The molecular and structural basis of advanced antiviral therapy for hepatitis C virus infection

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Abstract | The availability of the first molecular clone of the hepatitis C virus (HCV) genome allowed the identification and biochemical characterization of two viral enzymes that are targets for antiviral therapy: the protease NS3-4A and the RNA-dependent RNA polymerase NS5B. With the advent of cell culture systems that can recapitulate either the intracellular steps of the viral replication cycle or the complete cycle, additional drug targets have been identified, most notably the phosphoprotein NS5A, but also host cell factors that promote viral replication, such as cyclophilin A. Here, we review insights into the structures of these proteins and the mechanisms by which they contribute to the HCV replication cycle, and discuss how these insights have facilitated the development of new, directly acting antiviral compounds that have started to enter the clinic.

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Hepatitis C virus (HCV) infection has been called the silent epidemic — the majority of infections are asymptomatic, but in up to 80% of cases the virus persists, so many individuals infected with HCV are not aware of their infection[1]. For this reason, infected individuals do not receive medical care even though they have a high risk of developing serious liver disease, including fibrosis and liver cirrhosis, which is often a prelude to hepatocellular carcinoma[2]. With an estimated ~130 million chronically infected individuals worldwide, ~27% of all cases of liver cirrhosis and ~25% of hepatocellular carcinomas might be related to HCV infection[3], explaining why chronic hepatitis C has become one of the main indications for liver transplantation. Given this obvious medical need, tremendous efforts have been expended to develop efficacious prophylactic and therapeutic modalities for chronic hepatitis C. Unfortunately, prophylactic treatment to prevent HCV infection has met with limited success, and thus far, there is no vaccine in sight[4]. This is due, at least in part, to the high genomic variability of HCV, which has led to the distinction of seven genotypes (numbered 1–7), most of which have multiple subtypes (denoted a, b, and so on); these genotypes and subtypes differ in their nucleotide sequences by ~33% and ~25%, respectively[5]. This high variability explains the pronounced genotype-specific humoral and cellular immune response triggered by the virus, and thus the poor cross-genotype immunity[6].

Infections with genotype 1 are spread throughout the world. By contrast, genotype 2 and genotype 3 viruses are more prevalent in industrialized countries as well as in South America and Asia, whereas genotypes 4–6 are limited to distinct countries[7,8]. The prevalence of HCV ranges from 0.1% to 1.5% in many industrialized countries and reaches ~15% in some exceptional cases, such as Egypt, where viral spread has been ascribed primarily to the re-use of non-sterilized needles and syringes in the course of parenteral schistosomiasis therapy[9].

The majority of HCV infections are treated with a combination of pegylated interferon-α (PEG–IFNα) and ribavirin. This treatment leads to viral elimination in 20–80% of patients, depending primarily on disease stage, the genotype of the infecting virus and distinct genetic polymorphisms in the host interleukin-28B gene locus[10]. In fact, patients infected with genotype 2 and genotype 3 viruses clear the infection in up to ~85% of treated cases, but therapy is successful in only ~45% of cases for infections with genotype 1 viruses. Moreover, this treatment has several contra-indications that preclude patients from receiving the drugs, as well as numerous side effects such as flu-like symptoms, severe depression and haemolytic anaemia. Thus, more efficacious antiviral therapies, ideally independent of PEG–IFNα and ribavirin, are required. An important step in this direction has been the recent approval of two HCV-specific direct-acting antiviral agents (DAAs), the

Box 1 | **Cell culture systems for hepatitis C virus**

Three main categories of cell-based culture systems for hepatitis C virus (HCV) have been developed and are discussed below.

Replicons

The first culture system involves replicons, which are engineered 'mini-genomes'. Originally, these were composed of the 5′ non-translated region (NTR), a gene encoding a selection marker (for example, conferring G418 resistance), an internal ribosome entry site (IRES) from another virus to allow translation of the region encoding the replicase module (FIG. 1), and the 3′ NTR. Following transfection of such an RNA into human hepatoma cells and subsequent selection with G418, cell clones can be obtained that support stable and high-level replication of these HCV replicon RNAs[15]. Subsequent studies have led to enormous improvements in this system, for example, through the identification of replication-enhancing mutations that are essential for efficient amplification of these RNAs in cultured cells[137,138], as well as through the construction of functional replicons encoding easily measurable reporter genes that are suitable for high-throughput screening[139]. In addition to replicons derived from HCV genotype 1 and HCV genotype 2 isolates, subgenomic replicons derived from genotype 3 and genotype 4 isolates have been established more recently[140–142]. Although the replicon system has been enormously helpful for drug development, the disadvantage of the system is that it recapitulates only the intracellular steps of the HCV replication cycle. Because most replication-enhancing mutations interfere with virus assembly[143], for a long time it was not possible to turn this system into a fully competent HCV cell culture model.

The HCVpp system

The second cell-based HCV culture system exploits pseudoparticle technology, hence the name HCV pseudoparticles (HCVpps)[144,145]. These pseudoparticles consist of a retroviral core surrounded by a lipid envelope into which authentic HCV envelope glycoprotein E1–E2 complexes are embedded. The retroviral core contains a retroviral genome that encodes an easily measurable reporter protein such as luciferase or GFP. The HCVpp system mimics the infection process (that is, particle binding to the host cell, entry and uncoating) because it is driven by the envelope glycoproteins themselves. The HCVpp system has been instrumental for studying, for example, the neutralization of HCV infection by antibodies, the functionality of patient-derived HCV E1–E2 sequences, and the identification and characterization of HCV receptors[146]. The disadvantage of the HCVpp system is that it recapitulates only the very early steps of the HCV replication cycle.

The HCVcc system

The most recently developed cell culture system for HCV recapitulates the complete viral replication cycle in cultured human hepatoma cells. This cell culture-derived HCV (HCVcc) system became possible with the identification of a particular HCV isolate that was cloned from a Japanese patient with fulminant hepatitis (isolate JFH-1)[147]. Compared with other HCV isolates, the JFH-1 strain replicates to exceptionally high levels in the absence of replication-enhancing mutations. *In vitro* transcripts derived from a cloned JFH-1 genome can be transfected into human hepatoma cells, and this leads to the production of HCV particles that are infectious both in cell culture and *in vivo*[148–150]. In the past few years, numerous improvements have been made to the HCVcc system, including viruses with reporter genes for easy read-out as well as chimaeras composed of the JFH-1 replicase module and an assembly module derived from other HCV isolates of the same or different genotypes[151]. More recently, highly cell culture-adapted HCV isolates derived from genotypes 1a, 2a and 2b were developed that also support the complete viral replication cycle in cultured human hepatoma cells[152–154].

protease inhibitors telaprevir (VX-950) and boceprevir, but these drugs are still given in combination with PEG–IFNα and ribavirin and are also prone to selecting for drug resistance. More efficacious DAAs are in clinical development, raising hope for an IFN-sparing treatment regimen in the not too distant future.

The development of these DAAs has been greatly fostered by insights into the molecular virology of HCV and the interaction of the virus with the host cell, which in turn were linked to the establishment of robust cell culture systems (BOX 1). In this Review, we summarize the current status of HCV molecular virology and focus on the structural and functional properties of the primary drug targets: the serine-type protease residing in the NS3–NS4A heterodimer, the phosphoprotein NS5A and the RNA-dependent RNA polymerase (RdRp) NS5B. We also briefly discuss the most promising host cell targets for HCV-specific therapy and summarize the various DAA classes. For more detailed descriptions of the HCV replication cycle and virus–host cell interactions, we refer the reader to recent reviews[11–13].

HCV genome organization

HCV is grouped in the genus Hepacivirus within the family *Flaviviridae*[5]. These viruses have a positive-sense RNA genome that contains one long ORF flanked by highly structured non-translated regions (NTRs) that are essential for RNA replication (FIG. 1a). The 5′ NTR also contains a type III internal ribosome entry site that directs translation of the RNA genome in a cap-independent manner. The ORF encodes a polyprotein of ~3,000 amino acids. This polyprotein is cleaved co- and post-translationally into ten different proteins that are invariably associated with intracellular membranes (FIG. 1b). Proteins generated from the amino-terminal region of the polyprotein build up the virus particle (core protein (C), and the envelope glycoproteins E1 and E2) or support particle assembly while not being incorporated into the particle (p7 and NS2)[14]. The remaining polyprotein cleavage products, NS3, NS4A, NS4B, NS5A and NS5B, are sufficient to support viral RNA replication[15]. In this respect, the HCV genome encodes two functional modules (FIG. 1a), an assembly module (C–NS2) and a replication module (NS3–NS5B; although most, if not all, replicase proteins contribute to virion formation).

