



Hepatitis C Virus Replication

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Replication and amplification of the viral genome is a key process for all viruses. For hepatitis C virus (HCV), a positive-strand RNA virus, amplification of the viral genome requires the synthesis of a negative-sense RNA template, which is in turn used for the production of new genomic RNA. This process is governed by numerous proteins, both host and viral, as well as distinct lipids and specific RNA elements within the positive- and negative-strand RNAs. Moreover, this process requires specific changes to host cell ultrastructure to create micro-environments conducive to viral replication. This review will focus on describing the processes and factors involved in facilitating or regulating HCV genome replication.

The ~9600-nucleotide hepatitis C virus (HCV) RNA genome possesses one large open reading frame (ORF) that is flanked by highly structured 5' and 3' untranslated regions (UTRs) (Fig. 1). *cis*-acting RNA elements (CREs) present within the UTRs as well as the protein-coding region contribute to RNA translation and/or genome replication (for review, see Adams et al. 2017). Synthesis of the HCV proteins is mediated by the internal ribosome entry site (IRES) located in the 5'UTR and facilitated by distinct elements in the 3'UTR as well as CREs within the protein-coding region (Fig. 1). The IRES is composed of three stem-loop (SL) domains. Of these, SLII and SLIII reside in the 5'UTR and adopt an extended structure; SLIV overlaps with the 5' end of the core coding region and forms a short, rather unstable stem

that contains the start codon of the HCV ORF (Fig. 1; Pérard et al. 2013; Quade et al. 2015). Mechanistically, SLIII plays an important role in IRES function because it facilitates the IRES–40S ribosome subunit interaction by a conserved base-pairing between the 18S rRNA and a sequence in SLIII (Matsuda and Mauro 2014). In addition, SLII tightly associates with the head of the 40S subunit, whereas SLIII displaces eIF3 to allow the assembly of a translation-competent ribosome (Hashem et al. 2013; Yamamoto et al. 2015). Apart from the SL domains in the 5'UTR, several elements downstream of the IRES impact RNA translation, including SL47 and SL87 (also called SLV and SLVI, respectively) in the core coding region (see below) (McMullan et al. 2007; Vassilaki et al. 2008). RNA translation is also modulated by the liv-

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er-specific microRNA (miR)-122 that binds to numerous sites within the viral genome (Jopling et al. 2005).

Translation of the viral RNA leads to the production of an ~3000 amino acid polyprotein from which the individual viral proteins are liberated through the cumulative activity of both host and viral proteases (Fig. 1; Table 1). The structural proteins (i.e., core and the envelope glycoproteins E1 and E2) are the main constituents of HCV particles, whereas the viroporin p7 and nonstructural protein (NS) 2 are involved in virion assembly but are not incorporated into the virus particle (see Shimotohno

2019). The remaining nonstructural proteins (i.e., NS3, NS4A, NS4B, NS5A, and NS5B) have specific roles in viral genome amplification. NS3 has several functions. The amino-terminal domain, together with the cofactor NS4A, is a serine-type protease required for polyprotein cleavage and proteolytic processing of host cell factors (Failla et al. 1994; Bartenschlager et al. 1995; Meylan et al. 2005). The carboxy-terminal NS3 domain is a helicase and possesses an additional NTPase activity that is required for RNA unwinding (see below). Moreover, NS3, via interaction with the NS2 protease domain, is involved in the assembly of HCV particles

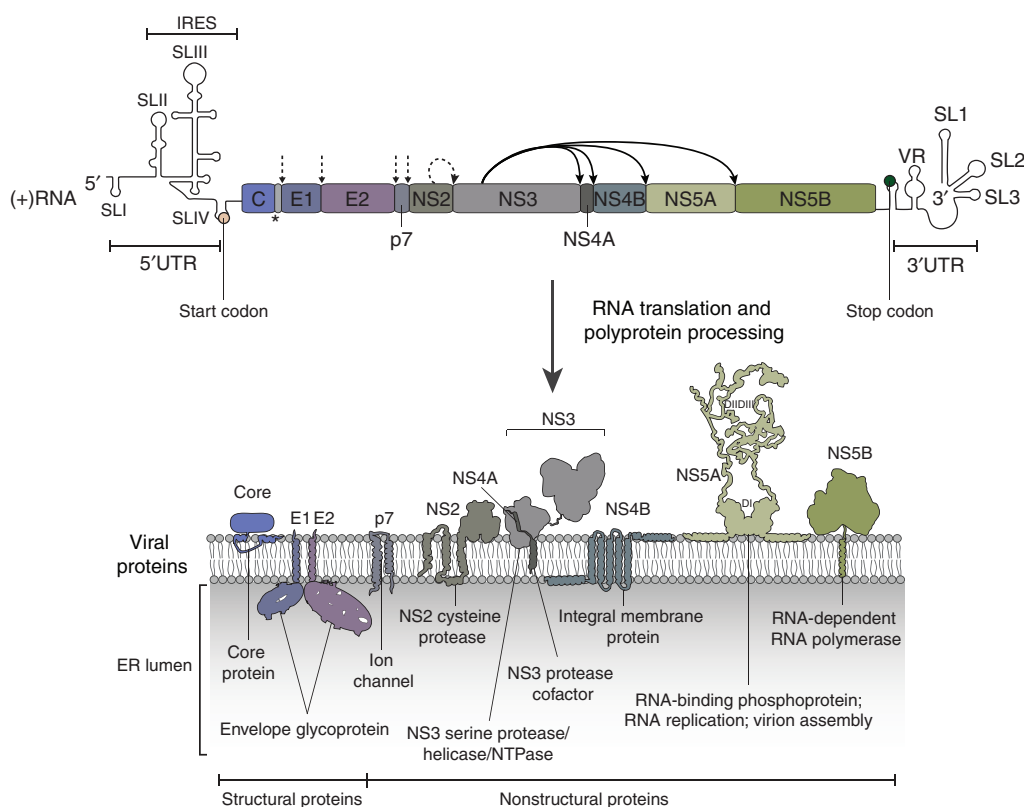


Figure 1. Hepatitis C virus (HCV) genome organization and membrane topology of viral proteins. The open reading frame (ORF) encoding the HCV polyprotein and the predicted secondary structures of the flanking 5' and 3' untranslated region (UTR) are depicted at the *top*. Membrane topology of mature viral proteins and their function are shown at the *bottom*. Co- and posttranslational cleavage of the viral polyprotein are indicated as follows: (dashed vertical arrows) signal peptidase, (star) signal peptide peptidase removing the E1 signal sequence from the carboxyl terminus of core, (dashed curved arrow) NS2-3 protease, (solid arrows) NS3-4A protease. Note that only NS5A is shown as a dimer, but other viral proteins also may form homo- and heterodimers or oligomeric complexes. (D) domain, (ER) endoplasmic reticulum. (Figure adapted from data in Bartenschlager et al. 2013, with permission, from the authors.)

