Replication of hepatitis C virus

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Abstract | Exciting progress has recently been made in understanding the replication of hepatitis C virus, a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma worldwide. The development of complete cell-culture systems should now enable the systematic dissection of the entire viral lifecycle, providing insights into the hitherto difficult-to-study early and late steps. These efforts have already translated into the identification of novel antiviral targets and the development of new therapeutic strategies, some of which are currently undergoing clinical evaluation.

Viral half-life
The time taken for half of the viruses to be cleared.

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Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) worldwide. A protective vaccine is not yet available and therapeutic options are still limited. Current standard therapy, which is pegylated interferon-α combined with ribavirin, is often difficult to tolerate and results in a sustained virological response in only 50% of patients. As a consequence, the number of patients presenting with the long-term sequelae of chronic hepatitis C, including HCC, is expected to increase further over the next 20 years[1]. Given this scenario, there is an urgent need to develop more effective and better tolerated therapies for chronic hepatitis C. A detailed understanding of the viral lifecycle underpins these efforts.

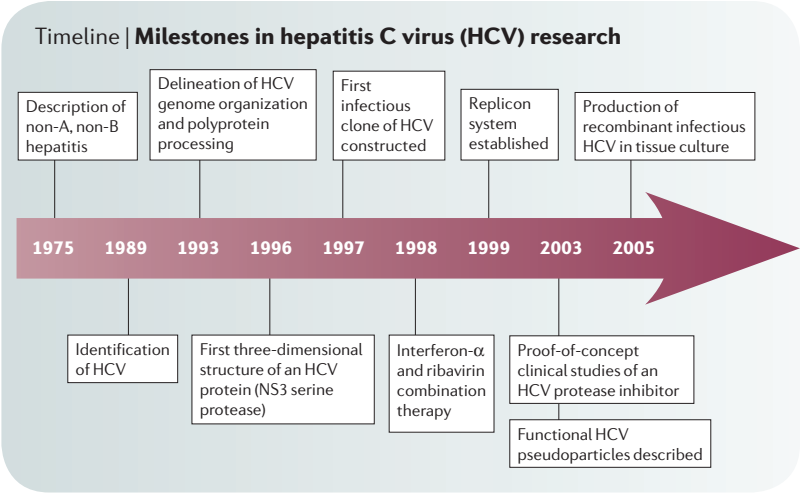
HCV was identified in 1989 by immunoscreening an expression library with serum from a patient with post-transfusion non-A, non-B hepatitis[2]. However, the virus was not visualized conclusively, the low viral titres in serum and liver tissue precluded biochemical characterization of native viral products and, most importantly, it was not possible to culture HCV efficiently *in vitro*, impeding elucidation of the viral lifecycle and the development of specific antiviral agents and preventive vaccines. Despite these obstacles, great progress has been made in the study of HCV over the past 18 years using heterologous expression systems[3,4], functional cDNA clones that are infectious *in vivo* in chimpanzees[5], a replicon system[6,7], pseudoparticles (engineered retroviral particles bearing functional HCV envelope proteins) that enable the study of viral entry under reproducible and conveniently measurable conditions[8,9] and, most recently, complete cell-culture systems[10–14]. Selected milestones in HCV research are shown in the TIMELINE. Here we highlight recent advances in the molecular biology of HCV. The reader is referred to REFS 15–18 for more comprehensive reviews of the molecular virology and pathogenesis of hepatitis C.

Classification and genetic variability

HCV is classified in the *Hepacivirus* genus within the *Flaviviridae* family, which includes the classical flaviviruses (for example, yellow fever, dengue and tick-borne encephalitis viruses), the animal pestiviruses (for example, bovine viral diarrhoea virus) and GB viruses A (GBV-A), GBV-B and GBV-C[19]. HCV has a positive-strand RNA genome that is composed of a 5′-non-coding region (NCR), which includes an internal ribosome entry site (IRES), an open reading frame that encodes structural and non-structural proteins, and a 3′-NCR. The structural proteins, which form the viral particle, include the core protein and the envelope glycoproteins E1 and E2. The non-structural proteins include the p7 ion channel, the NS2-3 protease, the NS3 serine protease and RNA helicase, the NS4A polypeptide, the NS4B and NS5A proteins and the NS5B RNA-dependent RNA polymerase (RdRp).

HCV infection is a highly dynamic process with a viral half-life of only a few hours and production and clearance of an estimated 10^{12} virions per day in a given individual[20]. This high replicative activity, together with the lack of a proof-reading function of the viral RdRp, is the basis of the high genetic variability of HCV. These properties are similar to those of HIV infection and provide a strong rationale for the development and implementation of antiviral combination therapies.

HCV isolates can be classified into genotypes and subtypes[21]. There are 6 major genotypes that differ in their nucleotide sequence by 30–35%. Patients infected with genotype 1 do not respond as well to interferon-α-based therapy as those infected with genotype 2 or 3. Within an HCV genotype, several subtypes (designated a, b, c and so on) can be defined that differ in their nucleotide sequence by 20–25%. The term quasispecies refers to the genetic heterogeneity of the population of HCV genomes that coexist in an infected individual[21].



More than 40,000 HCV sequences have been deposited in databases. Three complementary databases are specifically dedicated to HCV: the European HCV Database, the Los Alamos National Laboratory HCV Database and the Japanese Hepatitis Virus Database[22]. These offer several specialized features, including useful tools for HCV sequence comparisons, structural analyses of viral proteins and $CD4^+$ and $CD8^+$ T-cell epitope information.

Model systems

Different model systems have been used to study specific aspects of the viral lifecycle[18] (BOX 1). The development of a replicon system for HCV was a particularly important breakthrough[6]. The prototype subgenomic replicon was a bicistronic RNA in which the portion of the HCV genome encoding the structural and some of the non-structural proteins was replaced by the neomycin phosphotransferase gene. Translation of the non-structural proteins 3–5B was initiated by a second, heterologous IRES. With this success it became possible, for the first time, to study HCV RNA replication in Huh-7 human HCC cells *in vitro*.

Use of the replicon system has allowed the genetic dissection of HCV RNA elements and proteins, provided material for biochemical and ultrastructural characterization of the viral replication complex, and facilitated drug discovery, including investigations of antiviral resistance. Moreover, it has been exploited to study interactions between viral components and the host cell.

Since the original reports of functional genotype 1b replicons, replicons for genotypes 1a and 2a have also been constructed, as well as derivatives that express easily quantifiable marker enzymes in a separate cistron. In addition, full-length replicons and HCV genomes that replicate efficiently in tissue culture have been developed, and the range of permissive host cells has been expanded to other hepatic as well as non-hepatic cell lines. Finally, replicons have been established that harbour the green fluorescent protein in permissive sites within NS5A and allow tracking of functional HCV replication complexes in living cells (REF. 23 and references therein).

Interestingly, selected amino-acid substitutions (cell culture adaptive mutations) were found to increase the efficiency of replication by several orders of magnitude[7,24]. In addition, the efficiency of replicon RNA amplification was found to be determined by selection for particularly permissive cells within a given population of Huh-7 cells[25,26]. However, one puzzling and disappointing observation was that full-length HCV genomes with adaptive mutations were incapable of producing infectious virus. It was hypothesized that either the host cell lacked some factor(s) critical for particle formation and release, or that the adaptive mutations required for efficient replication in tissue culture interfered with packaging, assembly or release of virus. In support of the second idea an inverse correlation was found between mutations that permit efficient replication of HCV RNA in Huh-7 cells *in vitro* and productive replication after intrahepatic inoculation into chimpanzees *in vivo*[27].

An unexpected twist came when an HCV genotype 2a clone isolated from a Japanese patient with a rare case of fulminant hepatitis C, designated as JFH-1 (for Japanese fulminant hepatitis 1) was found to replicate in Huh-7 and other cell lines without the requirement for adaptive mutations[28]. Subsequently, it was found that cloned JFH-1 genomes transfected into Huh-7 cells produced virus (designated as HCVcc for cell-culture-derived HCV) that is infectious for naive Huh-7 cells, allowing, for the first time, *in vitro* studies of the complete lifecycle of HCV[11]. Efficiency was improved by using highly permissive Huh-7 sub-lines[10,12] and chimaeric clones that consisted of the 5′- and 3′-NCRs, as well as the NS3-5B region of JFH-1 and the C-NS2 region of a different genotype 2a isolate designated J6 (REFS 10,14). Virus produced in tissue culture was infectious *in vivo* in chimpanzees[11,13] and in immunodeficient mice with partially human livers[13]. Viral inocula derived from these animal models were infectious for naive Huh-7 cells in culture[13]. Thus, 17 years after the identification of HCV, Koch's postulates for this infectious agent were

Box 1 | *In vitro* and *in vivo* models to study HCV

***In vitro* models**
- Transient cellular expression systems
- Stable, transfected cell lines (constitutive or inducible expression)
- Replicons (subgenomic or full-length; selectable or transient)
- Related viruses (for example, GBV-B (GB virus B) or BVDV (bovine viral diarrhoea virus))
- Retroviral pseudoparticles displaying functional HCV (hepatitis C virus) glycoproteins
- Recombinant infectious HCV

***In vivo* models**
- Chimpanzee (*Pan troglodytes*)
- Transgenic mice
- Immunodeficient mice or hepatocellular reconstitution models
- Related viruses (for example, GBV-B or BVDV)

Permissive cell
A cell that can be infected by, or supports the replication of, a virus.

