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Ultrastructural and biochemical basis for hepatitis C virus morphogenesis

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Abstract Chronic infection with HCV is a leading cause of cirrhosis, hepatocellular carcinoma and liver failure. One of the least understood steps in the HCV life cycle is the morphogenesis of new viral particles. HCV infection alters the lipid metabolism and generates a variety of microenvironments in the cell cytoplasm that protect viral proteins and RNA promoting viral replication and assembly. Lipid droplets (LDs) have been proposed to link viral RNA synthesis and virion assembly by physically associating these viral processes. HCV assembly, envelopment, and maturation have been shown to take place at specialized detergent-resistant membranes in the ER, rich in cholesterol and sphingolipids, supporting the synthesis of luminal LDs-containing ApoE. HCV assembly involves a regulated allocation of viral and host factors to viral assembly sites. Then, virus budding takes place through encapsidation of the HCV genome and viral envelopment in the ER. Interaction of ApoE with envelope proteins supports the viral particle acquisition of lipids and maturation. HCV secretion has been suggested to entail the ion channel activity of viral p7, several components of the classical trafficking and autophagy pathways, ESCRT, and exosome-mediated export of viral RNA. Here, we review the most recent advances in virus morphogenesis and the interplay between viral and host factors required for the formation of HCV virions.

Keywords Hepatitis C virus · Viral life cycle · Virus replication · Virus morphogenesis · Virus–host interactions

Abbreviations

aa	Amino acid
ABHD5	α/β-hydrolase domain-containing protein 5
ANXA3	Annexin A3
Apo	Apolipoprotein
AP1	Clathrin adaptor protein complex 1
AP2	Clathrin adaptor protein complex 2
AP2M1	AP2 medium subunit
Ch	Cholesterol
CIDEB	Cell death-inducing DFFA-like effector B
CM	Convoluted ER membranes
Core protein	HCV core protein
DDX3X	DEAD Box Helicase 3, X-Linked
DGAT1	Diacylglycerol acyltransferase-1
DMVs	Double-membrane vesicles (Main constituents of the MW)
EM	Electron microscopy
ER	Endoplasmic reticulum
ESCRT	Endosomal-sorting complex required for transport
E1	HCV envelope protein 1
E2	HCV envelope protein 2
FAPP2	Four-phosphate adaptor protein 2

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HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCVcc	Cell culture produced HCV
HCVtcp	Trans-complemented HCV particles
HNRNPK	Heterogeneous nuclear ribonucleoprotein K
hVAP	Human VAMP-associated proteins
IKKα	IKappaB Kinase Alpha
IRES	Internal ribosome entry site
LDL	Low-density lipoproteins
LDs	Lipid droplets
cLDs	Cytoplasmic LDs
luLDs	Luminal LDs
LVPs	Lipo-viro-particles
JFH-1	Genotype 2a strain of HCV obtained from a Japanese patient with fulminant hepatitis
MAMs	Mitochondria-associated ER membranes
MTTP	Microsomal triglyceride transfer protein
MVBs	Multivesicular bodies
MW	Membranous web (Specific membrane alteration consisting of locally confined membranous vesicles that serves as a scaffold for the HCV replication complex)
NS	Non-structural
NS2	HCV non-structural protein 2
NS3	HCV non-structural protein 3
NS4A	HCV non-structural protein 4A
NS4B	HCV non-structural protein 4B
NS5A	HCV non-structural protein 5A
NS5B	HCV non-structural protein 5B
NPC	Nuclear pore complex proteins
NTFs	Nuclear transport factors
NTRs	Non-translated regions
Nup	Nucleoporin
ORF	Open reading frame
OSBP	Oxysterol-binding protein
PI4KIIIα	Phosphatidylinositol 4-kinase IIIα
PI4P	Phosphatidylinositol 4-phosphate
PLA1A	Phosphatidylserine-specific phospholipase A1
P7	HCV p7 protein
Rab	Ras-related in brain
SAR1A	Secretion associated Ras related GTPase 1A
SGs	Stress granules
SMYD3	Lysine methyltransferase SET and MYND domain-containing protein 3
SP	Cellular signal peptidase
SPP	Signal peptide peptidase
SREBPs	Sterol regulatory element-binding proteins
TGN	Trans-golgi network
TIP47	Tail-Interacting Protein 47
VAMP	Vesicle-associated membrane protein
VLDL	Very low-density lipoproteins

Introduction

Hepatitis C virus (HCV) infection is a major health problem (130–170 million people are estimated to be chronically infected) (Reviewed in [1]). Most of the infected persons develop chronic disease (70–80%) that can progress to cirrhosis, hepatocellular carcinoma (HCC), and liver failure [1]. In addition, chronic infection with HCV is the leading cause of end-stage liver disease, HCC, and liver-related death in the Western world (Reviewed in [2, 3]). Despite advances in the treatment of HCV with the recent introduction of new HCV-specific directly acting antiviral agents, development of HCV vaccines is an area of unmet clinical need (Reviewed in [4]). Screening of cDNA expression libraries, made from chimpanzees infected with serum from a patient with post-transfusion non-A, non-B hepatitis, allowed the first isolation and cloning of HCV genomes [5]. HCV is an enveloped RNA virus classified as the type member of the genus *Hepacivirus* in the family *Flaviviridae* (Reviewed in [6]). The HCV genome is a 9.6 kb single-stranded positive-sense RNA that contains a single open reading frame (ORF) encoding a polyprotein of 3010–3030 amino acid (aa) residues (Fig. 1) (Reviewed in [7]). The ORF is flanked by 5′- and 3′-terminal non-translated regions (NTRs) that are important for viral RNA translation and replication. The 5′-NTR contains an internal ribosome entry site allowing translation of the RNA genome in the absence of a cap structure (Reviewed in [8]). Upon its synthesis in the endoplasmic reticulum (ER), the HCV polyprotein is co- and post-translationally processed by cellular and viral proteases into 10 mature cleavage products, including structural (core, E1, and E2) and non-structural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Fig. 1). HCV isolates show substantial genetic diversity and have been grouped into seven genotypes and several subtypes [6].

