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HCV Virology

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Abstract

HCV was discovered and its sequence cloned in 1989, the acme of the pioneering work of Michael Houghton's group (Choo et al., 1989). Since then, studies of the HCV replication cycle have long been hampered by the following facts : (i) HCV is a strictly human pathogen that causes a chronic infection; (ii) the target cells of HCV replication are solely hepatocytes; (iii) virions isolated from the serum of HCV-positive patients could not infect cell culture systems in a productive manner; (iv) viral titration (assay of viral proteins and/or genome) could not be related to infectivity. Chimpanzees are permissive to HCV, but their use comes at prohibitive costs, and features of the disease are not similar to human clinical signs. Three major breakthroughs allowed to overcome these restrictions : the first one came with the discovery and implementation of the HCV replicon system in cell culture based on a full-length consensus genome from a viral RNA isolated from an infected human liver. This genome was then used to construct subgenomic replicons, which, upon transfection into a human hepatoma cell line, replicated to high levels (Lohmann et al., 1999). With this invaluable tool, scientists in the field of hepatitis C were able to address fundamental questions related to viral replication *in vitro*, and this also fuelled intensive research on antiviral molecules. The second breakthrough came with the molecular design of viral particles able to mimic the first stages of the interaction of HCV with its target cells: virion attachment to the cell surface, recognition of specific receptor molecules, internalization in intracellular compartments, viral fusion. This tool, so-called HCV pseudoparticles (HCVpp), was based on retroviral capsids harboring the glycoproteins E1 and E2 of HCV at the virion surface. HCVpp were subsequently used to study viral entry, fusion and humoral immune responses and to dissect the molecular details of the steps by which HCV initiated infection (Bartosch et al., 2003a). The third breakthrough emerged from a clinical case report in Japan of a fulminant hepatitis caused by HCV. This unusual feature for HCV led to the isolation and discovery of a new HCV clone, displaying a full replication cycle in cell culture settings. This clone was called JFH-1 (for Japanese Fulminant Hepatitis), and produced infectious viral particles capable of several rounds of *in vitro* infection, called HCVcc (HCV grown in Cell Culture) (Wakita et al., 2005, Lindenbach et al., 2005). These particles are used for structural and functional studies, and were successfully applied to the discovery of antiviral therapies now licensed and capable of HCV eradication.

Experimental systems to study HCV life cycle

In parallel to these key discoveries, adequate and improved *in vitro* and *in vivo* models were developed. Sophisticated cell culture systems were designed to facilitate HCV propagation and establish long-term culture systems. Human hepatoma cells Huh7 were used as the base to design Huh7-derived clones highly permissive to infection, producing high levels of infectious virions and maintaining persistent infection over several passages (Blight et al., 2002, Zhong et al., 2005). The use of these cells led to the discovery of two of the four essential receptors required for HCV entry : claudin-1 (Evans et al., 2007) and occludin (Liu et al., 2009a, Ploss et al., 2009), and led to the identification of other receptor molecules and entry factors [the Niemann-Pick C1L1 molecule : (Sainz et al., 2012); the receptor of the epidermal growth factor: (Lupberger et al., 2011); the heparan sulfate proteoglycan syndecan-1 (Shi et al., 2013b, Grigorov et al., 2016). Human primary hepatocytes are the most physiologically relevant cells to perform *in vitro* infections, closely reproducing the *in vivo* situation (Gondeau et al., 2014), but their survival in culture is

limited and their capacity to sustain productive infection is low. To overcome these limitations, human embryonic pluripotent stem cell-derived hepatocytes (hESC) were isolated, differentiated into hepatocyte-like cells (HLC) and successfully infected with HCV, able to complete a full replication cycle (Roelandt et al., 2012). Similar successful infection experiments could be mounted with human induced pluripotent stem cells (hiPSC) derived from patients (Carpentier et al., 2014).

HCV *in vivo* studies were long hampered by the lack of (small) animal models able to sustain HCV infection and develop human-like disease symptoms. Chimpanzees were instrumental to the discovery and identification of HCV as the etiologic agent for non-A non-B viral hepatitis (Choo et al., 1989). However the natural course of infection in chimpanzees differs from that in humans, since very few develop chronic HCV infection, no fibrosis and only one hepatocellular carcinoma (HCC) case has been observed (Muchmore et al., 1988). Moreover, availability, cost, and ethical concerns limit the use of these primates for HCV research. Support for invasive research on chimpanzees was recently discontinued in the USA. Small animal (rodent) models were therefore developed. The first fully permissive murine model that supported long-term HCV infection was produced by intrasplenic injection of primary human hepatocytes into immunodeficient mice with diseased liver, such as Alb-uPA/SCID (urokinase-type plasminogen activator transgenic severe combined immunodeficiency) and FRG (fumarylacetoacetate hydrolase-recombination activating gene 2-interleukin-2 receptor, common γ -chain knockout) (Mercer et al., 2001, Meuleman et al., 2005, Bissig et al., 2010). These mice with human chimeric liver secrete human serum albumin levels similar to those observed in humans (Calattini et al., 2015), and display a human-like blood lipoprotein profile (Steenbergen et al., 2010) that renders them highly susceptible for infection with human hepatotropic pathogens such as HCV and the hepatitis B virus, thus enabling the study of HCV biology, the evaluation of different antiviral strategies and the occurrence of antiviral resistance (Bissig et al., 2010, Shi et al., 2013a). However, these mice are immunodeficient, and active research was conducted to develop a fully immunocompetent mouse model of HCV infection with hepato-pathological manifestations (Chen et al., 2014).

