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Basic

Virology and cell biology of the hepatitis C virus life cycle – An update

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Summary

Hepatitis C virus (HCV) is an important human pathogen that causes hepatitis, liver cirrhosis and hepatocellular carcinoma. It imposes a serious problem to public health in the world as the population of chronically infected HCV patients who are at risk of progressive liver disease is projected to increase significantly in the next decades. However, the arrival of new antiviral molecules is progressively changing the landscape of hepatitis C treatment. The search for new anti-HCV therapies has also been a driving force to better understand how HCV interacts with its host, and major progresses have been made on the various steps of the HCV life cycle. Here, we review the most recent advances in the fast growing knowledge on HCV life cycle and interaction with host factors and pathways.
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Introduction

Hepatitis C virus (HCV) constitutes a significant health burden worldwide. Indeed, this virus has a high propensity for establishing a chronic infection and it is estimated that 130–170 million people suffer from chronic hepatitis C. In the long-term, this can lead to advanced liver fibrosis, cirrhosis and hepatocellular carcinoma. As a consequence, HCV is the most common indication for liver transplantation in developed countries [1]. For more than two decades, interferon has been the basis for HCV treatment. Responses to treatment were improved in 1998 by the addition of ribavirin and then in 2001–2002 by linking the interferon molecule to polyethylene glycol [2–4]. Recently, there have been major advances in hepatitis C treatments with the licensing of the first direct-acting antivirals (DAAs) and large numbers of ongoing trials with various DAAs showing high potency, favourable tolerability profile, higher barrier to resistance, shortened treatment duration, and all oral regimen [5]. However, there is no vaccine yet available.

Discovered in 1989 [6], HCV is a positive sense, single-stranded RNA virus of the *Flaviviridae* family, which also includes many arthropod-borne human pathogens of the *Flavivirus* genus such as yellow fever virus, West Nile virus and dengue virus. HCV genome organization is presented in Fig. 1. Together with the GBV-B virus and the recently identified non-primate, rodent and bat hepaciviruses, HCV has been grouped in the *Hepacivirus* genus [7–10]. HCV isolates have been grouped into seven genotypes and a number of subtypes [11] with distinct geographic distributions and sensitivity to interferon-based treatment [12,13]. The only true HCV animal model is the chimpanzee, which has been crucial in studies of HCV immunity and pathogenesis [14]. In addition, human-liver chimeric and genetically modified HCV-permissive mouse models have also been developed [15]. For a long time, the lack of a cell culture system has been a major obstacle to study the HCV life cycle. However, selectable replicon systems [16] and retrovirus-based pseudotyped particles [17] have been major tools to understand HCV genomic replication and virus entry, respectively. Finally, since 2005, the full viral life cycle can be investigated with the help of complete viral replication systems [18–20]. It should be noted that these cell culture systems usually use HuH-7-derived hepatoma cells. However, this cell line lacks many features of hepatocytes [21]. Primary human hepatocytes or human liver slices have therefore been developed to validate some experiments in more physiological models [22–24]. In this review, we will discuss recent advances in the virology and cell biology of the HCV life cycle.

Keywords: Hepatitis C virus; Viral life cycle; Virus entry; Virus-host interactions.
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Basic

Key Points

- The HCV life cycle is tightly linked to the lipid metabolism of the hepatocyte
- The HCV particle exhibits an unusual low buoyant density and is associated with lipoproteins
- ApoE is associated with infectious HCV particles and is involved in HCV assembly and entry
- HCV envelope glycoproteins are the major viral determinants of HCV entry into hepatocytes
- HCV entry into hepatocytes is a highly complex process involving a series of host cell factors, including SRB1, CD81, CLDN1 and OCLN as the major entry factors
- HCV replication induces massive rearrangements of intracellular membranes to create a micro-environment in the cytoplasm, called “membranous web”
- HCV replication takes place in double-membrane vesicles associated with the membranous web
- HCV recruits many host cell factors to replicate its genome, with PI4KIII playing a central role
- HCV assembly takes place at the proximity of cytosolic lipid droplets with the help of cellular factors, which include among others DGAT1 and PLA2G4
- HCV assembly is tightly connected to the biogenesis of VLDL

The HCV particle

Despite substantial progress in producing viral particles in cell culture and several biochemical and morphological studies, the structure of the HCV virion remains poorly characterized. This contrasts with the well-characterized flavivirus viral particles. A striking and unique feature of HCV biology is its association with lipoproteins, which exhibit an unusually low buoyant density [25–27]. HCV particles are 50–80 nm in diameter [28] and contain the single-stranded RNA genome, core and the envelope glycoproteins, E1 and E2 [29]. 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 the envelope glycoproteins are anchored. Importantly, due to virion association with lipoproteins, apolipoproteins such as apoE, apoB, apoA1, apoC1, apoC2, and apoC3 can also be found in association with HCV particles [25,28,30–32]. Furthermore, a characterization of cell culture-produced particles indicates that their lipid composition resembles very-low density lipoproteins (VLDL) and low-density lipoproteins (LDL) with cholesteryl esters accounting for almost half of the total HCV lipids [33]. Electron microscopy analyses of purified infectious virions confirm the pleomorphic nature of HCV particles and show virions with a rather smooth and even surface [28].

