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Entangled in a Membranous Web: ER and Lipid Droplet Reorganization during Hepatitis C Virus Infection

Nathan L. Meyers[*], **Krystal A. Fontaine**[*], **G. Renuka Kumar**, and **Melanie Ott**

Gladstone Institutes, University of California San Francisco, 1650 Owens Street, San Francisco, CA 94941

Abstract

Hepatitis C virus (HCV) is a major cause of liver disease worldwide. To establish and maintain chronic infection, HCV extensively rearranges cellular organelles to generate distinct compartments for viral RNA replication and virion assembly. Here, we review our current knowledge of how HCV proliferates and remodels ER-derived membranes while preserving and expanding associated lipid droplets during viral infection. Unraveling the molecular mechanisms responsible for HCV-induced membrane reorganization will enhance our understanding of the HCV life-cycle, the associated liver pathology, and the biology of the ER:lipid droplet interface in general.

Introduction

Hepatitis C virus (HCV) is a significant health burden: 170–185 million individuals are infected worldwide [1,2]. Most infections result in a life-long condition that increases risk of liver cirrhosis and hepatocellular carcinoma [1]. New anti-HCV drugs that eradicate the virus do not reverse end-stage liver disease, are very expensive, and unavailable in resource-poor countries where most infected people live [3].

HCV is an enveloped, positive-strand RNA virus of the family of *Flaviviridae* [4]. Unlike other *Flaviviridae*, such as yellow fever virus and dengue virus, HCV is not mosquito-borne and infects mainly hepatocytes after contact with infected blood. The virus replicates continuously to high titers within the cytoplasm of infected cells, a process that revolves around the endoplasmic reticulum (ER) and associated lipid droplets (LDs). HCV engages these organelles in multiple ways (Figure 1): 1) After viral entry, the ~9.6-kb single-stranded HCV RNA genome is translated at the rough ER into a single large polyprotein that is proteolytically processed into 10 functional HCV proteins, which are all, except the NS3 protease, firmly integrated in or associated with the ER membrane (Box 1). 2) The C-terminal region of the polyprotein gives rise to nonstructural viral proteins, which form a replicase complex that propagates the viral RNA genome within a newly formed ER-derived web-like membranous compartment that consumes considerable space and membrane resources in the cytoplasm of infected cells. 3) The N-terminal part of the polyprotein

Correspondence to: Melanie Ott, MD, PhD, Gladstone Institutes, University of California San Francisco, 1650 Owens Street, San Francisco, CA 94941, Tel: 415-505-4097, Mott@gladstone.ucsf.edu, http://www.ottlab.ucsf.edu/.

[*]Authors contributed equally.

produces structural viral proteins that reside on the surface of LDs or are firmly anchored within the ER membrane to assemble immature progeny virions that eventually bud into the ER. 4) Within the ER lumen, progeny virion production intersects with intraluminal LDs to produce mature “lipoviroparticles” that are released from producer cells via the lipoprotein pathway [3].

Here, we focus on points 2 and 3 and on recent insights into how HCV infection induces proliferation of ER membranes and manipulates LDs within the cytoplasm of infected hepatocytes.

Molecular architecture of the membranous web

Positive-strand RNA viruses induce extensive cytoplasmic membrane proliferation and remodeling [5]. Induced membrane structures provide favorable microenvironments for compartmentalization of viral RNA replicase complexes. Here, the local concentration of host and viral factors required for efficient RNA replication is increased, and replication is protected from nuclease-mediated degradation and host cell antiviral responses [3,6–9]. HCV is unique as it induces a matrix of cytoplasmic double-membrane vesicles (DMVs) (Figure 1) [10,11]. This is different from other flaviviruses, which mainly form invaginated vesicles within the ER [5,12]. DMVs have diameters of 150–200 nm [13,14] and contain active replicase complexes, supporting the model that they form to support viral RNA replication [8,13]. The outer membranes of ~50% of DMVs connect to the ER membrane via a neck-like structure [13,15]. Biochemical analyses of purified membranes reveal ER-resident calnexin and calreticulin proteins, confirming the ER as a major membrane source for DMVs [15–18].