The HCV replication cycle: a short summary

The predominant feature of HCV particles is their tight association with components of low-density and very-low-density lipoproteins (LDLs and VLDLs, respectively)[14,16,17] (FIG. 2). Various apolipoproteins, most notably apoE, are part of these lipoviroparticles[17,18]. A high lipid association of HCV correlates with high infectivity[19], indicating that lipoprotein components contribute to efficient entry of HCV into the host cell. At least four host proteins are essential for infection: scavenger receptor class B member 1 (SRB1), the tetraspanin CD81 and the two tight junction proteins claudin 1 (CLDN1) and occludin (OCLN) (reviewed in REF. 20). Virus particles are thought to enter the host cell in a coordinated manner involving the formation of receptor complexes followed by receptor-mediated endocytosis in a clathrin-dependent manner[21]. In addition, HCV spreads by cell-to-cell transmission, which might be the predominant route of spread *in vivo*[20].

Following uncoating and release of the RNA genome into the cytoplasm, the RNA is translated at the rough ER, which contains the cellular proteases that cleave part of the polyprotein N-terminal region (reviewed in REFS 13,22) (FIG. 1a). The structural proteins, as well as p7, are generated by host signal peptidase- and host signal peptide peptidase-mediated cleavages. The remainder

Internal ribosome entry site
An RNA sequence that allows ribosomes to bind an mRNA internally, independently of a cap structure, and that thus mediates translation of a downstream ORF.

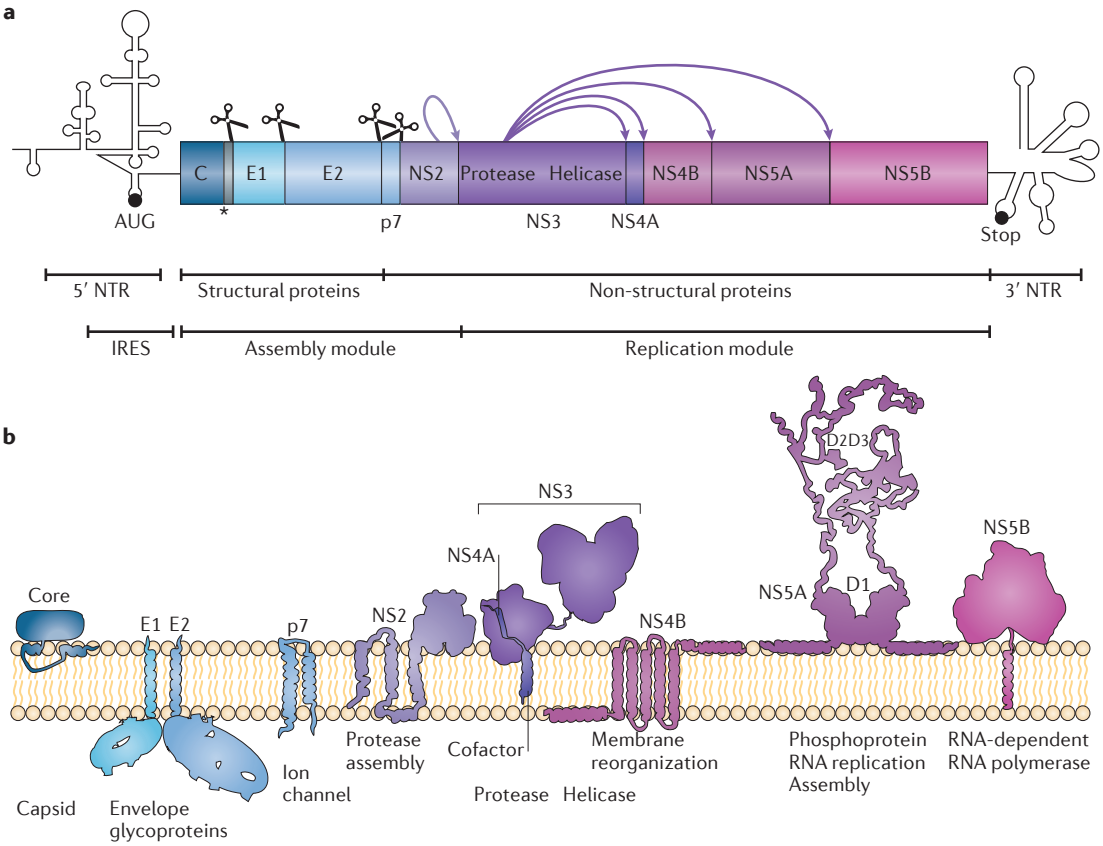

Figure 1 | **Hepatitis C virus genome organization and the membrane topology of cleaved viral proteins.** **a** | The single long hepatitis C virus (HCV) ORF encoding the polyprotein, and the predicted secondary structures of the flanking 5′ and 3′ non-translated regions (NTRs). Start and stop codons of the ORF are indicated. The 5′ NTR contains an internal ribosome entry site (IRES). The structural core (C) protein and the two envelope glycoproteins (E1 and E2), together with p7 and NS2, are required for virus assembly (the so-called assembly module). The remainder of the non-structural proteins are required for RNA replication (the so-called replication module). Polyprotein cleavage by cellular signal peptidases is indicated by scissors at the corresponding ORF position. The cleavage removing the carboxy-terminal region of the core protein, mediated by cellular signal peptide peptidase, is indicated by an asterisk. Arrows refer to cleavage by the viral proteases. Ribosomes can initiate low-frequency translation of HCV RNA internally at codons within the core-coding region or can generate alternative proteins by ribosomal +1 frameshifting. The physiological relevance of these 'minicore' or 'core+1' proteins (not shown) remains to be determined[155]. **b** | Membrane topologies and major functions of the HCV polyprotein cleavage products. Each protein is tethered to intracellular membranes by one or several transmembrane segments or, in the case of the core protein and NS5A, by amphipathic α-helices. NS3 is bound to membranes via a small α-helix and via the cofactor NS4A intercalating into the amino-terminal protease domain of NS3. Note that only NS5A is shown as a dimer, but most, if not all, HCV proteins form homo- or heterodimers (for example, the core protein and E1–E2, respectively) or oligomeric complexes (for example, p7).

Low-density and very-low-density lipoproteins
Lipoproteins that are made in the liver from triglycerides, cholesterol and apolipoproteins and are used to transport lipids in the blood.

Membranous web
Originally, a term describing a discrete accumulation of the membranous vesicles that have been detected in cells containing replicating hepatitis C virus (HCV) RNA. More recent studies have shown that this web is composed of single-, double- and multi-membraned vesicles, complex ER membrane rearrangements and lipid droplets. However, in most reports, the term is used as a synonym for the membranous HCV replication compartment, although firm proof of exactly where HCV RNA replication takes place is not available.

Cyclophilin A
A highly abundant protein that catalyses the *cis–trans* isomerization of peptide bonds at Pro residues and thus facilitates protein folding. Cyclophilin A binds to the immunosuppressive drug cyclosporin A and is involved in numerous biological processes.

miR-122
A short, non-coding RNA that is highly expressed in hepatocytes, where it regulates the translation and turnover of mRNAs involved in numerous activities, such as iron and cholesterol homeostasis. In addition, miR-122 was found to act as a tumour suppressor.

of the polyprotein is processed by the viral cysteine protease NS2-3 and the serine-type protease activity of the viral NS3–NS4A complex. HCV replicase proteins, presumably in concert with host cell factors, induce massive rearrangements of intracellular membranes, including the formation of double-membraned vesicles[23,24] (FIG. 2). These membranous structures manifest as vesicle clusters that are designated the membranous web and constitute the sites of HCV RNA replication[25]. RNA synthesis is catalysed by the viral RdRp activity of NS5B and supported by other viral NS proteins, as well as by host factors, including cyclophilin A (CYPA; also known as PPIase A) and the microRNA miR-122 (REF. 26). After synthesis of a negative-sense RNA intermediate, multiple positive-sense progeny RNAs are generated from this template and either used for RNA translation and replication or incorporated into virus particles. The latter process might initiate on the surface of lipid droplets that are targeted by the HCV core protein[27]. It is assumed that nucleocapsids form in close proximity to the ER membrane, where E1 and E2 accumulate in conjunction with p7, NS2 and, eventually, host cell factors[14,16]. The viral envelope is acquired by budding at the ER membrane, and this process appears to be linked to the VLDL machinery[28]. Virus particles are thought to be released via the secretory pathway[29,30].