The exact nature of the interactions involved between HCV virion components and the lipoproteins remains undetermined. It has been suggested that the HCV virion could be a hybrid particle composed of a virion moiety and a lipoprotein moiety [34]. 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 [35]. In both particle types, the interaction with lipoproteins could contribute to the shielding of HCV glycoproteins from the host immune response and explain the poor detection or availability of HCV glycoproteins at the virion surface [28,33,36]. Importantly, apolipoprotein(s) associated with HCV virion play a role in HCV entry (see below).

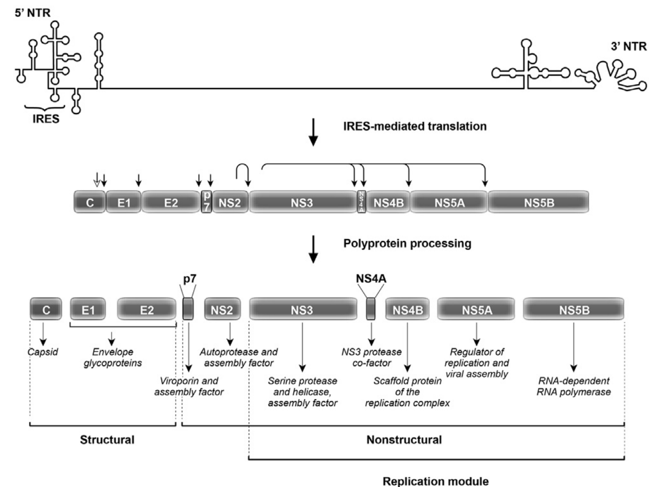

Fig. 1. HCV genome and proteins. HCV genome contains a single open reading frame flanked by 5′ and 3′ non-translated regions (NTRs). The 5′ NTR contains an internal ribosome entry site (IRES). After its synthesis, HCV polyprotein is cleaved by viral and host encoded proteases. Cleavage in the N-terminal part of the polyprotein is mediated by cellular signal peptidases as indicated by individual vertical arrows. An additional cleavage removing the carboxy-terminal region of the core protein is mediated by cellular signal peptide peptidase, as indicated by an open arrow. The linked arrows indicate the cleavages by the viral proteases NS2 and NS3/4A. The functions of the individual proteins are indicated at the bottom of the figure.

HCV envelope glycoproteins are the major viral determinants of HCV entry. They indeed play a role in receptor binding and mediate the fusion process between the viral envelope and an endosomal host cell membrane. HCV glycoproteins E1 and E2 are type I transmembrane proteins, which form a non-covalent heterodimer within infected cells, whereas they assemble as large covalent complexes stabilized by disulfide bonds on the viral particle [37]. Within the E1E2 heterodimer, the E2 glycoprotein has been shown to interact with receptors or co-receptors on target cells [38,39]. Based on the hypothesis that the structure of the fusion protein should be conserved within the *Flaviviridae* family, E2 had also initially been proposed to be the fusion protein responsible for the fusion between HCV envelope and a host-cell membrane [40]. However, recently published crystal structures of the E2 glycoprotein core domain do not confirm this hypothesis [41,42]. Furthermore, instead of forming an elongated shape, composed of three domains as previously predicted [40], E2 forms a compact globular structure distinct from any known viral fusion protein. Interestingly, the masking of neutralizing epitopes by glycans and the definition of the CD81 binding site in this new structure confirms previous experimental results [43,44]. Furthermore, these new data strongly suggest that E1

should be the fusion protein or, at least, a fusion partner of an E1E2 fusion complex formed upon conformational rearrangements [45,46].

HCV entry and uncoating

Viral entry plays an important role for hepatocyte tropism of HCV. During a primary infection, HCV particles are transported by the blood stream and come into contact with hepatocytes after crossing the fenestrated endothelium of the liver sinusoids. In the space of Disse, virions have direct contact with the basolateral surface of hepatocytes. This allows them to interact with attachment factors and receptors on the surface of these cells. Initial attachment of HCV particles onto hepatocytes is mediated by the heparan sulfate proteoglycan syndecan-1 or syndecan-4 [47,48] or by the scavenger receptor B1 (SRB1) [36], which depends on virion density. It was initially thought that HCV glycoproteins are responsible for virion binding to heparan sulfate proteoglycans [49] or SRB1 [39]. However, more recent data suggest that ApoE, rather than HCV glycoproteins themselves, could be involved in this initial contact [36,50]. Due to HCV particle interaction with lipoproteins, the LDL receptor (LDLR) has also been proposed to play a role in the early phase of HCV entry [51]. However, HCV-LDLR interaction seems to involve a non-productive entry pathway that can potentially lead to viral particle degradation [52].