Fulminant hepatitis C
Fulminant hepatic failure refers to the rapid development of severe acute liver injury, with impaired synthetic function and encephalopathy, in a person who previously had a normal liver or had well-compensated liver disease.

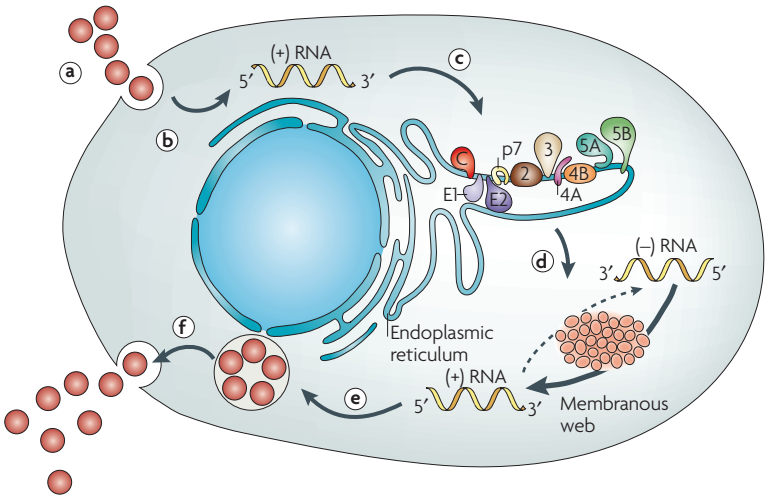


Figure 1 | **Lifecycle of hepatitis C virus (HCV).** Virus binding and internalization (**a**); cytoplasmic release and uncoating (**b**); IRES-mediated translation and polyprotein processing (**c**); RNA replication (**d**); packaging and assembly (**e**); virion maturation and release (**f**). The topology of HCV structural and non-structural proteins at the endoplasmic reticulum membrane is shown schematically. HCV RNA replication occurs in a specific membrane alteration, the membranous web. Note that IRES-mediated translation and polyprotein processing, as well as membranous web formation and RNA replication, which are illustrated here as separate steps for simplicity, might occur in a tightly coupled fashion. IRES, internal ribosome entry site.

finally fulfilled. Classical virological techniques can now be applied to the study of previously unexplored lifecycle steps, including viral entry, genome packaging, virion assembly, maturation and release. Although low levels of infectious virus have been obtained from a genotype 1a isolate with five adaptive mutations[29], the challenge remains to develop robust systems for all HCV genotypes.

The virus and its lifecycle

The lifecycle of HCV is illustrated in FIG. 1.

Virion structure. Although exciting progress has been made towards determining virion structures of some of the related alphaviruses and flaviviruses, for example, dengue virus[30], HCV has not been definitively visualized and its structure remains to be elucidated. Based on filtration and electron microscopic studies, HCV particles are 40–70 nm in diameter (REF. 11 and references therein). It is thought that the core protein and the envelope glycoproteins E1 and E2 are the principal protein components of the virion. E1 and E2 are presumably anchored to a host cell-derived double-layer lipid envelope that surrounds a nucleocapsid composed of multiple copies of the core protein and the genomic RNA.

HCV circulates in various forms in the infected host and can be associated with low-density lipoproteins (LDL) and very-low-density lipoproteins (VLDL), both of which seem to represent the infectious fraction, and also circulates as virions bound to immunoglobulins and free virions (reviewed in REF. 31). This feature may explain its unusually heterogenous and low buoyant density (peak infectivity near 1.10 g per ml).

Viral entry. HCV only infects humans and chimpanzees. Hepatocytes are the main target cells but infection of B cells, dendritic cells and other cell types has also been reported. CD81, a tetraspanin protein that is found on the surface of many cell types, including hepatocytes[32], the LDL receptor (LDLR)[33], scavenger receptor class B type I (SR-BI)[34] and, most recently, claudin-1 (REF. 35) have, among others, been proposed as HCV receptors (REFS 36,37; FIG. 2).

The LDLR is an attractive candidate receptor because of the association of HCV with LDL and VLDL. However, its precise role remains to be determined. Together with glycosaminoglycans, the LDLR and other cell surface proteins involved in serum lipoprotein binding and metabolism might serve as primary collectors of HCV particles for further targeting to CD81 and additional receptor components (FIG. 2). HCV E2 also binds to DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) and L-SIGN (liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin). L-SIGN is a calcium-dependent lectin expressed on liver sinusoidal endothelial cells that may facilitate the infection process by trapping the virus for subsequent interaction with hepatocytes.

Both CD81 and SR-BI bind E2 and are necessary but not sufficient for HCV entry. Expression of CD81 in CD81-negative hepatocyte-derived cell lines confers susceptibility to HCV pseudoparticles (HCVpp) and HCVcc. Blocking antibodies against CD81 or SR-BI, recombinant soluble CD81, oxidized LDL or siRNA-mediated down-regulation of CD81 expression reduce infectivity. However, expression of CD81 and SR-BI in non-hepatocyte-derived cell lines does not confer susceptibility to HCV infection, indicating that additional hepatocyte-specific factors must be required for HCV entry.

Using an iterative expression cloning approach the tight junction component claudin-1 was recently identified as an HCV co-receptor[35]. Claudin-1 was found to be essential for HCV entry into hepatic cells and rendered non-hepatic cells permissive to HCV infection. However, certain cell types were found to be non-permissive despite expression of CD81, SR-BI and claudin-1, indicating that one or more additional HCV entry factor(s) remain to be discovered. Claudin-1 acts at a late stage of the entry process, after virus binding and interaction with CD81. Interestingly, other tight junction-associated molecules have been shown to serve as viral (co-)receptors. In this context, coxsackievirus B entry requires initial binding to a primary receptor (decay-accelerating factor) on the lumenal cell surface, followed by lateral migration of the virus–receptor complex to the tight junction, where interaction with the co-receptor CAR (coxsackievirus and adenovirus receptor) and uptake into the host cell occur[38]. Future studies should clarify if HCV follows a similar pathway for cell entry.

HCV enters by clathrin-mediated endocytosis[39], with transit through an endosomal, low pH compartment[40,41] and presumably endosomal membrane fusion. The structural basis for low pH-induced membrane fusion has been elucidated for related flaviviruses and alphaviruses, including dengue virus and Semliki Forest

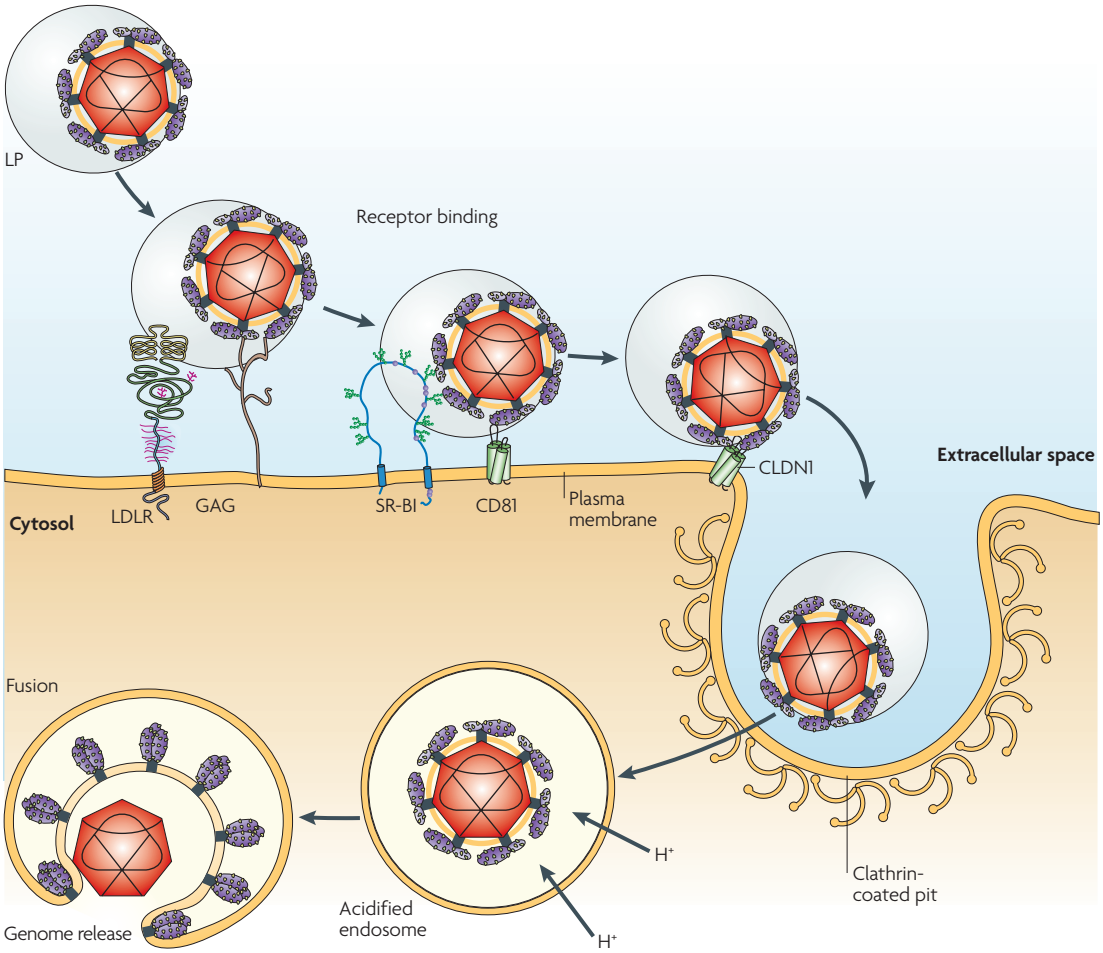


Figure 2 | **Current model for hepatitis C virus (HCV) entry.** Circulating HCV particles can be associated with low- and very-low-density lipoproteins (LP). Virus binding to the cell surface and entry may involve the low density lipoprotein receptor (LDLR), glycosaminoglycans (GAG), scavenger receptor class B type I (SR-BI), the tetraspanin protein CD81 and claudin-1 (CLDN1). CLDN1 functions at a late stage of cell entry, possibly at tight junctions of polarized hepatocytes. Internalization depends on clathrin-mediated endocytosis. Acidification of the endosome induces HCV glycoprotein membrane fusion. Little is known about the uncoating process, which results in genome release into the cytosol.