Table 1. Viral RNA elements, selected cellular proteins, and lipids involved in hepatitis C virus (HCV) replication covered herein

	Function in virus replication	References
Viral RNA element		
SLI	Involved in genome replication	Friebe et al. 2001
SLII	Associates tightly with the head of the 40S subunit to enable RNA translation	Friebe et al. 2001; Hashem et al. 2013; Yamamoto et al. 2015
SLIII	Involved in genome replication Facilitates the interaction between the IRES and the 40S subunit of the ribosome Displaces eIF3 to allow the assembly of a translation-competent ribosome	Hashem et al. 2013; Matsuda and Mauro 2014; Yamamoto et al. 2015
SL47	Impacts RNA translation	McMullan et al. 2007; Vassilaki et al. 2008
SL87	Impacts RNA translation and replication	McMullan et al. 2007; Vassilaki et al. 2008
SL248	Interactions with SL87 might serve as a molecular switch between genome replication and packaging	Pirakitikulr et al. 2016
5BSL3.2	Forms a kissing loop interaction with the 3'X SL2, which is essential for replication	Lee et al. 2004; You et al. 2004; Friebe et al. 2005; Romero-López and Berzal-Herranz 2009, 2012, 2017
3'X and poly(U/UC)	Forms long-range interactions with SLIII in the 5'UTR and has been proposed to regulate switching between replication and translation The 3'X and a minimal poly(U/UC) region are essential for genome replication	Tanaka et al. 1995, 1996; Kolykhalov et al. 1996, 2000; Blight and Rice 1997; Yanagi et al. 1999; Friebe and Bartschlagler 2002; Smith et al. 2002; Yi and Lemon 2003a,b; Friebe et al. 2005; You and Rice 2008
Variable region	Initiation of negative-strand RNA synthesis occurs at the terminal uridine that is base-paired to a guanosine in the 3'X SL1 Contributes to efficient genome replication	Yi and Lemon 2003a
Host protein		
PI4KA	Produces PI4P at HCV protein-containing membranes	Berger et al. 2011; Reiss et al. 2011
VAP-A	Interacts with a specific NS5A phosphoform	Evans et al. 2004
VAP-B	Interacts with NS5A and NS5B	Hamamoto et al. 2005
OSBP	Releases cholesterol in exchange for PI4P into the membrane of DMVs	Wang et al. 2014
NPC1 and NPC2	Transport unesterified cholesterol to DMVs	Stoeck et al. 2017
FAPP2	Might release glucosylceramide in exchange for PI4P into the membrane of DMVs	Khan et al. 2014

Continued

Table 1. *Continued*

	Function in virus replication	References
SREBP	Activates transcription of target lipogenic genes	Waris et al. 2007; Park et al. 2009
DDX3X	Activates a signaling cascade that induces the expression of SREBP	Li et al. 2013a
CypA	Catalyzes the isomerization of peptidyl-prolyl bonds in NS5A domain I and domain II stimulating the RNA-binding capacity of NS5A	Rosnoble et al. 2012; Madan et al. 2014; Ngure et al. 2016; Badillo et al. 2017
Sec14L2	Might be involved in the formation of RO	Saeed et al. 2015
<i>Lipids</i>	Enhances vitamin E-mediated inhibition of lipid peroxidation	
PI4P	Recruits OSBP and FAPP2	Khan et al. 2014; Wang et al. 2014
Cholesterol	Required for replicase activity and impacts replication complex architecture	Paul et al. 2013
Sphingolipids	Stimulates replicase activity	Weng et al. 2010; Hirata et al. 2012

(IRES) internal ribosome entry site, (UTR) untranslated region, (DMVs) double-membrane vesicles, (RO) replication organelle.



(Counihan et al. 2011). NS4B is a highly hydrophobic protein involved in inducing membrane alterations that are required for the biogenesis of the viral replication organelle (RO). NS5A is a multifunctional phosphoprotein required for both RNA replication and assembly. NS5B shows RNA-dependent RNA polymerase activity and therefore plays the central role for the amplification of the viral genome. Each of the viral proteins are bound to intracellular membranes by various means, including complex transmembrane domains (e.g., NS2, NS4B), a monotopic α -helix (e.g., NS5A), or a single transmembrane helix (e.g., NS5B) (Fig. 1). Therefore, the HCV replication cycle occurs on membrane surfaces, thus reducing dimensionality.

HCV-INDUCED MEMBRANE ALTERATIONS

Early electron microscopy (EM) studies of liver tissues from infected patients or chimpanzees indicated that HCV induces membrane alterations in infected hepatocytes (Jackson et al. 1979; Shimizu et al. 1990; Shimizu 1992), which is a hallmark of all positive-strand RNA viruses. With the advent of robust cell culture models for HCV, it became possible to study structure–function relationships of HCV proteins. It was found that the expression of individual viral proteins induced membrane alterations (Egger et al. 2002). Notably, the sole expression of NS4B induces a condensed membrane structure, consisting of single-membrane vesicles in a membranous matrix. This structure was given the designation “membranous web,” and it was also found in cells containing a subgenomic replicon of moderate replication competence (Gosert et al. 2003). However, in hepatoma cells containing a highly replication-competent HCV isolate, designated JFH-1, because it was isolated from a Japanese patient with fulminant hepatitis (Kato et al. 2003), the predominant virus-induced membrane structure is double-membrane vesicles (DMVs) that accumulate in the cytoplasm, often in close proximity of lipid droplets (Fig. 2). Although single-membrane vesicles were detected rather sporadically, multi-membrane vesicles were observed primarily at

late time points after infection and are thought to reflect a stress response induced by high-level virus replication (Ferraris et al. 2010; Romero-Brey et al. 2012; Paul et al. 2013). DMVs are heterogeneous in size, with an average diameter of \sim 200 nm, and are morphologically similar to membrane alterations identified in cells infected with coronaviruses (Gosert et al. 2002; Knoops et al. 2008) or picornaviruses (Belov et al. 2012). In the case of HCV, double-stranded RNA (dsRNA) and nonstructural proteins have been found in association with DMV membranes (Ferraris et al. 2010; Romero-Brey et al. 2012; Paul et al. 2013). This result and the observed correlation between DMV abundance and viral RNA replication argue that DMVs are the sites of viral genome replication (Ferraris et al. 2010; Romero-Brey et al. 2012; Paul et al. 2013). However, it is still unclear whether all DMVs are engaged in HCV replication and whether RNA replication occurs on the interior or exterior membrane surface of the DMV.