After the initial attachment to the cell surface, the following steps of HCV entry are only partially understood and they involve a series of specific cellular entry factors (Fig. 2). It emerges that the coordinated action of at least four major cellular factors is essential for HCV entry. They include SRB1 [39], tetraspanin CD81 [38] and tight-junction proteins claudin-1 (CLDN1) [53], and occludin (OCLN) [54]. Due to its dual interaction with HCV

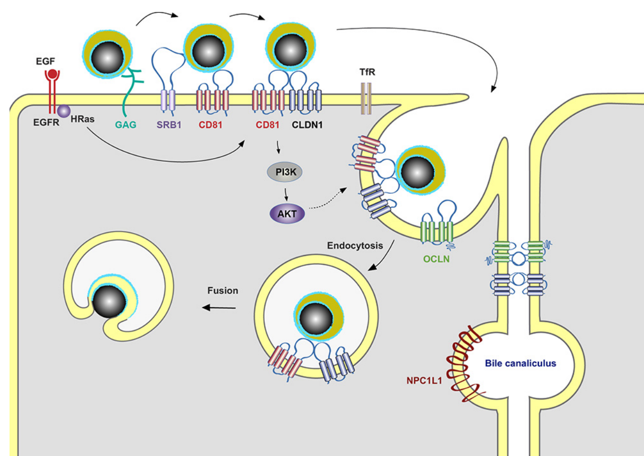

Fig. 2. HCV entry. The HCV virion is tightly associated with lipoproteins to form a complex particle that has been called lipoviroparticle. It initiates its life cycle by binding to glycosaminoglycans (GAGs) and SRB1. Then the virus follows a complex multistep process, involving a series of specific cellular entry factors, which include SRB1, CD81, tight-junction proteins, CLDN1 and OCLN, EGFR, transferrin receptor (TfR), and NPC1L1 as well as signaling proteins (see text for details). After binding to several components of the host cell, HCV particle is internalized by clathrin-mediated endocytosis and fusion takes place in early endosomes.

glycoprotein E2 and lipoproteins, SRB1 could be a first entry factor, interacting with the virion after initial cell attachment. The role of SRB1 in HCV entry was first suggested by its ability to mediate E2 binding and the hypervariable region 1 (HVR1) of E2 is essential for this interaction [39]. However, as above discussed, SRB1 also seems to contribute to virus attachment through interaction with virus-associated lipoproteins [36,55] and HCV mutants, harbouring HVR1 deletion or mutation that prevent E2 binding to SRB1, remain dependent on SRB1 for entry into cells [36]. One elegant hypothesis is that SRB1, through its lipid transfer activity, could modify the lipid composition of the lipoprotein moiety of the virion, which would lead to a better exposure of the CD81 binding site on E2 glycoprotein, as suggested by the observation that SRB1 mediates a post-binding event important for productive viral entry [36,56]. Alternatively, SRB1 interaction with HVR1 could also unmask the CD81 binding site of E2, as suggested by the reduced dependency on SRB1 of HVR1-deleted mutant viruses [57–59]. Whatever the mechanism involved, HCV virion seems to be primed to interact with CD81 after SRB1 binding.

Among HCV entry factors, the tetraspanin CD81 is undoubtedly a key player in the HCV lifecycle [60]. Amino acid residues involved in CD81 binding are located at the surface of the core of E2 protein [41]. This interaction seems to prime HCV envelope proteins for low pH-dependent fusion [61]. CD81 is highly dynamic at the cell surface and is enriched in membrane areas that form stable platforms, which are in permanent exchange with the rest of the membrane, and the balance of these dynamic exchanges in the cell membrane are essential to the process of HCV entry [62,63]. In particular, it is believed that CD81 molecules that freely diffuse and are therefore not engaged in static microdomains are used by HCV during its entry step [62,63]. CD81 has also been shown to interact with CLDN1 [64,65], another essential entry factor, to form a co-receptor complex involved in downstream events of HCV entry [66], and the viral particle might also potentially interact with CLDN1 [45]. CD81-CLDN1 association appears to be regulated by multiple signalling pathways. This interaction seems to be promoted by epidermal growth factor receptor (EGFR) and also potentially by protein kinase A [67,68]. Following EGFR stimulation, the Ras/MEK/ERK pathway is activated, which could lead to the activation of MAPK interacting serine/threonine kinase 1 (MKNK1), a kinase which facilitates HCV entry downstream of EGFR activation [69]. Furthermore, activation of EGFR also stimulates HRas, which in turn associates with CD81, and this interaction is required for CD81 lateral diffusion, allowing CD81-CLDN1 to associate [70]. Thus, it is believed that EGFR promotes CD81-CLDN1 complex formation by inducing CD81 diffusion through HRas activation and facilitates CD81-CLDN1 co-internalization with HCV particles. After interaction with the CD81-CLDN1 complex, the HCV particle also transiently activates the PI3K-AKT pathway to facilitate its entry [71]. Finally, it has also been shown that E2 binding to CD81 induces Rho GTPases signalling which in turn leads to a rearrangement of the actin cytoskeleton and could therefore also affect CD81 mobility at the plasma membrane [72].