virus[42,43]. The envelope proteins of these viruses have an internal fusion peptide that is exposed during low pH-mediated domain rearrangement and trimerization of the protein. The scaffolds of these so called class II fusion proteins are remarkably similar, suggesting that entry of all viruses in the *Flaviviridae* family, including HCV, may include a class II fusion step. However, the mechanisms involved in activating HCV for low pH-induced fusion, the fusion step, and the identity of the fusion peptide(s) have not yet been characterized.

Genome organization

HCV contains a 9.6-kb positive-strand RNA genome composed of a 5′-NCR, a long open reading frame encoding a polyprotein precursor of about 3,000 amino acids, and a 3′-NCR (FIG. 3). The mechanisms regulating translation, replication and packaging of the viral genome are unknown. Current evidence indicates that NS5A might function as a molecular switch between replication and assembly, as the phosphorylation state of this protein affects HCV RNA replication, and there is an inverse correlation between NS5A adaptive mutations facilitating replication and virus production (see below).

The 5′-NCR is highly conserved among different HCV isolates and contains an IRES that is essential for cap-independent translation of the viral RNA. The 5′-NCR is composed of four highly ordered domains numbered I to IV (FIG. 3). Domain I is not required for IRES activity, but domains I and II are both essential for HCV RNA replication[44]. Recent research showed that an abundant liver-specific microRNA (miRNA), miR-122, bound to the HCV 5′-NCR and enhanced viral RNA replication[45]. This finding provided the first example of a virus that exploits a cellular miRNA and uncovered a new angle for possible antiviral intervention by antagonizing or abrogating the function of miR-122.

The 3′-NCR is composed of a short variable region, a poly(U/UC) tract with an average length of 80 nucleotides, and an almost invariant 98 nucleotide RNA element, designated the X-tail[46,47]. The conserved elements in the 3′-NCR, including a minimal poly(U) tract of about 25 bases, are essential for replication in cell culture[48,49] and *in vivo*[50,51].

Pseudoknot
A pseudoknot is an RNA secondary structure containing two stem-loop structures in which the first stem's loop forms part of the second stem.

Besides the 5′- and 3′-NCRs, an essential *cis*-acting replication element (CRE) was identified in the sequence that encodes the C-terminal region of NS5B[52]. An essential stem-loop, designated 5B-SL3.2, was identified within a larger cruciform RNA element, designated 5B-SL3 (FIG. 3). The upper loop of 5B-SL3.2 was found to interact with a stem-loop in the X-tail, suggesting that a pseudoknot is formed at the 3′-end of the HCV genome that is essential for RNA replication[53].

IRES-mediated translation initiation

Domains II, III and IV of the 5′-NCR, together with the first 24–40 nucleotides of the core coding region, constitute the IRES. HCV translation initiation occurs through the formation of a binary complex between the IRES and the 40S ribosomal subunit, followed by the assembly of a 48S-like complex at the AUG initiation codon after the association of eukaryotic translation initiation factor 3 (eIF3) and ternary complex (eIF2•Met-tRNA$_i$•GTP). Finally, the rate limiting step is the GTP-dependent association of the 60S subunit to form an 80S complex[54].

High-resolution structures are available for the critical elements of the HCV IRES[55,56]. In addition, three-dimensional structures of the HCV IRES bound to the 40S ribosomal subunit and to eIF3 were resolved by cryoelectron microscopy[57,58]. It was found that IRES binding induces a significant conformational change in the 40S subunit that seems to be required for assembly of the 80S ribosome. In addition, modelling of the eIF3•IRES•40S complex revealed that eIF3 uses the HCV IRES or, in cap-dependent translation, the canonical translation initiation factor eIF4F in structurally similar ways to position the positive-strand viral RNA or a cellular mRNA, respectively, near the exit site of the 40S ribosomal subunit, promoting initiation complex assembly[58].

Polyprotein processing

Translation of the HCV open reading frame yields a polyprotein precursor that is co- and post-translationally processed by cellular and viral proteases into the mature structural and non-structural proteins (FIG. 3). The structural proteins and the p7 polypeptide are processed by the endoplasmic reticulum (ER) signal peptidase whereas the non-structural proteins are processed by two viral proteases, the NS2–3 protease and the NS3–4A serine protease.

Structure and function of the viral proteins

Core. The first structural protein encoded by the HCV open reading frame is the core protein which forms the viral nucleocapsid. An internal signal sequence located between the core and E1 sequences targets the nascent polypeptide to the ER membrane for translocation of the E1 ectodomain into the ER lumen. Cleavage of the signal sequence by signal peptidase yields an immature 191-amino-acid core protein. Further C-terminal processing by signal peptide peptidase yields the mature 21-kDa core protein of 173–179 amino acids[59].

The N-terminal hydrophilic domain (D1) of the core protein contains a high proportion of basic amino-acid residues and has been implicated both in RNA binding and homo-oligomerization. The core protein is an

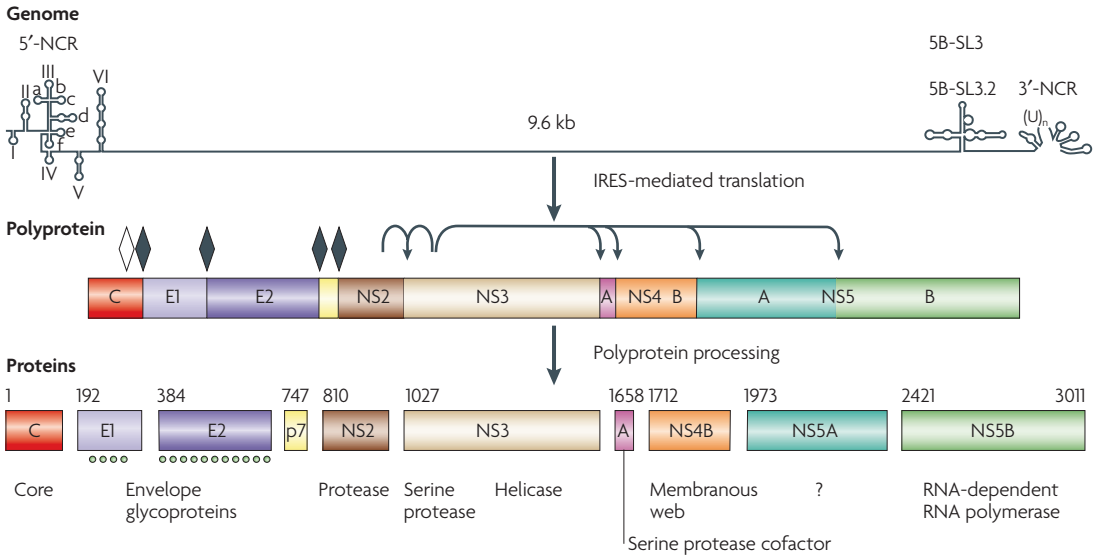


Figure 3 | **Genetic organization and polyprotein processing of hepatitis C virus (HCV).** The 9.6-kb positive-strand RNA genome is schematically depicted at the top. Simplified RNA secondary structures in the 5′- and 3′-non-coding regions (NCRs) and the core gene, as well as the NS5B stem-loop 3 *cis*-acting replication element (5B-SL3) are shown. Internal ribosome entry site (IRES)-mediated translation yields a polyprotein precursor that is processed into the mature structural and non-structural proteins. Amino-acid numbers are shown above each protein (HCV H strain; genotype 1a; GenBank accession number AF009606). Solid diamonds denote cleavage sites of the HCV polyprotein precursor by the endoplasmic reticulum signal peptidase. The open diamond indicates further C-terminal processing of the core protein by signal peptide peptidase. Arrows indicate cleavages by the HCV NS2–3 and NS3–4A proteases. Dots in E1 and E2 indicate the glycosylation of the envelope proteins (4 and 11 *N*-linked glycans, respectively, in the HCV H strain). Note that polyprotein processing, illustrated here as a separate step for simplicity, occurs co- and post-translationally.