Several studies have reported that viral proteins and RNA associated with the viral ROs are protected from exogenously added proteases or nucleases, indicating that RNA replication occurs in a membranous environment that is segregated from the surrounding cytoplasm (Miyazari et al. 2003; Quinkert et al. 2005; Hsu et al. 2010; Paul et al. 2013). Interestingly, most DMVs appear to be closed structures and only a minority (\sim 8%) have an opening pore toward the cytosol (Fig. 2; Romero-Brey et al. 2012). This morphology suggests that if replication occurs on the interior surface of DMVs, then a transport mechanism must be present to allow import of metabolites required for replication as well as export of viral RNA for translation or virion assembly. Alternatively, in a model in which replication occurs on the outer surface of DMVs or on open DMVs, a more complex architecture of the RO must exist to explain the protection of viral RNA from attack by exogenously added nucleases. In either case, the protected nature of the viral RNA indicates a transport mechanism to mediate the movement of macromolecules between cellular compartments. This model is supported by observations that HCV hijacks specific cellular components involved in nucleocytoplasmic transport,

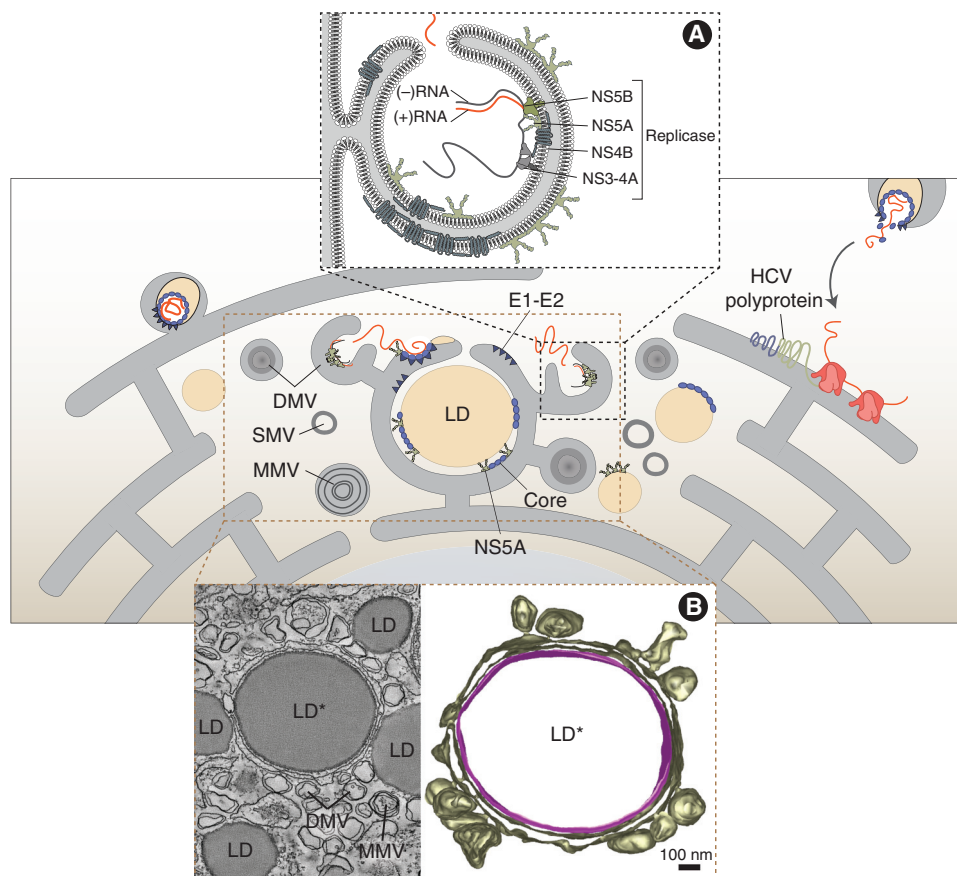


Figure 2. HCV replication organelle. After entering the cell, the HCV genome is released into the cytosol and translated at the rough endoplasmic reticulum (ER). Viral proteins, in cooperation with host factors, induce intracellular membrane alterations consisting of double-membrane vesicles (DMVs), single-membrane vesicles (SMVs), and multimembrane vesicles (MMVs). DMVs, usually found in close association with lipid droplets (LDs), are protrusions of the ER that contain nonstructural proteins required for genome amplification (*inset A*). These vesicles are open toward the cytosol or are closed (represented as gray shaded vesicles), possibly reflecting different stages of DMV “maturation” (early and late, respectively). Viral RNA amplification may occur inside DMVs, which would allow the exit of newly synthesized viral genomes as long as the DMV is open. RNA molecules might be delivered by NS5A and NS3 to nearby assembly sites enriched in core protein and E1-E2 envelope glycoprotein complexes that are associated with p7 and NS2. Alternatively, replication might occur on the outer surface of DMVs (not represented). Particles are formed by budding into the lumen of the ER. (*Inset B*) DMVs emanate from ER membranes that are tightly wrapped around LDs as revealed by a combination of live cell imaging and electron tomography. (*Left*) Single tomographic slice of an HCV-infected cell revealing two classes of LDs. First, an LD (LD*) that is tightly wrapped by the ER and that stains positive for E2 and NS5A as revealed by fluorescence microscopy (not shown) and, second, several LDs that are not wrapped by the ER and that do not stain for E2 and NS5A (LD) suggesting that HCV proteins trigger LD wrapping by ER membranes. (*Right*) 3D reconstruction of the membranes surrounding LD*. ER membrane and DMVs are shown in yellow-gray; the LD monolayer membrane is shown in violet. Note the DMVs originating from the wrapping ER membrane. In some cases, a stalk-like connection between DMVs and the ER is visible. Assuming that RNA replication occurs in these DMVs, only short-distance trafficking of viral RNA would be required to the ER lumen to allow virus budding (as indicated in the schematic above). (Images in *inset B* are adapted from images in Lee et al. 2019 under the terms of the Creative Commons Attribution License [CC BY].)

and that these cellular factors are involved in maintaining a selective barrier between the cytosol and the interior of viral ROs (Neufeldt et al. 2013, 2016). However, the mechanisms underlying this transport process as well as the nature of the barrier between replication compartments and the surrounding cytosol remain unclear.

Viral Factors Involved in HCV Replication Organelle Formation

Although individual expression of the nonstructural proteins induces membrane alterations, NS5A was found to be the only protein capable of inducing DMV formation on its own (Egger et al. 2002; Romero-Brey et al. 2012). However, the efficiency of DMV formation is rather low, but is greatly enhanced when NS5A is expressed as part of an NS3–NS5B polyprotein fragment. Mutation analyses identified important motifs in HCV proteins required for DMV formation. These include the helicase domain in NS3, glycine zipper motifs in NS4B, the amino-terminal NS5A membrane anchor, and domain I of NS5A (Romero-Brey et al. 2015; Paul et al. 2018). The important role of NS5A in HCV RO formation is best illustrated by the observation that highly active NS5A inhibitors such as daclatasvir block formation of the HCV RO independent of RNA replication (Berger et al. 2014). Molecular docking studies and the positioning of daclatasvir resistance mutations suggest that the drug binds to the membrane-proximal side of NS5A domain I (Berger et al. 2014; Nettles et al. 2014). Therefore, NS5A inhibitors appear to block formation of the HCV RO through disturbing the positioning, folding, and/or flexibility of the linker segment connecting the amino-terminal α -helix and domain I.