In addition to CLDN1, another tight junction protein, OCLN is also an essential HCV entry factor [54]. However, the precise role of OCLN in the HCV life cycle remains poorly understood. It seems to play a role at a late entry step [73,74]. Recently, it has been reported that activated macrophages produce TNFα that increases the diffusion coefficient of CD81 and relocalizes OCLN

at the basolateral membrane, thereby facilitating HCV entry [75]. OCLN depletion does not perturb CLDN1 expression or localization, suggesting that both entry factors function separately during HCV infection [73,76]. It is worth noting that, together with CD81, OCLN determines the tropism of HCV for human cells [54,77]. Since CLDN1 and OCLN are tight junction proteins, it was first believed that after binding to CD81, the HCV virion would migrate to tight junctions for internalization. However, CLDN1 associates with CD81 at the basolateral membrane of polarized HepG2 cells, whereas pools of CLDN1 in tight junctions show only a minimal association with CD81 [64,65]. Furthermore, disruption of tight junctions by calcium depletion of polarized HepG2 cells increases HCV entry [78,79]. Finally, live cell imaging experiments indicate that HCV particles do not migrate to cell-cell contact areas after binding, supporting the idea that tight junctions might not be required for HCV entry, at least in non-polarized hepatoma cells [80].

Finally, since the HCV virion is rich in cholesterol, the role of the cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) was investigated and NPC1L1 identified as an additional entry factor [81]. Furthermore, transferrin receptor 1 has also been reported to be involved in HCV entry [82]. However, the precise roles of these additional factors in HCV entry remain to be determined.

Whether entry of HCV is restricted to hepatocytes still remains an unresolved issue. The CD81 tetraspanin [38] is ubiquitously expressed and, in particular, is found in B-lymphocytes where it acts as a co-stimulatory molecule. However, CD81 interaction with its partner EWI-2wint may also represent an important molecular determinant contributing to the restricted tropism of HCV, since EWI-2wint is expressed in several tissues or cell types (such as brain or immune cells) but not in hepatocytes [83]. SRB1 is detected on many cell types, although it is highly expressed in the liver and steroidogenic tissues [84]. NPC1L1 is expressed on the apical surface of human hepatocytes but also in enterocytes [85]. The other entry factors, i.e., the tight junction proteins CLDN1 [53] and OCLN [54] have more restricted expression profiles in epithelial tissues along with high-level expression in hepatocytes. Thus, in addition to the tissue-restricted expression of miR-122, which favours HCV genome expression (see below), the combined presence of these receptors and entry factors in hepatocytes, their expression rates, and their ability to interact is one element that may explain why the liver constitutes the main target for HCV particles [86].

HCV is known to be endocytosed by a clathrin-dependent process [87]. Interestingly, it has been shown that CD81-CLDN1 complexes are internalized in a clathrin- and dynamin-dependent manner [66], consistent with a single particle imaging study, reporting that entering HCV particles are associated with CD81 and CLDN1 [80]. Furthermore, this analysis also indicates that the HCV particle binds cells on filopodia and reaches the cell body by a mechanism that relies on retrograde actin transport [80]. After internalization, the virion is transported to Rab5a positive early endosomes along actin stress fibers, where fusion seems to take place. Following fusion, the HCV genome is presumably released into the cytosol, where it is directly translated to produce viral proteins and initiate viral replication.

In the liver of patients, infected cells occur in clusters, pointing to a cell-to-cell spread as the predominant mode of HCV transmission [88]. Interestingly, direct cell-to-cell transmission is also observed in cell culture [89–92]. Although it seems to involve most HCV entry factors identified so far, the mechanism governing this process remains unknown, but might involve exosomes [93].

HCV RNA translation and replication

RNA translation is initiated with the help of cellular factors [94]. The HCV genome contains a single open reading frame (ORF), which is flanked by 5′ and 3′ non-translated regions (NTRs). These NTRs contain highly structured RNA elements that are critical for genome translation and HCV RNA replication [95]. The 5′NTR contains an internal ribosomal entry site (IRES), which initiates translation of the HCV genome into a single polyprotein (Fig. 1). Viral and host encoded proteases process the viral polyprotein into the 10 mature proteins, core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B [96]. Signal peptidase and signal peptide peptidase mediate cleavage of the structural proteins, core, E1, E2, and the p7/NS2 junction. NS3 mediates cleavage of NS4A from itself and NS4B, after which NS4A associates with the N-terminus of NS3. The resulting NS3/4A protease complex can then cleave at the NS4B/5A and NS5A/5B junctions, whereas the cleavage between NS2 and NS3 is mediated by the NS2 cysteine protease whose function is strongly enhanced by the N-terminal one-third of NS3.