For some time, viral NS4B protein, an integral membrane protein in the ER, was believed to induce DMVs [12]. NS4B expression induces membrane remodeling via a mechanism dependent on NS4B oligomerization, mediated by its N- and C-terminal α-helices [10,19,20]. However, more sophisticated EM techniques showed that NS4B induces formation of single-membrane vesicles (SMVs) [13]. Only combined expression of all HCV replicase proteins induces DMVs morphologically similar to HCV-infected cells [13]. 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 [13,14].

Critical players in DMV biogenesis are NS5A and the prolyl-peptidyl *cis-trans* isomerase cyclophilin A that binds to the D2 domain of NS5A [21–24], an intrinsically unstructured domain required for efficient HCV replication [25]. Cellular expression of NS5A alone induces small DMV formation albeit with low efficiency [9,13]. Pharmacological inhibition of cyclophilin A or treatment with antiviral compounds targeting NS5A abrogates *de novo* DMV formation in cells expressing the viral replicase complex, underscoring the critical role of both factors in membranous web formation [9,13,24,26,27]. The compounds likely prevent a conformational change in NS5A required for membrane rearrangements otherwise induced by active cyclophilin A. In support of this model, catalytically active cyclophilin A is required to induce DMVs [24,27], and the NS5A N-terminal amphipathic helix exhibits

membranolytic properties, suggesting a direct role in membrane remodeling [28–30]. NS4B and NS5A interact with the proline-serine-threonine phosphatase-interacting protein 2 (PSTPIP2), a host protein that induces positive membrane curvature and is required for DMV formation [31].

Lipid signaling and viral RNA replication

Local lipid concentrations recently emerged as a second regulatory layer of membranous web formation. During HCV infection, phosphatidylinositol-4-phosphate-3 kinase (PI4KIIIα), a critical enzyme within the phosphoinositide synthesis pathway, is trafficked from the Golgi apparatus and the plasma membrane to the ER via interaction with NS5A [32–35]. Intracellular phosphatidylinositol 4-phosphate (PI4P), the product of PI4KIIIα activity and the ligand of pleckstrin-homology domains present in many cellular coat and accessory proteins, increases in concentration concomitantly with this redistribution, particularly at local sites of HCV RNA replication [32,36]. Transient depletion or inhibition of PI4KIIIα suppresses viral RNA replication and causes aggregation of atypically small DMVs [32–34]. This phenotype is mirrored by NS5A mutations that impair interaction with PI4KIIIα, supporting the concept that NS5A recruits PI4KIIIα to the ER, where PI4P accumulates to enhance proper DMV formation and HCV replication [35].

One consequence is delivery of sphingholipids, cholesterol, and fatty acids to budding DMVs and formation of detergent-resistant lipid rafts [33,37]. Lipid rafts are membranous microdomains involved in compartmentalization of HCV replicase complexes within DMVs and signal transduction to modulate host-cellular processes [37]. Glycosphingolipids access PI4P-enriched membranes by interacting with four-phosphate adaptor protein 2 (FAPP2), which contains functional binding domains for PI4P and glycosphingolipids [38]. FAPP2 knockdown impairs HCV replication and prevents formation of DMVs, while addition of exogenous glycosphingolipids restores viral RNA replication [38].

In one model, viral proteins interact with specific host factors (i.e, PI4KIIIα) to induce lipid changes that recruit host factors (i.e., FAPP2 and associated lipids) to promote membrane curvature and DMV biogenesis (Figure 1). Cholesterol is delivered to DMVs by interaction of oxysterol-binding protein (OSBP) with PI4P and vesicle-associated membrane protein-associated protein A (VAPA), a host protein interacting with NS5A and NS4A [39–41]. Inhibitiing OSBP or depleting cholesterol results in aggregated, atypically small DMVs in HCV-infected cells, underscoring the critical contribution of this process to web formation [8,39]. Fatty acid synthase co-localizes with the viral NS5B polymerase within lipid rafts, and its transient knockdown or inhibition also impairs HCV replication [42].

