpp entry (RLU)
150
100
50
0
siCTL
siLC3
siATG7
siATG12
E
HCV replication/translation (RLU)
JFH1/Fluc-GND
JFH1/Fluc
100
50
0
***
***
***
siCTL
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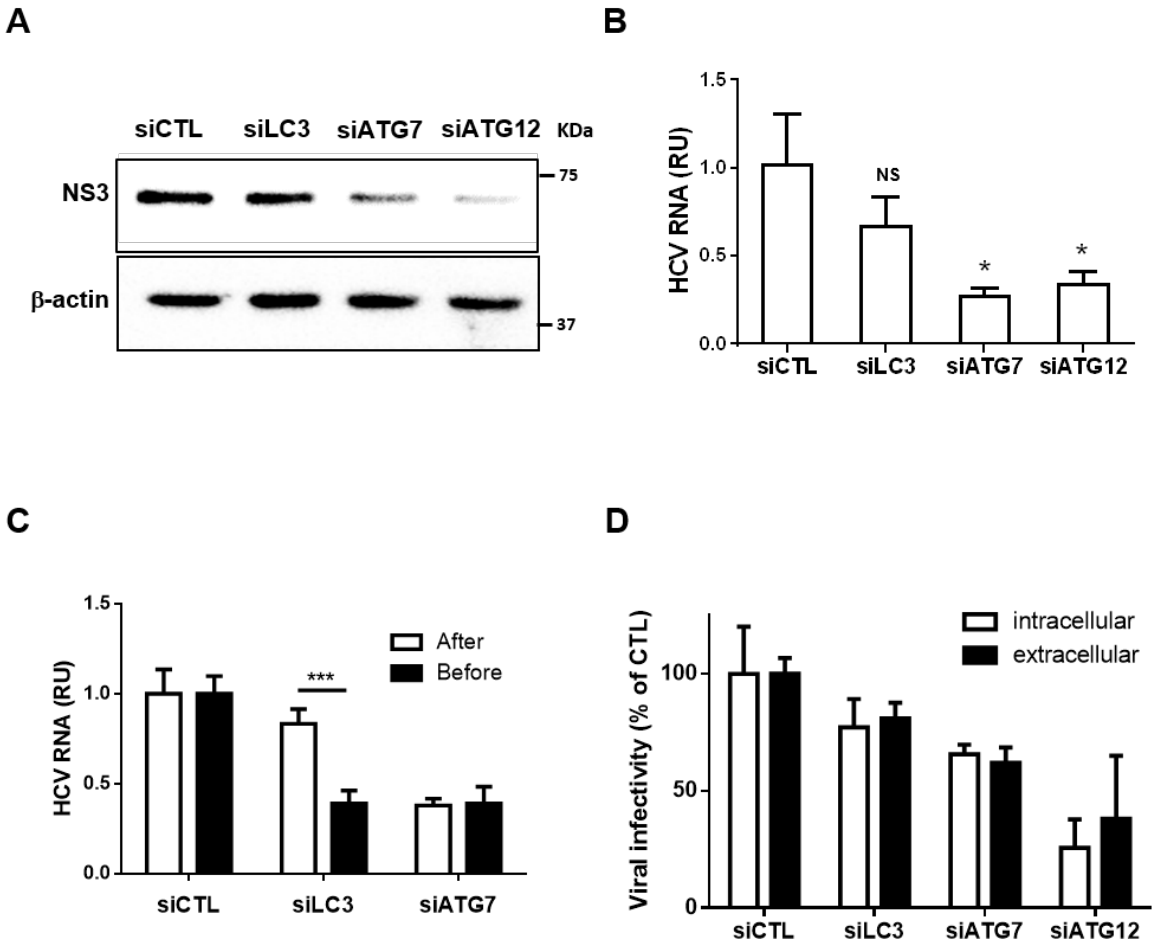
siATG7
siATG12

Figure 3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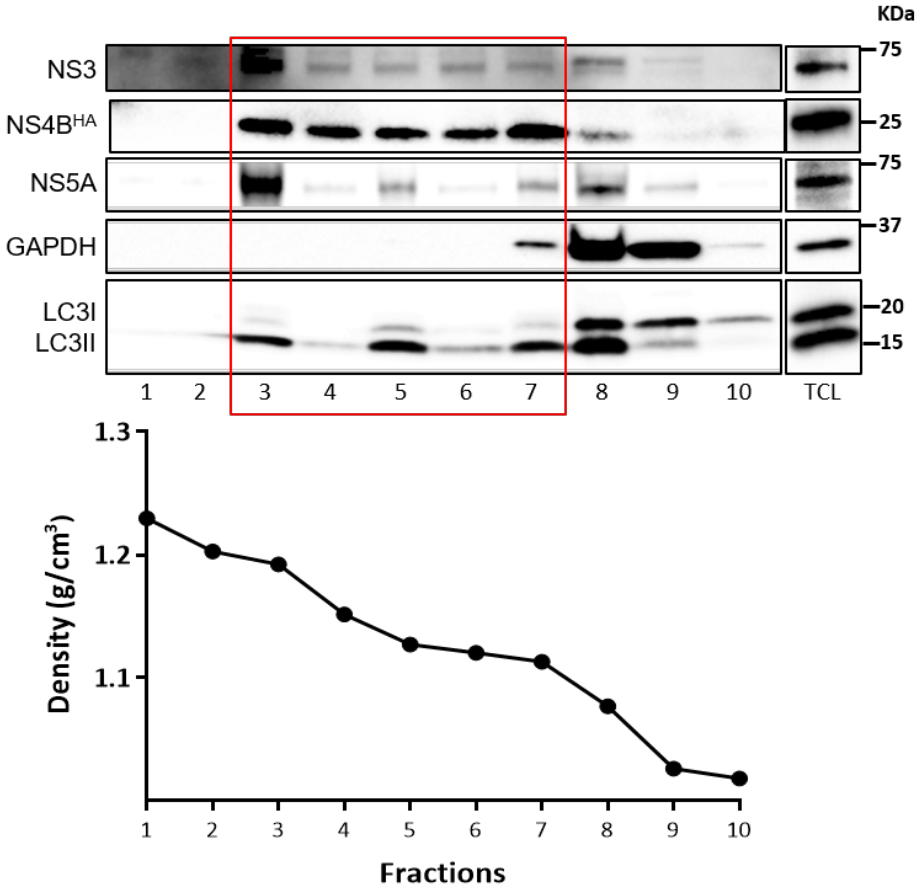
siCTL
siLC3
siATG7
siATG12
KDa
NS3
β-actin
75
37
B
HCV RNA (RU)
1.5
1.0
0.5
0.0
NS
*
*
siCTL
siLC3
siATG7
siATG12
C
HCV RNA (RU)
1.5
1.0
0.5
0.0
After
Before
***
siCTL
siLC3
siATG7
D
Viral infectivity (% of CTL)
100
50
0
intracellular
extracellular
siCTL
siLC3
siATG7
siATG12

Figure 4

A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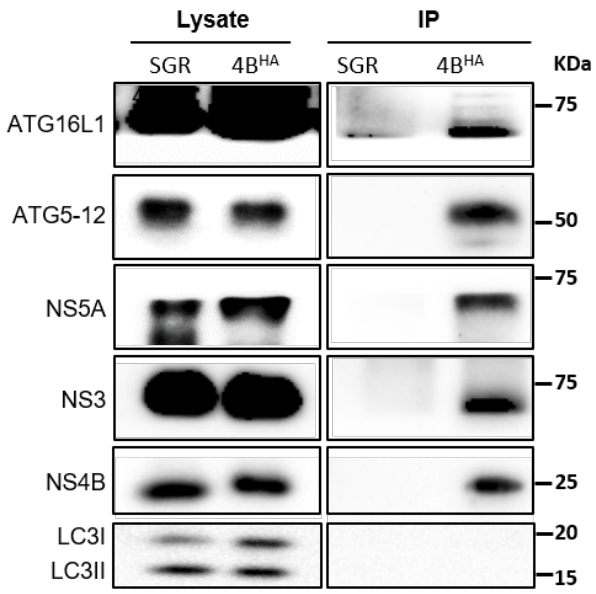
NS3
NS4B^HA
NS5A
GAPDH
LC3I
LC3II
75
25
75
37
20
15
1 2 3 4 5 6 7 8 9 10 TCL
Density (g/cm³)
1.3
1.2
1.1
1 2 3 4 5 6 7 8 9 10
Fractions

B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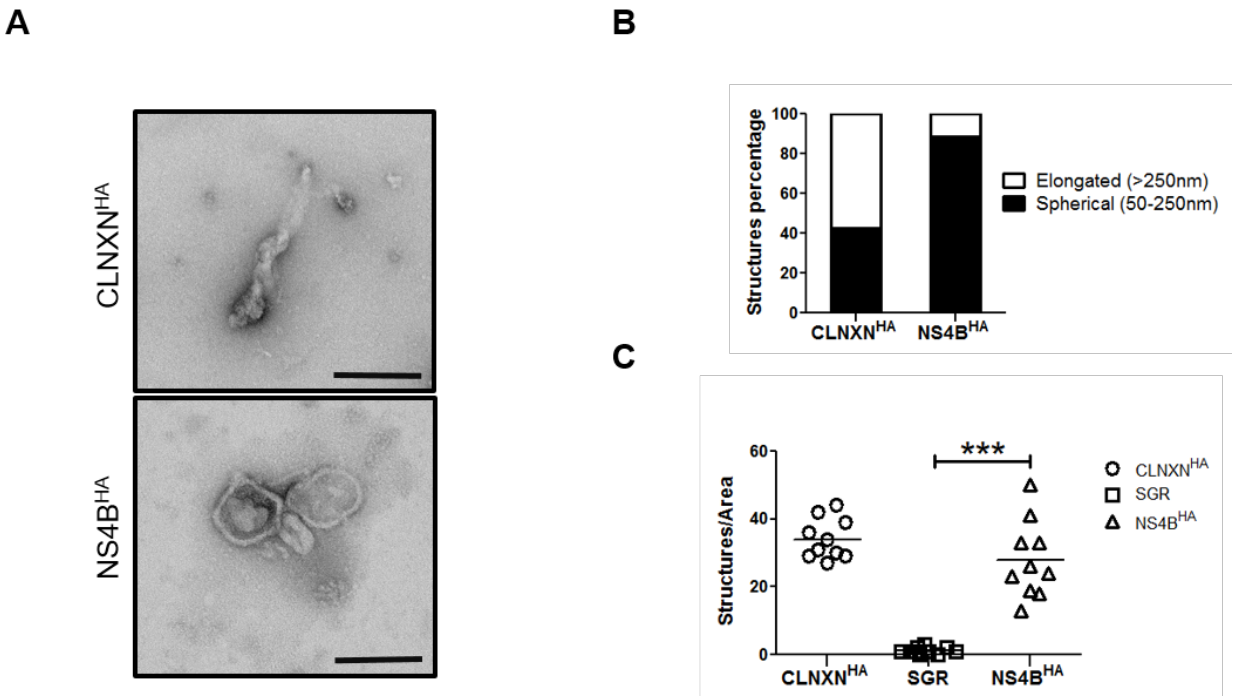
SGR
4B^HA
SGR
4B^HA
KDa
ATG16L1
ATG5-12
NS5A
NS3
NS4B
LC3I
LC3II
75
50
75
75
25
20
15