A major advance in HCV research occurred in 2005 with the discovery of the cell culture HCV (HCVcc) system based on the HCV genome derived from the JFH-1 clinical isolate (genotype 2a) produced in hepatoma cell lines [9–11]. In addition, fully infectious clones of HCV have been generated from other HCV strains and genotypes (Reviewed in [12]). Nevertheless, to study different genotypes and strains most studies have used chimeric genomes (including intra- and inter-genotypic chimeras) incorporating the HCV assembly module (Core to NS2) from the heterologous strain in the JFH-1 replication module (NS3 to NS5B) [12]. Particularly, constructs such as FL-J6/JFH-1 or Jc1 (using the replication module of JFH-1 (NS3-NS5B) and the core-NS2 region from genotype 2a isolate, J6) have been widely used because they reach high viral titers [9, 13]. Improvement of HCVcc systems is of great

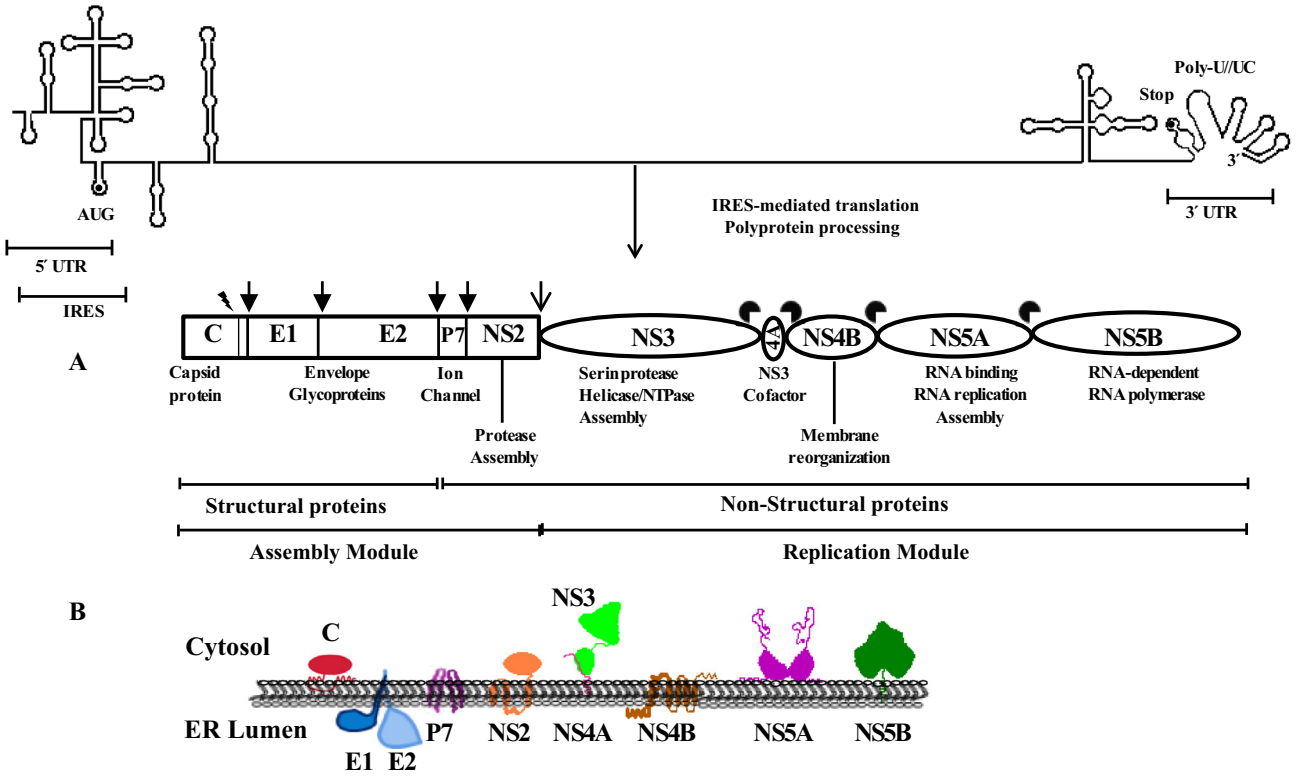


Fig. 1 Hepatitis C virus (HCV) genome organization and functions of viral proteins. **A** HCV RNA genome contains a single open reading frame flanked by 5′and 3′ non-translated regions (UTRs). Predicted secondary structures of UTRs, core and NS5B RNA regions are shown. The 5′ UTR contains an internal ribosome entry site (IRES) and the 3′ UTR contains a poly-U/UC region. Start and stop codons of the ORF are indicated. Processing by viral (NS2/NS3 (↑) and NS3/4A ( )) and host encoded proteases (cellular signal peptidases (SP:) and cellular signal peptide peptidase (SPP:)) releases mature viral proteins from the polyprotein precursor. Core (C), E1, E2, p7, and NS2 are primarily involved in HCV assembly (assembly module) while NS3, NS4A (4A), NS4B, NS5A, and NS5B are primarily involved in the viral RNA replication (replication module). Some of the functions of the individual proteins are indicated at the bottom of the figure. **B** Endoplasmic reticulum (ER) membrane association of HCV proteins

importance for vaccine development and characterization of viral particles, including morphological studies regarding epitope exposure and conformation (Reviewed in [14].

It has been shown that the HCV assembly module can be supplied in trans to HCV subgenomic replicons (assembly deficient but replication-competent HCV constructs containing the NS3–NS5B region) to produce trans-complemented HCV particles (HCVtcp) [15–17]. These HCVtcp are infectious but support only single-round infection and are unable to spread. Single-round infectious viral particles, generated by transpackaging systems, are valuable for studying entry into target cells, assembly, and release of infectious particles. On the other hand, budding of the HCV capsid can be viewed by electron microscopy (EM) in heterologous expression systems that overexpress either the core protein or HCV structural proteins [18–20]. Regardless of the abortive nature of HCV-like particles budding, this model has been used for ultrastructural analysis of early nucleocapsid assembly events [19, 20]. Detection and localization of HCV proteins and RNA in the liver of HCV-infected patients have also been shown to be helpful to study both HCV and host–viral interactions at the cellular level [21–26]. One of the least understood steps in the HCV life cycle is the morphogenesis of new viral particles. This review describes the phases of HCV life cycle that are involved in virus morphogenesis and the interplay between viral and host factors required for the formation of HCV virions.

HCV virions

Particles derived from either sera or hepatocytes of HCV-infected patients are spherical and heterogeneous (diameters between 30 and 80 nm and densities from 1.03 to 1.20 g/cm^3) [21–24, 27–31]. In addition, low-density viruses are more infectious than high-density particles and associate with different lipoprotein components that are involved in viral attachment and entry (such as apolipoprotein (Apo) AI, ApoB, ApoC1, and ApoE) [21, 30]. Such HCV particles, called LVPs for lipo-viro-

particles, resemble very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) (Fig. 5) (Reviewed in [32–34]). Detection of ApoB-48 (an isoform of ApoB exclusively generated from the small intestine) in LVPs and the fact that the buoyant density of HCV particles in serum rapidly shifts in relation to dietary triglycerides suggest that interaction of HCV particles with serum lipoproteins is transient and exchangeable [27, 35].