α-helical protein[60] that is found on membranes of the ER, in membranous webs (see below) and on the surface of lipid droplets. The association with lipid droplets, which is mediated by the central, relatively hydrophobic domain (D2) and ensures its folding[60], may have a role during viral replication and/or virion morphogenesis. In addition, it has been speculated that the interaction of the core protein with lipid droplets might affect lipid metabolism, contributing to the development of liver steatosis, which is often seen in hepatitis C, particularly in patients infected with genotype 3 (REF. 61).

Envelope glycoproteins. The envelope proteins E1 and E2 are glycosylated and form a non-covalent complex, which is believed to be the building block for the viral envelope[62]. HCV glycoprotein maturation and folding is a complex process that involves the ER chaperone machinery and depends on disulphide bond formation as well as glycosylation. The transmembrane domains of E1 and E2, located at their C-termini, are involved in heterodimerization and have ER retention properties. Each of them is composed of two stretches of hydrophobic amino-acid residues that are separated by a short polar segment. The second hydrophobic patch functions as an internal signal peptide for the downstream E2 and p7 proteins. Before signal sequence cleavage, the E1 and E2 transmembrane domains are proposed to adopt a hairpin structure at the translocon. After cleavage, the signal sequence is reorientated towards the cytosol, resulting in a single transmembrane passage. Determination of the three-dimensional structures of E1 and E2 will be key to elucidating the receptor binding and fusion processes that are mediated by these proteins.

p7. p7 is a 63-amino-acid polypeptide that is often incompletely cleaved from E2. It has two transmembrane segments connected by a short cytoplasmic loop, and the N terminus and C terminus are orientated toward the ER lumen[63]. HCV p7 is not required for RNA replication *in vitro* but is essential for productive infection *in vivo*[64]. It has been reported to form oligomers and to have cation channel activity[65,66], suggesting that it belongs to the viroporin family, and could have an important role in viral particle maturation and release. It might therefore be an attractive target for antiviral intervention.

NS2–3 protease. The NS2–3 protease, also known as the autoprotease, has been one of the difficult-to-study HCV proteins. It is dispensable for RNA replication *in vitro* but is essential for the complete replication cycle *in vitro* and *in vivo*[14,51]. The catalytic activity of the NS2–3 protease resides in the C-terminal half of NS2 and the N-terminal one-third of NS3 (REFS 67,68). Site-directed mutagenesis has shown that amino acids His143, Glu163 and Cys184 are essential for proteolytic activity[67,68]. As with all of the HCV proteins, NS2 is associated with intracellular membranes. Its N-terminal domain contains at least one (possibly three) transmembrane segments. Recombinant proteins lacking this domain retain enzymatic activity[69,70]. The crystal structure of the NS2 protease domain (amino-acid residues 94–217, $NS2^{pro}$) has recently been solved[71]. $NS2^{pro}$ forms a dimer that has two composite active sites (FIG. 4). Each active site is composed of residues from the two monomers, that is, residues His143 and Glu163 are contributed by one monomer and Cys184 by the other.

This structure changed the current view of HCV polyprotein processing, as NS2–3 cleavage was previously thought to occur as a unimolecular reaction in *cis* (hence the designation autoprotease). However, the requirement for dimerization to form an active NS2-3 protease suggests that NS2–3 cleavage and formation of an active replication complex may be regulated by the concentration of NS2. Solving the structure of the N-terminal membrane domain of NS2 might yield insights into additional functions of NS2, which was recently found to be essential for the production of infectious virus, possibly by affecting a late step of the viral lifecycle[14]. Interestingly, efficient HCV chimaeras were constructed using a fusion site in NS2 (see above). Plus, intra- and intergenotypic recombinants with crossover sites in NS2 have been identified in patients infected with HCV[72,73]. Clearly, the availability of complete cell-culture systems for HCV should now permit the different functions of NS2 in the viral lifecycle to be explored.

NS3–4A complex. NS3 is a multifunctional protein, with a serine protease located in the N-terminal one-third and an RNA helicase/NTPase located in the C-terminal two-thirds of the protein. Both enzyme activities have been well characterized, and high-resolution structures have been solved (REF. 74 and references therein). The NS4A polypeptide functions as a cofactor for the NS3 serine protease. Its central portion is incorporated as an integral component into the enzyme core, and its N-terminal portion is responsible for membrane association of the NS3–4A complex[75].

The NS3–4A serine protease has emerged as a prime target for the design of specific inhibitors as antiviral agents[76]. Its catalytic triad is formed by His57, Asp81 and Ser139. Determinants of substrate specificity include an acidic amino-acid residue at the P6 position, a P1 cysteine (both *trans*-cleavage sites) or threonine (*cis*-cleavage site between NS3 and NS4A), and an amino-acid residue with a small side chain, that is, alanine or serine, at the P1′ position (consensus cleavage sequence D/EXXXXC/T↓S/AXXX). The NS3–4A serine protease has an unusually shallow substrate-binding pocket and therefore requires extended interaction surfaces with the substrate. This posed a challenge to the development of efficient and specific inhibitors. It has recently been shown that the NS3–4A serine protease cleaves and thereby inactivates two crucial adaptor proteins in innate immune sensing, namely Trif[77] (also known as TICAM-1) and Cardif[78] (also known as MAVS[79], IPS-1 (REF. 80) and VISA[81]). Cardif is a mitochondrial outer membrane protein[79]. It was previously observed that a minor fraction of NS3-4A localizes to mitochondria[75]. These findings have major implications for the persistence and pathogenesis of HCV[82].

The NS3 helicase is a member of the superfamily 2 DExH/D-box helicases. It couples unwinding of double-stranded RNA, or of single-stranded RNA regions with