Figure 5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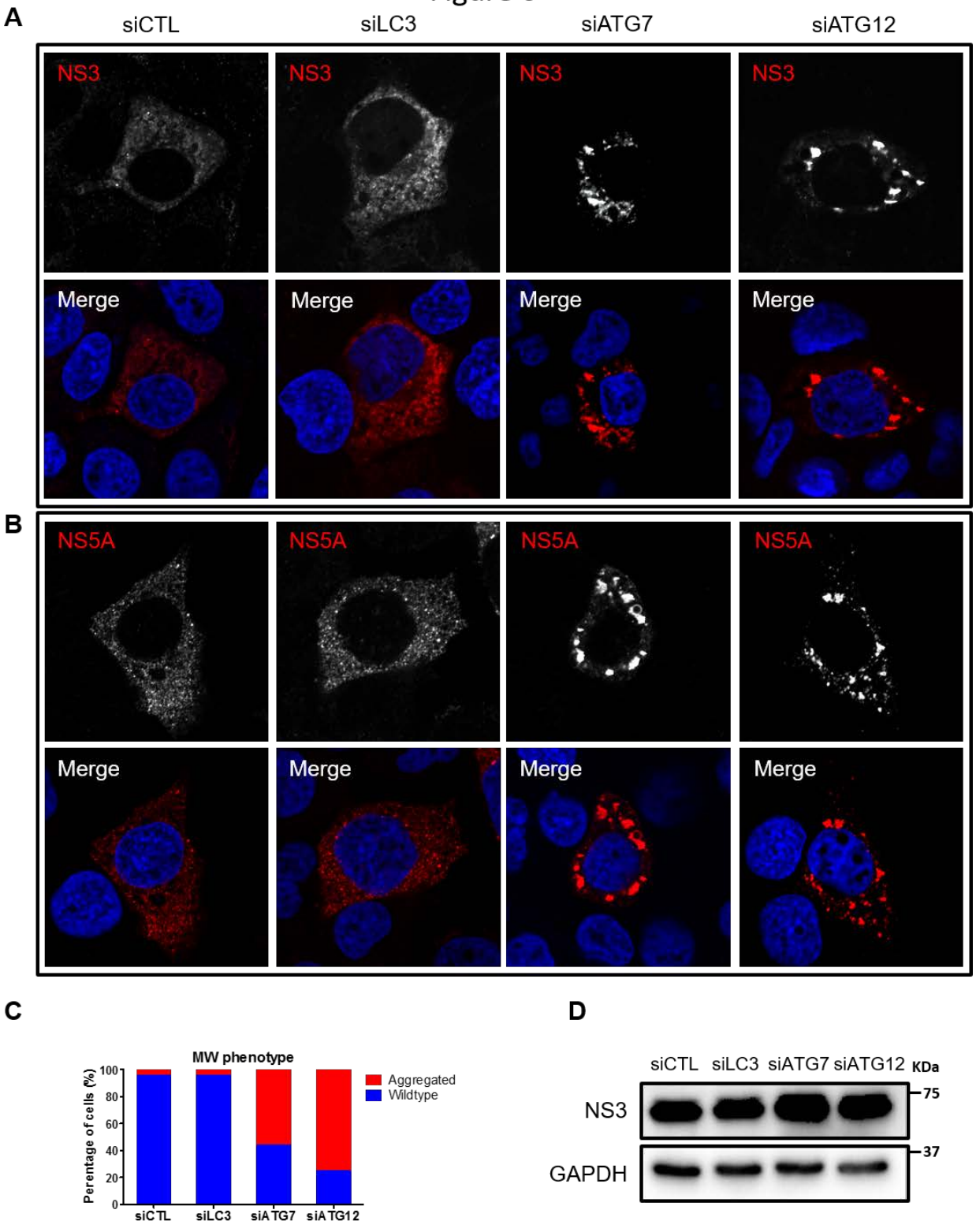
B
C
CLNXN^HA
NS4B^HA
Structures percentage
100
80
60
40
20
0
Elongated (>250nm)
Spherical (50-250nm)
CLNXN^HA
NS4B^HA
Structures/Area
60
40
20
0
***
CLNXN^HA
SGR
NS4B^HA
CLNXN^HA
SGR
NS4B^HA

Figure 6


A
siCTL
siLC3
siATG7
siATG12
NS3
Merge
B
NS5A
Merge
C
MW phenotype
Perentage of cells (%)
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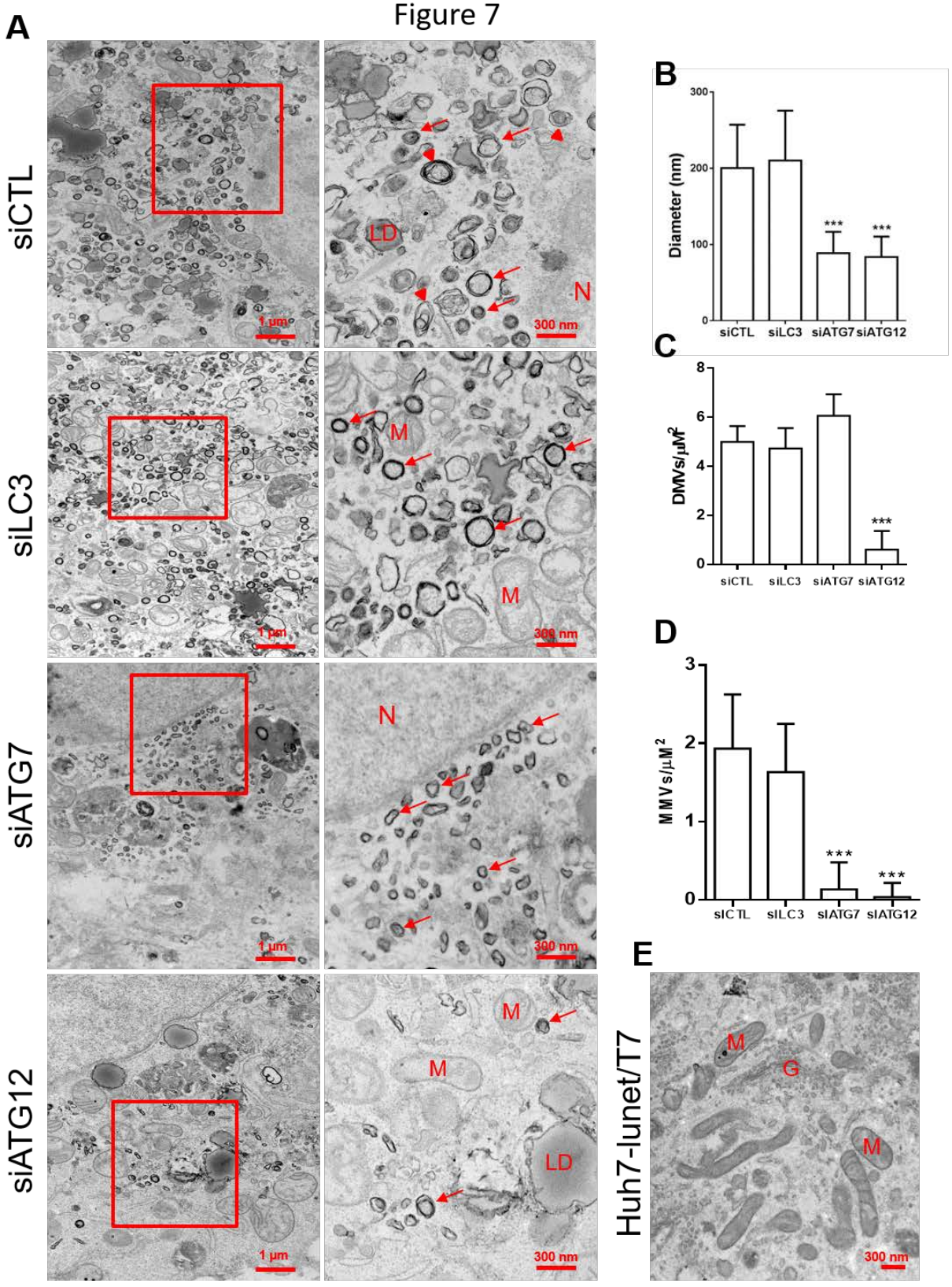
80
60
40
20
0
siCTL
siLC3
siATG7
siATG12
Aggregated
Wildtype
D
siCTL siLC3 siATG7 siATG12 KDa
NS3
GAPDH
75
37

Figure 7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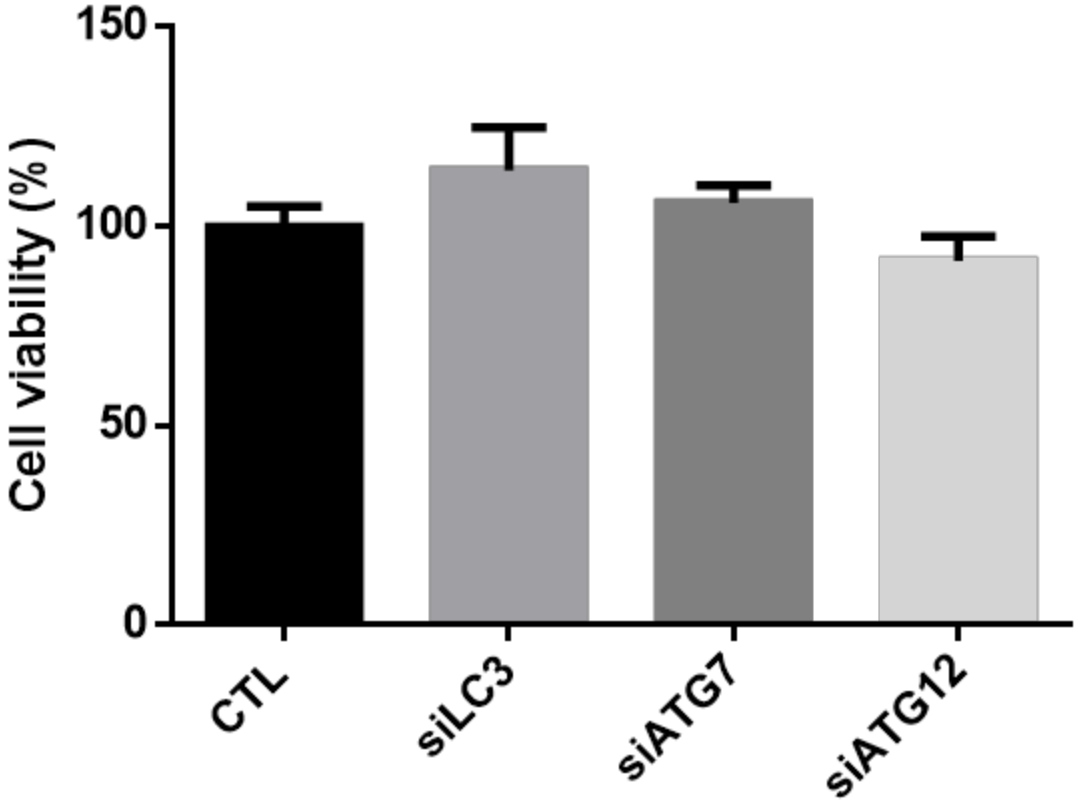
siLC3
siATG7
siATG12
1 µm
300 nm
LD
N
M
B
Diameter (nm)
300
200
100
0
siCTL
siLC3
siATG7
siATG12
***
C
DMVs/µM²
8
6
4
2
0
D
MMVs/µM²
3
2
1
0
E
Huh7-lunet/T7
M
G

S1



CHAPTER 3: DISCUSSION

One unique pathway of cellular membrane trafficking is autophagy. Various signaling pathways were shown to regulate this multifactorial process. In the past few decades research on autophagy has been massively expanded because it is a vital process in maintaining cellular homeostasis as well as it's profound connections to diseases. Moreover, it represents an essential part of both the innate and adaptive immune systems. Xenophagy specifically recognizes intracellular pathogens, including viruses, and physically targets them to the lysosome for degradation. Consequently, some pathogens have evolved different strategies to suppress autophagy, whereas others utilize autophagosomal membranes and the autophagic machinery to enhance their own replication.