However, fatty acids play a two-faced role in HCV infection. In hepatoma cells and primary hepatocytes, adding polyunsaturated fatty acids (PUFA), targets of reactive oxygen species in lipid peroxidation reactions, inhibits RNA replication of HCV strains that are not adapted to replication in cell culture [43,44]. Conversely, adding antioxidants, such as vitamin E, enhances replication of the same strains [43,44]. Expressing the vitamin E transporter SEC14L2, naturally lacking in hepatoma cells, or inhibiting sphingosine kinase 2 (SPHK2) phenocopies this effect, pointing to lipid peroxidation as a critical barrier to viral RNA replication in *in vitro* cultures [44,45]. The mechanism by which SPHK2 regulates HCV

replication remains unknown but it is likely that SEC14L2 expression serves to locally deliver vitamin E to sites of HCV replication. In some strains, resistance to PUFA treatment maps to adaptive mutations located in membrane-proximal residues of viral NS3/4A or NS5B proteins, suggesting that peroxidation could impair viral RNA replication by preventing viral protein oligomerization or generally by altering membrane fluidity [44].

Lipid storage and virion assembly

Packaging of the HCV genome into nucleocapsids, a process called assembly, also depends on lipids [46]. In particular, HCV assembly is dependent on lipid droplets (LDs), which are cytosolic lipid storage organelles involved in many cellular processes [47,48]. LDs 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 of the ER. Juxtaposed to the ER within the membranous web, LDs are regarded as platforms for HCV virion assembly (Figure 1) [12].

Two HCV-encoded proteins, the nucleocapsid core and NS5A, associate with LDs during virion assembly; NS5A may traffic the RNA genome out of DMVs to the LD surface where encapsidation by core is initiated (Figure 1) [46]. Notably, in HCV-infected or core-expressing cells, LDs cluster around the nucleus, which may further condense the sites of viral RNA replication and virion assembly [49,50]. Why LDs are required for assembly is unclear, as virion formation does not involve LDs directly, but instead occurs at adjacent ER membranes where envelope proteins E1 and E2 reside [51]. Mutations that prevent core from localizing to LDs inhibit HCV assembly [47,48,52], but viral p7 and NS2 are thought to eventually recruit LD-bound core to the ER to enable infectious virion formation [53].

Core undergoes two proteolytic cleavages at positions 191 and 179 that generate mature core protein (179 amino acids), which is loosely anchored within the cytosolic leaflet of the ER via a C-terminal signal peptide [54]. It then traffics onto the surface of LDs [55–58] and other closely associated organelles, such as the mitochondria [59,60]. Localization of core to the ER, mitochondria, or LDs is dependent on its D2 domain, which harbors an amphipathic helix-turn-helix motif found in other LD-binding proteins [60,61].

NS5A attaches to intracellular membranes through its N-terminal amphipathic helix [62]. This helix interacts with the LD-resident protein tail-interacting protein 47 (TIP47), which connects ER and LD membranes and supports HCV RNA replication [63–66]. This and the phosphorylation status of NS5A might serve as an important rheostat for NS5A's dual function in viral RNA replication and virion assembly (Figure 1) [67,68]. Interestingly, transfer of core and NS5A to LDs is linked via common host factors (e.g., diacylglycerol acyltransferase-1 (DGAT1), Ras-related protein 18 (Rab18) and apolipoprotein J).

DGAT1 is one of two known enzymes that catalyze the final step in triglyceride synthesis and fuel LD generation. Core and NS5A bind DGAT1 within the ER and associate with DGAT1-generated LDs, a process that enables proper colocalization of both factors to support assembly [69,70]. Similarly, Rab18 binds NS5A and promotes its association with LDs [63], while the role for Rab18 in the trafficking of core to LDs remains unclear [63,71].

Apolipoprotein J, a very low-density lipid (VLDL)-associated molecular chaperone, stabilizes the core-NS5A complex and supports infectious virion production [72].

LDs also harbor antiviral host factors such as viperin, an interferon-induced host factor involved in the antiviral immune response [73]. Viperin interacts with core and NS5A and is thought to disrupt the interaction of NS5A with the host VAPA protein at replication sites, thus suppressing viral RNA replication [74]. Heterologous nuclear ribonucleoprotein K (HNRNPK) is a poly(C)-binding protein exerting anti-HCV effects through localization near the ER and LDs [75]. Silencing of HNRNPK reduced HCV entry/replication steps, but enhanced virus assembly/release steps, suggesting complex involvement of the factor in HCV infection [75–78]. One model is that HNRNPK is redistributed to sites of viral particle production where it sequesters HCV RNA, blocking HCV assembly [75].