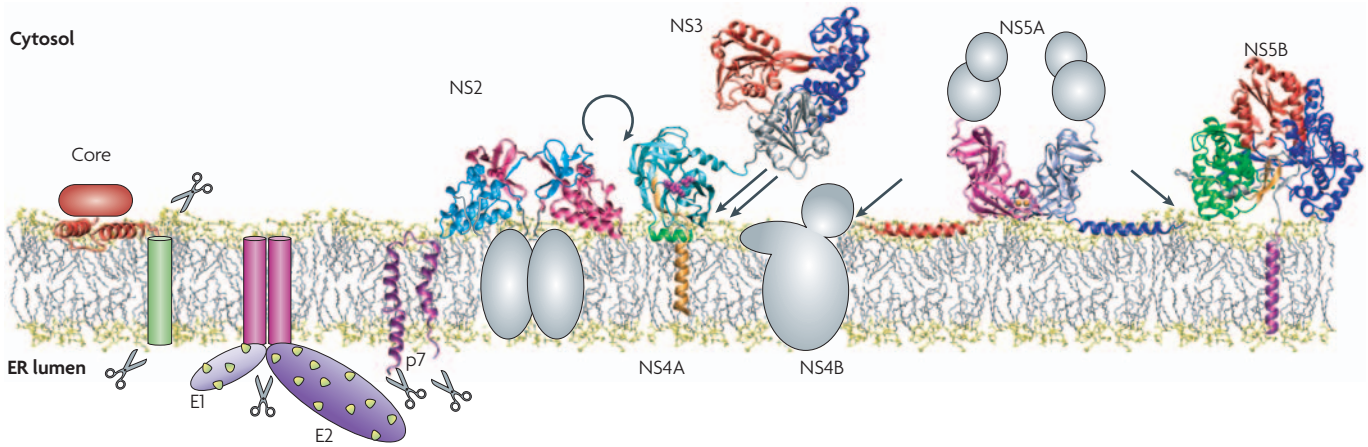


Figure 4 | **Structures and membrane association of hepatitis C virus (HCV) proteins.** Scissors indicate cleavages by the endoplasmic reticulum (ER) signal peptidase, except on the cytosolic side where it indicates the processing of the core protein by signal peptide peptidase. The cyclic arrow denotes cleavage by the NS2–3 protease. Black arrows indicate processing by the NS3–4A protease complex. Known protein structures are shown as ribbon diagrams. The structures and the membrane bilayer are shown at the same scale. Proteins or protein segments of unresolved structures are represented as coloured spheres or cylinders with their approximate sizes. The core protein (red) includes two amphipathic α-helices connected by a hydrophobic loop (D2 domain[60]). The E1 signal peptide (green) is cleaved by signal peptide peptidase[59]. The E1-E2 glycoprotein heterodimer is associated by the C-terminal transmembrane domains[62]; green spots denote glycosylation of the envelope proteins. The p7 viroporin monomer is shown in violet (F.P. *et al.*, unpublished data) and the NS2 catalytic domain has dimer subunits shown in blue and magenta connected to their N-terminal membrane domains[71] (protein data bank (PDB) entry 2HD0). The active-site residues His143, Glu163 and Cys184 are represented as spheres. The NS3 serine protease domain (cyan) is shown, associated with the central NS4A protease activation domain and the N-terminal NS4A transmembrane domain (yellow). The catalytic triad of the NS3 serine protease (His57, Asp81 and Ser139) is represented by spheres (magenta). NS3 helicase domains I, II and III are coloured in silver, red and blue, respectively. This representation of NS3 (derived from PDB entry 1CU1 (REF. 74)) indicates that the helicase domain can no longer interact with the NS3 protease domain when the latter is associated with the membrane through its amphipathic α-helix 11–21 (green) and the transmembrane domain of NS4A (D.M. and F.P., unpublished data). NS4B, with the N-terminal domain interacting in-plane with the membrane (D.M. and F.P., unpublished data), the central transmembrane domain and the C-terminal cytosolic domain are shown. The NS5A domain I dimer (PDB entry 1ZH1 (REF. 98), subunits coloured in magenta and ice blue) and the N-terminal amphipathic α-helix in-plane membrane anchor (PDB entry 1R7E[96], helices coloured in red and blue) are shown in relative position to a phospholipid membrane (adapted from REF. 98). Domains II and III of NS5A are symbolized by grey spheres. The NS5B RNA-dependent RNA polymerase (RdRp) catalytic domain (PDB entry 1GX6 (REF. 104)) is associated with the membrane by its C-terminal transmembrane segment (F.P. and D.M., unpublished data). The fingers, palm and thumb subdomains of the catalytic domain are coloured in blue, red and green, respectively. The catalytic site of the RdRp lies within the centre of the cytosolic domain and the RNA template-binding cleft is located vertically on the right along the thumb subdomain β-loop (orange) and the C-terminal part of segment 545–562 (silver), connecting the cytosolic domain to the transmembrane segment (magenta). The membrane is represented as a simulated model of a 1-palmitoyl-2-oleoyl-3-*sn*-glycero-3-phospholcholine (POPC) bilayer (obtained from D.P. Tieleman). Polar heads and hydrophobic tails of phospholipids (stick structure) are coloured in light yellow and light grey, respectively. The positions of the NS5A in-plane membrane helices at the membrane interface, as well as that of the transmembrane domain of NS5B and that of the p7 monomer, were deduced from molecular dynamics simulations in the POPC bilayer (F. P. and D.M. *et al.*, unpublished data). The positioning of the NS3–4A membrane segments is tentative. The figure was generated from the structure coordinates deposited in the PDB using Visual Molecular Dynamics and rendered with POV-Ray.

extensive secondary structures, to ATP hydrolysis. It was recently shown that NS3 unwinds RNA through a highly coordinated cycle of fast ripping and local pausing that occurs with regular spacing along the duplex substrate, suggesting that nucleic acid motors can function in a manner analogous to cytoskeletal motor proteins[83,84]. Data obtained using optical tweezers revealed that HCV helicase unwinds RNA in steps of 11 base pairs (step size) on average[85]. Although appealing as an antiviral target, specific inhibitors of the HCV NS3 helicase have only recently been described[86]. It is unknown why the serine protease and RNA helicase activities are physically linked but evidence for cross-talk between these two essential enzymatic activities is emerging[87].

NS4B. NS4B is a relatively poorly characterized 27-kDa protein. One of its functions is to induce the formation of the membranous web, the specific membrane alteration that serves as a scaffold for the HCV replication complex[88] (see below). It is predicted to contain four transmembrane segments and has recently been reported to be palmitoylated at two C-terminal cysteine residues and to form oligomers[89]. However, the detailed membrane topology and the role of oligomerization in NS4B function remain to be elucidated.

NS5A. NS5A is a phosphoprotein that can be found in basally phosphorylated (56 kDa) and hyperphosphorylated (58 kDa) forms. Recent biochemical and

functional studies suggest that the α-isoform of protein kinase CKI may be responsible for NS5A hyperphosphorylation[90]. However, it is unknown if additional cellular kinases are involved in generating the different phosphoforms of NS5A.

Phosphorylation of NS5A is a conserved feature among hepaciviruses and pestiviruses and is also found in flavivirus NS5 proteins, arguing that it has an important role in the HCV lifecycle[91]. Indeed, cell culture-adaptive mutations often affect centrally located serine residues that are required for hyperphosphorylation, suggesting that the phosphorylation state of NS5A modulates the efficiency of HCV RNA replication[92–94]. According to one model, hyperphosphorylation of NS5A reduces interaction with the human vesicle-associated membrane protein-associated protein A (hVAP-A)[92]. This vesicle sorting protein has been implicated in directing non-structural proteins to lipid rafts that might be involved in viral RNA replication[95].

NS5A is a monotopic protein anchored to the membrane by an N-terminal amphipathic α-helix embedded in-plane into the cytosolic leaflet of the membrane bilayer (REF. 96 and references therein). This helix has a hydrophobic, tryptophan-rich face that is embedded in the cytosolic membrane interface, whereas the polar, charged face is exposed to the cytosol and is probably involved in specific protein–protein interactions that are essential for the formation of a functional HCV replication complex[96].

Comparative sequence analyses and limited proteolysis of recombinant NS5A have defined three domains[97]. The crystal structure of the conserved domain I immediately following the membrane-anchoring α-helix revealed a dimeric structure and defined surface properties that may be involved in interactions with viral and cellular proteins, membranes and RNA[98] (FIG. 4). In fact, the groove generated by the 'claw-like' dimer is hypothesized to face away from the membrane, where it could accommodate either single- or double-stranded RNA. The deep, highly basic portion of the groove could favour contacts with RNA whereas the 'arms' extending out past this groove are more acidic, and might serve to prevent RNA from exiting the groove. RNA binding properties of NS5A have been confirmed biochemically[99]. According to one hypothesis, multiple NS5A dimers could form a two-dimensional array on intracellular membranes, thereby creating a 'basic railway' that would allow the sliding of RNA. Other regions of NS5A would protect it from degradation by cellular RNAses, or from recognition by double-strand RNA-induced antiviral defence mechanisms. According to this model, NS5A would tether the viral RNA onto intracellular membranes and coordinate its different fates during HCV replication. Given that numerous core protein molecules are needed to form a nucleocapsid for incorporation of one genomic RNA, a large excess of non-structural proteins are produced in an infected cell. So it is plausible that these proteins have additional functions as arrays or lattices on membranes. In fact, it has been shown that only a small proportion of HCV non-structural proteins expressed in cells harbouring HCV replicons are actively engaged in RNA replication[100,101]. As discussed above, accumulation of excess non-structural proteins might also be important for antagonizing host cell innate antiviral pathways prior to initiating RNA replication.

NS5B. HCV replication proceeds by the synthesis of a complementary negative-strand RNA using the genome as a template and the subsequent synthesis of genomic positive-strand RNA from this negative-strand RNA template. The key enzyme responsible for both of these steps is the NS5B RdRp. This viral enzyme has been extensively characterized (REFS 102–104 and references therein), and NS5B has emerged as a major target for antiviral intervention. The HCV NS5B protein contains motifs that are shared by all RdRps, including the hallmark GDD sequence within motif C, and has, based on the similarity of the enzyme structure with the shape of a right hand, the classical fingers, palm and thumb subdomains (FIG. 4). A special feature of the HCV RdRp is that extensive interactions between the fingers and thumb subdomains result in a completely enclosed active site. This feature is shared by other RdRps[105]. Similar to poliovirus RdRp[106], oligomerization of HCV NS5B has been reported to be important for cooperative RNA synthesis activity[107].

The HCV RdRp is a so called 'tail-anchored protein'. Membrane association is mediated by the C-terminal 21 amino-acid residues, which are dispensable for polymerase activity *in vitro* but indispensable for RNA replication in cells (REF. 108 and references therein). Membrane targeting occurs by a post-translational mechanism and results in integral membrane association of NS5B (FIG. 4).

ARFP/F proteins. An alternative reading frame (ARF) was identified in the HCV core coding region that, as a result of a –2/+1 ribosomal frameshift in genotype 1a, has the potential to encode a protein of up to 160 amino acids, designated ARFP (alternative reading frame protein) or F (frameshift) protein (reviewed in REF. 109). Amino-acid sequencing indicated that the frameshift probably occurs at, or near to, codon 11 of the core protein sequence. However, multiple, and in part isolate-dependent, frameshifting events, as well as internal translation initiation, have also been reported. Detection of antibodies and T cells that are specific for the ARFP/F proteins in patients with hepatitis C suggests that these proteins are expressed during HCV infection. Yet the ARFP/F proteins are not required for HCV RNA replication *in vitro* or *in vivo*[110]. Recent evidence suggests that one or more conserved and functionally important RNA elements are present in the core gene/ARF[110]. However, the functions, if any, of the ARFP/F proteins in the lifecycle and pathogenesis of HCV remain to be elucidated.

The viral replication complex

Formation of a membrane-associated replication complex, composed of viral proteins, replicating RNA and altered cellular membranes, is a hallmark of all positive-strand RNA viruses investigated so far (reviewed in REFS 111,112). Depending on the virus, replication might occur on altered membranes derived from the ER, Golgi apparatus, mitochondria or even lysosomes. The role of membranes in viral RNA synthesis is not

well understood. It may include: the physical support and organization of the RNA replication complex[106]; the compartmentalization and local concentration of viral products[113]; tethering of the viral RNA during unwinding; provision of lipid constituents important for replication; and protection of the viral RNA from double-strand RNA-mediated host defences or RNA interference.

A specific membrane alteration, named the membranous web, was identified as the site of RNA replication in Huh-7 cells containing subgenomic HCV replicons[114] (FIG. 5). Formation of the membranous web was induced by NS4B alone and was very similar to the 'sponge-like inclusions' previously observed using electron microscopy in the liver of HCV-infected chimpanzees[88]. It is currently hypothesized that the membranous web is derived from ER membranes. Ongoing studies are aimed at characterizing the host factors and cellular processes involved in the formation of the HCV replication complex.