Primary targets for HCV-specific DAAs

In principle, every step of the HCV replication cycle is a potential target for antiviral therapy. However, owing to

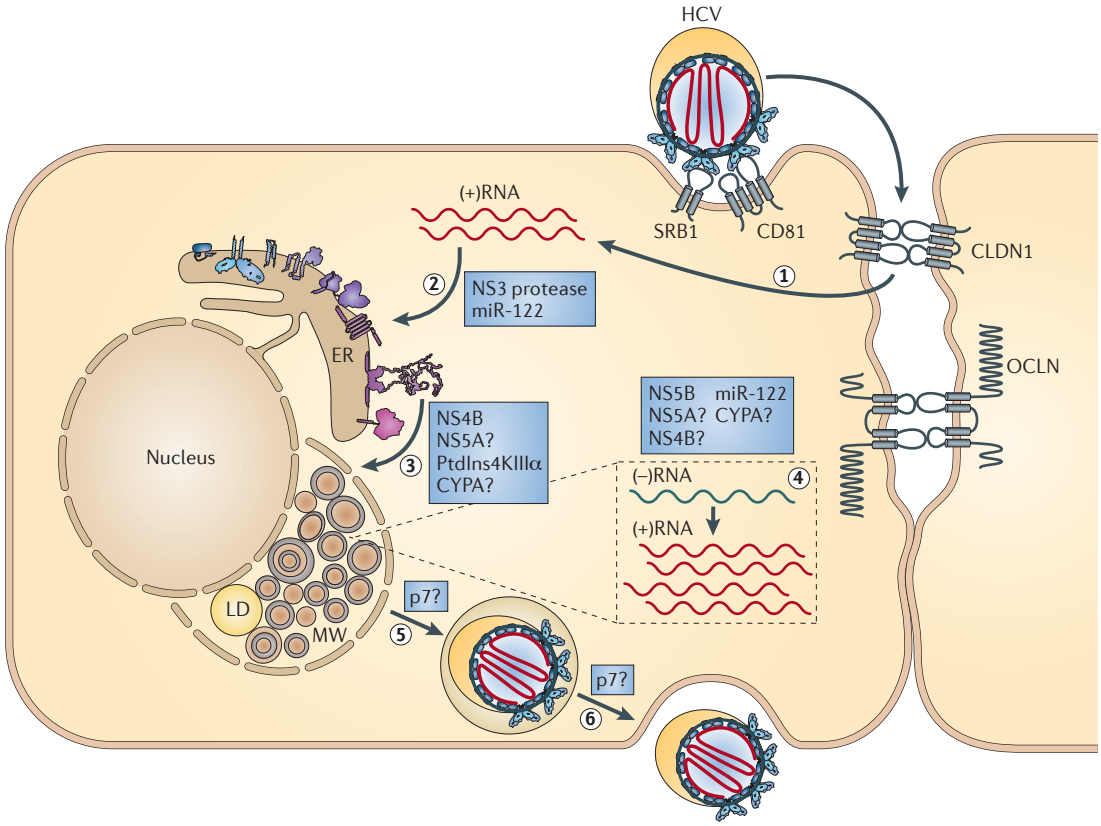

Figure 2 | **The hepatitis C virus replication cycle.** Following initial binding of the hepatitis C virus (HCV) particle to scavenger receptor class B member 1 (SRB1) and CD81, the particle engages in further interactions with the tight junction proteins claudin 1 (CLDN1) and occludin (OCLN) and finally enters cells by receptor-mediated endocytosis (step 1). The viral RNA genome is released into the cytoplasm and translated at the rough ER, giving rise to a polyprotein that is cleaved into mature proteins (step 2). Viral proteins, in conjunction with host cell factors, induce the formation of a membranous compartment (designated the membranous web (MW)) composed of single-, double- and multi-membraned vesicles as well as lipid droplets (LDs) (step 3). RNA replication occurs at an unspecified site within the membranous web and proceeds via a negative-sense copy ((–)RNA) that serves as a template for the production of excess amounts of positive-sense progeny RNAs ((+)RNA) (step 4). Assembly of HCV particles probably initiates in close proximity to the ER and lipid droplets, where core protein and viral RNA accumulate. The viral envelope is acquired by budding through the ER membrane in a process that is linked to lipoprotein synthesis (step 5). HCV particles are thought to be released via the constitutive secretory pathway (step 6). Viral and host cell factors that are targeted by inhibitors discussed in this Review are indicated in boxes. The steps of the replication cycle that are promoted by these factors are indicated. CYPA, cyclophilin A; PtdIns4KIIIα, phosphatidylinositol 4-kinase IIIα.

difficulties in establishing robust cell-based HCV replication systems and inspired by the success of protease inhibitors for the treatment of HIV infections, initial efforts focused on the protease NS3-4A. With the availability of recombinant NS5B polymerase[31,32] and cell-based replication systems[15], the HCV RdRp also became an attractive drug target. Finally, by using high-throughput screens in the replicon system (BOX 1), highly active inhibitors targeting NS5A have been developed. Below, we discuss these three major drug targets and briefly mention selected examples of DAAs. Readers who are interested in an up-to-date status of clinical trials and a comprehensive overview of continued and discontinued HCV-specific DAAs are referred to recent reviews (for example, REF. 33) or specialized websites such as the HCV Advocate's News and Pipeline Blog, the National AIDS Treatment Advocacy Project and the HIV–HCV–TB Pipeline Report.

The protease NS3-4A. NS3 is a multifunctional protein in which the N-terminal ~180 amino acids, together with the cofactor NS4A, constitute the serine-type protease NS3-4A, and the carboxy-terminal region constitutes a superfamily 2 DExH/D-box RNA helicase that also has NTPase activity[34] (FIG. 3). Both activities are essential for HCV replication, but only the protease has been successfully pursued as a drug target. Development of inhibitors of the NS3 helicase has met with limited success, at least in part owing to the similarities between NS3 and cellular RNA helicases.

The NS3 protease region adopts a chymotrypsin-like fold composed of two β-barrel subdomains separated by a cleft containing the active site (comprising His57, Asp81 and Ser139) (FIG. 3b). The C-terminal subdomain of the NS3 protease is stabilized by a Zn^{2+} ion, whereas the N-terminal subdomain contributes seven strands to an eight-stranded β-barrel, with the eighth

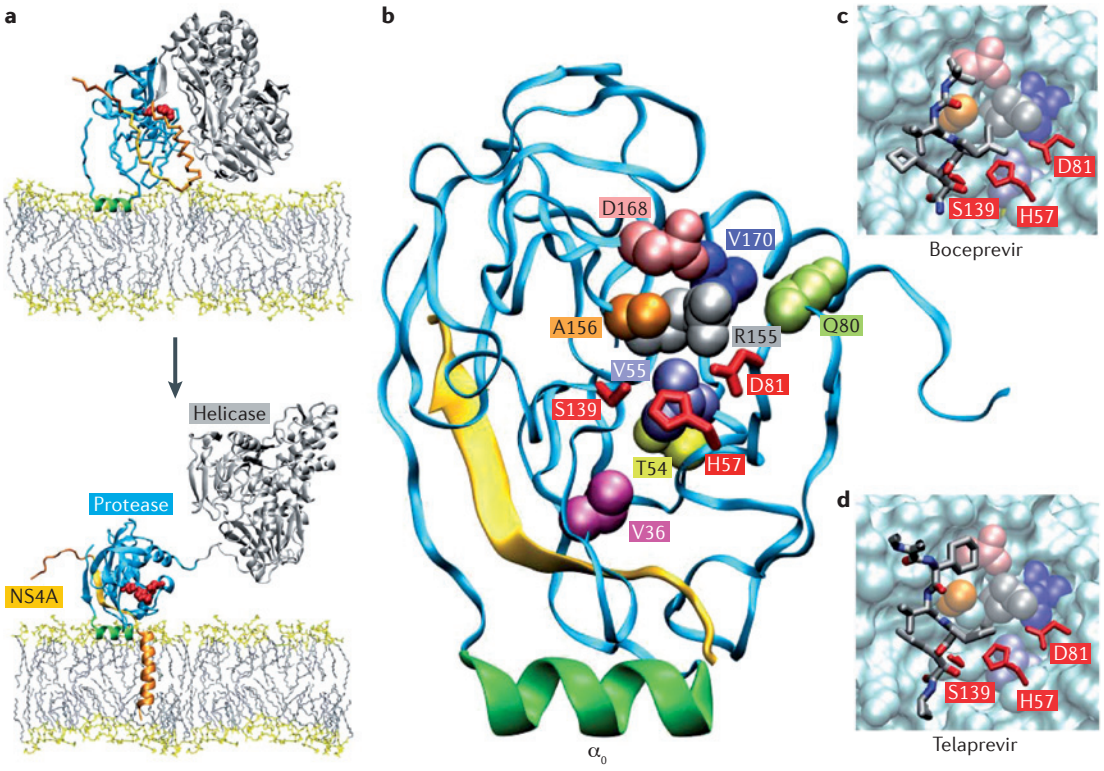

Figure 3 | **Membrane topology of NS3-4A and positions of mutations that confer resistance to NS3-4A inhibitors.** **a** | The presumed membrane orientation of the NS3-4A complex (ribbon representation) during polyprotein synthesis and before self-cleavage at the junction between NS3 and NS4A (upper panel). The topology of NS3-4A changes after self-cleavage (lower panel). Note the profound structural changes, especially the membrane insertion of the carboxy-terminal tail of NS4A and the repositioning of the C-terminal NS3 helicase domain. The membrane is represented as a simulated model of a 1-palmitoyl-2-oleoyl-3-*sn*-glycero-3-phosphocholine bilayer. Polar heads and hydrophobic tails of phospholipids (stick structures) are coloured light yellow and light grey, respectively. **b** | A ribbon diagram of the NS3 protease domain (Protein Data Bank (PDB) accession 1CU1) with the central NS4A activation domain (yellow). The α_0 helix serves as an additional membrane anchor of NS3. The catalytic triad (His57, Asp81 and Ser139) is indicated in red sticks. Mutation of certain residues confers resistance to NS3-4A inhibitors, and the side chains of these residues are represented as coloured van der Waals spheres. **c** | The structure of boceprevir (stick representation) complexed to the active site of NS3 (surface representation) (PDB accession 2OC8). **d** | The structure of telaprevir (stick representation) complexed to the active site of NS3 (surface representation) (PDB accession 3SV6). The figures were generated using Visual Molecular Dynamics and the structural coordinates deposited in the PDB, and were rendered with POV-Ray. Part **a** images are reproduced, with permission, from REF. 38 © (2008) National Academy of Sciences, USA.