After translation, the HCV proteins are associated with membranes derived from the endoplasmic reticulum (ER) (Fig. 3). Together, NS3/4A, NS4B, NS5A, and NS5B constitute the viral proteins of the replication machinery, which replicates the positive sense RNA genome through a negative strand intermediate [95]. The viral RNA-dependent RNA polymerase NS5B is the key enzyme of RNA synthesis. Nascent RNA genomes are translated to produce new viral proteins, serve as new/additional RNA templates for further RNA replication and are progressively assembled to form infectious virions. HCV replication is dependent on microRNA 122 (miR-122) [97], a liver-specific microRNA that recruits Argonaute 2 to the 5′ end of the viral genome [98], stabilizing it and slowing its degradation by the 5′ exonuclease Xrn1 [99].

To replicate its genome, HCV induces massive rearrangements of intracellular membranes to create in the cytoplasm a micro-environment, called the "membranous web" [95]. Analyses with electron microscopy and 3D reconstructions of the membranous web show predominantly double-membrane vesicles (DMVs) exhibiting an average diameter of 150 nm and accumulating in parallel to the peak of RNA replication [100,101]. At later time points of infection, double-membrane tubules and multi-membrane vesicles appear, the latter presumably reflecting a stress-induced host cell response. The presence of DMVs in the membranous web is in line with the proposed role of autophagy in HCV replication [102]. However, the exact functional role of autophagy in HCV replication remains debated [103]. Due to its capacity to oligomerize, the membrane protein NS4B has been proposed to form the scaffold of membranous vesicles [104]. Furthermore, while NS3/4A, NS4B, NS5A or NS5B expressed alone can induce some membrane remodelling, only NS5A is capable to induce DMVs [100,105,106]. However, none of these proteins expressed alone is capable to induce a complete membranous web structure, suggesting that the global membrane rearrangements observed in HCV infected cells require the concerted action of most if not all replicase proteins.