In addition to NS5A, NS4B also plays a central role in HCV RO biogenesis. NS4B has a complex membrane topology that is comprised of four amphipathic α -helices, two at the amino-terminal and two at the carboxy-terminal region, which flank four transmembrane-spanning α -helices (Fig. 1; for review, see Bartenschlager et al. 2013). These terminal protein domains can alter membrane properties and likely undergo posttranslational membrane

topology changes (Palomares-Jerez et al. 2012, 2013), presumably in an NS5A-regulated manner (Lundin et al. 2006). Additionally, NS4B forms oligomeric complexes via self-interaction, which is mediated in part by a glycine zipper within transmembrane segments 2 and 3 (Paul et al. 2018). This self-interaction is required for HCV RNA replication and the formation of functional ROs (Gouttenoire et al. 2010, 2014; Paul et al. 2011).

The insertion of the amino-terminal amphipathic α -helix of NS5A, which was shown to alter model membranes in vitro (Palomares-Jerez et al. 2010), into just one membrane layer and the formation of conically shaped protein complexes in the membrane, as might be the case with NS4B, could facilitate membrane curvature required for RO formations (McMahon and Gallop 2005; McMahon and Boucrot 2015). In addition to these direct roles, viral proteins, especially NS5A, might contribute to membrane alterations by recruiting host factors required for RO biogenesis.

Host Factors and Processes Involved in HCV Replication Organelle Formation

In addition to viral proteins and the RNA genome, host cell factors and machineries also contribute to DMV biogenesis. One example is macro-autophagy, the bulk degradation and recycling of cytosolic proteins or organelles. It was shown that HCV induces autophagy, and that the depletion of specific autophagy factors impairs HCV RNA replication and reduces the number of DMVs (for review, see Wang and Ou 2018). Moreover, HCV-induced DMVs have a striking morphological similarity to autophagosomes, which are also cytosolic double-membrane structures. However, DMVs induced by HCV have an average diameter of \sim 200 nm and thus are smaller than autophagosomes, which have a diameter of 500–1000 nm. It may be that HCV induces DMV formation by using only specific components of the autophagy pathway, in combination with viral proteins, leading to the formation of vesicular structures that have similar membrane topology but differ in overall size.

Although HCV-induced DMVs are derived from the ER membrane, their lipid composition is different from the originating membrane, having much higher levels of cholesterol and sphingolipids. NS5A recruits and activates the lipid kinase phosphatidylinositol 4-kinase III α (PI4KA) (Berger et al. 2011; Reiss et al. 2011),

which produces phosphatidylinositol 4-phosphate (PI4P) at HCV protein-containing membranes (Fig. 3, inset). NS5A also binds to and recruits, likely via VAP-A/B, lipid-transfer proteins, most notably oxysterol-binding protein (Wang et al. 2014). By analogy to results obtained in the yeast system (Mesmin et al. 2013,

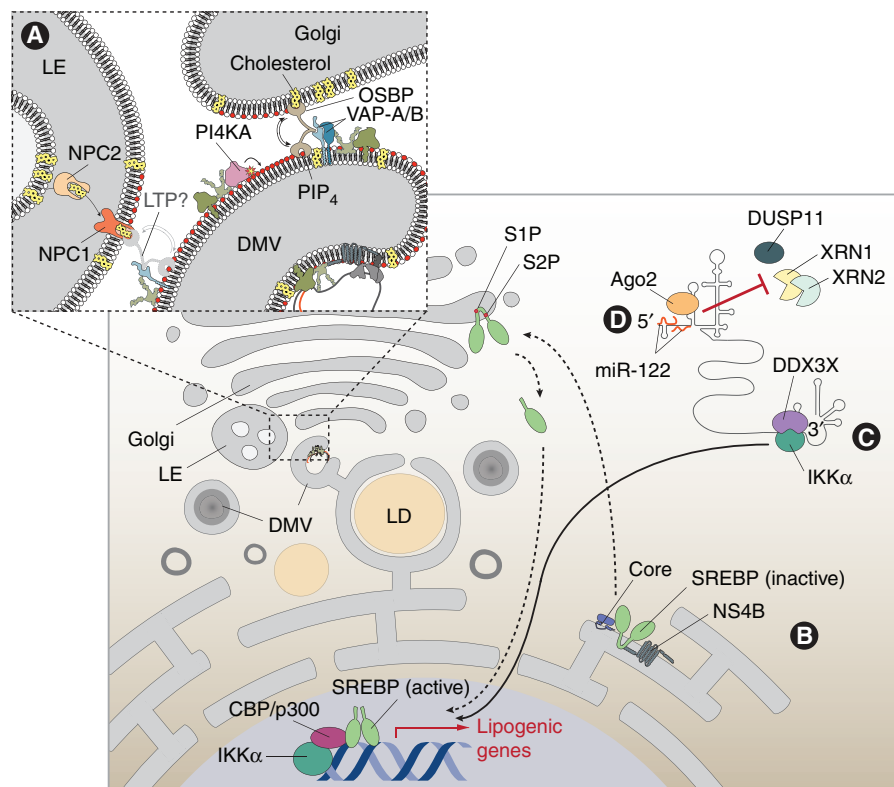


Figure 3. Exploitation of lipid pathways and miR-122 by hepatitis C virus (HCV). (A) HCV alters the lipid composition of rearranged membranes. NS5A and NS5B recruit and activate phosphatidylinositol 4-kinase- α (PI4KA) to produce a local accumulation of phosphatidylinositol 4-phosphate (PI4P). This may determine the directionality of cholesterol transfer by lipid transfer proteins (LTPs), such as oxysterol-binding protein (OSBP), which is recruited by NS5A via VAP-A/B and releases cholesterol in exchange for PI4P at these membrane contact sites. VAP proteins might serve as anchors for additional host proteins promoting the formation of endoplasmic reticulum (ER)–late endosome (LE) membrane contacts. Here NPC1, possibly in coordination with NPC2, mediates the export of unesterified cholesterol that might be accepted by lipid transfer proteins recruited by HCV (indicated with a question mark). (B,C) HCV infection activates the transcription of lipogenic genes by two distinct pathways. (B) The inactive SREBP precursor traffics from the ER to the Golgi on HCV infection or expression of core or NS4B. There, the transcriptionally active amino-terminal segment is released after two-step proteolytic processing by the site 1 protease (S1P) and S2P. Upon dimerization, the active SREBP enters the nucleus and activates the transcription of lipogenic genes. (C) The HCV 3'UTR interacts with DEAD box polypeptide 3X-linked (DDX3X). This RNA-binding protein activates IKK- α , which stimulates CBP-p300 to promote SREBP-mediated transcription. (D) miR-122, in association with Ago2, binds to the HCV 5'UTR and protects the viral genome from 5' triphosphate removal and nucleolytic degradation by 5'-3' exonucleases 1 (XRN1) and XRN2.

2017), we hypothesize that oxysterol-binding protein (OSBP) delivers cholesterol into the DMV membrane in exchange for PI4P (Paul and Bartenschlager 2015). Another lipid-transfer protein, NPC1, possibly together with NPC2 transports unesterified cholesterol to DMVs and contributes to the establishment of a microenvironment conducive for efficient HCV RNA replication (Fig. 3, inset; Stoeck et al. 2017). Additionally, the glucosylceramide transfer protein FAPP2 might be recruited to DMVs to release lipids in exchange for PI4P into the membrane of DMVs (Khan et al. 2014). Overall, these cellular lipid transfer proteins contribute to the enrichment of distinct lipids that could form lipid rafts assumed to be required for high-level HCV replicase activity and possibly the assembly of infectious HCV particles.