Ups and downs of lipid droplets

Remarkably, cellular LDs are preserved and enriched in HCV infection [79]. This is in contrast to the consumption of LDs that is observed during infection with the related dengue virus [80]. HCV infection is often characterized by liver steatosis, a complex feature involving aberrant accumulation of LDs recapitulated in HCV-infected or core-expressing cells [46]. Steatosis is more frequently associated with HCV genotype 3, and genotype 3a core protein induces formation of large LDs by downregulating phosphatase and tensin homolog (PTEN) [81] or increasing LD cholesterol ester content with sphingolipid biosynthesis [82]. Core expression in mouse liver induces expression of sterol regulatory element binding protein 1c (SREBP-1c), a critical transcription factor in fatty acid, triglyceride, and phospholipid biosynthesis [83]. Moreover, SREBP transcription is induced upon HCV infection via interaction of the 3′-UTR of the viral genome with the host protein DEAD box polypeptide 3, X-linked and the IκB kinase-α, an innate immune response that couples lipogenesis and inflammation [84,85]. NS5A and core associate with apolipoproteins A1 and A2, respectively, which regulate triglyceride content in hepatocytes [58,86], and core interacts with nuclear receptor retinoid X receptor alpha to promote lipogenesis [87]. Also, core modulates expression of two transcription factors regulating liver lipid metabolism, peroxisome proliferator-activated receptors (PPAR) alpha and gamma [88,89], and inhibits the microsomal triglyceride transfer protein, preventing lipid secretion through the VLDL pathway in hepatocytes [90].

Cellular LD homeostasis is maintained through a balance between lipogenesis and lipase-dependent processing of LDs (lipolysis). Lipolysis produces free fatty acids, which are transported into the mitochondria to undergo β-oxidation. Reports on β-oxidation during HCV infection are conflicting: HCV infection and core expression downregulate PPARα [88,91–93] and the cellular energy sensor AMP-activated protein kinase [94,95], two important activators of β-oxidation. In addition, levels of two enzymes involved in β-oxidation, medium-chain acyl coenzyme A dehydrogenase (MCAD) and short-chain acyl coenzyme A dehydrogenase (SCAD), decrease following HCV infection or core expression, supporting a model where β-oxidation is downregulated during HCV infection and LD content is increased [96]. However, Diamond et al. reported a paradoxical increase in both fatty acid oxidation and lipid biosynthesis in HCV-infected cells, which points to a more

complex regulation of both processes during HCV infection [97]. Furthermore, HCV particle production depends on the activity of dodecenoyl coenzyme A delta isomerase, an inner mitochondrial enzyme that catalyzes the breakdown of long-chain fatty acids during β-oxidation [98]. Similarly, inhibiting the mitochondrial import of fatty acids blocks HCV replication, further linking intact β-oxidation to efficient HCV infection [80,98].

Core directly interferes with lipolysis in cultured cells and murine livers [99,100]. Lipases linked to HCV infection are the hormone-sensitive lipase (HSL) [96], the putative lipase arylacetamide deactylase (AADAC) [101], and the adipose triglyceride lipase (ATGL) [102]. ATGL directly hydrolyzes triglycerides in LDs, but this activity is inhibited by core when both proteins are located on the same LD surface *in vitro* [102]. Core expression strengthens the interaction between ATGL and its activator CGI-58 and increases recruitment of the complex to core-coated LDs, a paradoxical finding pointing to the dynamics of lipase recruitment to LDs as a critical regulator of its activity [102]. Interestingly, a variant (I148M) of the closely related patatin-like phospholipase family 3 protein (PNPLA3) is linked to nonalcoholic fatty liver disease in genome-wide association studies [103] and increases steatosis risk in HCV-infected individuals in some, but not all, studies [104–108]. Mice carrying a knockin of the *pnpla3I148M* variant develop liver steatosis accompanied by a marked accumulation of the enzyme on LDs similar to what is observed with ATGL in core-expressing cells [109]. The possibility exists that lipases abnormally residing at the LD surface sequester an essential lipolytic factor, leading to an overall decrease in LD turnover [109].