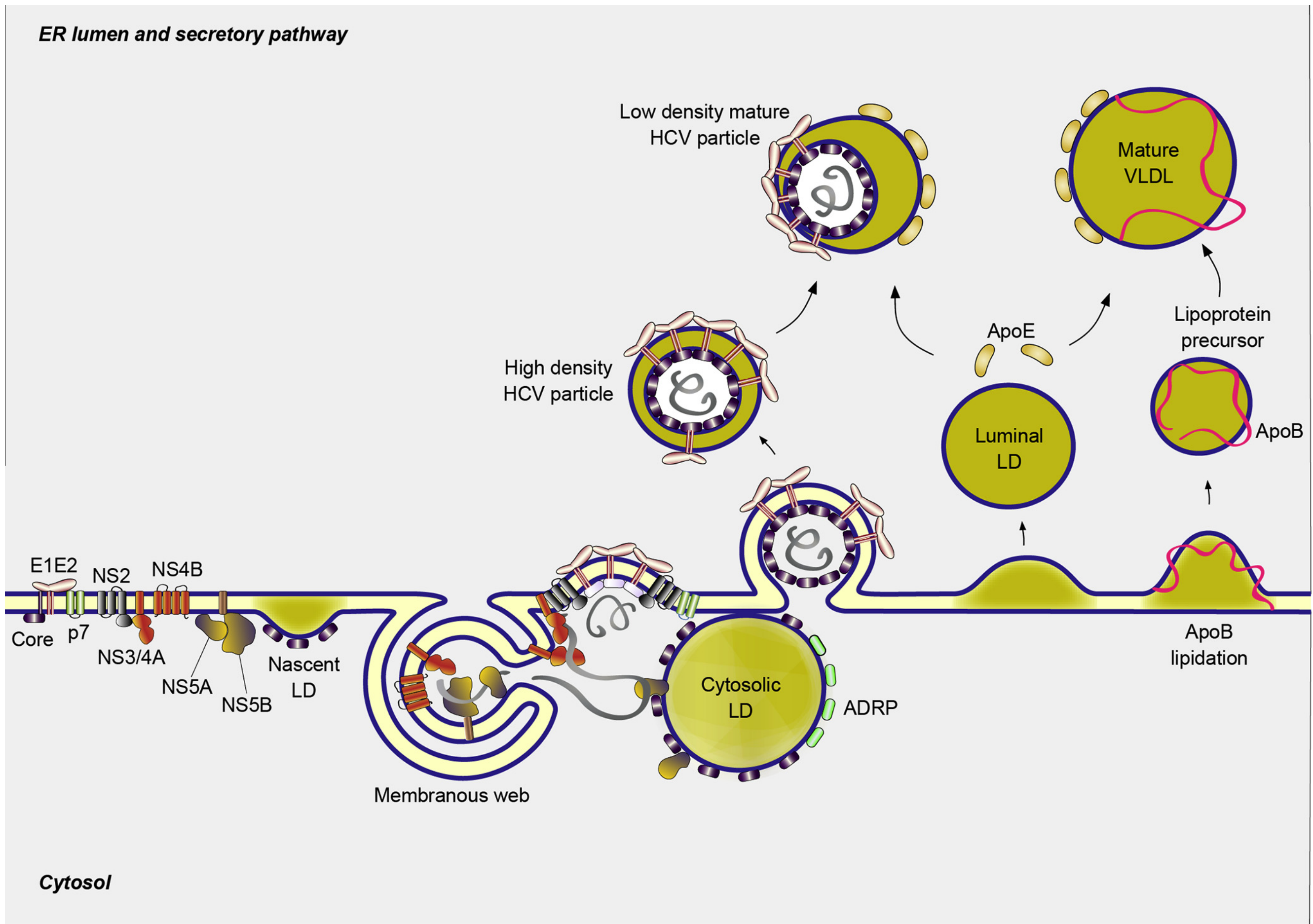

Fig. 3. HCV replication and assembly. Upon cleavage of the polyprotein, HCV non-structural proteins form the replication complex in association with cellular factors, which leads to the formation of double-membrane vesicles, also called the membranous web, where replication takes place. After cleavage of its C-terminus, core protein is loaded onto LDs. The junction between core-loaded LDs and the replication-complex-rich ER membranes is the site of virion assembly. Newly replicated viral genomes are transferred to the assembly sites via NS3/4A or NS5A, and NS2, and p7 connect replication complexes and core proteins to the glycoproteins. The next steps in HCV virion morphogenesis are tightly linked to the metabolism of VLDL assembly. However, the details of the intersection between HCV assembly and VLDL biogenesis remain poorly understood. It has been demonstrated that HCV particle contains apoE and neutral lipids, but how these components are acquired during HCV morphogenesis remains speculative, and this figure represents a hypothetical model.

Although a large number of host factors influencing translation and replication have been identified, using RNAi-screening and mass spectrometry interactome approaches [107,108], only a selection of them are discussed below. Phosphatidyl-inositol-4-kinase-III (PI4KIII) has been identified as a central host factor involved in HCV replication and NS5A interaction with this kinase induces accumulation of phosphatidylinositol-4-phosphate (PI4P) within the membranous web [106]. The absence of PI4KIIIα activity or alteration of its interaction with NS5A induces a dramatic change in the ultrastructural morphology of the membranous web [106,109]. NS5A has also been shown to hijack ARF-GAP1, a GTPase-activating protein for ARF1, which plays a central role of cargo sorting in COPI transport. NS5A-ARFGAP1 interaction has been proposed to help to maintain a PI4P-enriched microenvironment by removing PI4P phosphatase Sac1 from the site of viral replication [110]. Other cellular proteins, such as vesicle-associated membrane protein-associated protein A (VAP-A) and VAP-B, which are crucial for viral RNA replication, as well as cholesterol have also been found in enriched fractions of HCV-induced DMVs [111]. Interestingly, the accumulation of cholesterol in the membranous web is mediated by the interplay between PI4KIII and oxysterol-binding protein [112]. In addition to cholesterol, other lipids also likely play some role in HCV replication. In this regard, it is worth noting that HCV alters the expression of genes involved in cellular lipid metabolism, resulting in the accumulation of intracellular lipids [113]. It has also been reported that *de novo* formation of DMVs is blocked by inhibitors of cyclophilin A, which is another partner of NS5A that is critical for RNA replication [114]. Unexpectedly, nuclear pore complex proteins and nuclear transport factors are also involved in the membranous web formation [115]. Indeed, transport cargo proteins normally targeted to the nucleus are capable of entering regions of the membranous web and HCV proteins have been shown to interact with nuclear pore complex proteins and nuclear transport factors. Based on these observations, it has been hypothesized that in HCV infected cells, cytoplasmically positioned nuclear pore complexes could form channels across DMVs structures of the membranous web. These nuclear pore complexes could then facilitate the movement of nuclear localization signal (NLS)-containing proteins into the membranous

web while excluding proteins lacking NLS sequences such as pattern recognition receptors (PRRs).