Flaviviruses are among the viruses that have been shown to induce autophagy (Khakpoor *et al.*, 2009, Jin-Kun Li *et al.*, 2012, McLean *et al.*, 2011). The role of autophagy in flavivirus infection have been associated with variety of functions including inhibition of apoptosis(McLean *et al.*, 2011), lipid metabolism modulation for efficient viral replication (Heaton *et al.*, 2010), evasion of innate immune response (Jin *et al.*, 2013), or provision of suitable membranous scaffold for viral replication during infection (Khakpoor *et al.*, 2009).

Among these flaviviruses, HCV has been shown to modulate autophagy. Moreover, autophagy has been shown to be required for efficient HCV replication. Although, the exact role of autophagy in HCV replication is still elusive. One potential host mechanism that may represent a target for eradicating HCV replication is autophagy. Indeed, autophagy has been shown to be required for efficient HCV replication. However, the exact role of autophagy in HCV replication is still elusive. One suggested mechanism is that HCV may utilize autophagosomes as membranous platform to facilitate replication. Nevertheless, the capability of HCV to replicate within autophagosmes is still questionable as no colocalization has been observed between autophagosome, represented by LC3II marker, and HCV replication complex in HCV-infected cells (Dreux *et al.*, 2009b). In a previous report, our laboratory has suggested a role for the autophagy factor ATG5 in HCV replication. ATG5 was found to interacts with the HCV RNA-dependent RNA polymerase, NS5B. In addition, ATG5 colocalized with the viral nonstructural protein NS4B, a marker of the MW, in Huh7 cells harboring HCV SGR.

Moreover, inhibition of ATG5 by using siRNA approach has been shown to attenuate HCV replication (Guevin *et al.*, 2010). The interaction between ATG5 and NS5B has led us to assume that HCV utilizes either membranous structures and/or autophagic factors that precede LC3II recruitment to the autophagosome formation site, the phagophore assembly site (PAS). One major event in the biogenesis of the autophagosome that occurs before LC3II engagement is the recruitment of ATG5-12/16L1 complex to the growing phagophore which helps in the completion of autophagosome formation and the lipidation of LC3. ATG5 is known to conjugate with ATG12 and, in turn, this conjugate forms a multimeric complex with ATG16L1 named as the autophagy elongation complex (ATG5-12/16L1). Collectively, these observations drew our attention to execute a deeper investigation of the role of the autophagy elongation complex in HCV replication cycle.

4.1 ATG5-12/16L1 complex formation in HCV-infected cells

The initial step in this project was to evaluate the formation of ATG5-12/16L1 complex in human hepatocellular carcinoma-derived cells, namely Huh7. In normal conditions, ATG5 is found conjugated to ATG12 and the monomeric form of these proteins are usually undetectable (Hosokawa *et al.*, 2006). Indeed, we were able to detect only the conjugated form of ATG5 and ATG12 (55 kDa) in uninfected Huh7 cells suggesting that the majority of the ATG5 is readily conjugated to ATG12 in these cells as previously described for other cell types (Hosokawa *et al.*, 2006). Furthermore, our analysis of ATG5-12 conjugate formation in infected Huh7 cells revealed that HCV infection did not perturb the ATG5 conjugation to ATG12. Moreover, we showed that ATG5-12/16L1 complex was detectable in both HCV-infected and uninfected cells which indicates that HCV infection did not hamper the formation of ATG5-12/16L1 complex. To confirm the formation of such complex *in situ* at endogenous level of the proteins, the formation of ATG5-12/16L1 was examined by using proximity ligation assay (PLA). These results may imply that the previously described interaction of ATG5 with viral NS5B could, in fact, be between ATG5-12 conjugate or ATG5-12/16L1 complex and NS5B rather than the monomeric form of ATG5.

Remarkably, HCV infection has induced the accumulation of lipidated LC3. This is in line with several other reports that have shown the modulation of autophagy either by inducing autophagy or by blocking the maturation of the autophagosome (Dreux *et al.*, 2009b, H. Huang *et al.*, 2013, Ke *et al.*, 2011, Mohl *et al.*, 2012, Sir *et al.*, 2008, J. Wang *et al.*, 2014a, L. Wang *et al.*, 2015).

4.2 ATG5-12/16L1 colocalizes with HCV replication complex

Following the assessment of ATG5-12/16L1 complex formation in infected cells, we aimed at exploring the localization of ATG5-12 conjugates in HCV-infected cells. Our confocal analysis showed a noticeable localization of ATG5-12 conjugate on distinct membrane- like structures. These structures were also positive for HCV NS3, NS5A, NS4B, and NS5B. The colocalization between ATG5-12 conjugates and HCV nonstructural proteins were also confirmed by using PLA. The detected PLA signals were specific since no interaction between ATG5-12 and core was observed using the same technique. The unique membrane-localization of ATG5-12 conjugate was not observed in uninfected cells suggesting that HCV recruits ATG5-12 conjugate to membrane-like structure where HCV replication occurs (See Figure 2 in publication 1).

Since the formation of ATG5-12/16L1 complex is undeniably required to allow the expansion of the autophagosomal membrane and the completion of autophagosome (Kharaziha *et al.*, 2017, Walczak *et al.*, 2013), we investigated weather ATG16L1 also colocalizes with HCV replicase. As observed with ATG5-12 conjugate, ATG16L1 colocalized nicely with several HCV nonstructural proteins that constitute the viral replicase. In contrast, this colocalization was not translated into *in situ* interaction suggesting that the binding between ATG5-12/16L1 complex and viral NS3 is via ATG5-12 and not ATG16L1. Our results suggest that HCV recruits ATG5-12 and ATG16L1 as a complex to enhance HCV replication.

4.3 ATG5-12/16L1 complex is recruited to HCV MW

The translation of HCV ORF occurs at the ER and yield a single polyprotein that is cleaved by a combination of host and viral proteases into viral structural and nonstructural proteins. The nonstructural proteins then form what is called the viral replicase which produces the viral RNA genome within newly synthesized web-like membranous vesicles of ER origin (Paul *et al.*, 2014). Our next aim was to investigate whether ATG5-12/16L1 is recruited to HCV replicase at RNA replication site or to the site where HCV RNA translation takes place. For this purpose, we utilized several biochemical assays to show the localization of ATG5-12/16L1 in HCV -infected cells. First, we performed iodixanol density gradient to segregate the replicase from ER fractions by using the chaperone protein calnexin to mark fractions that constitute ER. A considerable amount of ATG5-12 co-fractionated with ATG16L1 and the viral replicase proteins NS3 and NS5B at lower density fractions (Figure 6 in publication 1, fractions 11-13).

During the multi-step HCV RNA replication, a negative-sense RNA strand is synthesized from the positive-sense RNA genome to serve as template for the generation of progeny RNA genomes (Meyers *et al.*, 2016). Thus, by specifically detecting the negative-sense RNA in our fractions, we would be able to refer to the location of the HCV replicase in these fractions. Indeed, the fractions (11-13) contained HCV RNA positive-strand and most of the HCV negative-strand RNA, indicating that ATG5-12/16L1 might be present at the HCV replication site.

The presence of ATG5-12/16L1 in the same fractions where HCV replicase exist might not reflect their intracellular localization. To resolve this issue, we showed that ATG5-12 and ATG16L1 were colocalizing with the HCV dsRNA, a replication intermediate generated during active HCV RNA replication, in HCV-infected cells.

Despite the broad knowledge about the three-dimensional architecture of DMVs in HCV-infected cells, determination of their viral and cellular composition and their exact role in the replication cycle of HCV is elusive. This is attributed to difficulties in producing sufficient quantities of highly purified HCV-remodeled membranes to be analyzed biochemically. Recently, a major technical breakthrough was achieved by the laboratory of Dr. Ralf Bartenschlager where his group has developed a method by which HCV MW can be specifically isolated form HCV SGR cells. They generated functional stable

replicon cells containing a hemagglutinin (HA) affinity tag in NS4B, the scaffold protein that trigger the viral replication complex formation. By using HA-specific affinity purification, they were able to isolate NS4B-containing membranes from stable replicon cells (Paul *et al.*, 2013). Following their protocol, we were able to pull-down the MW and detect the presence of ATG5-12/16L1, but not LC3II, in these membranes by using western blotting. Our EM analysis revealed that the isolated membranes were highly enriched in DMVs. This comes in line with our previous knowledge about the nature of MW membranes and confirmed our success in isolating HCV MW.

Our inability to detect LC3II in the isolated membranes was intriguing since in canonical autophagy, the recruitment of ATG5-12/16L1 to phagophore is followed by the lipidation of LC3 and its localization to the isolation membrane. Futhermore, HCV infection has been shown to induce the appearance of LC3II puncta throughout the infected cell. Apparently, our confocal analysis showed no detectable colocalization between LC3 and HCV replication complex. There are two main possible ways to explain the exclusion of LC3II from the HCV replication site. The first is that HCV may hijack ATG5-12/16L1 from the phagophore assembly site and recruits it to the MW where it might functionally help either in viral replication or in the construction of the MW. This assumption is supported by the recent finding that some autophagy key players, as ATG9 and ATG16L1, can traffic within the cytoplasm of a cell in vesicles that are not autophagosomes (Itakura *et al.*, 2012a). The other possible explanation is that HCV might co-opt phagophores or autophagosomes that are LC3II negative to utilize them in the build-up of the MW. This assumption is also valid since it was recently reported that incomplete autophagosome formation can exist in the absence of LC3II (Tsuboyama *et al.*, 2016). Interestingly, these incomplete autophagosomes were positive for ATG5 which may increase the likelihood for this to occur in case of HCV infection (Tsuboyama *et al.*, 2016). Altogether, our findings suggest that HCV co-opts ATG5-12/16L1 or ATG5-12/16L1-positive membranes to the replication site where it act as a proviral factor.

4.4 ATG12 conjugation to ATG5, but not LC3 lipidation, is required for HCV replication

The absence of LC3II from the HCV-induced MW has tempted us to further investigate the role of LC3 lipidation in HCV replication. For this purpose, we overexpressed a dominant negative form of ATG4B (ATG4BDN), a protein that cleaves LC3 pre-protein to prepare it for lipidation, in HCV-infected cells. In these LC3II-attenuated cells HCV replication was maintained with no decrease either on HCV proteins expression or viral RNA level. These results along with our earlier findings suggest that LC3II formation is not required for HCV replication.