Although numerous studies have provided important insights into the biogenesis and function of HCV-induced ROs, many questions related to DMV formation remain unanswered. For instance, what is the exact site of HCV RNA replication? Is it SMVs or DMVs? What is the relationship between these two vesicle species? Assuming that DMVs are the site of RNA replication, are they all engaged in replication or only a subpopulation? The observation that only ~8% of DMVs analyzed at a given time point have a pore-like opening argues that the latter is correct. Alternatively, DMVs might be transient structures that are actively engaged in RNA replication as long as they have an opening to the cytosol, but on closure might become inactive and released out of the cell as extracellular vesicles (Grünvogel et al. 2018). Other questions relate to the molecular mechanisms by which DMVs and MMVs are formed from intracellular (ER-derived) membranes. What is the role of autophagy in this process? Are MMVs a by-product resulting from a cellular stress response induced by HCV? And how is viral cargo transported between HCV-induced subcellular compartments (i.e., sites of RNA translation, RNA replication, and virion assembly)? How is this transport coordinated? These questions illustrate that more studies are required to clearly define the roles of virus-induced membrane alterations in the replication

cycle of HCV, and positive-strand RNA viruses in general.

GENOME REPLICATION

RNA Elements Involved in Genome Amplification

Amplification of the viral genome requires a concerted effort from viral proteins and RNA elements as well as specific host factors. Outside the context of protein production, RNA structural elements found in both the positive-sense viral genome and the negative-strand replication intermediate are essential for viral genome replication. RNA structures found in the 5'UTR of the positive-strand RNA are primarily involved in translation initiation, but several SL structures designated SLI and SLII have been linked to genome replication (Fig. 4; Friebe et al. 2001; Friebe and Bartenschlager 2002). This link is likely associated with the 3' end of the negative-strand RNA, which forms distinct secondary structures from those found in the positive strand (Fig. 4). In fact, several independent studies have confirmed SL elements in the 3' terminal region of the negative-strand RNA, including SLI', SLIIz', SLIIy', SLIIa', and SLIIb'; additional elements have been suggested but specific structures and functions remain uncertain (Schuster et al. 2002; Dutkiewicz et al. 2008). Sequence pertaining to the regions of SLI' and SLIIz' has been genetically mapped as the minimal requirements for synthesis of the positive-strand RNA from the negative-strand RNA template, and functional assays show that SLIIy' is also required for RNA synthesis (Friebe et al. 2001; Friebe and Bartenschlager 2009). The importance of the SLI' element has also been confirmed by *in vitro* approaches, which also indicate a role for SLIIb' in promoting RNA synthesis. However, contrary to the *in cellulo* analysis, biochemical assays suggested that SLIIz' inhibits rather than promotes RNA synthesis (Astier-Gin et al. 2005; Masante et al. 2008; Mahias et al. 2010). This discrepancy may indicate the presence of additional control elements in host cells that are required to regulate the structure of the SLIIz' region or its function in viral genome replication.

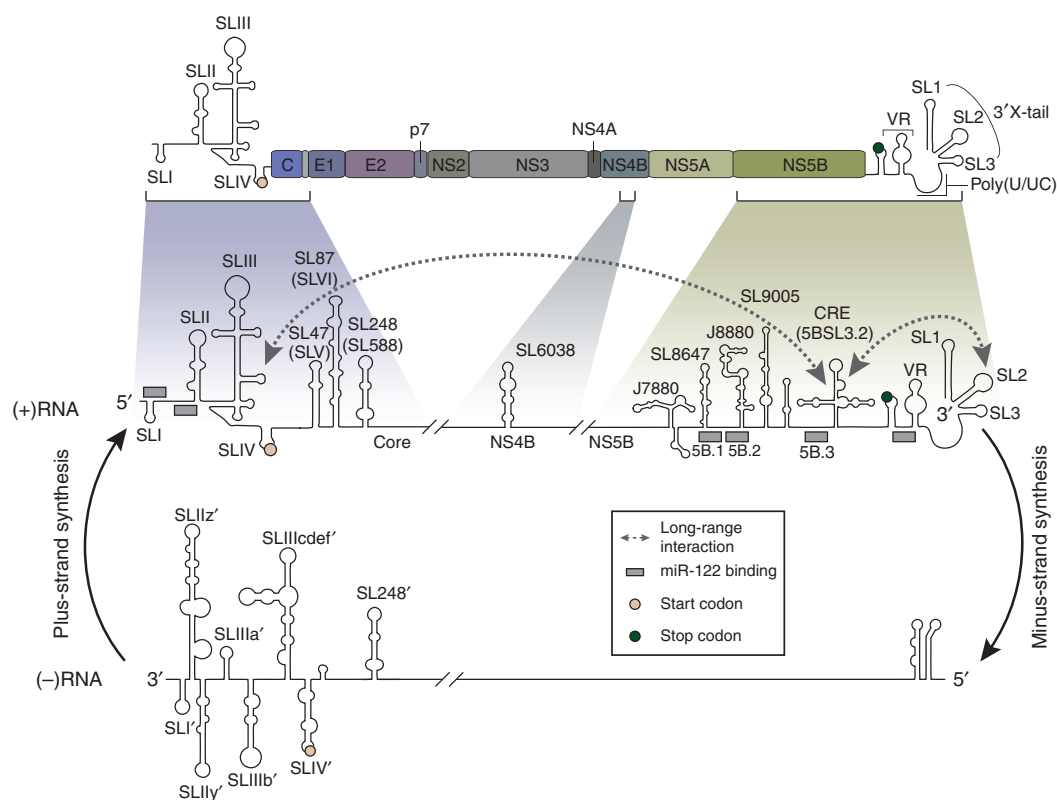


Figure 4. RNA elements within the positive-strand hepatitis C virus (HCV) genome and its negative-strand replication intermediate. The HCV genome organization is represented on the *top* as in Figure 1. A magnification of three regions within the positive-strand (+) RNA are illustrated *below*, each representing predicted RNA structures. Long-range RNA–RNA interactions are indicated with dashed arrows, whereas predicted binding sites of miR-122 are shown as gray rectangles. Structures within the negative-strand (–) RNA are shown on the bottom. Alternative nomenclatures of the structures are given in parentheses. (RNA stem-loop (SL) structures are adapted from data in Niepmann et al. 2018 and Bartenschlager et al. 2013.)

Downstream of the positive-strand RNA 5'UTR, several RNA elements have been identified in the core coding region that promote full genome replication, including SL87 (also designated SLVI or SL427) and the SL248 element (also called SL588) (Fig. 4; McMullan et al. 2007; Vassilaki et al. 2008; Pirakitikulr et al. 2016). For SL87, which has previously been described as a translational promoter, this may indicate a dual role in both RNA translation and genome replication. The SL248 element has been linked to replication in the context of full genome virus, but genomes in which this structure has been genetically altered are still replication-competent and produce infectious virus (Vassilaki et al. 2008; Pirakitikulr et al. 2016).