strand contributed by the viral protease cofactor NS4A. This cofactor is a 54 amino acid protein that has several functions. First, the hydrophobic N-terminal transmembrane α-helix, together with the short α_0 helix in the N-terminal region of NS3 (amino acids 12–23), tethers NS3 to intracellular membranes (FIG. 3a); second, the central portion of NS4A (residues 21–32) intercalates into NS3 and activates the protease activity by stabilizing this protease subdomain and contributing to the substrate recognition site; third, the C-terminal acidic portion of NS4A interacts with the NS3 helicase and other HCV proteins and contributes to RNA replication as well as assembly[35].

The substrate specificity of NS3-4A is rather low and is determined primarily by an acidic residue at the P6 position (that is, 6 amino acids N-terminal to the cleavage site), a Cys at the P1 position and a small amino acid side chain at the P1′ site (that is, right after the cleavage site). This low specificity might facilitate NS3-4A-mediated cleavage of cellular substrates that often have non-canonical cleavage sites. Examples of well-characterized cellular proteins that are cleaved, and thus inactivated, by NS3-4A are the signalling molecules MAVS (mitochondrial antiviral-signalling protein; formerly known as CARDIF, VISA and IPS1)[36] and TRIF (TIR domain-containing adaptor inducing IFNβ)[37], two proteins that are crucially involved in the induction of an IFN response. It is thought that the membrane association of NS3-4A compensates for the low substrate specificity by proper positioning of the protease relative to the membrane-associated substrate[38].

A hallmark of NS3-4A is the unusual substrate recognition, which is mediated by a series of weak interactions along an extended surface. This posed a major challenge for the development of NS3-4A-targeting DAAs. Nevertheless, by exploiting the fact that after NS3-4A-mediated cleavage the product derived from the N-terminal fragment remains bound to the active

MAVS
(Mitochondrial antiviral-signalling protein). An important factor involved in the activation of a rapid interferon response following the triggering of intracellular RNA sensors such as RIG-I or MDA5.

TRIF
(TIR domain-containing adaptor inducing interferon-β). A protein involved in the induction of an interferon response following the activation of certain Toll-like receptors, for example Toll-like receptor 3.

site and thus blocks the enzyme[39,40], highly potent DAAs targeting the protease have been developed[41]. These DAAs can be divided into three classes on the basis of their physicochemical properties[42]. The first class comprises linear peptidomimetics that form covalent, but reversible, adducts with the enzyme. This compound class, to which telaprevir and boceprevir belong, binds to the active-site Ser (FIG. 3c,d). These 'Ser-trap' inhibitors antagonize the enzyme in a two-step reaction by forming a covalent enzyme–inhibitor adduct that dissociates with very slow kinetics. This peculiar reaction mechanism might contribute to antiviral potency and compensate for the rather weak binding of the inhibitors to the enzyme.

The second and third classes of NS3-4A-specific DAAs do not form covalent adducts with their targets. These DAAs are classified as linear peptidomimetics (the second class) or macrocyclic inhibitors (the third class) on the basis of their structure. Although these NS3-4A inhibitors are expected to cause fewer and less severe side effects than telaprevir and boceprevir, they do not target all HCV genotypes to the same extent and thus are not pan-genotypic. Moreover, the barrier to select for resistance against these first-generation inhibitors is low, and profound cross-resistance exists[43]. For instance, NS3 mutations Arg155(Lys/Thr/Gln), Ala156(Val/Thr) and Asp168(Ala/Val/Thr/His) confer cross-resistance to other NS3-4A inhibitors with low resistance barriers (FIG. 3b). These limitations are overcome in the more recently developed second-generation NS3-4A inhibitors, exemplified by the two macrocycles MK-5172 (REF. 44) and ACH-2684 (REF. 45), which are characterized by pan-genotypic activity and improved resistance profiles. For instance, MK-5172 retains antiviral activity against HCV variants containing resistance mutations at NS3 residue Arg155 because this compound interacts with a distinct conformation of the active site[46].

The replicase factor NS5A. NS5A is one of the most enigmatic HCV proteins. It is a ~450 amino acid phosphoprotein composed of an N-terminal amphipathic α-helix and three domains (D1–D3) that are separated by so-called low-complexity sequences (LCS1 and LCS2), a term that reflects the sensitivity of these sequences to limited proteolysis[47] (FIG. 4a). The N-terminal amphipathic α-helix is monotopic and tethers NS5A to the cytosolic leaflet of intracellular membrane bilayers[48]. D1 is primarily required for RNA replication, whereas D3 plays a major part in the assembly of infectious virus particles, probably by interacting with the core protein at lipid droplets[49,50]. In fact, D3 can be deleted without affecting RNA replication and tolerates the in-frame insertion of heterologous proteins such as GFP. Although D2 is also required for RNA replication, a large segment within D2 can be deleted with no significant effect on RNA replication and virus production in cultured cell lines[51].

The X-ray crystallographic structures of D1 reveal that the domain can take two alternative dimer forms (FIG. 4b,c). The structure of the D1 monomer is virtually identical in both forms, whereas the dimer interface is very different[52,53]. Biochemical studies suggest that the D1, LCS1 and D2 domains bind RNA, and that this binding promotes NS5A dimerization[54]. Although both dimers have RNA-binding motifs, the proposed RNA-binding residues identified in one study are exposed and distributed between two spatially separated and flat surfaces[53] (FIG. 4b). By contrast, in the 'claw-like' dimer structure identified in another study, the positively charged cleft formed by the two monomers can accommodate single- and double-stranded RNA[52] (FIG. 4c). On the basis of *in silico* modelling, it has been suggested that this dimer oligomerizes to form long chains that tether the viral RNA genome to intracellular membranes and eventually shield it from degradation as well as from sensing by pattern recognition receptors[55].

D2 and D3 of NS5A are intrinsically unfolded monomers[56,57] and as such are not true structural domains (FIG. 4a). This structural flexibility might explain the promiscuous interaction properties of NS5A, which has been reported to bind to numerous cellular proteins[58]. However, only few of these binding partners have been validated so far in authentic cell culture systems or *in vivo*. Two prominent examples are CYPA[59] (FIG. 4a) and phosphatidylinositol 4-kinase IIIα (PtdIns4KIIIα)[60,61], which are essential for HCV replication and have been pursued as antiviral drug targets (see below).

NS5A is produced as multiple phospho-variants. On the basis of their apparent molecular masses, the predominant forms are p56 and p58, designated the basal and hyperphosphorylated forms, respectively[62]. Phosphorylation is mediated by the α-isoform of casein kinase I[63] and by casein kinase II[64], although other kinases also seem to be involved[65]. Several potential phosphorylation sites have been identified in the central and C-terminal part of NS5A as well as in the LCS1 region. Mutations that enhance RNA replication in cell culture often affect hyperphosphorylation and impair virion assembly, arguing that the phosphorylation status of NS5A regulates these processes.

For a long time, it was thought that NS5A was not druggable, because it does not possess a known enzymatic activity[66]. Instead, this protein recruits viral and cellular proteins that mediate a given reaction, so interference with these cofactors or with their binding to NS5A should exert antiviral activity. Recently, lead compounds were identified using cell-based HCV replication systems (BOX 1) for high-content screening of large drug libraries. One class of these compounds has been found to target PtdIns4KIIIα[67]. Another class that could be refined into highly potent inhibitors with antiviral activities in the low picomolar range indeed targets NS5A. This compound class is exemplified by daclatasvir (BMS-790052)[68]. Daclatasvir is characterized by a highly symmetrical structure, and resistance mutations against daclatasvir and related compounds map to either D1 or the linker region between the N-terminal α-helix and D1 (FIG. 4b,c). The most prominent mutation affects Tyr93, which is positioned on the dimer interface in both X-ray crystallographic structures. Importantly, mutations at this site confer cross-resistance to several NS5A inhibitors and, in the case of daclatasvir, reduce drug sensitivity by ~20-fold for

Phosphatidylinositol 4-kinase IIIα (PtdIns4KIIIα). A lipid kinase that generates PtdIns 4-phosphate by adding a phosphate group to the 4-hydroxy group of PtdIns. PtdIns phosphates are membrane-localized metabolites that have important roles in intracellular signalling and membrane trafficking.