In contrast, when we overexpressed a dominant-negative form of ATG12 (ATG12-DN) lacking the C-terminal glycine that is essential for its conjugation with ATG5 in HCV-infected cells, a significant decrease in HCV RNA and proteins was observed even with the limited transfection efficiency of Huh7 cell, 40-50% transfection efficiency. Similar results were obtained when a dominant-negative form of ATG5 (ATG5-DN) was overexpressed. To confirm these results, we rescued the defective ATG12 by a co-transfection with ATG12 wild type. Indeed, ATG12 wild type co-expression was able to restore both HCV RNA and protein levels. These results confirm that the ATG5-12 conjugated form, rather than the monomeric form of these proteins, is important for HCV replication cycle.

4.5 The ATG5-12 conjugate is implicated in the HCV lifecycle at a post-translational step

The obvious effect of ATG5-12 disruption, but not LC3-impaired lipidation, on HCV replication has tempted us to further investigate the contribution of those two conjugation systems in different steps of HCV life cycle. For this purpose, we directly targeted each conjugation system using siRNA against ATG12 and LC3 or indirectly by the silencing of ATG7, which would inhibit both conjugation system at once. Indeed, we were able to efficiently attenuate the expression of these target proteins using their respective specific siRNA. To study the effect of siATG7, siATG12, and siLC3 on HCV entry, we utilized HCVpp that features unmodified HCV E1 and E2 glycoproteins which upon

internalization, a luciferase signal is emitted enabling us to specifically monitor HCV entry (Bartosch *et al.*, 2009). We showed that diminishing the expression of ATG7, ATG12 or LC3 had no effect on Luciferase expression which suggests that neither the ATG5-12/16L1 nor LC3 modulates viral entry.

It has been previously reported that the onset of HCV RNA translation is inhibited by the abrogation of LC3 conjugation using siRNA against ATG4B (Dreux *et al.*, 2009b). The initiation of translation was assessed by following the luciferase activity of a replication-defective subgenomic replicon RLuc/SGR harboring an inactivation mutation (GDD to GND) at the active site of the HCV polymerase NS5B (RLuc/SGR-GND) (Dreux *et al.*, 2009b). This observation suggests an implication of either autophagy machinery or LC3 lipidation in establishing HCV RNA translation. Using similar approach, when we knocked down LC3 expression the translation of electroporated replication-defective HCV replicon was significantly inhibited which confirms the role of LC3 in establishing the translation of viral RNA. In contrast, silencing of ATG12 had a little effect on replication-deficient virus whereas it strongly affected the replication of the JFH1/Fluc virus, thus indicating that ATG5-12/16L1 primarily modulates HCV replication in a stage beyond the viral RNA translation.

The post-translational modulation of HCV replication by ATG5-12/16L1 was further confirmed in cells stably expressing the HCV subgenomic replicon. In these cells, silencing of ATG7 or ATG12 significantly inhibited HCV replication while silencing of LC3 rendered no effect. Our observations that siLC3 impedes HCV only when performed before infection while siATG7 inhibits HCV when transfected before or after infection support our earlier results and suggested a specific role of LC3 in viral RNA translation and that the ATG5-12 conjugate, but not LC3, is important in viral replication.

The evaluation of the effects of siRNA treatment on intracellular and extracellular HCV infectious particle production in JFH1-infected cells revealed that HCV maturation and secretion were most likely not affected by siRNA treatment. Although silencing ATG12 led to a significant decrease in HCV particle formation, this effect is likely due to the severe reduction in viral replication exerted by the attenuation of this protein.

Collectively, while LC3 has a clear role in HCV RNA translation, the role of ATG5-12/16L1 appears to be linked to the stage of HCV RNA replication.

4.6 ATG5-12 conjugate is crucial for the proper morphology of HCV-induced MW

While several reports have suggested a role of autophagy in different stages of HCV replication cycle, none of them provided an evidence on the implication of autophagy/autophagic protein in the construction of HCV-induced MW. The mechanism by which HCV-induced MW are formed remains enigmatic. Recently, it was shown that they originate from ER membranes, similar to what has been found for other members of the *Flaviviridae* family (Romero-Brey *et al.*, 2014). Electron tomographic analysis revealed that these DMVs are frequently connected to the ER membrane via a neck-like structure (Romero-Brey *et al.*, 2012).

The involvement of autophagy in the architecture of the MW has been proposed based on three main observations. First, the morphological similarities between the autophagosome and the DMVs that constitute the majority of the MW vesicle content (Romero-Brey *et al.*, 2014). Second, both HCV-induced DMVs and, to some extent, autophagosomes share the same origin as both start as a protrusion from ER. Third, HCV proteins that have been shown to modulate autophagy, either by inducing or blocking the autophagic flux, and accumulate autophagosomes are found to promote the formation of the MW vesicles. It is noteworthy that, ATG5-12/16L1 has been shown to harbor a membrane-tethering activity that is independent of LC3 (Romanov *et al.*, 2012, Walczak *et al.*, 2013).

All these observations challenged us to investigate whether ATG5-12 conjugate is involved in the construction of the MW. For this purpose, we utilized a well-established T7-polymerase-based HCV RNA synthesis system in which continuous production of HCV polyproteins persists even in the absence of HCV RNA replication (Reiss *et al.*, 2011). By using this system, we were able to show that either silencing of ATG7 or ATG12 had a severe effect on the distribution of viral replication complex proteins, NS3 and NS5A. Normally, replicase proteins show a dispersed subcellular localization throughout the cytoplasm of a cell. Silencing of ATG7 or ATG12 has severely affected this distribution and led to the formation of large protein clusters within the cytoplasm when observed by

confocal microscopy. In contrary, silencing of LC3 did not change the subcellular distribution of HCV replicase proteins, NS3 and NS5A. These results may refer to a phenotypic change in the MW architecture exerted by the attenuation of ATG7 and ATG12 expression.

By exploring the morphological structure of HCV MW using EM. We were able to show that silencing of ATG7 has led to a decrease in DMVs size and a disappearance of MMVs that normally exist in wild-type MW. Furthermore, we showed that by silencing of ATG12 a more pronounced effect is exerted as DMVs have decreased both in size and number in addition to the disappearance of MMVs that was observed by knocking down ATG7. Again, LC3 silencing did not affect neither the number nor the size of DMVs while MMVs were still present in a comparable amount as in control cells. Our study suggested a novel role of ATG5-12/16L1 in HCV replication by participating in the formation of proper HCV-induced MW.

4.7 Autophagy as a potential target for HCV treatment

Impressive leaps in the understanding of HCV pathogenesis, replication, and protein functions have paved the way toward the development of direct acting antivirals (DAAs). These drugs showed strong potency and effectiveness in eradicating HCV infection which has led to the replacement of the traditional interferon with highly tolerated oral therapies that can cure more than 90% of patients (Afdhal *et al.*, 2014, Feld *et al.*, 2014). To date, inhibition of HCV replication has converged on three main viral targets: the NS3/4A protease (simeprevir, paritaprevir, and grazoprevir), the NS5A (daclatasvir, ledipasvir, ombitasvir, elbasvir, and velpatasvir), and the NS5B RNA-dependent RNA polymerase (the nucleotide analog sofosbuvir and the non-nucleoside inhibitor dasabuvir). Despite the high rates of sustained virological response achieved with these regimens, the infection is not eliminated from an increasing number of patients, 1%–15%, according to the patient group and regimen (Pawlotsky, 2014).

Another alternative treatment strategy could be by targeting cell components that contribute to the HCV life cycle using host-targeted antivirals (HTAs). The advantage of targeting a host factor is the likelihood of having a pangenotypic activity and a high barrier

to resistance. Cyclophilin inhibitors has been shown to inhibit HCV replication by blocking the peptidyl-prolyl cis-trans isomerase activity of cyclophilin A, which is required for efficient HCV replication (Coelmont *et al.*, 2009). In addition, targeting of microRNA 122 by using the microRNA 122 antagonist miravirsen, which inhibits binding of microRNA 122 to the 5'UTR of the HCV genome, has shown antiviral activity *in vitro* and *in vivo* (Janssen *et al.*, 2013, Lanford *et al.*, 2010).

One potential host mechanism that may represent a target for eradicating HCV replication is autophagy. Indeed, autophagy has been shown to be required for efficient HCV replication. Herein, we have shown that autophagy elongation complex plays a crucial role in HCV replication possibly via helping in the formation of the MW. Thus, targeting ATG5-12/16L1 by specific inhibitors may provide a new strategy to eradicate HCV infection.

CONCLUSION AND PERSPECTIVES

We revealed that HCV infection benefits from the autophagy elongation complex to allow efficient viral replication in vitro. Clearly, we showed that ATG5-12/16L1 complex, but not LC3II, is recruited to the HCV replication site and exist within the HCV-induced membranous web. Furthermore, analysis of the role of LC3 and ATG5-12 conjugate in HCV replication cycle has revealed the involvement of ATG5-12 in HCV RNA replication step whereas LC3 was required only for the onset of HCV RNA translation. ATG5-12 did not modulate neither HCV entry nor the quality of HCV infectious particles secreted. Moreover, we showed that the modulation of HCV RNA replication by ATG5-12 conjugate could be attributed to a morphological modification in the architecture of the MW as seen by confocal microscopy. Indeed, disrupting ATG5-12 conjugate by using siRNA approach has led to a dramatic decrease in size and number of DMVs associated with a disappearance of MMVs from HCV-induced MW. Altogether, our study highlights a new role of autophagic factors in HCV lifecycle which possibly could be extended to other flaviviruses.

The role of ATG5-12/16L1, but not LC3, in the formation of MW and possibly in HCV RNA replication may indicate the participation of other early autophagy events that precede LC3 recruitment. In order to investigate this, the involvement of other autophagy key players such as ATG9 and Beclin1, factors that precede the recruitment of LC3 and ATG5-12/16L1 to the growing phagophore (Webber *et al.*, 2010), should be studied.

In addition, non-canonical autophagy that can exist in the absence of key autophagic players such as ATG7 could be a potential mechanism induced by HCV infection. One useful inhibitor of this pathway is Brefeldin A, a lactone antibiotic that exerts its disruptive effect at the cis-Golgi. It is noteworthy that Brefeldin A was shown to inhibit HCV infection which may indicate the potential involvement of non-canonical autophagy in HCV replication and/or the formation of the MW. In addition, it can also demarcate the contribution of Golgi as membrane source for HCV MW.

Moreover, several attempts to investigate weather HCV induces a complete autophagic flux (Sir *et al.*, 2008). However, these trials were confecting and did not provide sufficient evidences to address this issue. Thus, this debate should be resolved by a thorough

assessment of the autophagic flux during HCV infection using powerful tools known to follow the completion of autophagy in mammalian cells such as by following the degradation of P62, a protein that has a specific propensity to be degraded in the lysosome through autophagy. This can also be studied by using overexpression of the mTagRFP-mWasabi-LC3 protein, which was recently shown to be a more accurate reporter for monitoring autophagic flux compared to mRFP-eGFP-LC3 due to higher sensitivity of the mWasabi over eGFP to the acidic environment of lysosome. Thus, the autophagic flux can be followed calorimetrically by using confocal microscope. One additional tool is long-lived protein degradation assay which allows the assessment of autophagic flux through following the degradation of long-lived proteins, specifically degraded via autophagy, by labeling with valine C^{14}.