HCVcc particles are spherical, with spike-like projections, and heterogeneous in size ranging from 40 to 100 nm in diameter [10, 36–39]. Extracellular HCVcc particles are distributed over a range of densities from 1.03 to 1.16 g/cm^3, and particles with intermediate densities (near 1.10 g/cm^3) have the highest specific infectivity [9, 37–39]. Besides, a similar composition of lipids and cholesterol has been observed in highly purified HCVcc virions and in LDL-VLDL particles [39]. Apolipoproteins such as ApoE, ApoB, ApoCI, and ApoA1 have also been reported to be associated with HCVcc [36, 39–44]. Interestingly, NS3 and several host factors (including nucleoporin (Nup) 98 kDa (Nup98)) have been identified in highly purified extracellular HCV virions [42]. Density of HCVcc particles may vary based on the cell type producing the virus [45–47]. Indeed, LVPs produced in human liver engrafted mice [45, 46] and in primary human hepatocytes [47] more closely resemble particles purified from patients infected with HCV with the lowest-density virions showing the highest specific infectivity.

Ultrastructure and biochemical basis for HCV replication

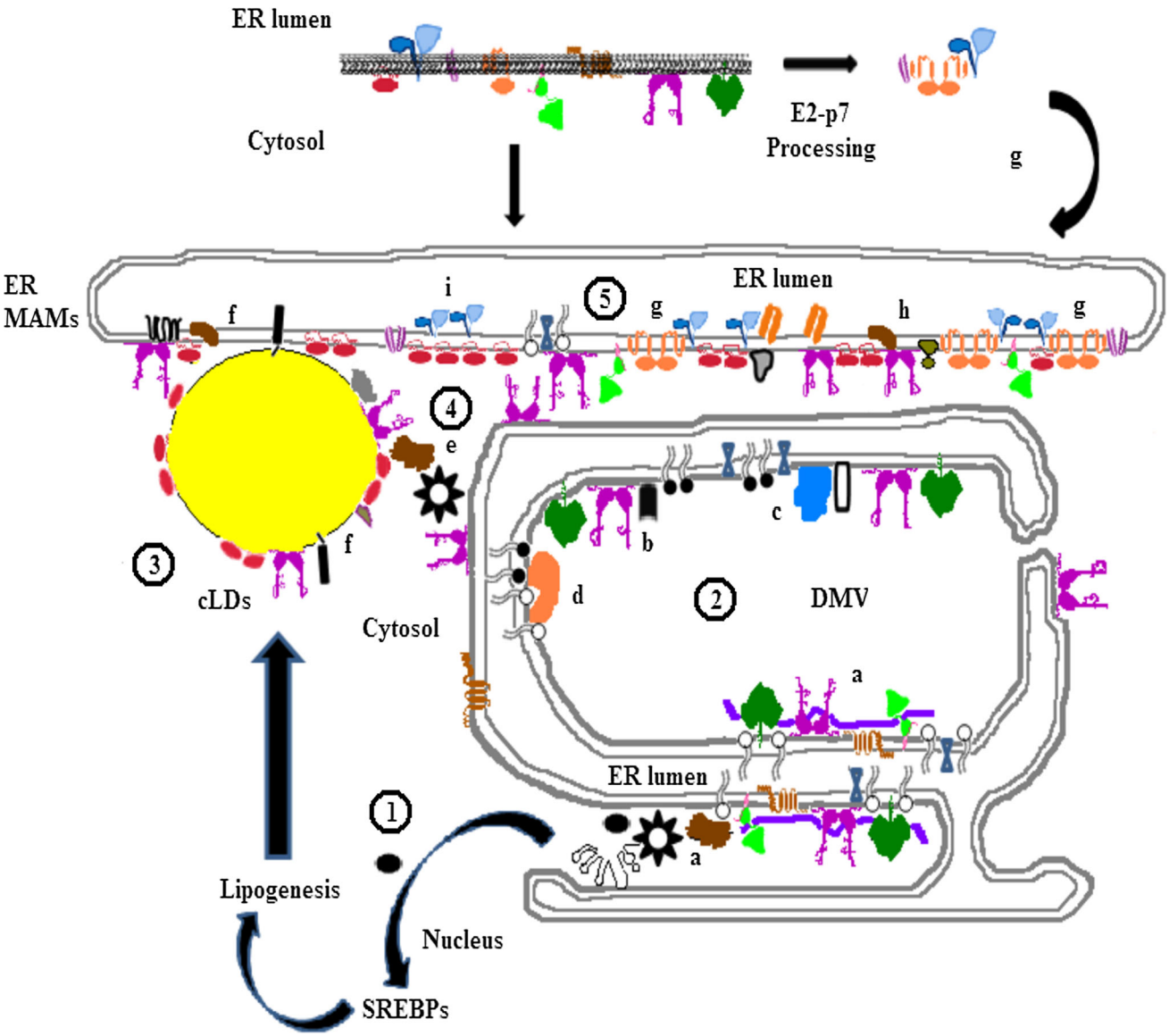
During viral replication, HCV induces massive remodeling of primarily ER-derived membranes to create a cytoplasmic microenvironment called the membranous web (MW) [48]. The MW has been proposed to constitute a virally encoded organelle protecting viral proteins and RNA and synchronizing virus replication and assembly [49, 50]. Similarly, alteration of ER-derived membranes (showing ER dilatation and abundant membrane vesicles) is an ultrastructural hallmark of hepatocytes from HCV-infected patients [22, 23, 51]. The MW contains large replication complexes showing very limited movement with a static internal architecture [52]. Interestingly, small replication complexes emerging early during HCV replication and showing high motility have been suggested to allow the spread of replication sites over the cytoplasm [52]. The main constituents of the MW are double-membrane vesicles (DMVs) that enclose functional RNA replicase complexes (Fig. 2) [53–55]. DMVs originate from the ER and require the coordinated interaction of the viral replication module with host proteins [48, 53, 55, 56]. NS5A is a key viral protein involved in DMVs formation [55]. NS5A is a monotopic phosphoprotein that is targeted to the ER by a unique N-terminal amphipathic α-helix sequence [57]. In addition, three domains have been defined in NS5A [58]. Domains 1 and 2 interact with RNA and lipid droplets (LDs), and have been implicated in RNA replication [56, 58]. On the other hand, domain 3 interacts with several host proteins and the core protein, and is involved in HCV assembly [59–61]. Major contributors of DMVs formation have recently been shown to include domain 1 and the N-terminal amphipathic α-helix of NS5A as well as the helicase domain of NS3 [56].