Lipid droplets (LDs) are also found in ultrastructural studies of the membranous web. LDs are organelles which function as a deposit of triacylglycerides and cholesteryl esters surrounded by a phospholipid monolayer that harbours numerous proteins. LDs are currently believed to play a central role in the coordination of viral RNA synthesis and virion morphogenesis [116]. Viral double-stranded RNA has been found surrounding LDs, suggesting that these organelles could play a role in RNA replication [117]. Furthermore, Rab18, a protein associated with LDs, appears to promote the physical association of NS5A and other replicase components with LDs, and modulation of Rab18 affects HCV RNA replication [118]. Another LD protein, Tail-Interacting Protein 47 (TIP47), also modulates HCV RNA replication by interacting with NS5A [119,120]. By interacting with different cellular partners, NS5A plays a central role in the formation of the membranous web and the recruitment of the replicase around LDs. It has also to be noted that within infected cells, NS5A motility and efficient HCV RNA replication require the microtubule network and the cytoplasmic motor dynein [121].

HCV assembly and release

HCV morphogenesis requires the accumulation of viral structural proteins and genomic RNA that are brought together in a temporally and spatially organized manner [35]. Assembling, budding or egressing virions are difficult to detect in infected cells, suggesting that these processes are either rare or rapid. One peculiar feature of HCV particle assembly shared with other members of the *Flaviviridae* family is the implication of the viral non-structural proteins in this process. Another major peculiarity of HCV morphogenesis is its intricate connection with lipid metabolism.

A major component of the viral particle is the core protein, which interacts with the genomic RNA to form the nucleocapsid. Following synthesis on ER membranes and cleavage by the signal peptide peptidase, the core protein homodimerizes [122] and is then transferred to cytosolic LDs [123,124] (Fig. 3). HCV core protein interaction with LDs is believed to be essential for the recruitments of other viral components involved in virion assembly [116]. The core protein has a C-terminal domain containing two amphipathic helices that interact with LDs [125], and core protein accumulation around LDs progressively leads to a profound change in LDs intracellular distribution [126]. Indeed, LDs are normally distributed throughout the cytoplasm in uninfected cells, whereas they accumulate in the perinuclear region upon HCV infection. Importantly, mutations that prevent core interaction with LDs strongly inhibit virus assembly [116,127,128]. Interestingly, live imaging analyses indicate that core is rapidly trafficked to LDs and slowly recruited into motile puncta that traffic on microtubules and likely represent virus particles within the secretory pathway [129]. Finally, it should be noted that the magnitude of core-LD accumulation inversely correlates with the efficiency of production of infectious viral particles, which could reflect the transient localization of core protein on LDs before it is transferred to ER-derived assembly sites [128,130].

The core-LD association can also be influenced by cellular proteins. Indeed, diacylglycerol acyltransferase-1 (DGAT1), an enzyme involved in LD morphogenesis has been demonstrated to interact with the core protein and to enable core-LD association and virus production [131]. Trafficking of core protein to LDs also requires the MAPK-regulated cytosolic phospholipase A2 (PLA2G4) and its product arachidonic acid [132]. Surprisingly, IκB kinase-α (IKK-α) has also been shown to be a crucial host factor for HCV assembly [133]. Indeed, it has been reported that HCV RNA activates IKK-α, which translocates into the nucleus and induces a CBP/p300-mediated transcriptional program involving the sterol regulatory element-binding proteins (SREBPs). This innate pathway induces lipogenic genes and enhances core-associated LD formation to facilitate viral assembly. The core-LD interaction is dynamic, and the core protein must be retrieved from the surface of LDs to move to the site of virus budding. The μ subunit of the clathrin adaptor protein complex 2 (AP2M1) has been reported to interact with a conserved YXXØ motif present in the HCV core protein and essential for retrieval of core from LDs, leading to virus assembly [134]. This observation is however difficult to reconcile with the low accessibility of the YXXØ motif present in domain II of the core protein [125]. Besides cellular factors, non-structural proteins p7 and NS2 also regulate in a coordinated fashion core trafficking to the site of virus assembly [130].

Another major component of the viral particle is the envelope glycoprotein complex. HCV glycoproteins E1 and E2 form a non-covalent heterodimer, which is retained in the ER [135]. However, this glycoprotein complex needs to migrate in close proximity of LDs where assembly takes place [116]. It has been shown that NS2 interacts with E1, E2, and p7 and these interactions are essential for the migration of E1E2 heterodimer at the virion assembly site [136–139]. Moreover, it has recently been reported that the cellular factor signal peptidase complex subunit 1 (SPCS1) is involved in HCV assembly by helping the formation of membrane associated NS2-E2 complex [140]. It has been proposed that E1E2 heterodimer, NS2 and p7 form a functional unit that migrates close to the LDs [138]. The presence of disulfide bridges between HCV envelope glycoproteins at the surface of HCV particle suggests that lateral protein-protein interactions, assisted by disulfide-bond formation, might play an active role in the budding process of the HCV particle [37]. Besides their role in helping in the transport of HCV envelope glycoproteins to the assembly site, p7 and NS2 might also play additional functions during the assembly process. P7 is indeed also necessary for the final steps of capsid assembly as well as for capsid envelopment [141].