Recent studies have revealed a complex interaction between HCV RNA replication and cellular lipid metabolism, presumably through the trafficking and association of viral and host proteins with intracellular membranes. In cell culture, HCV RNA replication is stimulated by saturated and mono-unsaturated fatty acids, and inhibited by polyunsaturated fatty acids or inhibition of fatty acid synthesis. These results suggest that membrane fluidity is important for the function of the replication complex. In addition, it was found that geranylgeranylation of one or more host proteins is required for HCV RNA replication[115,116]. One such protein is FBL-2 which contains an F-box motif and is likely to be involved in targeting proteins for degradation, although the natural substrates are currently unknown[117]. Such observations suggest that pharmacological manipulation of lipid metabolism might have therapeutic potential for hepatitis C infections[118].

Recent studies have identified additional host factors involved in HCV RNA replication. Starting with the observation that cyclosporin A (CsA) inhibits HCV RNA replication *in vitro*, cyclophilin B was identified as a target of CsA action[119]. It was shown that cyclophilin B interacts with NS5B, thereby stimulating its RNA binding activity. Cyclophilin B is a peptidyl-prolyl *cis-trans* isomerase. This enzymatic activity has an as-yet-undefined role in HCV RNA replication. Based on these findings, non-immunosuppressive CsA analogues are currently being developed as antivirals against hepatitis C[120]. More recently, it was shown that FKBP8 (a member of the FK506-binding protein family) and Hsp90 form a complex with NS5A and have an important role in HCV RNA replication[121]. However, FK506 (tacrolimus) does not bind to FKBP8 and does not inhibit HCV RNA replication.

Packaging, assembly and particle release

Little is known about the late steps of the viral lifecycle, as these have only recently become amenable to systematic study. NS2 and possibly other non-structural proteins, as well as yet-to-be defined RNA structures, are involved in these processes. Virions presumably form by budding into the ER, or an ER-derived compartment, and exit the cell through the secretory pathway. A possible link between lipoprotein metabolism and viral assembly and release has been proposed[31].

Implications for drug development

In principle, each step of the HCV lifecycle (FIG. 1) is a target for antiviral intervention[122]. Specific inhibitors of the biochemically and structurally well-characterized NS3–4A serine protease and NS5B RdRp are currently being developed as antiviral agents, and the first candidates have already been evaluated in clinical trials[76]. Serine protease inhibitors seem particularly promising, as they not only block viral polyprotein processing but might also reverse the inhibition of innate immune sensing by HCV. In addition, new targets have been uncovered by the

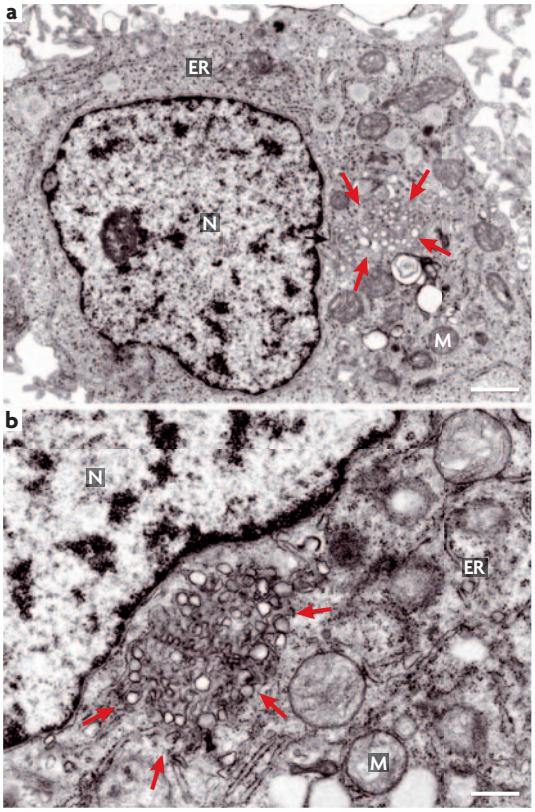


Figure 5 | **Hepatitis C virus (HCV) replication complex.** **a** | Low-power overview of a Huh-7 cell harbouring a subgenomic HCV replicon. A distinct membrane alteration, named the membranous web (arrows), is found in the juxtanuclear region of the cell. Note the circumscript nature of this specific membrane alteration and the otherwise unaltered cellular organelles. Scale bar represents 1 μm. **b** | Higher magnification of a membranous web (arrows), which is composed of small vesicles that are embedded in a membrane matrix. Note the close association of the membranous web with the rough endoplasmic reticulum. Scale bar represents 500 nm. The membranous web contains all HCV non-structural proteins and nascent viral RNA in Huh-7 cells that harbour subgenomic replicons[114], and therefore represents the subcellular site of HCV RNA replication. ER, endoplasmic reticulum; M, mitochondria; N, nucleus.

recent studies highlighted above, including, among others, the HCV 5′-NCR, viral entry and fusion, the p7 ion channel, the NS2–3 protease and NS5A. Moreover, drugs affecting host factors that are involved in HCV replication are being explored as antiviral agents. At this early stage it is already evident that the genetic variability of HCV, which facilitates the rapid development of antiviral resistance, is a major challenge to the clinical development of specific inhibitors and that, in common with HIV infection, combination therapy will be necessary for therapeutic success[123,124].

Conclusions and perspectives

The development of powerful model systems has enabled the dissection of the HCV lifecycle. Much work remains to be done with respect to understanding virion assembly and structure, the early and late steps of replication, the mechanism and regulation of RNA replication, and the pathogenesis of HCV-induced liver disease. Ultimately, these efforts should result in innovative preventive and therapeutic strategies for one of the most common causes of chronic hepatitis, liver cirrhosis and HCC worldwide.