To generate the membrane proliferations and LDs necessary for the viral life cycle, HCV alters the expression of genes involved in cellular lipid metabolism. This leads to the accumulation of intracellular lipids, recruitment of cholesterol (Ch), and specific subsets of phospholipids and sphingolipids (SL) to HCV replication and assembly complexes (Explained in Fig. 2). LDs are cellular storage organelles for triacylglycerides and cholesteryl esters surrounded by a phospholipid monolayer that is derived from the ER. LDs can be produced from droplet budding toward the cytosolic (cLDs) or the luminal (luLDs) side of ER membranes. Interestingly, the primary trigger of LDs biogenesis during HCVcc infection has been suggested to be the highly structured HCV 3′UTR [62]. Upon HCVcc infection, DEAD Box Helicase 3, X-Linked (DDX3X) interacts with the HCV 3′UTR and IKappaB Kinase Alpha (IKKα) to induce stress granules (SGs) formation, IKKα-mediated cellular lipogenesis, and virus production [62, 63]. SGs and processing bodies are compositionally related ribonucleoprotein granules that cooperatively regulate translation and decay of mRNAs (Reviewed in [64]), Notably, they have been involved in various stages of the HCV life cycle [63, 65–67]. A number of additional host factors have been reported to contribute to viral replication and DMVs formation, including the autophagy pathway, nuclear pore complex (NPC) proteins, and nuclear transport factors (NTFs) [53, 54, 68, 69]. It is interesting to note that several NPC proteins (including Nup98) and NTFs have been associated with HCV replication and assembly [42, 69, 70]. Moreover, NPC-like structures have been suggested to constitute a selective permeability barrier in the MW between viral production centers and the surrounding cytosol [69, 70].

Ultrastructural and biochemical basis for HCV assembly

Early stage of HCV assembly

It is thought that HCV RNA replication and virion assembly occur in distinct membranous compartments (Fig. 2). Hence, subcellular fractionation studies have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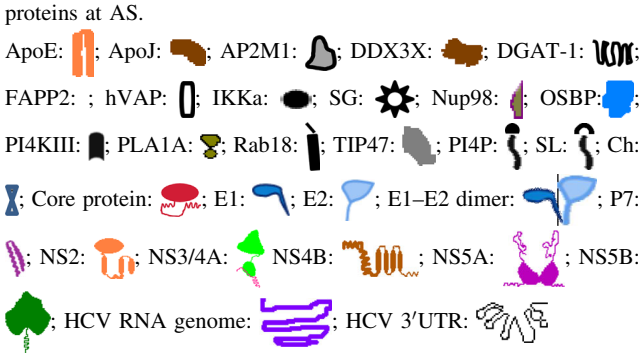
◀**Fig. 2** Hypothetical model of hepatitis C virus (HCV) replication and early assembly events. HCV replication takes place in double-membrane vesicles (DMVs) while viral assembly sites (AS) have been suggested to be specialized detergent-resistant membranes (DRMs) in the ER or in mitochondria-associated ER membranes (MAMs), rich in sphingolipids (SL) and cholesterol (Ch) [50, 74] (See *2* and *5*). Some of the host factors involved in these processes are shown in this model. *1* DDX3X interacts with both HCV 3′UTR and IKKα to induce stress granules (SGs) formation. Activated IKKα translocates to the nucleus to induce cellular lipogenesis through SREBPs and to enhance core protein-associated cLDs formation [62]. *2 a* Following translation of the RNA genome, non-structural proteins (NS3, NS4A, NS4B, NS5A, and NS5B) form the replication complex in association with cellular factors leading to the formation of DMVs that enclose functional viral RNA replicases. DDX3X colocalizes with NS5A and NS3 on SGs near ER membranes early during HCV infection. *b* NS5A and NS5B interact with PI4KIIIα increasing PI4P levels in DMVs and HCV replication [130, 131]. *c* OSBP associates with hVAP-A/B and NS5A [132]. *c*, *d* OSBP and FAPP2 are recruited to PI4P-containing membrane microdomains to mediate Ch and SL transport for viral replication [132, 133]. *a* SL activates NS5B through the enhancement of its binding to the viral template RNA [134]. *3* cLDs coordinate viral RNA synthesis and virion assembly by physically associating replication complexes and AS. *4 e* DDX3X, SGs, and NS proteins redistribute from ER to cLDs after core protein accumulation on cLDs. *f* NS5A, Rab18, TIP47, and Nup98 are involved in the recruitment of the replication complex around cLDs. NS5A interacts with core protein, DGAT1 supports their localization to cLDs, and ApoJ stabilizes the core–NS5A complex (*h*). *5* HCV assembly requires ongoing accumulation of viral proteins, HCV RNA, and host factors that are gathered together at the AS. *g* Core protein must be either retrieved from the surface of cLDs or directly migrate after its synthesis to the AS. p7, NS2, AP2M1, and NS2–NS3 interactions are necessary to induce the localization of core protein to AS. p7 also regulates NS2–E1–E2 localization to AS/DRMs [74]. *h* Interaction of NS5A with core protein and NS2 is important for the recruitment of E2 to AS. PLA1A enhances the formation of NS2–E2 and NS2–NS5A complexes [116]. *i* Accumulation of HCV structural proteins at AS.



identified key components of viral replication and assembly in two different cytoplasmic membrane fractions [50]. While NS5B, viral RNA, and phosphatidylinositol 4-kinase IIIα (PI4KIIIα) were mainly found in ER-derived microsomal membranes, assembly center components (such as ApoE, core protein, and infectious HCV) were enriched in cytoplasmic and mitochondria-associated ER membranes (MAMs). Moreover, HCV replication has been shown to promote the transfer of NS proteins and host factors implicated in HCV assembly (such as diacylglycerol acyltransferase-1 (DGAT1)) to MAMs [71, 72]. MAMs are specialized regions of the ER (enriched in lipid synthetic proteins and internal detergent-resistant membranes (DRMs)) that interact with mitochondria and are involved in various metabolic processes such as cell signaling, synthesis of phospholipids, and cholesterol trafficking (Reviewed in [73]). It is interesting to note that DMRs have been directly implicated in HCV assembly [74]. Furthermore, several host factors involved in lipid metabolism, HCV replication, and morphogenesis (such as microsomal triglyceride transfer protein (MTTP), ApoE, ApoB, ApoC, and non-opioid sigma-1 receptor) localize to MAMs [75, 76]. These experimental evidences suggest that MAMs and DRMs are involved in HCV morphogenesis.

cLDs, on the other hand, have been proposed to play a central role in the coordination of viral RNA synthesis and virion assembly in the HCVcc model, by physically associating replication and assembly sites [77, 78]. As mentioned above, HCV 3́UTR-mediated IKKa activation induces cellular lipogenesis and enhances core protein-associated cLDs formation to facilitate viral assembly [62, 63]. Maturation of HCV core on ER membranes by the intramembrane protease signal peptide peptidase is required for core protein homodimerization, localization to cLDs and virus production [79]. Accumulation of core protein on cLDs takes place early during HCV infection (>50% of the core protein colocalized with cLDs at about 12 h post-infection) [80]. At later times of HCV infection, however, association of core protein with cLDs is considerably reduced (<10% of the core protein colocalized with cLDs) except in cells infected with the wild-type HCVcc/JFH-1 (40% of the core protein colocalized with cLDs) [80, 81]. Interestingly, HCV 3′UTR-DDX3X-SGs and HCV NS proteins redistribute from ER to cLDs after core protein accumulation on cLDs [63, 65, 67]. NS5A has been proposed to regulate the transition between replication and assembly. Thus, recruitment of NS5A to either cLDs or assembly sites in the ER (through interaction with core protein) has been shown to be required for virus particle assembly [19, 60, 61, 78]. Localization of HCV core and NS5A to cLDs and virus production is supported by DGAT1, an enzyme involved in triglyceride synthesis and luLDs maturation, and the mitogen-activated protein kinase-regulated protein cytosolic phospholipase A2 and its product arachidonic acid [82–84]. In addition, NS5A supports both recruitment of the HCV replication complex to cLDs and delivering of the HCV genome to core protein [61, 85]. Phosphorylation of NS5A domain 3 by casein kinase IIα has also been described to be critical for assembly of infectious virus [86]. Interestingly, interaction of NS5A domain 3 with the lysine methyltransferase SET and MYND domain-containing protein 3 (SMYD3) has been proposed to counteract the inhibitory effect of