Interestingly, mutually exclusive interactions between SL248 and the adjacent SL87 might serve as a molecular switch between efficient replication and infectious virus production (Pirakitikulr et al. 2016). This suggests that RNA structures found throughout the viral genome contribute complex mechanisms involved in regulating different viral processes.

In addition to the 5' end of positive-strand RNA, several RNA elements present in the 3'UTR and the NS5B-coding region are essential for genome replication. The 3'UTR is comprised of a variable region, a poly(U/UC) tract of variable length and a highly conserved 3' region designated the 3'X-tail (Fig. 4; Tanaka et al. 1995; Kolykhalov et al. 1996). Each of these re-

gions contribute to viral replication with the 3'X and a minimal poly(U/UC) region being essential and the variable region contributing to efficient replication (Tanaka et al. 1995, 1996; Kolykhalov et al. 1996, 2000; Blight and Rice 1997; Yanagi et al. 1999; Friebe and Bartenschlager 2002; Smith et al. 2002; Yi and Lemon 2003a, b; Friebe et al. 2005; You and Rice 2008). Initiation of negative-strand RNA synthesis begins at the terminal uridine that is base-paired to a guanosine in the 3'X SL1. In this structure, access of the terminal nucleotide for the NS5B polymerase is limited, suggesting that alternative RNA structures are involved in regulating the initiation of RNA synthesis (Fricke et al. 2015). In addition, in the NS5B-coding region, a *cis*-acting RNA element (CRE; also called 5BSL3.2) has been identified as a crucifix-like structure and forms a kissing loop interaction with the 3'X SL2, which is essential for replication (You et al. 2004; Friebe et al. 2005). This CRE also forms long-range interactions with SLIII in the 5'UTR (Fig. 4) and has been proposed to regulate switching between replication and translation (Lee et al. 2004; You et al. 2004; Romero-López and Berzal-Herranz 2009, 2012, 2017). Additional long-range interactions between 3' and 5' elements, facilitated by *trans*-acting host factors, have been shown to potentiate viral genome replication (for review, see Niepmann et al. 2018). Three other SL elements within the coding region have also been linked to replication, SL6038, J7880, and J8880 (Fig. 4), but the specific functions of these elements remain to be determined (Mauger et al. 2015; Pirakitikulr et al. 2016).

Virus Protein Contributions to Genome Replication

NS5B Polymerase

A plethora of studies examining NS5B, which harbors RNA-dependent RNA polymerase (RdRp) activity (Behrens et al. 1996; Lohmann et al. 1997), have given us significant insights into its structural and functional properties. NS5B is a tail-anchored protein composed of an amino-terminal catalytic domain that makes

up the majority of NS5B, and a carboxy-terminal *trans*-membrane domain tethering the protein in the membrane (Fig. 1). The *trans*-membrane domain is essential for RNA replication in cells, most likely to allow proper insertion into the membranous replicase machinery, yet dispensable for enzymatic activity and is therefore deleted in most biochemical or structural assays to increase solubility of the protein. Like all viral RdRps, NS5B has a “right-hand” shape containing palm, thumb, and fingers domains (for review, see Sesmero and Thorpe 2015). Additionally, HCV NS5B contains a β -flap domain that is specific to *Flaviviridae* RdRps and a linker domain that is common to *de novo* initiating enzymes. Through well-defined mechanisms, each of these domains contributes to specific steps in viral RNA synthesis (for review, see Sesmero and Thorpe 2015).

RNA synthesis processes governed by NS5B can be divided into four steps: RNA binding, initiation, processive elongation, and termination at the end of the template. Although early reports show that NS5B can initiate RNA synthesis using both primer-based and *de novo* mechanisms, structural evidence suggests that NS5B uses *de novo* initiation to replicate the viral genome in cells (Behrens et al. 1996; Lohmann et al. 1997; Luo et al. 2000; Sun et al. 2000; Zhong et al. 2000). It is thought that initiation starts directly at the 3' end of the viral RNA genome and requires high levels of GTP that bind to an allosteric site in the enzyme to act as a structural support to prime the initiation process (Lohmann et al. 1999b). As discussed above, the 3' end of the positive-strand RNA is a poor template for *de novo* initiation, as it is concealed within an SL structure. In contrast, the 3' end of the negative-strand RNA consists of a stem loop with an overhang (Fig. 4) that serves as a highly efficient initiator of RNA synthesis. This difference likely has a role in regulation of the replication processes and might contribute to the 10-fold excess of positive- over negative-strand RNA. It is highly advantageous for HCV to regulate production of negative-strand RNA, as increased RNA synthesis interferes with translation and excess dsRNA intermediates would stimulate innate immune responses.

K. Tabata et al.

NS5B can bind to many RNA target sequences and initiate RNA syntheses both internally within the viral genome and on circular RNA templates, arguing for a lack of specificity for the viral genomic RNA ends (Lohmann et al. 1997; Shim et al. 2002; Ranjith-Kumar and Kao 2006). These properties indicate that the enzyme is not limited to the “closed” conformation that is suggested by most structural analysis. In fact, recent studies identified an NS5B open state that can also accommodate primer and template RNA, which is consistent with early reports that purified NS5B can initiate using both primer-based and de novo mechanisms. These observations argue that, in solution, there is equilibrium between open and closed states that may serve different functions in the replication cycle. Additionally, several reports have shown that NS5B can bind to 3'UTRs of specific host mRNAs (Yuhashi et al. 2014). In combination with low specificity for RNA targets, these observations lend to the hypothesis that NS5B can amplify host RNAs, which could be involved in regulation of host protein production or in activation of innate immune responses (Yu et al. 2012). Although NS5B alone does not seem to have specificity for the viral genome, genetic studies argue that an interaction between the NS3 helicase, NS5A, and NS5B is required for initiation of RNA synthesis (Binder et al. 2007) indicating that template specificity is conferred by a combination of viral factors. In addition, the HCV replicase is tethered in intracellular membranes, which limits access to cellular RNAs.

NS3-4A

HCV NS3 is a bifunctional enzymatic protein containing a carboxy-terminal DExD-box helicase domain (NS3h) and an amino-terminal protease domain that function in conjunction with the cofactor, NS4A (Fig. 1). Whereas the protease domain is indirectly involved in replication through its role in polyprotein processing, the helicase domain has a more direct role in RNA synthesis. Consistent with this assumption, specific NS3h mutations alter HCV replication fitness and a correlation between nucleic acid unwinding activity and replicative ability

was observed (Stross et al. 2016; Zhou et al. 2018). Several studies have indicated an allosteric mechanism that governs a switch between protease activity and helicase activity, with the “open or extended form” showing higher DNA unwinding function and representing the biologically relevant state for RNA replication (Ding et al. 2011; Saalau-Bethell et al. 2012). Thus, in the current model, the protease domain also contributes to the activity of the helicase domain (Beran et al. 2007). Although the DNA unwinding mechanism of HCV NS3h is well characterized (Gu and Rice 2010), its precise function within the viral replication cycle remains elusive. Specifically, the helicase activity could be required for the dissociation of highly structured single-stranded RNA elements before NS5B-mediated template-guided RNA synthesis or it could function in dissociating dsRNA following replication. In either case, the NS3 helicase activity is critically required for HCV RNA replication.