Interestingly, other flaviviruses, like Zika virus, have been shown to exploit autophagy for viral replication by unknown mechanisms. Due to the similarity among these group of viruses, especially in the massive membrane remodeling that they exert in the cell during infection, one could expect that some of them might hijack ATG5-12/16L1, as in HCV, for the formation of membranous replication factories. Thus, this should also be investigated taking Zika or any other related virus as a model.

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APPENDIX

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Effect of the pituitary adenylate cyclase-activating polypeptide on the autophagic activation observed in in vitro and in vivo models of Parkinson's disease.

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Effect of the pituitary adenylate cyclase-activating polypeptide on the autophagic activation observed in *in vitro* and *in vivo* models of Parkinson's disease

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder that leads to destruction of the midbrain dopaminergic (DA) neurons. This phenomenon is related to apoptosis and its activation can be blocked by the pituitary adenylate cyclase-activating polypeptide (PACAP). Growing evidence indicates that autophagy, a self-degradation activity that cleans up the cell, is induced during the course of neurodegenerative diseases. However, the role of autophagy in the pathogenesis of neuronal disorders is yet poorly understood and the potential ability of PACAP to modulate the related autophagic activation has never been significantly investigated. Hence, we explored the putative autophagy-modulating properties of PACAP in *in vitro* and *in vivo* models of PD, using the neurotoxic agents 1-methyl-4-phenylpyridinium (MPP^+) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), respectively, to trigger alterations of DA neurons. In both models, following the toxin exposure, PACAP reduced the autophagic activity as evaluated by the production of LC3 II, the modulation of the p62 protein levels, and the formation of autophagic vacuoles. The ability of PACAP to inhibit autophagy was also observed in an *in vitro* cell assay by the blocking of the p62-sequestration activity produced with the autophagy inducer rapamycin. Thus, the results demonstrated that autophagy is induced in PD experimental models and that PACAP exhibits not only anti-apoptotic but also anti-autophagic properties.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder with a significant prevalence in elderly people. It evolves slowly and is distinguished by the appearance of movement disorders characterized by instability, postural rigidity, tremor at rest, and bradykinesia/akinesia [1,2]. It is associated with a massive destruction of neurons in the brainstem, particularly the dopaminergic (DA) neurons of the *substantia nigra* (SN) [3]. Although neuronal death mechanisms remain poorly understood, several studies showed that loss of DA neurons is particularly caused by alteration of mitochondrial functions [4–6]. In fact, during the neurodegenerative course, reactive oxygen species (ROS) are generated and they subsequently alter cell components, thereby inducing cellular stress and giving rise to processes such as apoptosis and autophagy [7–9]. While apoptosis is a programmed cell death mechanism, autophagy is considered as a self-degradation action that occurs in normal physiological conditions to clean up the cell from defective intracellular components. Indeed, altered cytoplasmic organelles are processed by lysosomes to purify the cell through the autophagic process [10]. Hence, conditions associated with an increase in cellular stress promote changes in cellular organelles and might induce autophagy. Moreover, in more acute conditions of organelle alterations, autophagy might become deleterious to cell survival. Autophagy was shown to be involved in a variety of pathologies including cancer, infection and inflammation [11]. Furthermore, some studies showed that autophagy is observed during the development of neurodegenerative

Abbreviations: Akt, protein kinase B; Bcl-2, B-cell lymphoma 2 anti-apoptotic protein; DA, dopaminergic; DAPI, 2-(4-amidinophenyl)-1 H-indole-6-carboxamidine hydrochloride; FBS, fetal bovine serum; GFP, green fluorescent protein; JNK, c-Jun. N-terminal kinase; LC3 I and II, microtubule-associated protein light chain 3 I and II; MAPK, mitogen-activated protein kinase; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTP, mitochondrial transmembrane potential; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium; PAC1, PACAP receptor; PACAP, pituitary adenylate cyclase-activating polypeptide; PACAP38 or P38, 38-amino acid isoform of PACAP; PBS, phosphate buffer saline; PD, Parkinson's disease; PFA, paraformaldehyde; ROS, reactive oxygen species; SN, *substantia nigra*; SNpc, *substantia nigra pars compacta*; TBST, Tris-buffered saline with Tween 20; TH, tyrosine hydroxylase; VIP, vasoactive intestinal peptide; VPAC1, VIP/PACAP type 1 receptor; VPAC2, VIP/PACAP type 2 receptor.

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illnesses such as the Alzheimer's and Parkinson's diseases [12–14]. Nevertheless, the precise role of autophagy in the pathogenesis of neuronal disorders is yet inadequately understood. Indeed, in ischemia and brain injuries, as well as in neurodegenerative diseases, some reports suggested that activation of autophagy might be beneficial while others revealed that it would be harmful for the cell [15–18]. Similarly, other studies demonstrated that inhibition or activation of autophagy produced protective effects depending on the stress conditions [19–22].

The pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide that exerts a large array of actions *via* the activation of three different G protein-coupled receptors, *i.e.* PAC1, VPAC1 and VPAC2 [23]. This molecule is a potent anti-apoptotic, anti-inflammatory and vasodilating substance [24–27], and these biological activities are essentially mediated through PAC1, VPAC1 and VPAC2, respectively [28–30]. In particular, by reducing apoptosis, PACAP exhibits potent neuroprotective effects in experimental cellular and animal models of pathologies and neuronal damages, including cerebral ischemia and brain injuries, as well as Alzheimer's and Parkinson's diseases [31–34]. Hence, PACAP is able to protect against neurotoxic agents several cell types such as PC12 cells, GIRK2-positive and GIRK2-negative dopamine neurons of primary ventral midbrain cultures, as well as Neuro-2a neuroblastoma cells [35,31]. It has been clearly established that PACAP modulates several pathways involved in apoptosis to promote neuroprotective effects. Indeed, in many *in vitro* studies of neuronal injuries, PACAP induced Bcl-2 expression, stimulated the Akt/MAPK cascade, inhibited the c-Jun N-terminal kinase (JNK) pathway, and blocked caspase activation [36–40]. Moreover, as demonstrated by our previous work, selective PAC1/VPAC1 analogs exert anti-apoptotic effects against 1-methyl-4-phenylpyridinium (MPP^+)- and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicities [41,42]. The compound MPP^+ is a neurotoxic agent used to mimic *in vitro* features of Parkinson's disease, whereas its reduced form, MPTP, is utilized to reproduce *in vivo* Parkinsonian symptoms. Studies have demonstrated that MPP^+ inhibits the mitochondrial complex I, thus causing neuronal death [43,4,44]. Interestingly, it has also been reported that MPP^+ is able to modulate autophagy in different manners, according to the conditions of stress (*e.g.* hypoxia, serum depletion) and its duration [19,45,46]. Therefore, MPTP and MPP^+ cause not only neuronal death through apoptosis but also modulate autophagy [47]. Furthermore, it was shown that cyclic adenosine monophosphate (cAMP), a second messenger produced following the activation of adenylyl cyclase by PACAP, and protein kinase A (PKA), an enzyme with an activity dependent on the cellular level of cAMP, regulate MPP^+-induced autophagy [48]. Henceforth, in the present investigation, we assessed the activation of the autophagic response induced by MPP^+ and MPTP in *in vitro* and *in vivo* models that reproduce characteristics of PD and we evaluated the effect of exogenous PACAP on MPP^+- and MPTP-induced autophagy.

2. Materials and methods

2.1. Materials

Otherwise stated, chemicals and cell culture media were purchased from Sigma-Aldrich (Mississauga, ON, CAN) and Fisher Scientific (Nepean, ON, CAN). JC-1 was acquired from Life Technologies (Burlington, ON, CAN), and the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium (MTS) assay kit was obtained from Promega (Madison, WI, USA). PACAP was synthesized in our laboratory using a Rink-amide-AM resin as the solid support, and a standard Fmoc-based chemistry [49]. After acidic cleavage and purification, PACAP was characterized by MALDI-TOF mass spectrometry and analytical RP-HPLC (purity >95%). A LC3-green fluorescent protein expression vector construct (peGFP-LC3) was kindly provided by Dr. Tamotsu Yoshimori (Osaka University, JPN). Mouse monoclonal anti-p62 antibody was bought from Abnova (Walnut, CA, USA) while rabbit anti-tyrosine hydroxylase (TH), mouse monoclonal anti-β-tubulin as well as goat anti-mouse HRP-conjugated antibodies were from Millipore (Morsheim, FRA or Etobicoke, ON, CAN). Mouse monoclonal anti-actin and rabbit anti-LC3 antibodies were purchased from Sigma-Aldrich (Mississauga, ON, CAN). Mouse monoclonal anti-GAPDH antibody and goat anti-rabbit HRP-conjugated antibody were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Finally, AlexaFluor488-conjugated secondary antibody was from Life Technologies (Longjumeau, FRA or Burlington, ON, CAN).

2.2. Cell culture

The SH-SY5Y cells, which are human neuroblastoma cells naturally expressing PAC1 and VPAC2 receptors but not VPAC1 [50], were cultured in a 1:1 mixture of Ham's F12 Nutrient and MEM media supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 UI/mL each of penicillin and streptomycin, and 15% FBS. The cell line was maintained as a monolayer at 37 °C in a humidified atmosphere containing 5% CO_2 and cell passages were performed by trypsinization when cells were at 80% confluence.

2.3. Cell survival

Assessment of SH-SY5Y cell survival upon MPP^+ treatment, in the presence or not of PACAP, was carried out as previously published [42]. Briefly, cells were seeded in 96-well plates and incubated 48 h prior to the test. A concentration of 100 nM of PACAP38 was applied to the cells 4 h before a MPP^+ treatment (1.5 mM) of 24 h. Cell survival was evaluated with a MTS assay kit following the manufacturer's recommendations.

2.4. Mitochondrial transmembrane potential (MTP) assay

Change of MTP was measured using the fluorescent probe JC-1 following a procedure described in a previous publication [42]. Briefly, SH-SY5Y cells were treated as described above for the survival assay and then, incubated for 15 min, at 37 °C, with the JC-1 probe (10 μg/mL). JC-1 fluorescence was measured with a SpectraMax M Series multi-mode microplate reader using the SoftMax Pro Software (Molecular Devices, CA, USA).

2.5. Animals

Ten-week-old male C57Bl/6 mice were obtained from Charles Rivers Laboratories (L'Arbresle, FRA) and acclimatized for 1 week in the animal facility, in controlled temperature and lighting conditions (23 ± 1 °C, light on from 8 a.m. to 8 p.m.) with free access to food and water. Experiments were performed under the supervision of an authorized investigator (D.V.) in accordance with the French Ministry of Agriculture and the European communities' council directive 2010/63/UE of September 22, 2010 (approval number N/01-12-11/24/12-14).