SMYD3 on HCV assembly [59]. Moreover, association of LDs with viral replication and assembly sites is promoted by the interaction of core–NS5A with host factors including Nup98 and proteins associated with LDs and VLDLs (such as ras-related in brain (Rab) 18, tail-interacting protein 47 (TIP47), and ApoJ) [42, 82, 87–89].

Late stage of HCV assembly

Delivery of HCV RNA for encapsidation and envelopment

Late stages of HCV assembly entail a regulated allocation of viral and host factors to viral assembly sites. Available evidences suggest that virus budding takes place through encapsidation of the HCV genome and viral envelopment in the ER. Early viral RNA encapsidation has been proposed to begin either on cLDs or in assembly sites at ER membranes near cLDs (Reviewed in [33, 34]). HCV core associates with viral RNA to form the nucleocapsid. However, delivery of the HCV genome for encapsidation and virus envelopment are tightly regulated (Fig. 3). It has to be pointed out that NS5A is necessary for these processes [61]. Consequently, HCVcc mutations in two regions of NS5A domain 3 (affecting either NS5A–core protein or NS5A–HCV RNA interactions) produce an early accumulation of slow sedimenting core protein that partially associates with viral RNA [61]. In contrast, this early peak of slow sedimenting core protein disappears at later times of wild-type HCVcc infection when the core protein assembles into fast sedimenting complexes representing enveloped viruses [61]. Further characterization of cells transfected with these NS5A mutant viruses demonstrated that impairment in HCV envelopment was associated with a strong reduction in HCV RNA–core protein interaction [61]. These results indicated that association of NS5A domain 3 with both core protein and HCV genome regulated the assembly process at two distinct steps. First, through recruitment of replication complexes to assembly sites and then, throughout HCV RNA delivery to core protein, thus promoting viral genome encapsidation and particle envelopment. NS3 has also been suggested to interact with core protein and to be involved in packaging viral RNA [34, 90]. Notably, NS3 and Nup98 have been found in MAMs fractions that are enriched in core protein, ApoE, and infectious HCV as well as in purified HCVcc virions [42, 50, 69]. Interestingly, the NS3–NS4A protease that is localized at MAMs, cleaves and inactivates a key innate immune signaling protein [71].

Remarkably, early accumulation of the slow sedimenting core protein and reduced HCV RNA–core protein interaction have also been observed in cells transfected with HCVcc mutants lacking either p7 or the E1–E2 region (HCVccΔE1E2) [61, 91]. Moreover, inter-genotypic chimeras (carrying E1–E2 genes of HCV genotype 1a in the genotype 2a genome) have been shown to modulate virus production and to induce the accumulation of slow sedimenting non-enveloped core protein structures [92]. Taken together these studies implicate p7 and E1–E2 in the association of HCV RNA with core protein, subsequent nucleocapsid assembly, and viral envelopment. E1 and E2 reside on the virion surface and are type I trans-membrane proteins with an N-terminal ectodomain and a C-terminal trans-membrane domain (Reviewed in [93, 94]). During their synthesis, N-terminal ectodomains are targeted to the ER lumen and then, E1 and E2 assemble as non-covalent heterodimer complexes that are mostly retained in the ER [95]. A possible involvement of E1–E2 in capsid assembly and envelopment may be related to their folding and with core protein–E1–E2 interactions (Described in Fig. 3).

Allocation of viral and host factors to viral assembly sites

Although the exact mechanism linking nucleocapsid assembly with envelop acquisition is unknown, p7 and NS2 have been proposed to play a critical role in the migration of core protein and E1–E2 heterodimers to the virion assembly site (Fig. 2) [74, 96–101]. It has been suggested that core protein must be retrieved from the surface of cLDs to move to the ER site of virus assembly and budding [78, 91, 102, 103]. Thus, increased interaction of HCV core with cLDs has been associated with reduced assembly of infectious particles [80, 104]. Notably, compatibilities between p7 and the first NS2 trans-membrane domain have been shown to be required for core protein localization in the ER [96]. In addition, the interaction between NS2 and NS3 was described to be important for core protein recruitment to motile puncta that traffic on microtubules during HCV morphogenesis [102, 105]. Although the authors suggested that core protein traffics from cLDs to ER, they could not observe a direct transfer of HCV core from cLDs to either ER membranes or motile puncta during live imaging studies [102]. Interestingly, the μ subunit of clathrin adaptor protein complex 2 (AP2M1) promotes virus assembly through interaction with a specific YXXø motif in the core protein and subsequent AP2M1-mediated HCV core intracellular trafficking [103]. Consequently, disruption of core-AP2M1 binding was associated with increased accumulation of core protein on cLDs, reduced colocalization of core-E2, and reduced core protein localization to the trans-Golgi network (TGN) [103]. Additional experimental evidences support the view that early steps of viral RNA encapsidation occur in the ER and that core protein can directly migrate to assembly sites immediately after its synthesis [19, 96, 106]. Accordingly, subcellular fractionation studies have shown that loss of core protein in LDs-containing fractions and enrichment of HCV core in subcellular ER–E2-containing fractions are associated with