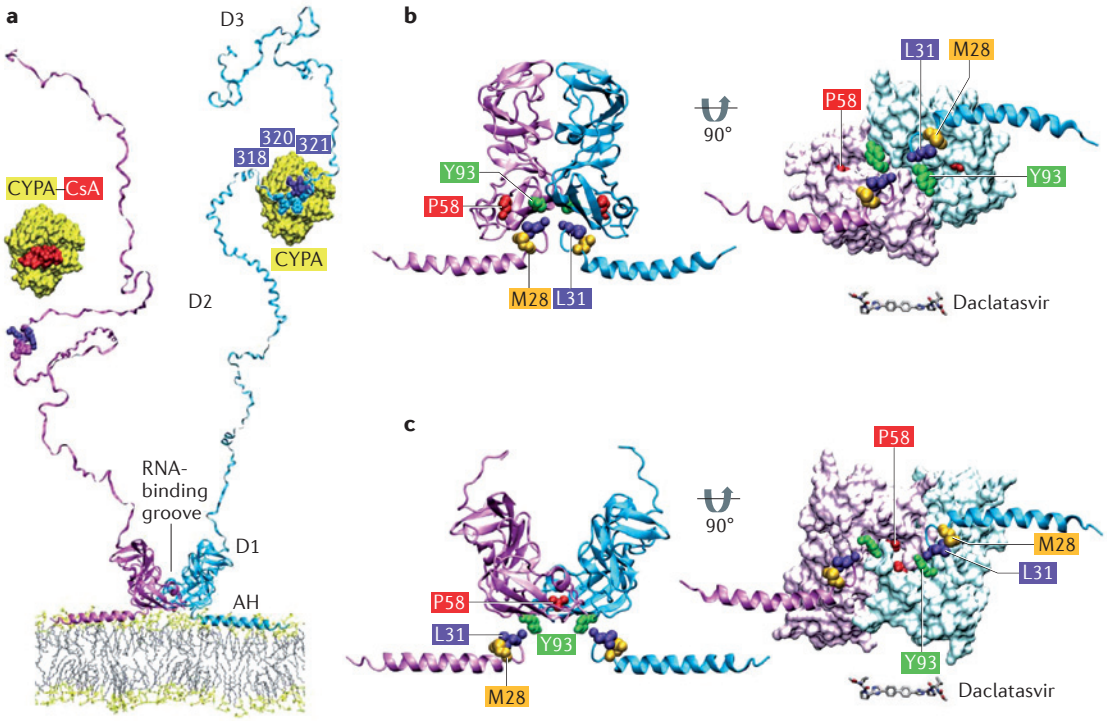

Figure 4 | **Structure of the NS5A dimer. a** | Ribbon diagram of a model of the full-length NS5A dimer associated with a phospholipid membrane. Each subunit (lilac and cyan, respectively) consists of the amino-terminal amphipathic α-helix (AH; Protein Data Bank (PDB) accession 1R7E), the highly structured domain 1 (D1; PDB accession 1ZH1) — which is shown in position relative to a 1-palmitoyl-2-oleoyl-3-*sn*-glycero-3-phospholcholine membrane bilayer, according to data published in REF. 52 — and the intrinsically unfolded 'domains' D2 and D3, representative conformers of which are also shown (F.P., unpublished observations). A surface representation of cyclophilin A (CYPA) in complex with cyclosporin A (CsA) is shown on the upper left (PDB accession 1CWA). A putative structure for CYPA and its main binding site in NS5A D2 is shown on the upper right. Note that all domains and structures are drawn to scale to illustrate the length and flexibility of the 'arms' formed by D2 and D3 and the relative sizes of CYPA, the CYPA–CsA complex and NS5A. **b**,**c** | Structure of the NS5A D1 dimer according to data pubished in REF. 53 (PDB accession 3FQQ) (part **b**) and REF. 52 (part **c**) , and the positions of mutations that confer resistance to NS5A inhibitors. The left panels show side views, and the right panels show views of the putative membrane-interacting surface. Note that the position of the amphipathic α-helix relative to D1 in the right panels is arbitrary and assumes that resistance mutations observed in D1 and the amphipathic α-helix would be close to each other. Also note the supposed membrane-proximal positions of the resistance mutations in both dimer structures. Mutation of Pro58 has been associated with secondary resistance to daclatasvir but does not confer resistance by itself[156]; this residue is shown to highlight the alternative orientations of the monomers in the different dimer structures. The structure of daclatasvir (stick representation) is shown, drawn to scale, in the right panels of parts **b**,**c**.

genotype 1b subgenomic replicons and ~1,800-fold for genotype 1a subgenomic replicons[68]. This loss of drug sensitivity might be even higher, as daclatasvir seems to affect not only RNA replication as tested with such replicons, but also virion assembly and/or virus particle secretion[69]. In any case, these results and the direct binding of a daclatasvir-related compound to NS5A identify daclatasvir as an NS5A inhibitor, but the mode of action is currently unknown. It has been reported that daclatasvir reduces NS5A hyperphosphorylation, but as this phenotype is pleiotropic and caused by many different perturbations (such as mutations in NS5A or treatment with protease inhibitors), reduced amounts of p58 are probably an epiphenomenon rather than a reason behind the inhibitory potency of daclatasvir. The same might apply to the observed redistribution of NS5A from the ER to lipid droplets following inhibitor treatment[70]. Another possibility is that daclatasvir and related drugs block dimerization or oligomerization of NS5A. In fact, the position of the Try93 resistance mutation near the dimer interface in both X-ray crystallographic structures argues that daclatasvir might disturb dimer formation. Alternatively, structural changes induced by daclatasvir might abrogate functionally important interactions with host factors such as PtdIns4KIIIα. Further studies will be required to discriminate between these possibilities.

The RdRp NS5B. The RdRp NS5B is the key enzyme mediating viral RNA synthesis. The structure of the catalytic core can be compared to a right hand with 'fingers', 'palm' and 'thumb' subdomains[71–73] (FIG. 5). The active site is highly conserved and located in the palm subdomain (FIG. 5a). The catalytic domain precedes a linker and a C-terminal membrane insertion sequence, the latter being essential for RNA replication in cell culture but not for *in vitro* enzymatic activity[31,32,74]. This membrane insertion sequence is omitted in all structural studies, so detailed information on the