NS5A

NS5A is a promiscuous viral protein that has roles at several stages of the viral life cycle (for review, see Ross-Thriepland and Harris 2015). NS5A can bind to RNA and forms homodimers and probably also higher-order structures. It contains three defined domains, linked by low-complexity sequences (LCSS), and an amino-terminal amphipathic α -helix that facilitates membrane association and is essential for genome replication (Fig. 1; Penin et al. 2004). Domain I contains an RNA-binding motif and is linked to virus replication as well as RO biogenesis (Huang et al. 2005; Romero-Brey et al. 2015), whereas domain III functions primarily in virion assembly (Appel et al. 2008). Although the major part of domain II is dispensable for replication, specific residues in the carboxyl terminus are required for genome replication (Appel et al. 2008; Tellinghuisen et al. 2008; Ross-Thriepland and Harris 2014). These different NS5A functions seem to be regulated via differential phosphorylation states. Early studies described two prominent phospho-isoforms for NS5A that could be separated by sodium



dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were designated the basally phosphorylated p56 and the hyperphosphorylated p58 (Kaneko et al. 1994). However, this view has been challenged by opposing data sets obtained from experiments performed with genotype 1b versus 2a HCV strains. Additionally, recent reports showed that the NS5A p58 isoform consists of several independently phosphorylated species that perform different functions and argue for an essential role of p58 in both assembly and RNA replication (Masaki et al. 2014; Harak et al. 2016; Schenk et al. 2018). In any case, reports have consistently linked several phospho-acceptor-sites in the LCS1 region to viral genome replication, and showed that limiting phosphorylation of these sites by blocking the casein kinase I isoform α (CKI α) limits replication and virion assembly (Appel et al. 2005; Quintavalle et al. 2006; Pietschmann et al. 2009; Masaki et al. 2014; Harak et al. 2016; Goonawardane et al. 2018). This suggests that phosphorylation in this region may function as a regulatory switch between RNA replication and virion assembly. The combined data on NS5A highlight the multipurpose nature of this protein and the complexity of regulating its different functions through multiple phosphorylation events. This complexity makes experimental characterization of specific NS5A functions exceedingly difficult. At the same time, the multiple functions exerted by NS5A might explain the exceptional antiviral potency of NS5A inhibitors that most likely block several steps of the viral life cycle such as RNA replication and virion assembly (Berger et al. 2014; McGivern et al. 2014). Although the precise mechanisms for how this multifunctionality is achieved are unknown, it is tempting to speculate that various NS5A phosphovariants bind to distinct host cell factors, such as PI4KA, VAPA/B, or apolipoprotein E, that exert the required function.

HOST CELL FACTORS OF RELEVANCE TO HCV GENOME REPLICATION

We can assume that for each individual step of the HCV life cycle, host cell factors are required.

These can be proteins, lipids, and/or nucleic acids. During the last years, numerous factors of this kind have been identified and characterized, but only a few key examples can be mentioned here because of space limitations. The reader interested in more in-depth discussion of this aspect is referred to more recent reviews (Ross-Thrieland and Harris 2015; Sarnow and Sagan 2016; Wang and Tai 2016).

miRNAs are a class of small noncoding RNAs with an approximate length of 22 nucleotides, which are involved in the posttranscriptional regulation of gene expression. Certain miRNAs are expressed ubiquitously, whereas miR-122 is specifically expressed in the liver. Interestingly, binding sites for miR-122 are present in the 5'UTR, 3'UTR, and the NS5B-coding regions of the HCV positive-strand RNA genome (Fig. 4) and are linked to various viral processes (for reviews, see Sarnow and Sagan 2016; Bernier and Sagan 2018). Original studies showed that HCV RNA mutated in miR-122-targeting 3'UTR sequence was replicated as well as wild-type, whereas HCV RNA mutated in 5'UTR target sequence affected RNA accumulation and translation (Jopling et al. 2005), suggesting that HCV replication is dependent on miR-122 binding. On one hand, in typical interactions of miRNAs with mRNA, miRNAs promote translational repression and/or degradation of the target RNA. On the other hand, in the case of HCV, miR-122 has a positive effect by binding to the viral RNA genome in association with Ago2 and protecting the viral genome from nucleolytic degradation by host 5'-3' exoribonucleases 1 (XRN1) and XRN2 (Fig. 3; Shimakami et al. 2012; Li et al. 2013b, 2015). XRN activity is usually specific to 5' monophosphate transcripts and not the 5' triphosphate product of viral polymerase. In this case, the 5' RNA triphosphatase, DUSP11, functions together with XRNs to restrict HCV, a process that is prevented by miR-122 binding (Fig. 3; Amador-Cañizares et al. 2018a; Kincaid et al. 2018). It is also reported that miR-122 binding contributes to the folding of a functional IRES by suppressing energetically favorable alternative secondary structures (Amador-Cañizares et al. 2018b; Schult et al. 2018; Chahal



et al. 2019). Additionally, a recent study of the miR-122-binding sites in the NS5B-coding region correlated miR-122 binding with genome replication (Gerresheim et al. 2017). Specifically, mutations in the miR-122-binding site denoted 5B.2 (Fig. 4) caused a significant decrease in HCV RNA accumulation. However, compensatory mutations in this region that restore miR-122 binding failed to rescue viral RNA accumulation indicating that this RNA region has a role beyond that of direct miR-122 binding (Bernier and Sagan 2019). Together, these studies show that miR-122 is intricately involved in regulating different stages of the HCV infection cycle. It is an essential HCV host dependency factor and its depletion renders cells nonpermissive for this virus under physiological conditions.

Another host factor required for HCV replication is the prolyl-peptidyl *cis-trans* isomerase cyclophilin A (CypA), which alters the conformation of proteins by interconverting the *cis* and *trans* isomers of peptide bonds with the amino acid proline. CypA is ubiquitously expressed in tissues and inhibited by cyclosporine A (CsA) or nonimmunosuppressive compounds binding tightly to CypA. Initial studies showed that CsA treatment efficiently suppressed viral replication (Watashi et al. 2003). Subsequent studies showed that CypA interacts with NS5A, and that binding appears to catalyze the isomerization of peptidyl-prolyl bonds in NS5A domain I and domain II, resulting in conformational changes in NS5A (Badillo et al. 2017, and references cited therein). These alterations may stimulate the RNA-binding capacity of NS5A and enhance viral replication. Interestingly, CypA and NS5B competitively bind to a similar region of NS5A suggesting a regulatory role for CypA in the formation of virus protein interactions and genome replication (Rosnoblet et al. 2012; Ngure et al. 2016). CypA activity also seems to be required for the formation of HCV Ros, because CypA antagonists such as cyclosporine D block de novo formation of ROs (Madan et al. 2014).