2.6. In vivo neuroprotection assessment

This set of experiments was based on a publication by Deguil et al. [51]. Four groups of 7 animals were formed: Control, MPTP, PACAP, and MPTP + PACAP. From day 1 to day 5, animals received 2 different injections: a 100 μL intraperitoneal (i.p.) injection of MPTP (30 mg/kg/day) and then 1 h later a 100 μL intravenous (i.v.) injection of 3.5 nmol/kg/day of PACAP38, *i.e.* 15.7 μg/kg/day. Whenever a compound was not given, a saline injection of the same volume was performed. Mice from each group were sacrificed 3 days after the last injection and the brain proteins/tissues were used for Western blot and immunohistochemistry experiments.

2.7. Immunohistochemistry

Three animals from each group described in Section 2.6 were used to perform immunohistochemistry on brain tissue slices. Animals were anesthetized with pentobarbital (40 mg/kg body weight i.p.) and an intracardiac saline perfusion was performed to evacuate blood. A 4% paraformaldehyde (PFA) solution prepared in PBS was then perfused for prefixation of tissues. Animals were decapitated and the brain was removed and incubated overnight at 4 °C in a 4% PFA solution. To prepare tissues for cryopreservation, two consecutive 24 h treatments in 15% and 30% sucrose/PBS solutions, respectively, were performed and finally brains were stored in Tissue-Tek O.C.T. (Sakura Finetek, Villeneuve d'Ascq, FRA) at −80 °C until use. Tissues were cut with a cryostat (Leica CM3050; Leica Microsystems, Nanterre, FRA), and mounted onto gelatin-coated glass slides. Brain slices were blocked with normal donkey or goat serum for 90 min at room temperature and then incubated overnight at 4 °C with a rabbit anti-LC3 primary antibody (1:200 dilution). This was followed by a second incubation with an AlexaFluor 488®-conjugated donkey anti-rabbit antibody or an AlexaFluor 488®-conjugated goat anti-rabbit antibody, respectively. Images were acquired using a Zeiss LSM 780 laser scanning confocal microscope.

2.8. Autophagic flux monitoring

SH-SY5Y cells were seeded into 6-well plates, at a density of 5×10^5 cells per well and incubated 24 h to ensure cell adhesion. Then, they were transfected with peGFP-LC3, an expression vector for GFP-labeled LC3, using the Trans IT-2020 transfection reagent, as recommended by the supplier (Mirus Bio, Madison, WI, USA). After 24 h, cells were trypsinized and seeded in 24-well plates over coverslips. Cells were then treated with PACAP and/or MPP^+, as described for the survival assay. Coverslips were finally submitted to an immunofluorescence protocol with appropriate antibodies, as described below.

2.9. Immunofluorescence protocol

SH-SY5Y cells were seeded into 24-well plates containing coverslips, at a density of 75×10^3 cells per well, and incubated 48 h to ensure cell adhesion. After being submitted to the desired cell treatment, the medium was removed and the coverslips with cells were washed twice with PBS before being fixed with 4% PFA in PBS for 10 min. Next, coverslips were incubated in blocking buffer (PBS, 3% bovine serum albumin, 10% FBS, 0.1% Triton X-100) for 30 min at RT. After washing with PBS, the coverslips were incubated for 1 h at RT with a primary antibody diluted in blocking buffer. Coverslips were then washed in PBS and incubated with either Alexa fluor™-(488 or 568) goat anti-mouse IgG or Alexa fluor™-(488 or 568) goat anti-rabbit IgG for 1 h at RT. Thereafter, coverslips were washed twice with PBS and were incubated 5 min with DAPI to stain the nuclei. Finally, coverslips were washed again twice with PBS and were mounted on glass slides with Prolong™ Antifade (Life Technologies, Burlington, ON, CAN) to be examined.

2.10. Western blot analyses

Proteins from SH-SY5Y cells or SN proteins obtained from brains of each animal group were prepared as described previously [42]. Protein samples (20 μg) were subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane (Amersham, Les Ulis, FRA). Blocking was performed in a solution composed of 5% skim milk in 50 mM Tris-buffered saline containing 0.1% Tween 20 (TBST), and membranes were incubated overnight at 4 °C with primary antibodies (1:1000 dilution) against LC3, p62, actin, TH or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:4000 dilution) prepared in the same blocking solution. Afterwards, membranes were washed with TBST and then incubated 1 h at RT, in PBS containing 5% skim milk, with goat-anti-rabbit IgG ($1{:}10^4$ dilution) conjugated to horseradish peroxidase. Protein bands were visualized with either the Super Signal West-Pico or -Femto chemiluminescence substrates (Pierce-ThermoFisher, Nepean, ON, CAN).

2.11. Statistical analysis

All data are from 3 to 4 independent experiments. Prism software (Graphpad Software, CA, USA) was used to analyze data. Data are expressed as mean ± S.E.M. and statistical evaluation of the results was performed by ANOVA followed by a Bonferroni's test. Results were considered significant with $p \leq 0.05$.

3. Results

3.1. Effect of PACAP on MPP^+-induced alterations and death of SH-SY5Y cells

According to a protocol used previously, SH-SY5Y cells were treated with PACAP38 (100 nM) 4 h prior to MPP^+ exposure (1.5 mM) [42]. In such conditions, MPP^+, a neurotoxic agent that produces similar cellular

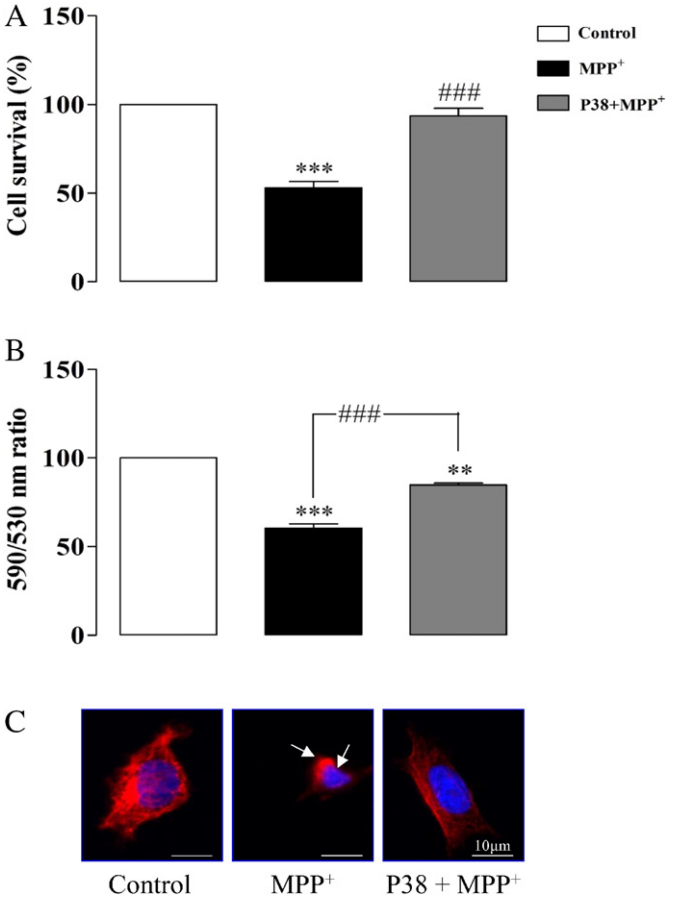

Fig. 1. Neuroprotective effect of PACAP38 (P38) in an *in vitro* model of Parkinson's disease. (A) Effect of MPP^+ (1.5 mM) with or without a 4 h pre-treatment with PACAP38 (100 nM) on SH-SY5Y cell survival. (B) Effect of PACAP38 on MPP^+-induced alteration of mitochondrial membrane potential in SH-SY5Y cells in the same conditions as in Fig. 1A. Mitochondrial transmembrane potential was assessed using the JC-1 probe, and the ratio of fluorescence emissions 590/530 nm was measured as an index of proper function. (C) Immunofluorescence images in which microtubules were revealed with an anti-tubulin antibody and nuclei were stained with DAPI. The photographs illustrate the beneficial effect of PACAP on MPP^+-induced morphological changes of SH-SY5Y cells treated in the same conditions as described in Fig. 1A. Each value represents the mean ± S.E.M. of at least 3 independent assays performed in octuplicate. Statistical analyses were carried out using an ANOVA followed by the Bonferroni's test. $^{**}p < 0.01$; $^{***}p < 0.001$ *vs* control. $^{\#\#\#}p < 0.001$ *vs* MPP^+-treated cells.

features as those observed in PD, caused approximately 50% cell death (Fig. 1A) but this effect was almost fully counteracted with PACAP pre-treatment (Fig. 1A). Considering that MPP^+ produces permeabilization of the mitochondrial outer membrane, we examined the ability of PACAP to prevent the deleterious effect of MPP^+ on mitochondrial transmembrane potential (MTP) using the fluorescent ratiometric probe JC-1. Treatment of SH-SY5Y cells with MPP^+ (1.5 mM) for 24 h induced a significant reduction of the 590/530 nm fluorescence emission ratio, thereby indicating that the mitochondrial integrity was severely altered by the toxic molecule (Fig. 1B). In this assay, PACAP had no effect on the fluorescence emission ratio per se (data not shown), but it decreased the effect of MPP^+ on MTP when added 4 h prior to the toxin treatment (Fig. 1B). Next, in order to study the effect of PACAP on MPP^+-induced morphological alterations of SH-SY5Y cells, we evaluated by immunofluorescence their structural changes in the presence of the peptide and/or the neurotoxic agent (Fig. 1C). Treatment with MPP^+ (1.5 mM) for 24 h induced a marked destabilization of microtubules, which was assessed by immunostaining of β-tubulin III, a member of the tubulin protein family located in the cytoplasm of neurons. This effect was markedly reduced by PACAP (Fig. 1C). As a matter of fact, the results showed that the control neurons exhibited a streaked cytoskeleton around the nucleus and dendritic extensions, whereas MPP^+-treated cells displayed cytoplasmic shrinkage and absence of dendritic extensions. Nevertheless, PACAP was able to counteract the morphological changes exerted by MPP^+ and restored the healthy appearance of the cell.