1. Williams, R. Global challenges in liver disease. *Hepatology* **44**, 521–526 (2006).
2. Choo, Q.-L. *et al.* Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**, 359–362 (1989).
3. Grakoui, A., McCourt, D. W., Wychowski, C., Feinstone, S. M. & Rice, C. M. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. *J. Virol.* **67**, 2832–2843 (1993).
4. Bartenschlager, R., Ahlborn-Laake, L., Yasargil, K., Mous, J. & Jacobsen, H. Kinetic and structural analyses of hepatitis C virus polyprotein processing. *J. Virol.* **68**, 5045–5055 (1994).
5. Kolykhalov, A. A. *et al.* Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. *Science* **277**, 570–574 (1997).
6. Lohmann, V. *et al.* Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110–113 (1999).
7. Blight, K. J., Kolykhalov, A. A. & Rice, C. M. Efficient initiation of HCV RNA replication in cell culture. *Science* **290**, 1972–1974 (2000).
8. Bartosch, B., Dubuisson, J. & Cosset, F. L. Infectious hepatitis C virus pseudo-particles containing functional E1–E2 envelope protein complexes. *J. Exp. Med.* **197**, 633–642 (2003).
9. Hsu, M. *et al.* Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proc. Natl Acad. Sci. USA* **100**, 7271–7276 (2003).
10. Lindenbach, B. D. *et al.* Complete replication of hepatitis C virus in cell culture. *Science* **309**, 623–626 (2005).
11. Wakita, T. *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nature Med.* **11**, 791–796 (2005).
12. Zhong, J. *et al.* Robust hepatitis C virus infection *in vitro*. *Proc. Natl Acad. Sci. USA* **102**, 9294–9299 (2005).
13. Lindenbach, B. D. *et al.* Cell culture-grown hepatitis C virus is infectious *in vivo* and can be recultured *in vitro*. *Proc. Natl Acad. Sci. USA* **103**, 3805–3809 (2006).
14. Pietschmann, T. *et al.* Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc. Natl Acad. Sci. USA* **103**, 7408–7413 (2006).
15. Penin, F., Dubuisson, J., Rey, F. A., Moradpour, D. & Pawlotsky, J. M. Structural biology of hepatitis C virus. *Hepatology* **39**, 5–19 (2004).
16. Lindenbach, B. D. & Rice, C. M. Unravelling hepatitis C virus replication from genome to function. *Nature* **436**, 933–938 (2005).
17. Appel, N., Schaller, T., Penin, F. & Bartenschlager, R. From structure to function: new insights into hepatitis C virus RNA replication. *J. Biol. Chem.* **281**, 9833–9836 (2006).
18. Moradpour, D. & Rice, C. M. in *Hepatology. A textbook of liver disease* (eds Boyer, T. D., Wright, T. L. & Manns, M. P.) 125–147 (Elsevier Science, 2006).
19. Thiel, H. J. *et al.* in *Virus taxonomy. VIIIth Report of the International Committee on Taxonomy of Viruses* (eds Fauquet, C. M., Mayo, M. A., Maniloff, J., Desselberger, U. & Ball, L. A.) 979–996 (Academic Press, 2005).
20. Neumann, A. U. *et al.* Hepatitis C viral dynamics *in vivo* and the antiviral efficacy of interferon-α therapy. *Science* **282**, 103–107 (1998).
21. Simmonds, P. *et al.* Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* **42**, 962–973 (2005).
22. Kuiken, C. *et al.* Hepatitis C databases, principles and utility to researchers. *Hepatology* **43**, 1157–1165 (2006).
23. Moradpour, D. *et al.* Insertion of green fluorescent protein into nonstructural protein 5A allows direct visualization of functional hepatitis C virus replication complexes. *J. Virol.* **78**, 7400–7409 (2004).
24. Lohmann, V., Körner, F., Dobierzewska, A. & Bartenschlager, R. Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *J. Virol.* **75**, 1437–1449 (2001).
25. Blight, K. J., McKeating, J. A. & Rice, C. M. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J. Virol.* **76**, 13001–13014 (2002).
26. Lohmann, V., Hoffmann, S., Herian, U., Penin, F. & Bartenschlager, R. Viral and cellular determinants of hepatitis C virus RNA replication in cell culture. *J. Virol.* **77**, 3007–3019 (2003).
27. Bukh, J. *et al.* Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees. *Proc. Natl Acad. Sci. USA* **99**, 14416–14421 (2002).
28. Kato, T. *et al.* Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology* **125**, 1808–1817 (2003).
29. Yi, M., Villanueva, R. A., Thomas, D. L., Wakita, T. & Lemon, S. M. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc. Natl Acad. Sci. USA* **103**, 2310–2315 (2006).
30. Kuhn, R. J. *et al.* Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell* **108**, 717–725 (2002).
31. André, P., Perlemuter, G., Budkowska, A., Bréchot, C. & Lotteau, V. Hepatitis C virus particles and lipoprotein metabolism. *Semin. Liver Dis.* **25**, 93–104 (2005).
32. Pileri, P. *et al.* Binding of hepatitis C virus to CD81. *Science* **282**, 938–941 (1998).
33. Agnello, V., Abel, G., Elfahal, M., Knight, G. B. & Zhang, Q. X. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. *Proc. Natl Acad. Sci. USA* **96**, 12766–12771 (1999).
34. Scarselli, E. *et al.* The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J.* **21**, 5017–5025 (2002).
35. Evans, M. J. *et al.* Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* **446**, 801–805 (2007).
36. Bartosch, B. & Cosset, F. L. Cell entry of hepatitis C virus. *Virology* **348**, 1–12 (2006).
37. Cocquerel, L., Voisset, C. & Dubuisson, J. Hepatitis C virus entry: potential receptors and their biological functions. *J. Gen. Virol.* **87**, 1075–1084 (2006).
38. Coyne, C. B. & Bergelson, J. M. Virus-induced Abl and Fyn kinase signals permit coxsackievirus entry through epithelial tight junctions. *Cell* **124**, 119–131 (2006).
39. Blanchard, E. *et al.* Hepatitis C virus entry depends on clathrin-mediated endocytosis. *J. Virol.* **80**, 6964–6972 (2006).
40. Koutsoudakis, G. *et al.* Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. *J. Virol.* **80**, 5308–5320 (2006).
41. Tscherne, D. M. *et al.* Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. *J. Virol.* **80**, 1734–1741 (2006).
42. Modis, Y., Ogata, S., Clements, D. & Harrison, S. C. Structure of the dengue virus envelope protein after membrane fusion. *Nature* **427**, 313–319 (2004).
43. Gibbons, D. L. *et al.* Conformational change and protein–protein interactions of the fusion protein of Semliki Forest virus. *Nature* **427**, 320–325 (2004).
44. Friebe, P., Lohmann, V., Krieger, N. & Bartenschlager, R. Sequences in the 5′ nontranslated region of hepatitis C virus required for RNA replication. *J. Virol.* **75**, 12047–12057 (2001).
45. Jopling, C. L., Yi, M., Lancaster, A. M., Lemon, S. M. & Sarnow, P. Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA. *Science* **309**, 1577–1581 (2005).
46. Kolykhalov, A. A., Feinstone, S. M. & Rice, C. M. Identification of a highly conserved sequence element at the 3′ terminus of hepatitis C virus genome RNA. *J. Virol.* **70**, 3363–3371 (1996).
47. Tanaka, T., Kato, N., Cho, M.-J., Sugiyama, K. & Shimotohno, K. Structure of the 3′ terminus of the hepatitis C virus genome. *J. Virol.* **70**, 3307–3312 (1996).
48. Friebe, P. & Bartenschlager, R. Genetic analysis of sequences in the 3′ nontranslated region of hepatitis C virus that are important for RNA replication. *J. Virol.* **76**, 5326–5338 (2002).
49. Yi, M. & Lemon, S. M. 3′ nontranslated RNA signals required for replication of hepatitis C virus RNA. *J. Virol.* **77**, 3557–3568 (2003).
50. Yanagi, M., St Claire, M., Emerson, S. U., Purcell, R. H. & Bukh, J. *In vivo* analysis of the 3′ untranslated region of the hepatitis C virus after *in vitro* mutagenesis of an infectious cDNA clone. *Proc. Natl Acad. Sci. USA* **96**, 2291–2295 (1999).
51. Kolykhalov, A. A., Mihalik, K., Feinstone, S. M. & Rice, C. M. Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3′ nontranslated region are essential for virus replication *in vivo*. *J. Virol.* **74**, 2046–2051 (2000).
52. You, S., Stump, D. D., Branch, A. D. & Rice, C. M. A *cis*-acting replication element in the sequence encoding the NS5B RNA-dependent RNA polymerase is required for hepatitis C virus RNA replication. *J. Virol.* **78**, 1352–1366 (2004).
53. Friebe, P., Boudet, J., Simorre, J.-P. & Bartenschlager, R. A kissing loop interaction in the 3′ end of the hepatitis C virus genome essential for RNA replication. *J. Virol.* **79**, 380–392 (2005).
54. Otto, G. A. & Puglisi, J. D. The pathway of HCV IRES-mediated translation initiation. *Cell* **119**, 369–380 (2004).
55. Kieft, J. S., Zhou, K., Grech, A., Jubin, R. & Doudna, J. A. Crystal structure of an RNA tertiary domain essential to HCV IRES-mediated translation initiation. *Nature Struct. Biol.* **9**, 370–374 (2002).
56. Lukavsky, P. J., Kim, I., Otto, G. A. & Puglisi, J. D. Structure of HCV IRES domain II determined by NMR. *Nature Struct. Biol.* **10**, 1033–1038 (2003).
57. Spahn, C. M. *et al.* Hepatitis C virus IRES RNA-induced changes in the conformation of the 40S ribosomal subunit. *Science* **291**, 1959–1962 (2001).
58. Siridechadilok, B., Fraser, C. S., Hall, R. J., Doudna, J. A. & Nogales, E. Structural roles for human translation factor eIF3 in initiation of protein synthesis. *Science* **310**, 1513–1515 (2005).
59. McLauchlan, J., Lemberg, M. K., Hope, G. & Martoglio, B. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *EMBO J.* **21**, 3980–3988 (2002).
60. Boulant, S. *et al.* Structural determinants that target the hepatitis C virus core protein to lipid droplets. *J. Biol. Chem.* **281**, 22236–22247 (2006).
61. Asselah, T., Rubbia-Brandt, L., Marcellin, P. & Negro, F. Steatosis in chronic hepatitis C: why does it really matter? *Gut* **55**, 123–130 (2006).