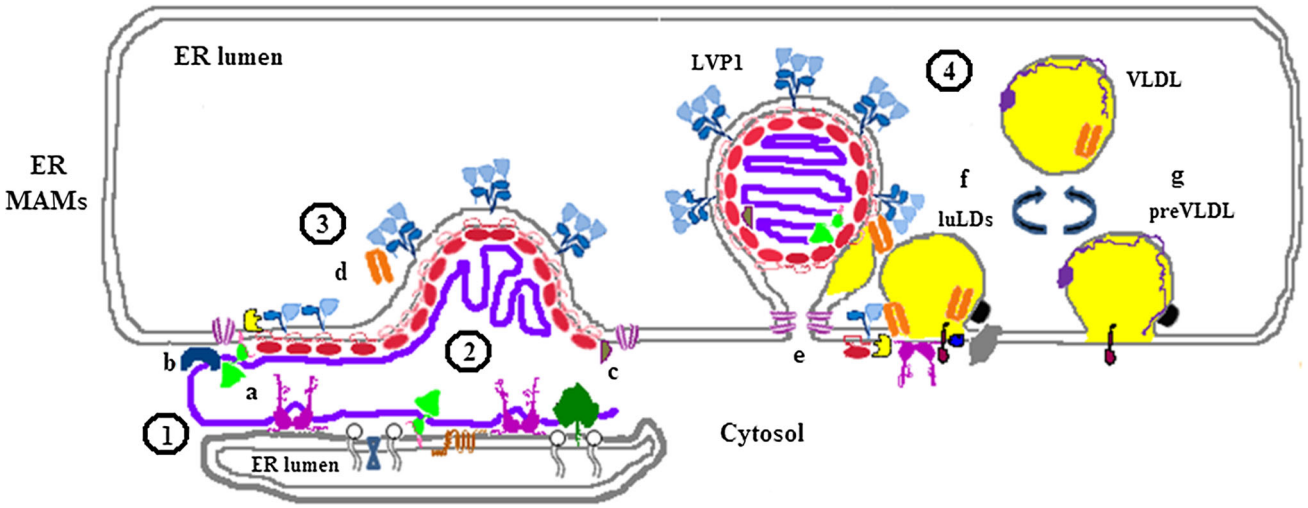


Fig. 3 Hypothetical model of late hepatitis C virus (HCV) assembly events and early maturation in the ER. This hypothetical model proposes that viral genome encapsidation occurs after structural proteins have accumulated at viral assembly sites (AS) in the ER. It is also assumed that HCV RNA is transferred directly from replication complexes at the ER to the AS. Other putative models have proposed that encapsidation of the viral RNA starts on cLDs while virus buds into the ER and that the HCV genome can be delivered from NS5A on cLDs to core protein (Reviewed in [33, 135]). *1* Delivery of HCV RNA for encapsidation and virus envelopment are tightly regulated. NS5A and NS3/4A are involved in transferring newly replicated HCV genomes to the core protein (*a*). *b* HNRNPK interacts with HCV RNA, NS3, and core protein regulating the availability of the viral genome for incorporation into nucleocapsids [136]. *c* Nup98 promotes HCV assembly and has been found with NS3 in purified HCVcc virions. *2* HCV core associates with viral RNA to form the nucleocapsid [61]. *3* The envelope glycoprotein complex is a major component of the viral particle. *d* A possible involvement of E1–E2 in capsid assembly and envelopment may be related to core protein–E1–E2 interactions that can be mediated through ANXA3 [114, 137]. In addition, folding and glycosylation of E1 and E2, lateral interactions of E1–E2 ectodomains, and disulfide bond formation have been involved in viral assembly and budding [138–141]. Moreover, rearrangement of E1–E2 heterodimer complexes during HCV assembly leads to the formation of a trimeric form of the E1–E2 heterodimer [142]. *e* p7 has been suggested to play a role during the final steps of capsid envelopment by supporting membrane-to-membrane adhesion [143]. *4* HCV particle maturation in the ER. Please note that in this hypothetical model virus budding and/or early virus maturation is associated with luLDs development. Alternatively, HCV budding and early maturation have been proposed to be linked to VLDL precursors [40]. *f* ApoE is required for HCV morphogenesis. Interactions between ApoE and NS5A promote the assembly of infectious virus and CIDEB supports the association of ApoE with both NS5A and HCVcc particles [110, 144, 145]. Moreover, ANXA3 promotes the interaction of E2 with ApoE and core protein [114]. Association of ApoE with the envelope proteins is involved in HCV maturation and the viral particle acquisition of lipids to form a lipoviral particle (LVP1) [118]. Notably, ABHD5 promotes LDs consumption and HCV infectious virus production [112]. TIP47 has a high affinity to lipoproteins and interacts with HCV particles [128]. *g* ApoB is translocated into the ER and loaded with phospholipids and triglycerides by MTTP to produce a VLDL precursor (preVLDL) [111, 146]. preVLDL undergoes further lipidation steps acquiring exchangeable ApoE and ApoC to form VLDL particles. ABHD5 ; ANXA3: ; ApoE: ; ApoB: ; CIDEB: ; HNRNPK: ; MTTP: ; Nup98: ; TIP47: ; SL: ; Ch: ; Core protein: ; E1–E2: ; P7: ; NS2: ; NS3/4A: ; NS4B: ; NS5A: ; NS5B: ; HCV RNA genome: ; E1–E2 trimers:

increased viral production [96]. Besides, cells infected with HCVcc (enclosing a modified core protein with increased ability to multimerize and to self-assemble into viral particles) have shown a predominant NS5A–core protein colocalization at ER membranes but not at the surface of cLDs [19]. Moreover, the core protein from a recently developed infectious genotype 3a HCVcc does not localize to cLDs in infected cells [106].

Trans-membrane domains of NS2 are also critical for E2–NS2 interaction, colocalization with NS5A, and viral assembly [100]. It is worth to point out that processing of E2–p7 and p7 have been shown to regulate the localization of NS2–E1–E2 at DRMs [74]. These DRMs were enriched in NS3, core protein, and ApoE. Specially, efficient E2–p7 cleavage and infectious virus production require the N-terminal helical region of p7 [107]. Noteworthy, disruption of DMRs inhibited recruitment of NS2, E2, and core protein to assembly sites and virus assembly suggesting the involvement of DMRs in early HCV morphogenesis [74]. Interaction of NS5A with core protein and NS2 have also been implicated in the recruitment of E2 to viral assembly sites [61, 97]. Remarkably, NS2 interactions with other NS proteins have been described to mediate the redistribution of TGN membranes and components to LD-, ApoE-, and core protein-positive compartments in HCV-infected cells [108]. Interestingly, several TGN–endosomal

adaptors were involved in HCV assembly and secretion [108]. In addition, ApoJ has been shown to reallocate from the TGN to the vicinity of cLDs [87]. ApoJ was described to stabilize the core–NS5A complex and to support infectious virus production.