3.2. In vitro effect of PACAP on MPP^+-induced LC3 II formation and p62/SQSTM1 degradation

As shown in several studies related to neurodegenerative diseases, MPP^+ induces cell death and autophagy [19,46,52,53]. In order to explore the effect of PACAP on autophagy induced in a PD model, we investigated the mechanism involved in MPP^+-induced autophagy and studied the action of PACAP on the autophagic flux. Along with the reduction of cell viability induced by 1.5 mM MPP^+ [42], it was observed that this treatment produced a significant accumulation of microtubule-associated protein light chain 3 II (LC3 II), which is related to autophagy induction (Fig. 2A and B, 3rd column *vs* control). In parallel, p62/SQSTM1, an adaptor protein involved in the engulfment process of ubiquitinated proteins by phagophore to form autophagosomes, which thereafter fuse with lysosomes to produce the degradation of the ingested material, became much less abundant after MPP^+ treatment (Fig. 2A and C, 3rd column *vs* control). PACAP exposure restored the level of LC3 II to basal level (Fig. 2A and B, 4th column *vs* control), and stopped p62 degradation induced by the MPP^+ treatment (Fig. 2A and C, 4th column *vs* control). Interestingly, albeit it was not significant, PACAP itself seemed to slightly increase the LC3 II and p62 levels (Fig. 2A, B and C, 2nd column *vs* control). These findings strongly suggest that PACAP inhibits MPP^+-induced autophagy. In order to support these results, SH-SY5Y cells were treated with rapamycin, an inducer of autophagy, in the presence or absence of PACAP. As expected, rapamycin treatment induced p62 protein degradation. However, this effect was

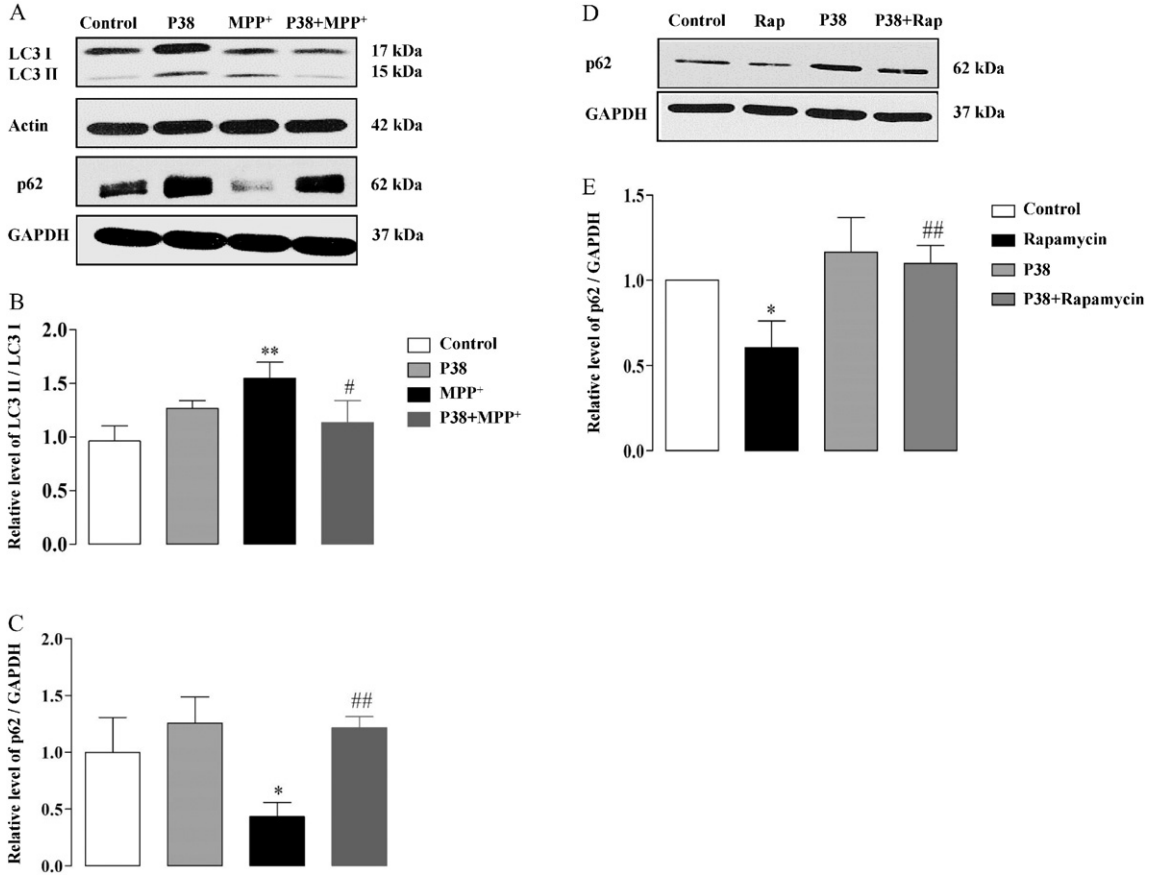

Fig. 2. Effects of PACAP38 (P38), MPP^+, and P38 + MPP^+ on the autophagy-related proteins LC3 and p62/SQSTM1 in SH-SY5Y cells. (A) Digital photographs illustrating the expression of LC3 I and II, and p62/SQSTM1 after immunoblotting. (B) Bar graphs displaying the relative abundance of LC3 II *vs* I measured by densitometry of the bands obtained on immunoblots, and standardized with actin. (C) Bar graphs displaying the relative abundance of p62/SQSTM1 measured by densitometry of the bands obtained in immunoblots. (D) Digital photographs of immunoblots illustrating the expression of p62/SQSTM1 in SH-SY5Y cells in presence of 200 nM rapamycin (Rap), an autophagy inducer, PACAP38 (P38 – 100 nM), or P38 + Rap. (E) Bar graphs displaying the relative abundance of p62/SQSTM1 measured by densitometry of the bands obtained in immunoblots. Each value represents the mean ± S.E.M. of three independent experiments. Statistical analyses were carried out using an ANOVA followed by the Bonferroni's test. $^*p < 0.05$; $^{**}p < 0.01$; *vs* control. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ *vs* MPP^+ or rapamycin-treated cells.

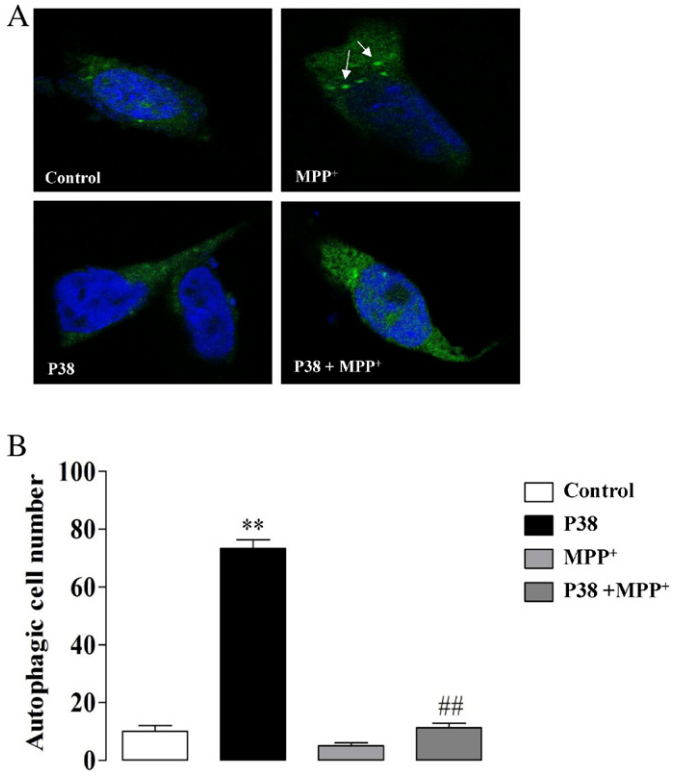

Fig. 3. Effect of PACAP38 against MPP^+-induced autophagic vacuoles (pointed out with white arrows). (A) Representative images of SH-SY5Y cells transfected with a GFP-LC3-encoding plasmid, cultured in complete medium for 24 h, and pretreated (or not) for 4 h with 100 nM PACAP38 and/or treated with 1.5 mM MPP^+. (B) Bar graphs displaying the relative abundance of autophagic cells calculated as percentage of total cells (in randomly selected 100 cells). A cell was considered in an autophagic state when it was containing >5 autophagosomes. Each value represents the mean ± S.E.M. of three independent experiments. Statistical analyses were carried out using an ANOVA followed by the Bonferroni's test. $^{**}p < 0.01$ *vs* control and $^{\#\#}p < 0.01$ *vs* MPP^+-treated cells.

abolished when cells were co-treated with PACAP (Fig. 2D and E). To further document this finding, autophagosome accumulation was assessed upon MPP^+ treatment. To achieve this, GFP-LC3 was expressed in SH-SY5Y cells before treatment with MPP^+ and/or PACAP (Fig. 3A and B). In line with our previous results, SH-SY5Y cells treated with MPP^+ showed a clear accumulation of autophagosomes, as indicated by GFP-LC3 puncta (Fig. 3A). However, treatment with PACAP prior to addition of MPP^+ diminished autophagosome formation as almost no LC3 puncta remained visible (Fig. 3A and B).

3.3. In vivo effect of PACAP on MPTP-induced LC3 II formation and p62/SQSTM1 degradation

A decrease of TH expression in DA neurons within the *substantia nigra* is a hallmark of PD. Accordingly, as described previously [42], TH expression in the SN *pars compacta* (SNpc) dopaminergic neurons was markedly reduced in mice that had received MPTP injection (Fig. 4A). Also, TH immunostaining in MPTP-treated mice that had received an i.v. injection of PACAP went back to normal, confirming the neuroprotective action of the peptide [42]. To investigate the possible involvement of autophagy in those effects, the impact of MPTP and PACAP on *in vivo* regulation of LC3 and p62 was also evaluated. In line with the *in vitro* findings, subchronic injections of MPTP increased LC3 II accumulation (Fig. 4B). However, surprisingly, they did not trigger significant degradation of p62 when compared to sham (Fig. 4B and C). Injections of PACAP had no obvious effects on LC3 II and p62 production (Fig. 4B and C) but this treatment appeared to counteract the effect of MPTP on LC3 II levels (Fig. 4B). To further confirm the inhibitory effect of PACAP on autophagy, brain slices of the SNpc region were immunostained with anti-LC3 II antibody in order to label autophagic vacuoles. After subchronic injections of MPTP, the SNpc revealed several puncta of LC3 indicative of the presence of autophagic vacuoles (Fig. 5). However, such LC3 II-associated puncta were not observed following treatments with PACAP alone or PACAP with MPTP (Fig. 5).

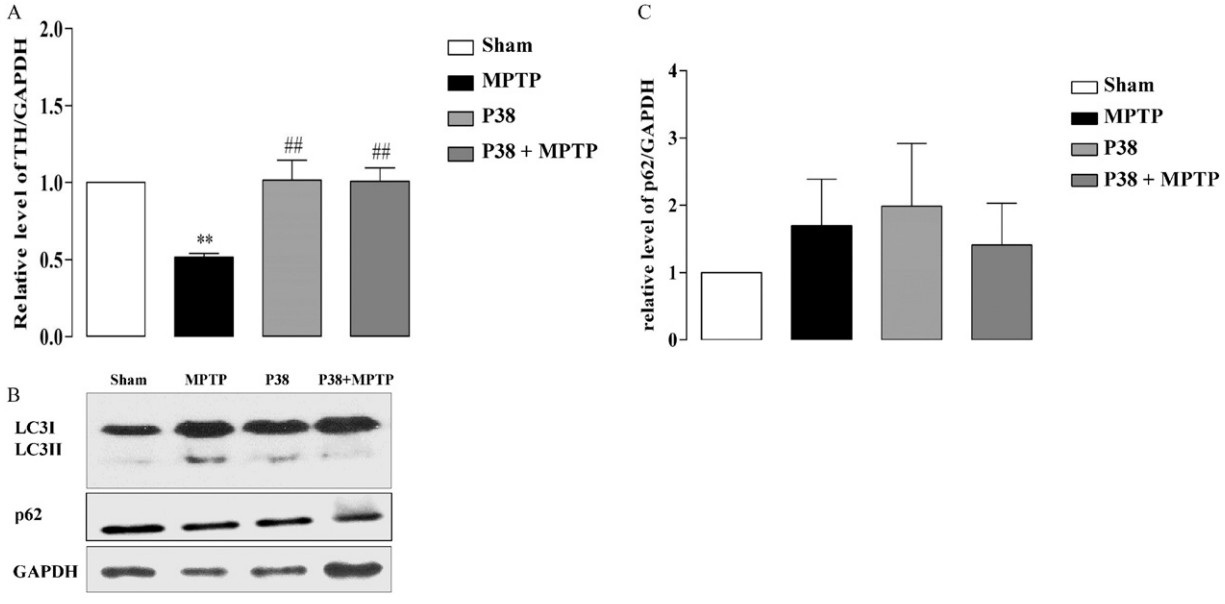

Fig. 4. Neuroprotective effect of PACAP38 on MPTP-induced decrease of tyrosine hydroxylase (TH) expression and autophagy in *substantia nigra pars compacta* (SNpc). (A) Densitometric analysis of TH protein expression in the SNpc of sham, MPTP-, PACAP38-, and MPTP + PACAP38-treated mice. (B) Digital photographs showing the expression of LC3 II and p62/SQSTM1, respectively, after immunoblotting. (C) Bar graphs displaying the relative abundance of p62/SQSTM1 measured by densitometry of the bands obtained in immunoblots, using AlphaEaseFC software (Alpha Innotech). For TH and p62/SQSTM1 expressions, values represent the relative optical density (OD) after normalization to GAPDH. All values are expressed as mean ± S.E.M. of at least three independent experiments. $^{**}p < 0.01$ is statistically significant compared to untreated animals; $^{\#\#}p < 0.01$ is statistically significant compared to MPTP-treated animals.