62. Dubuisson, J., Penin, F. & Moradpour, D. Interaction of hepatitis C virus proteins with host cell membranes and lipids. *Trends Cell Biol.* **12**, 517–523 (2002).
63. Carrère-Kremer, S. *et al.* Subcellular localization and topology of the p7 polypeptide of hepatitis C virus. *J. Virol.* **76**, 3720–3730 (2002).
64. Sakai, A. *et al.* The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences. *Proc. Natl Acad. Sci. USA* **100**, 11646–11651 (2003).
65. Griffin, S. D. *et al.* The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. *FEBS Lett.* **535**, 34–38 (2003).
66. Pavlovic, D. *et al.* The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. *Proc. Natl Acad. Sci. USA* **100**, 6104–6108 (2003).
67. Grakoui, A., McCourt, D. W., Wychowski, C., Feinstone, S. M. & Rice, C. M. A second hepatitis C virus-encoded proteinase. *Proc. Natl Acad. Sci. USA* **90**, 10583–10587 (1993).
68. Hijikata, M. *et al.* Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. *J. Virol.* **67**, 4665–4675 (1993).
69. Pallaoro, M. *et al.* Characterization of the hepatitis C virus NS2/3 processing reaction by using a purified precursor protein. *J. Virol.* **75**, 9939–9946 (2001).
70. Thibeault, D., Maurice, R., Pilote, L., Lamarre, D. & Pause, A. *In vitro* characterization of a purified NS2/3 protease variant of hepatitis C virus. *J. Biol. Chem.* **276**, 46678–46684 (2001).
71. Lorenz, I. C., Marcotrigiano, J., Dentzer, T. G. & Rice, C. M. Structure of the catalytic domain of the hepatitis C virus NS2–3 protease. *Nature* **442**, 831–835 (2006).
72. Kalinina, O., Norder, H., Mukomolov, S. & Magnius, L. O. A natural intergenotypic recombinant of hepatitis C virus identified in St. Petersburg. *J. Virol.* **76**, 4034–4043 (2002).
73. Noppornpanth, S. *et al.* Identification of a naturally occurring recombinant genotype 2/6 hepatitis C virus. *J. Virol.* **80**, 7569–7577 (2006).
74. Yao, N., Reichert, P., Taremi, S. S., Prosise, W. W. & Weber, P. C. Molecular views of viral polyprotein processing revealed by the crystal structure of the hepatitis C virus bifunctional protease-helicase. *Structure Fold. Des.* **7**, 1353–1363 (1999).
75. Wölk, B. *et al.* Subcellular localization, stability and *trans*-cleavage competence of the hepatitis C virus NS3–NS4A complex expressed in tetracycline-regulated cell lines. *J. Virol.* **74**, 2293–2304 (2000).
76. Lamarre, D. *et al.* An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. *Nature* **426**, 186–189 (2003).
77. Li, K. *et al.* Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc. Natl Acad. Sci. USA* **102**, 2992–2997 (2005).
78. Meylan, E. *et al.* Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* **437**, 1167–1172 (2005).
79. Seth, R. B., Sun, L., Ea, C. K. & Chen, Z. J. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-κB and IRF 3. *Cell* **122**, 669–682 (2005).
80. Kawai, T. *et al.* IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nature Immunol.* **6**, 981–988 (2005).
81. Xu, L. G. *et al.* VISA is an adapter protein required for virus-triggered IFN-β signaling. *Mol. Cell* **19**, 727–740 (2005).
82. Johnson, C. L. & Gale, M., Jr. CARD games between virus and host get a new player. *Trends Immunol.* **27**, 1–4 (2006).
83. Serebrov, V. & Pyle, A. M. Periodic cycles of RNA unwinding and pausing by hepatitis C virus NS3 helicase. *Nature* **430**, 476–480 (2004).
84. Levin, M. K., Gurjar, M. & Patel, S. S. A Brownian motor mechanism of translocation and strand separation by hepatitis C virus helicase. *Nature Struct. Mol. Biol.* **12**, 429–435 (2005).
85. Dumont, S. *et al.* RNA translocation and unwinding mechanism of HCV NS3 helicase and its coordination by ATP. *Nature* **439**, 105–108 (2006).
86. Kwong, A. D., Rao, B. G. & Jeang, K. T. Viral and cellular RNA helicases as antiviral targets. *Nature Rev. Drug Discov.* **4**, 845–853 (2005).
87. Frick, D. N., Rypma, R. S., Lam, A. M. & Gu, B. The nonstructural protein 3 protease/helicase requires an intact protease domain to unwind duplex RNA efficiently. *J. Biol. Chem.* **279**, 1269–1280 (2004).
88. Egger, D. *et al.* Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.* **76**, 5974–5984 (2002).
89. Yu, G. Y., Lee, K. J., Gao, L. & Lai, M. M. Palmitoylation and polymerization of hepatitis C virus NS4B protein. *J. Virol.* **80**, 6013–6023 (2006).
90. Quintavalle, M., Sambucini, S., Di Pietro, C., De Francesco, R. & Neddermann, P. The α-isoform of protein kinase CKI is responsible for hepatitis C virus NS5A hyperphosphorylation. *J. Virol.* **80**, 11305–11312 (2006).
91. Reed, K. E., Gorbalenya, A. E. & Rice, C. M. The NS5A/NS5 proteins of viruses from three genera of the family flaviviridae are phosphorylated by associated serine/threonine kinases. *J. Virol.* **72**, 6199–6206 (1998).
92. Evans, M. J., Rice, C. M. & Goff, S. P. Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. *Proc. Natl Acad. Sci. USA* **101**, 13038–13043 (2004).
93. Neddermann, P. *et al.* Reduction of hepatitis C virus NS5A hyperphosphorylation by selective inhibition of cellular kinases activates viral RNA replication in cell culture. *J. Virol.* **78**, 13306–13314 (2004).
94. Appel, N., Pietschmann, T. & Bartenschlager, R. Mutational analysis of hepatitis C virus nonstructural protein 5A: potential role of differential phosphorylation in RNA replication and identification of a genetically flexible domain. *J. Virol.* **79**, 3187–3194 (2005).
95. Gao, L., Aizaki, H., He, J. W. & Lai, M. M. Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. *J. Virol.* **78**, 3480–3488 (2004).
96. Penin, F. *et al.* Structure and function of the membrane anchor domain of hepatitis C virus nonstructural protein 5A. *J. Biol. Chem.* **279**, 40835–40843 (2004).
97. Tellinghuisen, T. L., Marcotrigiano, J., Gorbalenya, A. E. & Rice, C. M. The NS5A protein of hepatitis C virus is a zinc metalloprotein. *J. Biol. Chem.* **279**, 48576–48587 (2004).
98. Tellinghuisen, T. L., Marcotrigiano, J. & Rice, C. M. Structure of the zinc-binding domain of an essential replicase component of hepatitis C virus reveals a novel fold. *Nature* **435**, 375–379 (2005).
99. Huang, L. *et al.* Hepatitis C virus nonstructural protein 5A (NS5A) is an RNA-binding protein. *J. Biol. Chem.* **280**, 36417–36428 (2005).
100. Miyanari, Y. *et al.* Hepatitis C virus non-structural proteins in the probable membranous compartment function in viral RNA replication. *J. Biol. Chem.* **278**, 50301–50308 (2003).
101. Quinkert, D., Bartenschlager, R. & Lohmann, V. Quantitative analysis of the hepatitis C virus replication complex. *J. Virol.* **79**, 13594–13605 (2005).
102. Ago, H. *et al.* Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. *Struct. Fold. Des.* **7**, 1417–1426 (1999).
103. Lesburg, C. A. *et al.* Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. *Nature Struct. Biol.* **6**, 937–943 (1999).
104. Bressanelli, S., Tomei, L., Rey, F. A. & De Francesco, R. Structural analysis of the hepatitis C virus RNA polymerase in complex with ribonucleotides. *J. Virol.* **76**, 3482–3492 (2002).
105. Butcher, S. J., Grimes, J. M., Makeyev, E. V., Bamford, D. H. & Stuart, D. I. A mechanism for initiating RNA-dependent RNA polymerization. *Nature* **410**, 235–240 (2001).
106. Lyle, J. M., Bullitt, E., Bienz, K. & Kirkegaard, K. Visualization and functional analysis of RNA-dependent RNA polymerase lattices. *Science* **296**, 2218–2222 (2002).
107. Wang, Q. M. *et al.* Oligomerization and cooperative RNA synthesis activity of hepatitis C virus RNA-dependent RNA polymerase. *J. Virol.* **76**, 3865–3872 (2002).
108. Moradpour, D. *et al.* Membrane association of the RNA-dependent RNA polymerase is essential for hepatitis C virus RNA replication. *J. Virol.* **78**, 13278–13284 (2004).
109. Branch, A. D., Stump, D. D., Gutierrez, J. A., Eng, F. & Walewski, J. L. The hepatitis C virus alternate reading frame (ARF) and its family of novel products: the alternate reading frame protein/F-protein, the double-frameshift protein, and others. *Semin. Liver Dis.* **25**, 105–117 (2005).
110. McMullan, L. K. *et al.* Evidence for a functional RNA element in the hepatitis C virus core gene. *Proc. Natl Acad. Sci. USA* **104**, 2879–2884 (2007).
111. Salonen, A., Ahola, T. & Kääriäinen, L. Viral RNA replication in association with cellular membranes. *Curr. Top. Microbiol. Immunol.* **285**, 139–173 (2004).
112. Mackenzie, J. Wrapping things up about virus RNA replication. *Traffic* **6**, 967–977 (2005).
113. Schwartz, M. *et al.* A positive-strand RNA virus replication complex parallels form and function of retrovirus capsids. *Mol. Cell* **9**, 505–514 (2002).
114. Gosert, R. *et al.* Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. *J. Virol.* **77**, 5487–5492 (2003).
115. Ye, J. *et al.* Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. *Proc. Natl Acad. Sci. USA* **100**, 15865–15870 (2003).
116. Kapadia, S. B. & Chisari, F. V. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. *Proc. Natl Acad. Sci. USA* **102**, 2561–2566 (2005).
117. Wang, C. *et al.* Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. *Mol. Cell* **18**, 425–434 (2005).
118. Sakamoto, H. *et al.* Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. *Nature Chem. Biol.* **1**, 333–337 (2005).
119. Watashi, K. *et al.* Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol. Cell* **19**, 111–122 (2005).
120. Paeshuyse, J. *et al.* The non-immunosuppressive cyclosporin DEBIO-025 is a potent inhibitor of hepatitis C virus replication *in vitro*. *Hepatology* **43**, 761–770 (2006).
121. Okamoto, T. *et al.* Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. *EMBO J.* **25**, 5015–5025 (2006).
122. De Francesco, R. & Migliaccio, G. Challenges and successes in developing new therapies for hepatitis C. *Nature* **436**, 953–960 (2005).
123. Trozzi, C. *et al.* *In vitro* selection and characterization of hepatitis C virus serine protease variants resistant to an active-site peptide inhibitor. *J. Virol.* **77**, 3669–3679 (2003).
124. Yi, M. *et al.* Mutations conferring resistance to SCH6, a novel hepatitis C virus NS3/4A protease inhibitor. Reduced RNA replication fitness and partial rescue by second-site mutations. *J. Biol. Chem.* **281**, 8205–8215 (2006).

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Competing interests statement

The authors declare competing financial interests: see web version for details.

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