Ultrastructural evidences of HCV assembly

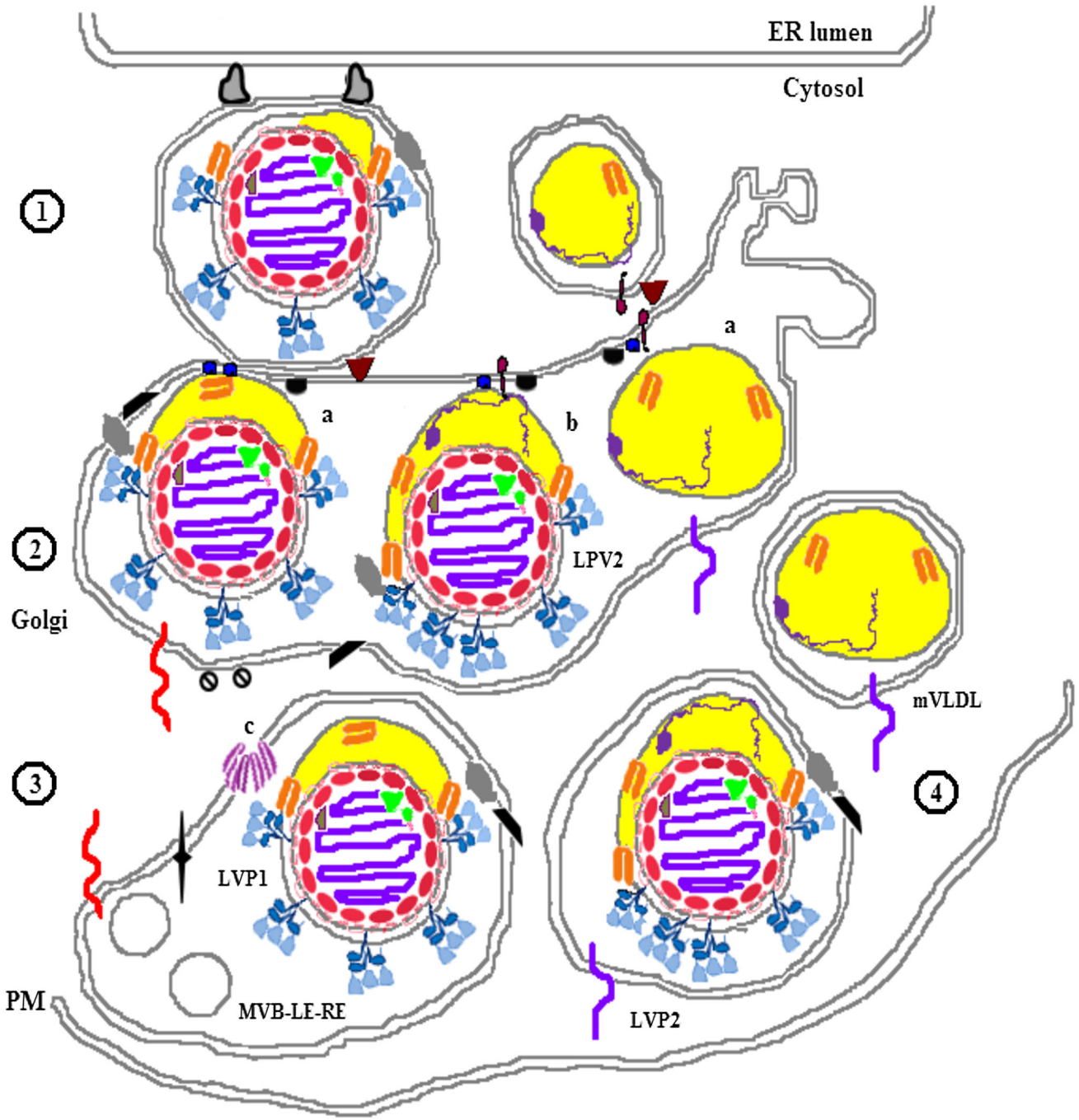
Ultrastructural features of HCV morphogenesis such as nucleocapsid assembly, viral budding, and virions have been difficult to detect in HCVcc-infected cells. Accordingly, it has been reported that only a subset of the assembled infectious precursors are rapidly secreted, whereas the rest are targeted for degradation suggesting that the structures and processes related to HCV morphogenesis are transient and rapid [41]. One study suggested that viral budding occurs at ER membranes directly apposed to lipid droplets in cells infected with HCVcc [78]. On the other hand, the capsid budding can be viewed by EM if the core protein is overproduced with heterologous expression systems [18, 20]. Therefore, various HCV core proteins have been described to induce the formation of convoluted ER membranes (CM) and tubular structures that are associated with multimerization of core protein, nucleocapsid assembly, and budding [18–20]. These studies demonstrated that while the core protein can localize on cLDs, HCV-like particles assemble at CM near cLDs [19, 20]. This is an interesting finding because localization of core protein in the ER has been described to require the NS proteins [96, 102]. Interestingly, replacement of 10 unusual amino acids of the JFH-1 core with the most conserved residues in HCVcc increased core protein localization in the ER and viral assembly in infected cells [19]. In addition, this modified core protein showed an augmented ability to multimerize and to self-assemble into HCV-like particles in heterologous expression systems inducing the formation of tubular structures and CM [19].

It is worth to mention that similar HCV-related CM and tubular structures have been observed in hepatocytes from HCV-infected humans and chimpanzees [22–24, 51]. These tubular structures have been immunolabelled with anti-HCV core and anti-envelope antibodies [22, 24]. Envelope proteins have also been detected in electron-dense ER membranes but not near cLDs in chimpanzee and human HCV-infected livers [22–24]. In addition, budding of HCV-like particles (immunolabelled with anti-core antibodies) has been shown in CM lacking cLDs and near the tubular structures in human HCV-infected livers [23, 24]. Moreover, HCV-like particles have been observed in intracellular vesicles of human hepatocytes [23, 109]. These experimental evidences support the findings in the HCVcc model suggesting that early HCV assembly takes place in the ER.

Maturation and release of viral particles

HCV morphogenesis is tightly linked to the VLDL assembly pathway (Depicted in Figs. 3 and 4). Several proteins involved in the VLDL pathway including MTTP, ApoB, ApoE, ApoJ, α/β hydrolase domain-containing protein 5 (ABHD5), and cell death-inducing DFFA-like effector B (CIDEB) have been shown to regulate the production of infectious HCV particles [40, 41, 87, 110–112]. However, the functional importance of ApoB for HCV assembly and infectivity is controversial, and several experimental evidences have indicated that ApoB is not essential for HCV particle biogenesis [105, 108]. On the other hand, ApoE has been illustrated to be vital in HCV morphogenesis [105, 113, 114]. Hence, microdomains in the ER that have been proposed to be involved in HCV assembly and maturation contain ApoE [39, 50, 74, 114–116]. Interestingly, it has been suggested that the formation of HCV nucleocapsids and their envelopment is not affected by ApoE depletion in HCV-infected cells [117, 118]. Instead, interaction of ApoE with E1–E2 has been shown to be required for a step after envelopment and prior to release (Described in Fig. 3) [113, 118]. In fact, nascent virus particles traffic through the secretory pathway in association with ApoE, but not with ApoB [105]. Importantly, reconstitution of HCV assembly in non-hepatocytic cell lines indicates that ApoE is the minimum apolipoprotein required to produce infectious HCVcc particles [113, 119]. Members of the ApoA and ApoC family can also complement HCV virus production in non-hepatocytic cell lines [117, 120]. In addition, domains of amphipathic alpha-helical repeats that are shared by these apolipoproteins have been involved in HCV assembly [117, 120]. These results point to an important role of exchangeable apolipoproteins (especially ApoE) in the maturation of HCV particles and their acquisition of lipids. They also suggest that production of infectious HCV particles may actually depend on ApoE-containing luLDs rather than on VLDL particle formation. In this way, HCV might assemble into virions that are secreted predominantly as LVPs lacking ApoB. Alternatively, ApoE-, ApoA-, and ApoC-associated HCV virions could fuse with VLDL precursors to form LPVs-containing ApoB (Figs. 4 and 5) [32, 33, 40, 41, 117, 120].