Conclusions and Future Perspectives

HCV biology is closely tied to the lipid biology of infected cells, resulting in rearrangement of membranes and LDs. The past few years have seen a prolific increase in our understanding of the molecular mechanisms governing these changes with implications far beyond HCV infection. This progress was possible after break-through discoveries in *in vitro* viral replication systems in 1999 (viral RNA replication system) and 2005 (full-length infectious clone). While these systems propelled research in the area of viral RNA replication and viral assembly, they remain limited to certain viral genotypes and a handful of cancer cell lines. The next step will be to examine all HCV genotypes in primary hepatocytes. The finding that core of genotype 3 in a new replication system is not found at LDs is an indication of system complexity [110]. Similarly, findings pointing to lipid peroxidation as a major limiting factor for viral RNA replication in primary or hepatoma cells might open the door for future studies.

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Box 1

HCV Proteins and Functions

Protein	Function
Structural Proteins	
Core	A multifunctional protein, core forms a viral capsid that protects HCV RNA. Its N-terminal domain (D1) contains three basic subdomains that bind RNA and other proteins, while its C-terminal domain (D2) is highly helical and mediates lipid binding. Mature core resides at the cytosolic side of the ER membrane and traffics to cLDs to facilitate viral assembly.
E1 E2	Viral surface glycoproteins, E1 and E2 form non-covalent heterodimers within infected cells, but assemble as large covalent complexes stabilized by disulfide bonds on viral particles. E2 mediates binding to receptors at hepatocyte surfaces to promote viral entry. E1 and E2 may mediate fusion between the viral envelope and endosomal host cell membranes.
p7	A short, 63-residue ion channel protein, p7 protects nascent HCV particles while in transit through acidic intracellular compartments to facilitate maturation and particle release. p7 also modulates capsid assembly and the envelopment of viral particles.
Non-Structural (NS) Proteins	
NS2	A Cys-protease that mediates cleavage at the NS2/NS3 junction of the viral polyprotein, NS2 is not required for RNA replication. Rather, NS2 co-localizes with core and NS5A at punctate sites near cLDs and recruits E2 to promote viral assembly.
NS3	A bifunctional molecule comprised of N-terminal serine-protease and C-terminal helicase domains. The NS3 protease domain catalyzes polyprotein cleavage between non-structural proteins, while the role of the helicase domain is unknown.
NS4A	A 54-residue transmembrane protein that is a cofactor for NS3. NS4A stimulates NS3 protease and helicase activities, and regulates NS3 localization to ER and mitochondrial membranes.
NS4B	A poorly-characterized protein with four transmembrane helices and N- and C-terminal amphipathic α-helices. NS4B is critical for efficient RNA replication and HCV particle assembly. The protein, as homo-oligomers, facilitates membranous web formation.
NS5A	A multifunctional protein that associates with membranes via an N-terminal amphipathic α-helix. NS5A localizes to both DMVs and cLDs and serves critical roles in both HCV RNA replication and virion assembly. A phosphoprotein, basal NS5A promotes HCV RNA replication, whereas hyperphosphorylated NS5A promotes HCV assembly.
NS5B	NS5B is the RNA-dependent RNA polymerase required for RNA replication. Its N-terminal catalytic domain connects to a transmembrane domain anchored to intracellular membranes.