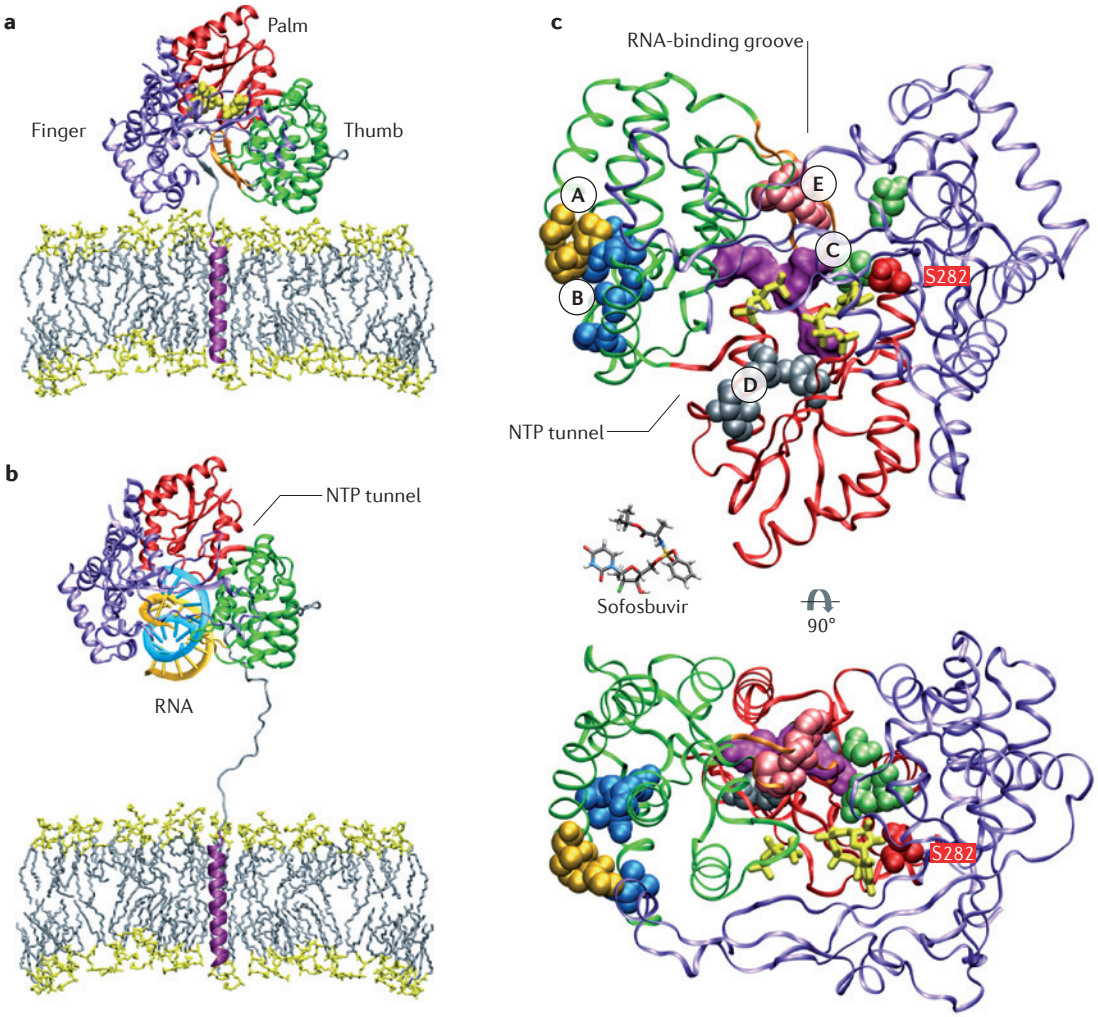

Figure 5 | **Structure of NS5B and positions of resistance mutations.** **a** | Ribbon diagram of full-length NS5B (Protein Data Bank (PDB) accession 1GX6) and the association with the membrane via the NS5B carboxy-terminal transmembrane tail. The finger, thumb and palm subdomains are indicated, and the so-called β-loop is shown in orange. The C-terminal linker sequence (grey) connects the core of the enzyme with the membrane insertion sequence (magenta). The structure indicates the proposed membrane topology of NS5B in the so-called closed conformation, on the basis of available X-ray crystallographic structures, and this is believed to represent the initiation state of the polymerase. The active site is highlighted by two priming nucleotides (yellow). In this conformation, the RNA-binding groove is hidden by the NS5B ectodomain that stacks to the membrane. **b** | A model of NS5B in a hypothetical elongation mode, which releases the RNA-binding groove. A double-stranded RNA replication intermediate was modelled into the active site according to data in REF. 77. Note the supposed membrane dislocation of the NS5B catalytic core, induced by the 'stretching' of the linker sequence on switching from initiation to elongation of RNA synthesis (F. P., unpublished observations). **c** | Positions of the target regions of five distinct classes of non-nucleoside NS5B inhibitors (A–E) and of some side chains of amino acid residues involved in resistance to these drugs. The position of the major resistance mutation against nucleotide and nucleoside inhibitors (at Ser282) is also indicated. The C-terminal membrane anchor and the linker have been removed for clarity. The active site is indicated by two priming nucleotides (yellow sticks). For a better comparison with already published structures, the orientation of the finger, palm and thumb subdomains has been rotated by 180° relative to parts **a**,**b**. The lower panel is a view onto the RNA-binding groove. Highlighted positions that confer drug resistance when they are mutated are: Pro495, Pro496 and Pro499 (site A; gold); Leu419, Met423, Ile482 and Val494 (site B; blue); Gly554, Ser556 and Asp559 (site C; green); Leu314, Ile363 and Ser365 (site D; grey); and Cys445 and Tyr452 (site E; light pink). Mutations affecting positions shown in magenta confer cross-resistance to several classes of non-nucleoside inhibitors: mutation of Cys316 and Tyr414 confers resistance to class C, D and E inhibitors, whereas mutation of Tyr448 confers resistance to class C and E inhibitors. The structure of the nucleotide analogue sofosbuvir, drawn to scale, is indicated by a stick representation. NTP, nucleoside 5′-triphosphate.

native conformation of membrane-associated NS5B is not available. Compared with other RdRps, NS5B has a rather closed conformation of the active centre (FIG. 5a). In this conformation, the C-terminal linker folds back into the active centre and might be involved in initiation of RNA synthesis[75]. As a consequence, the entire enzymatic core is tethered to the membrane, thus hiding the RNA-binding groove.

The closed conformation is supposed to represent the initiation state of the polymerase, as the catalytic core provides space for a single-stranded RNA template, but not for a double-stranded replication intermediate[76]. To switch from initiation to processive elongation, the enzyme must undergo a major conformational change resulting in an opening of the structure to generate a large cavity capable of binding double-stranded RNA[77] (FIG. 5b). In addition, the structure of the C-terminal linker is disordered in this open conformation, most probably repositioning the enzyme relative to the membrane.

With the availability of recombinant NS5B[31,32] and cell-based high-throughput screening systems, several candidate NS5B inhibitors have been identified[42]. They are grouped into two major classes on the basis of their mode of action: nucleoside and nucleotide inhibitors (NIs), which are mimics of the natural substrates of the polymerase and act at the active site of the enzyme, and non-nucleoside inhibitors (NNIs), which bind to different allosteric sites and inhibit conformational changes of the polymerase. Owing to the high degree of conservation of the active site, as compared to the allosteric binding sites, NIs are generally effective against a broader range of viral genotypes and have a higher barrier to resistance than NNIs.

NIs are derivatives of ribonucleosides or ribonucleotides and compete with natural ribonucleotide substrates for binding to the active site of the polymerase. Thus, NIs require high intracellular concentrations to be effective and have EC_{50} (effector concentration for half-maximum response) values in the low-micromolar range[78]. These drugs are typically delivered as non-phosphorylated prodrugs carrying chemical modifications to enhance uptake and bioavailability. Within the cell, these modifications are cleaved off, and the liberated nucleoside is converted into the active nucleoside 5′-triphosphate by cellular enzymes. As the initial phosphorylation of a nucleoside is rate limiting, NIs have been developed (so-called ProTides) that are prodrugs of nucleoside 5′-phosphates, thereby increasing the effective intracellular concentration of the drugs. After phosphorylation, the active drug competes with its natural nucleotide counterpart for incorporation into the growing RNA.

3′-deoxy-modified nucleosides are classic chain terminators that were shown to efficiently inhibit NS5B *in vitro*, but owing to inefficient phosphorylation, they are poorly active in cell culture[79]. Therefore, almost all NIs currently in clinical development retain the 3′ hydroxyl group, but are modified at the 2′ position of the ribose, probably causing chain termination by steric hindrance[42]. Owing to the structural similarity of 2′-modified NIs, a single NS5B mutation (Ser282Thr) confers resistance to all these NIs in cell culture[80] (FIG. 5c). However, this mutation confers only a low level of resistance and dramatically reduces viral fitness, thus explaining why it has not been observed in patients[81].

One possible drawback of HCV-targeting ribonucleoside analogues is their interference with human mitochondrial RNA polymerase[82]. In fact, ribonucleoside analogues containing 2′-*C*-methyl, 4′-methyl and 4′-azido substituents, which are often found in HCV-specific 2′-modified NIs, are inhibitors of this enzyme and thus inhibit mitochondrial gene expression. This property might explain why several ribonucleoside analogues, such as valopicitabine (NM283; a prodrug of 2′-*C*-methylcytidine) and balapiravir (RG1626; a prodrug of 4′-azidocytidine) exert serious side effects and could not be pursued in clinical trials[42].

Mericitabine (RG7128) is currently the clinically most advanced nucleoside analogue and has been shown to increase sustained virological response rates in patients infected with genotype 1 or genotype 4 viruses[83]. Sofosbuvir (PSI-7977)[84] (FIG. 5c), the most advanced nucleotide analogue, has demonstrated extraordinary efficacy against HCV genotypes 1–6 in combination with both PEG–IFNα and ribavirin[85], and even in IFN-free treatment regimens, particularly against HCV genotype 2 and HCV genotype 3 (REFS 85–87).

NNIs are chemically and functionally much more diverse than NIs and are currently grouped into 4–5 classes on the basis of their allosteric binding sites and the unique sets of mutations that confer resistance to the drugs[42,88] (FIG. 5c). The mode of action of NNIs is mostly non-competitive. Some are highly potent against genotype 1 viruses, but resistance can be readily selected and is typically not associated with a high fitness cost[89]. A number of NNIs are currently in clinical development, and we can therefore refer to only a few representative examples.

The inhibitor-binding sites A and B of NS5B (also known as thumb I and thumb II, respectively) are located in the thumb domain. Site A inhibitors such as BI 207127 bind at the junction of the thumb subdomain and the N-terminal finger loop, and inhibit productive binding of the polymerase to the template RNA[90]. Site B, at the base of the thumb domain, is targeted by a set of chemically diverse compounds (for example, lomibuvir (VX-222) and filibuvir (PF-868554)) that bind only to the closed conformation of NS5B[91] and thereby probably inhibit a conformational change that is required for elongation[92] (FIG. 5a,b). These inhibitors are mainly active against HCV genotype 1 strains, and mutations conferring resistance affect similar amino acid residues (FIG. 5c).