On the other hand, trafficking and release of infectious HCV have been shown to happen along the Golgi apparatus–endosome secretory pathway and/or recycling endosomes independently of VLDL secretion [105, 108]. Accordingly, host cell proteins involved in HCV secretion are part of the classical trafficking pathway including the Golgi–endosomal pathway, recycling endosomes, microtubules, and secretory vesicles [105, 108, 121]. Besides, HCVcc-associated E1–E2 contain both high mannose and


ER lumen
Cytosol
1
a
a
b
2
Golgi
LPV2
mVLDL
c
3
4
LVP1
PM
MVB-LE-RE
LVP2

◀**Fig. 4** Hypothetical model of hepatitis C virus (HCV) maturation in post-ER compartments through the secretory pathway. *1* Vesicular trafficking of LVP1 and VLDL. AP2M1 supports the traffic of HCV core to the Golgi apparatus. HCVcc particles possibly travel from the ER to the Golgi with SAR1A-COPII vesicles. VLDLs are transferred to Golgi in a VLDL transport vesicle in a CIDEB and COPII-dependent manner [147, 148]. *2* During HCV maturation, E1 and E2 acquire complex N-linked glycans [141]. Maturation of both LVP1 and VLDL might involve further lipidation steps by MTTP and/or CIDEB (*a*) [110, 111, 146]. ABHD5 colocalize with ApoE at the Golgi apparatus and may mobilize triglycerides for HCV maturation [112]. *b* LVP1 might fuse with VLDL to form a LPV containing both ApoE and ApoB (LPV2) [33, 40, 41, 111]. *3* Following maturation of particles in the Golgi complex, LVPs are sorted into secretory vesicles [possibly including multivesicular bodies (MVBs), late (LE) and recycling endosomes (RE)] en route to the plasma membrane (PM). Clathrin and clathrin adaptor protein complex 1 (AP1) are involved in viral secretion. Components of the ESCRT and exosomes as well as VAMP1 vesicles have been implicated in HCV secretion. TIP47–Rab9 interaction is involved in HCV secretion. *c* The ion channel activity of p7 has been suggested to protect virus glycoproteins and particles from premature exposure to acidification within the secretory and endolysosomal compartments during virus maturation and egress [127]. *4* LVP2 might be secreted either like LVP1 or similar to mature VLDL (mVLDL) through the VLDL pathway involving large vesicles containing VAMP 7 [149]. ApoE: ; ApoB: ; Rab9: ; CIDEB: ; Nup98: ; ABHD5: ; ESCRT complex: ; AP1: ; VAMP1: ; VAMP7: ; AP2M1: ; MTTP: ; SAR1A: ; TIP47: ; Core protein: ; E1–E2 trimers: ; P7 Ion chanel: ; NS3/4A: ; HCV RNA genome:

complex N-linked glycans, indicating that virus particles transit through the Golgi [141]. As mentioned above, AP2M1 supports the traffic of HCV core to the TGN [103]. In addition, secretion associated Ras related GTPase 1A (SAR1A) is involved in the production of extracellular HCV suggesting the transport of HCVcc particles from the ER to the Golgi with COPII secretory vesicles [105]. Moreover, the core protein has been shown to traffic with ApoE, markers of recycling endosomal compartments, and VAMP1 vesicles in HCV-producing cells, which is consistent with virus release through the secretory pathway into sorting endosomal compartments [103, 105, 122]. Furthermore, clathrin and the clathrin adaptor protein complex 1 have been implicated in viral secretion [105, 108, 121]. Taken together these evidences indicate that ApoE is involved at a step after viral envelopment and prior to release and that egress of apolipoproteins-containing virus diverges from the natural ApoE exocytic pathway, possibly following a clathrin-dependent transendosomal secretory route [105, 108, 118].

Several components of the endosomal-sorting complex required for transport (ESCRT) pathway (which are involved in multivesicular bodies (MVBs) biogenesis), exosomes, and the ion channel activity of p7 have also been implicated in HCV secretion [123–127]. Interestingly, the autophagy pathway has been involved in HCV production and exosome-mediated HCV release [128, 129]. In addition, the TIP47-GTP-Rab9 complex (functioning in the intracellular vesicular transport) has been described to interact with HCV particles protecting them from autophagosomal-mediated degradation and promoting the release of virions [128].

Conclusions

HCV alters the normal functions of genes involved in cellular lipid metabolism to induce LDs biogenesis and to remodel intracellular membranes establishing cytoplasmic replication factories. Viral assembly, budding, and release are tightly regulated and linked processes involving the redistribution of required viral proteins, HCV RNA, host

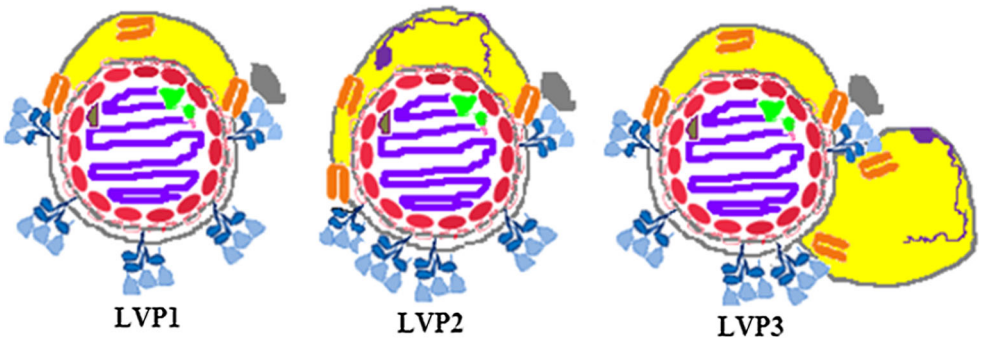


Fig. 5 Putative extracellular HCV particles. Putative lipoviral particles (LVP) LPV1 and LPV2 contain lipoproteins that have been incorporated within virions during intracellular morphogenesis [21, 31]. On the other hand, interaction between extracellular HCV virions and VLDL particles might form the LVP3 [27, 35]. Thus, LVP1 and LPV2 may represent hybrid lipoviral particles while LVP3 supports a two-particle model of lipoproteins peripherally associated with HCV virions (Reviewed in [32–34])

factors, and Golgi–endosomes components to LDs-, ApoE-, and core protein-positive compartments in HCV-infected cells. HCV morphogenesis is related to the VLDL assembly pathway and viral particles associate with various apolipoproteins. New improved HCV culture systems would be relevant to advance in the knowledge of the HCV life cycle, the structure and biochemical composition of infectious HCV particles as well as development of new antivirals and vaccines.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

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