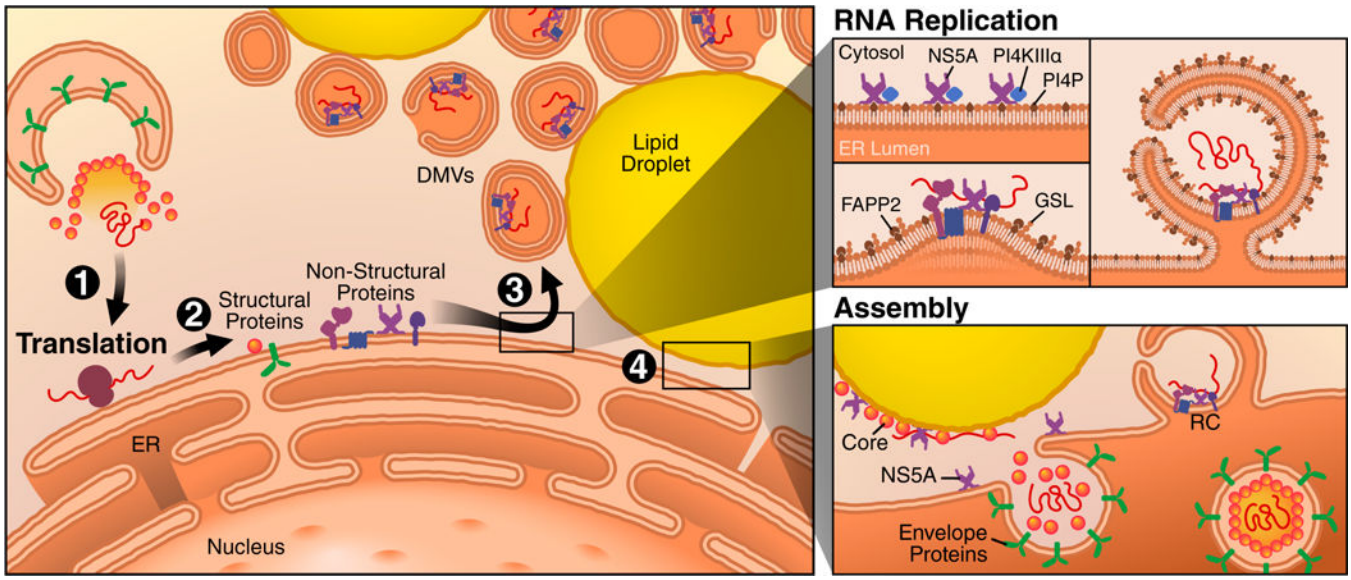

FIGURE 1. HCV replication and assembly are coordinated via intracellular organelles

Left, HCV infection stimulates production of DMVs and LDs. 1) After viral entry and uncoating, HCV RNA is released into the cytoplasm. 2) The HCV RNA genome is translated at the rough ER into a single large polyprotein, which is cleaved into structural and nonstructural proteins. 3) Viral proteins NS4B and NS5A, along with host factors, induce changes in the ER membrane to produce DMVs. DMVs remain attached to the ER or bud off into the cytosol and form a membranous web hosting viral RNA replicase complexes. 4) LD production increases during infection to serve as a scaffold for assembly. NS5A and core proteins are loaded onto LDs and promote HCV RNA translocation from DMVs.

Upper right, DMV formation from ER membranes. Following translation and processing at ER membranes, NS5A activates PI4KIIIα, locally enriching the membrane in PI4P. FAPP2 is recruited to these sites via interaction with PI4P recruiting associated glycosphingolipids. Membrane curvature is induced by these lipid changes, along with the coordinated actions of NS5A and NS4B.

Lower right, HCV assembly at LDs. The core-NS5A complex recruits replication complexes to ER membranes close to LDs. Eventually, LDs connect with ER regions containing the viral glycoproteins. Assembly begins when core and viral RNA, mediated by NS2 and NS3/4A, are transferred back to the cytosolic membrane of the ER.