Allosteric inhibitor-binding sites within the palm domain are overlapping (FIG. 5c; binding site C is in the palm I region, and sites D and E are in the palm II region), and some mutations have been identified that confer cross-resistance to different classes of inhibitors (FIG. 5c). Site C is located at the junction of the palm and thumb subdomains. Most inhibitors targeting this site (for example, ABT-333)[93] contain a benzothiadiazine scaffold and block a step before elongation. Site D is close to the active site and targeted by a class of benzofuranes (for example, nesbuvir (HCV-796))[94]. Binding of site E inhibitors (imidazopyridines such as tegobuvir (GS-9190))[95] involves the palm domain and the β-loop region (FIG. 5c). Interestingly, these compounds are not active against purified NS5B but are potent inhibitors of RNA replication in cells. Recent data indicate that tegobuvir binds covalently to the polymerase after an intracellular activation step mediated by cytochrome P450

Sustained virological response rates
The percentages of patients with undetectable hepatitis C virus RNA in the blood at least 6 months after the completion of antiviral therapy.

(REF. 96). However, clinical development of HCV-796 was halted owing to adverse effects[42], tegobuvir seems to have been discontinued owing to limited efficacy[33], and no other site D or site E inhibitor seems to be involved in any current Phase II or Phase III clinical trial.

Alternative viral drug targets

In addition to NS3-4A, NS5A and NS5B, several other viral proteins and steps of the HCV replication cycle have been pursued as drug targets, including polyprotein processing by the NS2-3 protease and RNA unwinding by the NS3 helicase region. Here, we briefly refer to the targets for which drug development is currently the most advanced, NS4B and p7.

NS4B. The highly hydrophobic protein NS4B is the main inducer of the membranous web[97]. NS4B has an unusual membrane association; two amphipathic α-helices (AH1 and AH2) form the N-terminal region and two α-helices within the C-terminal region contact the membrane, and there are four predicted transmembrane segments (FIG. 6a). In addition, the C terminus of NS4B appears to be palmitoylated, suggesting that the membrane association of NS4B is mediated both by a complex set of membrane-binding segments and by covalently attached fatty acid moieties[98]. The membrane topology of NS4B is dynamic, and AH2 is expected to traverse the membrane bilayer[99] (FIG. 6a). This might be triggered by NS4B oligomerization and reduced by interaction with other viral proteins, most notably NS5A[100]. Oligomerization of NS4B is critical for the formation of double-membraned vesicles, which are the main constituents of the membranous web. The formation of NS4B oligomers is probably mediated by several homo- and heterotypical interactions involving AH2 and the C-terminal helices[101,102]. Nevertheless, a recent study has demonstrated that formation of the membranous web is not mediated by NS4B alone, but rather appears to require the concerted action of several, if not all, HCV replicase proteins[24].

Apart from inducing membrane curvature, NS4B might contribute more directly to HCV RNA replication. This protein was reported to bind viral RNA with a preference for the 3′ terminal region of negative-sense RNA[103]. Moreover, NS4B might possess NTPase activity[104], but its role in the HCV replication cycle remains to be determined. Finally, NS4B mutants with enhanced virus production compared with wild type have been described, indicating that NS4B is probably also involved in assembly of infectious HCV particles[102,105].

At least two classes of NS4B inhibitors have been described. The first class is exemplified by clemizole hydrochloride, which inhibits the NS4B interaction with HCV RNA[106]. Interestingly, mutations conferring resistance to this compound map to the 5′ NTR of the genome or to NS4B, supporting the notion that the NS4B–RNA interaction is affected by clemizole. The second class of NS4B inhibitors appears to affect the NS4B–membrane interaction or NS4B self-interaction (compounds A2 and C4 in REF. 107, respectively). Silibinin (SIL) is a hepatoprotective therapeutic that has been approved for treatment of intoxications and is used to treat patients with chronic hepatitis C after they have had a liver transplant, as well as patients who do not respond to IFN; very recently, intravenous SIL was found to target NS4B as well[108]. Resistance mutations mapping to NS4B were found in cell culture and in a patient who underwent SIL treatment. Although the mode of action of SIL is not clear, the available data suggest that it disturbs the morphology of the membranous web as a result of impaired NS4B–NS3 interaction[108].

p7. The integral membrane protein p7 (63 amino acids) is composed of two transmembrane α-helices that are connected by a short flexible loop (FIG. 6b). p7 forms hexamers or heptamers with properties indicative of a cation channel function and is therefore regarded as a viroporin[109]. Electron microscopy of negative-stain samples in combination with single-particle reconstruction revealed that a p7 hexamer formed in a synthetic detergent has a flower-shaped three-dimensional structure[110]. However, molecular dynamics simulations suggest that p7 adopts a cylindrical, upright conformation when inserted into a synthetic phospholipid bilayer that mimics the thickness of the ER membrane[111] (FIG. 6b). The adaptability of the p7 conformation to the thickness of the membrane bilayer illustrates the structural plasticity of p7 and its minimalist ion channel architecture, and this is a general property of viroporins. Moreover, the conserved bulky hydrophobic residues at positions Phe25 and Ile32 (FIG. 6b) seem to play an essential part in p7 channel gating by forming a hydrophobic barrier that could be released by minor movements of the α-helical segments[111].

The exact function of p7 in the HCV replication cycle is not known, but most evidence points to a role in assembly and/or release of infectious virus particles. One attractive hypothesis is that p7 prevents premature acidification of virus-containing transport vesicles and thus premature induction of the conformational changes that are required in viral envelope proteins for viral entry[112]. Whatever the mechanism is, p7 forms stable complexes with E1–E2 and NS2 as part of a possible 'envelopment complex' before or during viral budding[14].

Owing to the ion channel activity of p7, *in vitro* studies and clinical trials have been conducted with amantadines and rimantadine, two well-described types of inhibitor of the influenza A virus M2 ion channel. However, conflicting results were obtained *in vitro* and in clinical studies[109]. The most clinically advanced p7 inhibitor is BIT225, which was developed by rational drug design based on amiloride derivatives. BIT225 inhibits p7 channel activity *in vitro*, blocks production of bovine viral diarrhoea virus (a relative of HCV) and appears to improve the antiviral response in patients receiving a combination of PEG–IFN, ribavirin and BIT225 (REF. 113).

Alternative host cell targets

Apart from targeting virally encoded proteins, host cell factors that are essential for HCV replication have emerged as promising targets for antiviral therapy. For example, inhibitors of SRB1 are currently being tested in clinical studies for their ability to block viral entry[114].

Viroporin
A virally encoded membrane protein that localizes mainly to the ER or the cell membrane of the host cell and forms an ion channel or pore.