The development of tools such as subgenomic replicons (Lohmann et al. 1999a), the infectious HCV cell culture system that was based on the unique viral isolate JFH-1 (Wakita et al. 2005) and highly permissive hepatoma cell

lines (Blight et al. 2002; Friebe et al. 2005; Zhong et al. 2005), enabled us to study different aspects of the HCV life cycle. However, replication of primary isolates contained in patient serum has been notoriously difficult. Recently, two discoveries have been made that provide explanations for this difficulty. The first discovery was reported by Saeed and colleagues who found that overexpression of SEC14L2 in Huh7.5 cells allowed some replication of nonadapted HCV isolates (Saeed et al. 2015). SEC14L2 is a phosphatidylinositol transfer protein involved in the regulation of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway, cholesterol synthesis, and vitamin E metabolism (Kempná et al. 2003; Mokashi and Porter 2005; Mokashi et al. 2005; Ni et al. 2005; Neuzil et al. 2006). Although the precise mode of action of SEC14L2 in the HCV replication cycle is unknown, part of the mechanism appears to be an enhanced vitamin E-mediated inhibition of lipid peroxidation (Saeed et al. 2015). Consistently, replication of HCV isolates resistant to lipid peroxidation, such as JFH-1, is not stimulated by SEC14L2 expression. The second discovery was made by Harak and coworkers, who studied the mechanism by which RNA replication-enhancing mutations that accumulate in natural HCV sequences on passage in cell culture stimulate HCV replication in hepatoma cell lines. The authors found that these genetic changes are loss-of-function mutations that attenuate the interaction between HCV NS5A and PI4KA (Harak et al. 2016). As alluded to above (see Host Factors and Processes Involved in HCV Replication Organelle Formation), this lipid kinase is required to render HCV-remodeled membranes conducive to RNA replication (Fig. 3, inset). Of note, the majority of hepatoma cell lines express high levels of PI4KA, which appears to be deleterious to robust HCV replication. This defect can be compensated either by mutations that impair the interaction with this lipid kinase, which is the case with HCV replication-enhancing mutations, or by pharmacological inhibition of PI4KA (Harak et al. 2016). Thus, enhanced HCV replication in cell culture is mediated, at least in part, by loss-of-function mutations.

LIPID-MODULATING HOST CELL FACTORS OF RELEVANCE TO HCV GENOME REPLICATION

The extensive remodeling of intracellular membranes induced by HCV and the assembly of highly lipidated HCV particles require the production and accumulation of distinct lipids. Consistently, lipidomic profiling of HCV-infected cells revealed distinct alterations of host cell lipid composition (Diamond et al. 2010). In addition to the importance of cholesterol in HCV replication described above, fatty acid biosynthetic pathways are also required for efficient HCV replication. Indeed, inhibition of fatty acid synthesis by blocking acetyl-CoA carboxylase decreases viral replication (Kapadia and Chisari 2005). Furthermore, increases in saturated and monounsaturated fatty acids enhance HCV replication, whereas increases in polyunsaturated fatty acids suppress replication (Kapadia and Chisari 2005). These results suggest that specific lipids and membrane fluidity are important for the function of the membranous HCV RO. Moreover, distinct lipids might form lipid rafts required for assembly and activity of the viral replicase. Consistent with this assumption, extraction of cholesterol from the replicase complex impairs replicase activity (Paul et al. 2013). In addition, sphingolipids were shown to stimulate HCV replicase activity (Weng et al. 2010; Hirata et al. 2012).

Lipid metabolism is regulated by a family of sterol regulatory element-binding proteins (SREBPs). SREBPs are transcription factors that activate the expression of more than 30 genes involved in biosynthesis or uptake of cholesterol, fatty acids, triglycerides, and phospholipids. In the case of HCV, virus-induced ER stress or viral proteins such as NS4B (Waris et al. 2007; Park et al. 2009) trigger the normally ER-localized inactive SREBP precursor to traffic to the Golgi, where it is proteolytically cleaved by site 1 protease (S1P) and S2P (Fig. 3). The released amino-terminal fragment is transported into the nucleus and activates transcription of target genes such as fatty acid synthase and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA), which is the rate-limiting

enzyme of the cholesterol biosynthetic mevalonate pathway. Moreover, SREBPs are also activated by the cellular RNA helicase DDX3X. It was shown that the 3'UTR in the HCV RNA genome binds to DDX3X, which acts as an intracellular sensor to activate a signaling cascade that induces, via CBP-p300, the expression of SREBP, which in turn activates the expression of lipogenic genes (Li et al. 2013a). Finally, NS5B was reported to bind to fatty acid synthase, which activates the viral polymerase; however, this activation appears to be mediated by direct protein-protein interaction rather than by lipids produced by the synthase (Huang et al. 2013). Although these results illustrate the profound effect of HCV on host cell lipid metabolism, further studies are needed to clarify how distinct lipids contribute to or regulate viral RNA replication.

CONCLUDING REMARKS

The plethora of studies focused on uncovering the mechanisms of HCV RNA replication have had a significant impact on both our basic understanding of these biological processes and in the production of HCV-specific direct-acting antiviral drugs. However, there remain many unanswered questions in regard to these viral processes, and addressing them will improve our understanding of the basic principles of HCV replication. Some of the key unresolved topics in HCV replication are the mechanisms driving RO biogenesis, the precise subcellular location of viral RNA replication, the 3D architecture and functionality of the viral replicase machinery, the mechanisms responsible for governing the fate of newly synthesized viral RNA, including the trafficking of this RNA between different viral compartments, the host cell factors involved in HCV RNA replication, the mechanism whereby NS5A exerts its multiplicity of functions, and how HCV replication intermediates are concealed from the immune response. A greater mechanistic understanding of these processes will not only give insight into HCV replication strategies but also will generate knowledge that might be applicable to a large range of human pathogens. For instance, all pos-



itive-strand RNA viruses induce the reorganization of cellular membranes, several of which, including coronaviruses and picornaviruses, induce membrane structures similar to HCV (Paul and Bartenschlager 2013; Neufeldt et al. 2018). The similarity of these structures invites speculation that similar mechanisms are operating with these viruses. Therefore, insights gained from the study of HCV replication and membrane manipulation have the potential for application to related viruses and could lead to the identification of novel strategies for development of broad-spectrum antivirals by targeting host cell pathways and factors that are commonly used by these viruses. Moreover, the study of how HCV interacts with its host to create an environment conducive to replication has also contributed to our understanding of basic cellular processes. Examples of this include important insights into polymerase or helicase structure and activity, cellular lipid metabolism and the role of distinct lipids for robust viral replication, ER membrane dynamics, exploitation of miRNAs to protect the viral genome from degradation and to alter RNA structure, and sensing of viral RNA and viral countermeasures to suppress innate immunity. Thus, the combined research on HCV replication has had an enormous impact on various fields, far beyond HCV itself. Taking into account the excellent toolbox that has been generated to study this virus, we can expect that continued research of HCV–host cell interaction will continue to make important discoveries with far-reaching implications.

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K. Tabata et al.

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K. Tabata et al.

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K. Tabata et al.

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