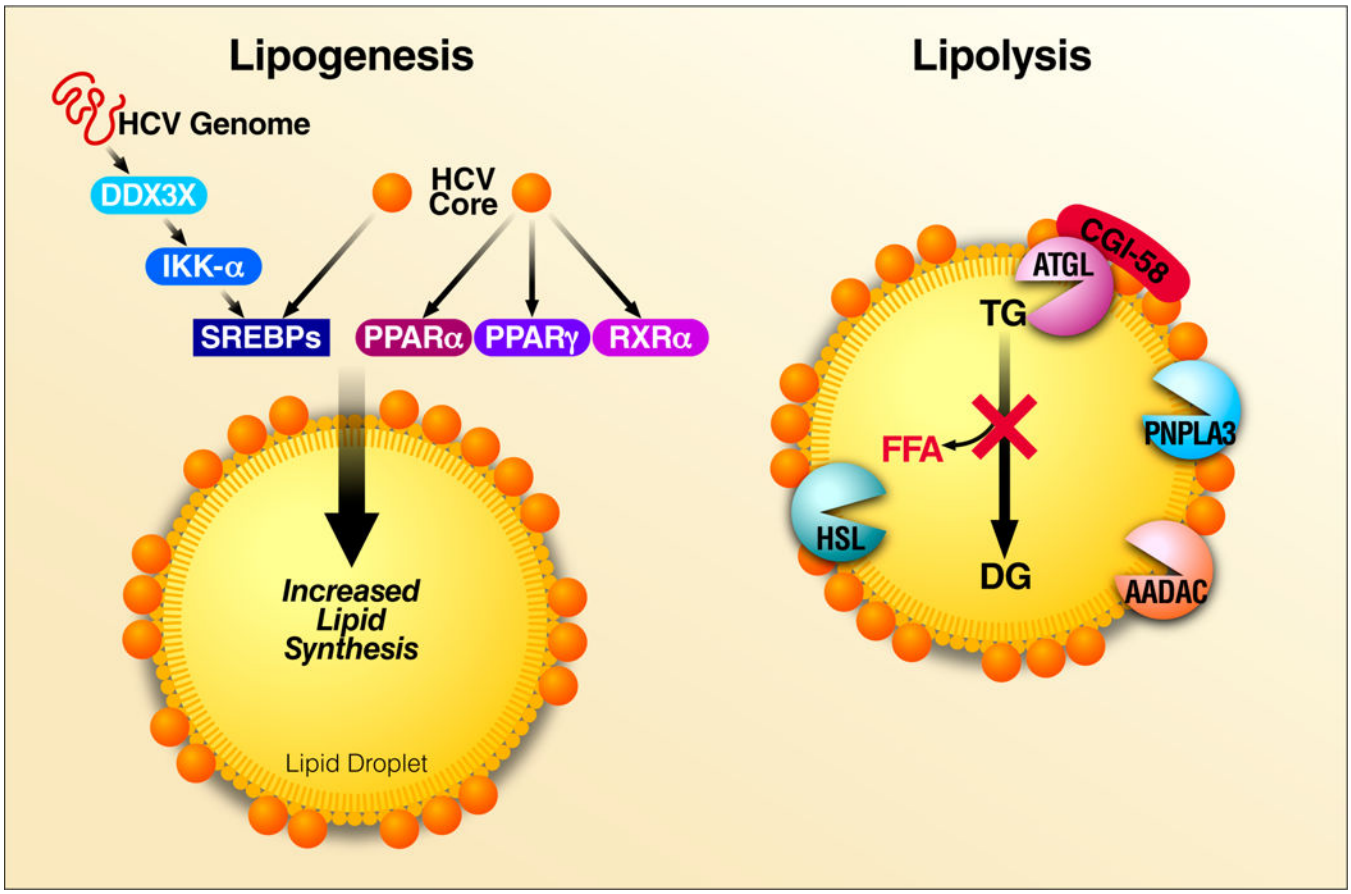

FIGURE 2. The modulation of lipogenesis and lipolysis by HCV

Left, Lipogenesis is induced during HCV infection. Interaction of the HCV genome 3′-UTR with DEAD box polypeptide 3, X-linked activates IκB kinase-α, which results in SREBP-mediated expression of lipogenic genes, such as fatty acid synthase (FASN).

Right, Lipolysis is inhibited during HCV infection. HCV infection has been linked to several lipases, including ATGL, HSL, AADAC and PNPL3. Core strengthens the interaction between ATGL and its activator CGI-58, and increases the recruitment of the complex to LDs.

Importantly, this interaction results in suppression of ATGL activity. Core expression and HCV infection modulate HSL phosphorylation to potentially impair its activity. HCV infection also regulates the temporal expression of the putative lipase AADAC. The I148M variant of PNPLA3 causes liver steatosis in mice, which is accompanied by accumulation of the enzyme on LDs, similar to what is observed with ATGL.