Besides p7 and NS2, the other non-structural proteins are also involved in the assembly process. Among these proteins, NS5A emerges as a central player in the transition between replication and assembly [35]. Indeed, although the C-terminal domain of this protein is dispensable for replication, it plays a major role in HCV assembly. This domain is essential for NS5A interaction with the LD-bound core protein, a key step in HCV assembly [116,142–144]. Specifically, phosphorylation of a specific serine residue within this region by casein kinase II is essential for regulation of virus assembly [144]. Furthermore, genetic and biochemical data indicate that the C-terminal domain of NS5A is also involved in the transient and weak association with the p7-NS2 complex [136–138,145]. Host factors have also been reported to affect the recruitment of NS5A around LDs. These cellular factors include DGAT1 and Rab18 [118,146]. It has to be pointed out that other host factors, not reported here, could also affect HCV assembly and/or release [35].

Within the replicase, the NS3/4A enzyme complex is also involved in HCV assembly. Indeed, mutations in the helicase domain of NS3 or in the C-terminus of NS4A cause defects in virus

assembly [147,148]. Furthermore, genetic and biochemical evidence indicates that interactions between the NS3 helicase domain and the core protein are essential for virus assembly [149,150]. Interestingly, the cellular factor Y-box-binding protein 1 (YB-1) has been shown to be a partner of NS3/4A that modulates the equilibrium between HCV RNA replication and the production of infectious particles [151]. In addition to NS3/4A and NS5A, the two remaining proteins of the replicase, NS4B and NS5B, have also been implicated in virus assembly [152,153]. However, it remains to be determined whether these viral proteins play a direct or an indirect role in HCV morphogenesis.

HCV virion biogenesis is closely related to the VLDL assembly pathway (Fig. 3). Indeed, inhibitors of the microsomal triglyceride transfer protein (MTP), a protein involved in VLDL biogenesis, block the production of viral particles [154–156]. In addition, long chain acyl-CoA synthetase 3 (ACSL3), another enzyme involved in VLDL assembly and hepatocyte nuclear factor 4α (HNF4α), a transcription factor that regulates the VLDL secretory pathway also regulate the production of infectious HCV particles [157,158]. Furthermore, apolipoproteins such as apoE, apoB, apoA1, apoC1, apoC2, and apoC3 can also be found in association with HCV particles [28,34], and the lipid composition of viral particles resembles the one of VLDL and LDL with cholesteryl esters accounting for almost half of the total HCV lipids [33]. Among HCV-associated apolipoproteins, there is a consensus about the involvement of apoE in HCV morphogenesis [159–161]. Importantly, reconstitution of HCV assembly in a non-hepatocytic cell line indicates that apoE is the minimum apolipoprotein required to produce infectious HCV particles [159,160]. Although initially reported as playing a role in HCV assembly and as being associated with the HCV virion, apoB might not be essential for HCV particle biogenesis [159–162].

The endosomal-sorting complex required for transport (ESCRT) pathway has also been proposed to play a role in HCV budding [163–165]. The ESCRT pathway is a cellular machinery involved in budding and fission of vesicles away from the cytoplasm and implicated in the formation of multivesicular bodies. This pathway is also exploited by many enveloped viruses for their budding and release from infected cells [166]. It is however not clear how HCV, which is supposed to bud into the lumen of the ER would exploit the ESCRT pathway [162]. Interestingly, this pathway is clearly involved in the biogenesis of exosomes that are released from infected cells and that contain HCV RNAs [167].

After assembly and budding in the ER, HCV particles are released from cells by transit through the secretory pathway [162]. During this process, HCV virions acquire their characteristic low buoyant density [154,168]. Furthermore, glycans associated with the viral envelope glycoproteins are also modified [37]. Finally, during egress, it has also been suggested that HCV particles depend on p7 to neutralize acidic compartments within the secretory pathway [169].

Concluding remarks

The characterization of HCV particles and replication during the last decade has witnessed highly original and specific features of this important human pathogen. Of particular interest are future studies of this virus, addressing molecular and cellular details of the host-HCV interactions. Noteworthy, a most remarkable feature of HCV lies in the formation of hybrid infectious particles that combine, during assembly and egress, viral and lipoprotein components and which consistently allow HCV to enter cells by using lipoprotein and cholesterol transfer receptors and to induce virus escape from neutralizing antibodies. Much remains to be understood about the structure and the biochemical composition of infectious HCV particles and about the steps of the virus life cycle, allowing entry, replication and production of infectious virus. Importantly, HCV propagation depends on and also shapes several aspects of lipid metabolism such as cholesterol transfer through different lipoprotein receptors during its entry into cells, lipid structures modulating HCV genome replication, cytosolic and luminal lipid droplets building viral particles. Unravelling these interconnections is key to define the infectivity parameters of HCV *in vivo* and its physio-pathological processes, but also to elaborate novel therapeutics.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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