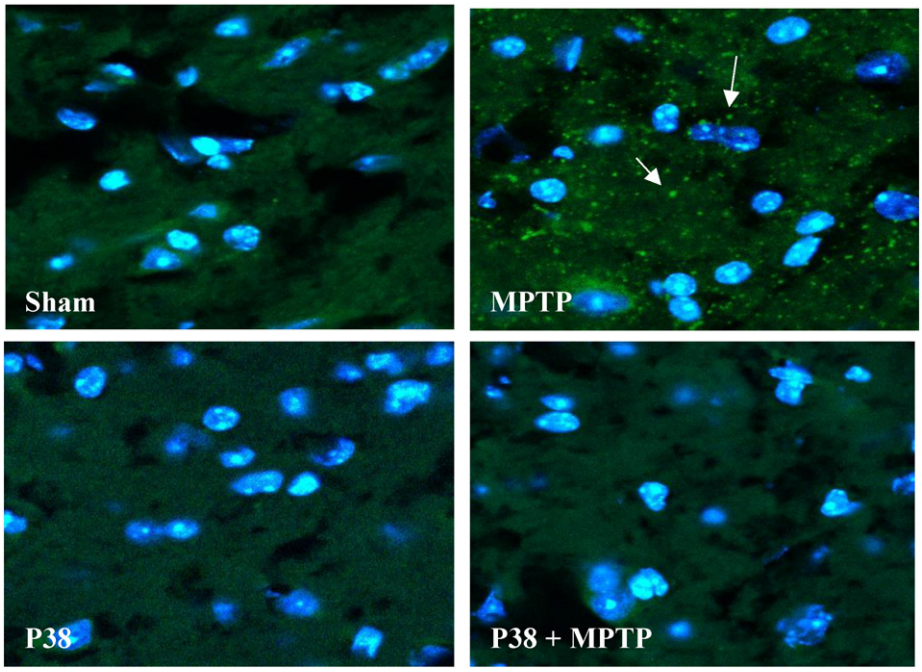

Fig. 5. Inhibitory effect of PACAP38 against the formation of MPTP-induced autophagic vacuoles in *substantia nigra pars compacta* (SNpc) of mice. Slices of SNpc were incubated with anti-LC3 (green color) and nuclei were stained with DAPI (blue color). The MPTP-treated animals showed in the DA cells the presence of many autophagic vacuoles (pointed out with white arrows). Compared to sham mice, PACAP38 itself had no effect whereas in MPTP-treated animals, the peptide prevented the formation of autophagic vacuoles.

4. Discussion

During the course of neurodegenerative processes, albeit numerous studies, cell death is the final outcome of a complex series of biological events that remain only partly understood. Accordingly, apoptosis and associated intracellular signaling have been intensively examined in various neurodegenerative diseases, including PD [54,55]. More recently, autophagy has also emerged as an important aspect of the etiology of these disorders and it has been reported that it is activated when neurons undergo aggressive stress [56–60]. There is still ongoing debate about the beneficial or detrimental outcome of autophagy activation during neurodegenerative diseases [56,57,59,61,62,22]. Nevertheless, dysregulated autophagy appears as a key element of these pathologies [11,7]. In particular, previous studies [19,20] reported an increase of the autophagic process in *in vitro* and *in vivo* PD models of neuron death induced by the toxic agents MPP^+ and MPTP. In addition, studies using siRNAs designed for silencing genes coding for autophagic proteins such as Atg8 (to which belongs LC3), Atg7 and Atg5 protected neurons from MPP^+-induced neurotoxicity [46,59], thereby adding evidence that the induction of the neurotoxic effect of MPP^+ involves activation of autophagy. Hence, SH-SY5Y neuroblastoma cells, which exhibit a dopaminergic neuronal phenotype, were exposed to an acute *in vitro* stress generated by MPP^+. The *in vitro* experiments showed that MPP^+ treatment produced the activation of the autophagic flux in these cells, as revealed by an increased formation of LC3 II. This result is consistent with those of Nopparat et al. [18] who showed that a concentration of 1.6 mM of MPP^+ was able to induce LC3 II accumulation and autophagosome formation in dopaminergic neurons. Also, MPP^+ treatment induced a potent decrease of the p62 protein level, which is suggestive of a complete autophagic flux. So far, these actions are notably associated with the removal of defective mitochondria [63] and accordingly, several reports revealed that during oxidative stress, autophagy is activated to eliminate defective cell components [64,65].

PACAP has been shown to reduce toxic agent-induced neurotoxicity in PD models based on neuroblastoma cells [66,67,31,41,42]. In particular, it reduced cell death, restored the mitochondrial activity, and preserved cell integrity. However, up to now, no study has investigated the impact of PACAP on autophagy produced following MPP^+ or MPTP treatments used to mimic PD characteristics. Hence, we described for the first time that *in vitro*, PACAP diminished MPP^+-induced accumulation of LC3 II, a marker of autophagy, in SH-SY5Y neuroblastoma cells. Furthermore, this led to the restoration of the p62 protein that was originally reduced by MPP^+. These observations suggest that PACAP down-regulates MPP^+-induced autophagy. Nonetheless, it was yet unclear if the anti-autophagic effect of the peptide was coming from a reduction of apoptosis or if PACAP was directly modulating the autophagic flux. To document this question, SH-SY5Y cells were treated with PACAP and rapamycin, a compound that activates autophagy by inhibiting mTOR. In such a condition, the peptide was able to restore significantly the level of p62, thereby confirming the inhibitory effect exerted by PACAP on autophagy. Moreover, SH-SY5Y cells transfected with GFP-LC3 demonstrated that MPP^+ triggered the formation of autophagic vacuoles, and that this phenomenon was reversed with PACAP. Overall, our data with MPP^+-treated SH-SY5Y cells showed that PACAP reduced cell death, maintained mitochondrial activity, diminished LC3 II formation, restored p62, and decreased autophagic vacuole formation. Based on these findings, it clearly appears that the neuroprotective peptide PACAP can modulate the autophagic process. The precise pathways regulated by PACAP to block the MPP^+-induced autophagy are still unknown. Nevertheless, PACAP possesses the ability to stimulate cAMP production [23] and intracellular cAMP negatively regulates autophagy [68]. Moreover, studies on PACAP anti-apoptotic activity have demonstrated that following PAC1 receptor activation, a cascade of signaling events occurs, which increases Bcl-2 levels and blocks Bax expression [69]. Thus, upon PACAP treatment, the intracellular environment would favor Bcl-2/Beclin interactions that inhibit autophagy and restrain Bax translocation to lysosomes or mitochondria membranes.

In vivo studies showed that MPTP, the precursor of MPP^+, reproduced features of PD. Accordingly, we observed that MPTP caused in mice the death of SNpc dopaminergic neurons. Neuronal loss was largely decreased when animals were injected with PACAP, and the peptide restored the expression of TH that was reduced by the neurotoxic agent. In addition, the results revealed that subchronic doses of

MPTP increased the formation of LC3 II and increased autophagosome formation in the SNpc. Surprisingly, in contrast to what was observed *in vitro*, MPTP alone did not induce the disappearance of p62, an occurrence suggesting that autophagic flux was disrupted [70]. It could be explained by the difference of paradigms (*in vitro* human neuroblastoma cells *versus in vivo* injection of MPTP in mice), as rodents show distinct sensitivity to MPTP neurotoxicity because of their particular oxidative metabolism [71]. Another explanation could be the difference in the stress duration on neurons and the way with which the cell reacted to the change. Indeed, under short stress conditions, cells activate autophagy to get rid of defective organelles. However, under prolonged stress, cells are overwhelmed with ROS and defective organelles and consequently, cell death through apoptosis probably becomes the dominant event. In fact, it has been demonstrated that prolonged stress could lead to a disruption of the autophagic flux [19]. Also, impairment of lysosomal function with disruption of the autophagic flux has been reported previously in a MPTP mice model of PD and this was directly associated with the pathogenesis of the disease because accumulation of autophagosomes in PD brain samples correlated with the presence of Lewy bodies [72]. Finally, we observed that PACAP inhibited the formation of LC3 II following a MPTP treatment and that it reduced the autophagosome formation in the SNpc.

In the context of PD, mutants of two proteins, namely PINK-1 and Parkin, have been identified in familial forms of the disease. These proteins play an essential role in the mitochondria turnover in cells. As a matter of fact, under normal physiological conditions, PINK-1 accumulates at the membrane of defective mitochondria and then Parkin is recruited to trigger selective autophagy [73]. Accordingly, mutation of these proteins gives rise to aberrant mitophagy. Moreover, abnormal expression of α-synuclein, which is a main component of Lewy bodies that are a pathological trait of PD, has been linked to increased autophagy while mutated forms of the protein have been found to co-localize within autophagosomes containing normal polarized mitochondria [57]. Altogether, it seems that cells affected by the pathology will increasingly suffer from a loss of mitochondrial function. In this point of view, treatment with PACAP could offer protection against increased autophagic and apoptotic mechanisms. Furthermore, a recent study has demonstrated that PACAP was able to up-regulate expression levels of Pgc1α, a transcriptional regulator of mitochondrial biogenesis [74]. Thus, treated cells would not only benefit from blockade of the cell death process but also from renewal of mitochondria.

In conclusion, we showed that PACAP, a neuropeptide known for its anti-apoptotic properties, could also negatively modulate MPP^+- and MPTP-induced autophagy. These findings provide additional information regarding the link between apoptosis and autophagy and highlight that PACAP exerts its potent neuroprotective activity through diverse complementary pathways. Nevertheless, further studies are required to deeper dissect the relationship between neuroprotection and inhibition of autophagy produced with PACAP.

Conflict of interest

No conflict of interest is reported.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

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