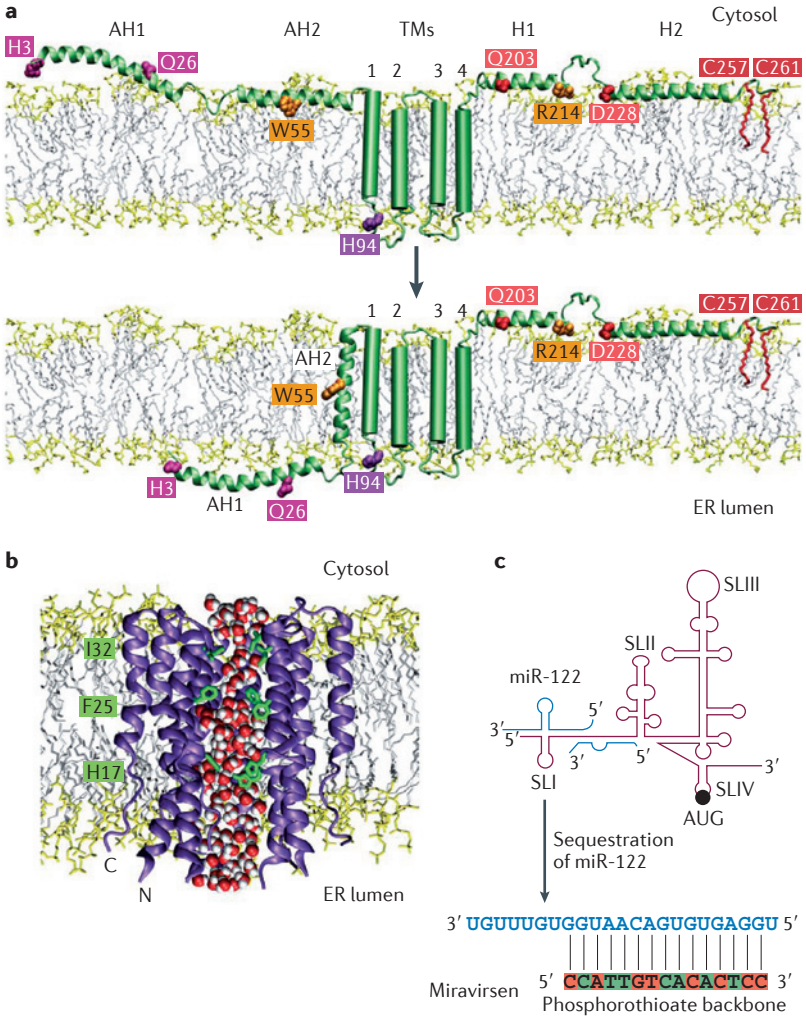

Figure 6 | **Alternative targets for hepatitis C virus-specific therapy. a** | The two alternative membrane topologies of hepatitis C virus (HCV) protein NS4B. Ribbon structures correspond to amphipathic α-helix 1 (AH1), AH2, α-helix1 (H1) and H2 as determined by NMR (Protein Data Bank (PDB) accessions 2JXF and 2KDR for AH2 and H2, respectively; F.P., unpublished observations for AH1 and H1). Green cylinders indicate the four predicted transmembrane helices (TMs)[157]. The two palmitic acid acyl chains attached to amino acids Cys257 and Cys261 are represented in red. Mutations at various residues (the side chains of which are shown as coloured van der Waals spheres) confer resistance to the following NS4B inhibitors: silibinin (Gln203Arg and Asp228Asn[108]), clemizole (Trp55Arg and Arg214Gln), an inhibitor from Bristol-Myers Squibb (His3Arg and Gln26Arg) and compound 2 from ViroPharma (His94Arg) (reviewed in REF. 158). The upper panel displays the NS4B membrane topology that is assumed to form during HCV polyprotein processing. The amino terminus of NS4B might then be translocated into the ER lumen, probably following oligomerization of AH2, thereby creating a fifth transmembrane passage (lower panel). **b** | Ribbon representation of a p7 hexamer model structure (side-view snapshot of the p7 pore in the open state) inserted into a fully hydrated phospholipid bilayer; this structure was obtained by combining NMR analyses and molecular dynamic simulations[111]. Water molecules inside the central pore are represented as van der Waals spheres (red and grey). The side chains of pore-lining residues His17, Phe25 and Ile32 are represented as green sticks. To visualize the water molecules in the central pore, one p7 monomer is omitted. **c** | Schematic of the 5′ non-translated region of the HCV genome, indicating the four major stem–loop (SL) structures, the initiator AUG codon of the polyprotein-coding region and the two binding sites for the microRNA miR-122. Sequestration of miR-122 by chemically modified antisense oligonucleotides (for example, miravirsen) impairs translation and replication of HCV RNA. In the phosphorothioate backbone of miravirsen, locked nucleic acid (LNA)-modified nucleotides are indicated in red, whereas deoxyribonucleotides are indicated in green.

Among the plethora of host factors contributing to the viral replication cycle, we briefly describe the two that have been explored in most detail as drug targets: CYPA and miR-122.

Cyclophilins. The discovery of CYPA as an essential HCV dependency factor started with the observation that the immunosuppressive drug cyclosporin A (CsA) inhibits HCV replication in cell culture[115]. This drug targets CYPA (FIG. 4a), the peptidyl prolyl isomerase that catalyses the *cis–trans* isomerization of peptide bonds at Pro residues. In addition, the CYPA–CsA complex inhibits calcineurin, the protein phosphatase required for the activation of T cells. Several subsequent studies identified CYPA, which is expressed at high abundance in the cytosol, as the primary host factor supporting HCV replication and targeted by CsA[59,116]. On the basis of these results, CYPA antagonists, often derived from CsA, have been developed. These compounds, such as alisporivir (formerly known as Debio 025), NIM811 and SCY635, lack immunosuppressive effects but retain high-affinity CYPA binding.

The way in which CYPA contributes to HCV replication is still not clear. It was found that CYPA binds to NS5A domains D2 and D3, which serve as a direct substrate for the isomerase[56,57] (FIG. 4a). Moreover, mutations conferring resistance to CYPA antagonists primarily map to the D2 domain of NS5A[117] or to the C-terminal region of D3 close to the NS5A–NS5B cleavage site[116]. It is therefore assumed that CYPA affects proper folding of NS5A, which in turn affects replicase activity — for example, by interfering with the interaction between NS5A and the RdRp NS5B or by altering the RNA-binding properties of NS5A. Alternatively, CYPA might be required for the recruitment of NS5B into the membranous HCV replicase complex[118]. In any case, all classes of CYPA inhibitors, including CsA derivatives such as alisporivir or the structurally distinct sanglifehrins, inhibit the NS5A–CYPA interaction in a dose-dependent manner. Importantly, these *in vitro* results hold true in clinical trials, as CYPA antagonists have antiviral activity against all HCV genotypes *in vivo* and exert a high genetic barrier towards drug resistance[119].

miR-122. The second important host cell factor that is pursued for treatment of chronic hepatitis C is miR-122, which is expressed preferentially in the liver. In contrast to the usual role of microRNAs in repressing mRNA translation, miR-122 enhances HCV replication[120]. Two copies of miR-122 bind to two regions in the 5′ NTR close to the 5′ end of the viral RNA genome[121] (FIG. 6c), whereas a third binding site for miR-122 in the 3′ NTR appears to be dispensable. Consistent with the general biogenesis of microRNAs, miR-122 binds to HCV RNA in a complex containing Argonaute proteins, which are part of the RNA-induced silencing complex (RISC)[122]. This explains the originally perplexing observation that knockdown of RISC components, as well as of Dicer and Drosha, reduces HCV replication rather than enhancing it[123]. miR-122 seems to stimulate HCV replication by several mechanisms: first, by stabilizing HCV RNA[124], in

RNA-induced silencing complex
A multiprotein complex that incorporates a microRNA to recognize complementary sequences in mRNAs and block protein expression.

part by protecting it from degradation by 5′–3′ exoribonuclease 1 (XRN1)[125]; second, by stimulating translation of the viral genome[126]; and third, by enhancing RNA replication[120]. Whatever the mechanism is, depleting miR-122 from cells using miR-122-specific antagomirs efficiently disrupts HCV replication *in vitro* and *in vivo*[127,128]. However, miR-122-knockout mice have recently been shown to develop steatohepatitis, fibrosis and liver tumours[129,130]. This tumour-suppressive property of miR-122 and the important role of this microRNA in hepatocyte lipid homeostasis have to be taken into account when considering the therapeutic use of miR-122 antagonists.

Concluding remarks

With the advent of cell culture models (BOX 1) that recapitulate the HCV replication cycle and with the determination of the structures of HCV proteins, tremendous progress has been made in our understanding of both the HCV–host cell interaction and the mode of action of viral and cellular factors that contribute to efficient viral replication. Without doubt, these insights have paved the way for the development of HCV-specific DAAs. First, they allowed the discovery of novel drug targets such as NS5A; second, they allow the improvement and validation of HCV-specific compounds that are developed using *in vitro* assays or surrogate systems; third, they enable us to study the mode of action of DAAs; and fourth, they are instrumental to identifying and understanding viral escape from DAAs. Today, ~25 years after the first molecular cloning of the HCV genome[131] and ~15 years after the establishment of the first cell culture system for this virus[15], a remarkable pipeline of HCV-specific DAAs is in advanced clinical trials, and we can expect that new therapies with fewer and less severe side effects, much lower dependency on HCV genotypes and higher success rates will become available in the next few years[33].

With the recent approval of the first-in-class DAAs, the two NS3-4A inhibitors telaprevir and boceprevir, an important first step has been made, as viral elimination rates for the HCV genotype 1 infections (the most common infections) have increased from ~45% to ~75%[132–135]. However, this therapy still relies on PEG–IFNα and ribavirin, and is associated with even more side effects than the dual combination without a protease inhibitor. Moreover, telaprevir and boceprevir require a very strict and complex dosing schedule, are limited to treatment of HCV genotype 1 infections and are associated with selection for antiviral resistance, especially in patients with a poor IFNα response[43]. Thus, the 'holy grail' will be a highly efficacious, all-oral and well-tolerated treatment that does not involve IFNα or ribavirin and is effective against all HCV genotypes. A few years ago, most people in the field would have regarded this goal as a dream, but recent clinical trials (for example, those described in REFS 85–87,93,136) have demonstrated that this can become a reality, and much faster than even optimists would have anticipated. One main question is which IFNα- and ribavirin-free combination will become the future standard of therapy. The answer is still up in the air, but it is obvious that DAAs with a high potency and a high genetic barrier to resistance will be required. Second-generation NS3-4A inhibitors, NS5A-targeting DAAs and inhibitors of the RdRp NS5B are the most likely candidates. In the case of the latter, NIs are expected to become an important component of future therapy although recent clinical trials have shown that NNIs, when given in combination with NS3-4A inhibitors and an NS5A inhibitor, are highly efficacious, inducing a sustained virological response in >90% of treated patients[33]. Whatever the outcome of these studies, treatment schedules are expected to become easier and better tolerated, raising hope that antiviral therapy will also become applicable to those difficult-to-treat patients who suffer most from chronic hepatitis C.

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Competing interests statement
The authors declare competing financial interests. See Web version for details.

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