Structural organization of viral particles

HCV isolated from the serum of infected patients, chimpanzees or mice with humanized livers can be fractionated into roughly three populations : a very compact and heavy fraction, a fraction of intermediate density, and a population of very light particles floating at the surface of density gradients (Andre et al., 2002). HCVcc display a similar biophysical behavior (Nielsen et al., 2006). Observed by transmission electron microscopy (TEM), the three populations appear respectively as : (i) 35-40 nm nonenveloped particles; (ii) 70 nm-spherical particles, comprising an electron-dense core and a lipid envelope identified as a bilayer, and considered as canonical viral particles; (iii) particles with the aspect of serum lipoproteins, called lipo-viro-particles (LVPs; see below and Fig 1).

All particles have in common the presence of a nucleocapsid composed of the single-stranded viral RNA compacted by the capsid protein called core. Particle populations of intermediate and light densities are enveloped fractions harbouring the glycoproteins E1 and E2 embedded in the lipid membrane surrounding the nucleocapsid. Core, E1 and E2 constitute the structural proteins of HCV. At the virion surface, E1 and E2 form a heterodimer assembled by covalent interactions and stabilized by disulfide bridges (Vieyres et al., 2010).

Nonenveloped nucleocapsids

A high density fraction of viral particles isolated from HCV-infected patients was found composed of the HCV core, associated with HCV RNA. Electron microscopy revealed particles of heterogeneous size, with the predominant population 38 to 43 nm in diameter (Maillard et al., 2011). Similar structures were found in HCV produced in cell cultures (HCVcc) (Gastaminza et al., 2010), with a mean diameter of 44 nm. However these fractions displayed only low infectivity, so they are unlikely to be infectious.

Canonical particle structure

This intermediate-density pool of viral particles constitutes ca. 50% of the total particle population of HCV grown in cell cultures (clone JFH-1). This pool has been characterized at the ultrastructural level by

cryo-TEM: analysis revealed particles of an average diameter of 60-64 nm, composed of a 5-6 nm thick electron-dense bilayer, surrounding the nucleocapsid viewed as the densest zone (Gastaminza et al., 2010). These particles are characterized by a high E2/core protein ratio, and contain HCV RNA. As a matter of fact, this fraction of *in vitro* produced particles had the highest infectivity. However particles produced *in vitro* from another clone (Jc1), or *in vivo* in mice with humanized livers displayed a lower but more homogenous density profile, and a higher specific infectivity than JFH-1 HCVcc, and were found enriched with apolipoprotein E (apoE; see below) (Merz et al., 2011, Calattini et al., 2015). The lipid envelope of JFH-1 HCVcc was highly enriched in cholesterol as compared to cellular membranes, and displayed a high cholesterol-to-phospholipid content (Aizaki et al., 2008). Together with sphingomyelin, virion-associated cholesterol played a key role in HCV infectivity. The envelope of Jc1 HCVcc showed similar cholesterol contents as those measured for cellular membranes, but was found enriched in sphingomyelin (Merz et al., 2011), a lipid composition comparable to that of low-density lipoproteins (LDL) of human serum.

LVP structure

A hallmark of HCV particles is their association with host-cell lipids and lipoproteins, mainly very low-density lipoproteins (VLDL) and LDL, which are known to impact virion assembly, infectivity and structure (Andre et al., 2002, Nielsen et al., 2006, Felmler et al., 2010, Lindenbach et al., 2006) (Fig1). In patient sera virions display a very low buoyant density and are associated with lipoproteins. Based on these findings, HCV virions have been called lipo-viro particles (LVPs) (Andre et al., 2002). Biochemical as well as electron microscopy-based approaches have confirmed LVPs as hybrid particles composed of viral components and cellular lipoprotein components including in particular apolipoproteins E, B100/48, CI, CII and CIII (Lussignol et al., 2016). The dependence of HCV assembly on the cellular lipid metabolism is probably responsible for the strong morphological heterogeneity and dynamic structural changes of LVPs that have been observed in response to changes in dietary triglycerides (Felmler et al., 2010, Piver et al., 2016). In serum HCV components have been identified in various forms: LVPs, composed of an electron-dense center encapsidating the HCV RNA genome surrounded by a detergent-sensitive lipid coat. Interestingly, nucleocapsids are frequently detected at the periphery of the LVP and not in the center. LVPs also contain, besides the envelope glycoprotein heterodimer E1/E2, ApoE, B100 at the surface. Subviral lipoprotein-like particles, which contained E1/E2 and ApoE and B but lacked the nucleocapsid and HCV genome, were also detected in patient sera (Scholtes et al., 2012). Interestingly, lipoprotein-like particles represent a predominant form of HCV in the blood suggesting that they may play a major role in HCV immune responses and evasion. Indeed, reversible association of ApoE with LVPs in the serum has been shown to protect viruses from neutralization and modify their interactions with heparan sulfate proteoglycans (Bankwitz et al., 2017). However, the exact roles of LVPs in persistence as well as transmission remain to be determined.

The viral proteins

The HCV particles contain a positive polarity RNA genome with 5' and 3' untranslated regions (UTR) and a long open reading frame encoding a polyprotein precursor of about 3,000 amino acids. UTRs constitute highly conserved, cis-acting RNA elements regulating viral genome translation and replication. Translation of the polyprotein is initiated by ribosome binding to an internal ribosome entry site (IRES), which spans most of the 5'-UTR and the first 24–40 nucleotides of the core coding region. This results in the production of a single precursor polyprotein, which is processed by cellular and viral proteases into 10 structural and non-structural proteins (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). Core protein and the envelope glycoproteins (E1 and E2) make up the structural components of the virion. Nonstructural proteins (from NS3 to NS5B) assemble into a membranous-web-associated HCV RNA replicase complex that catalyzes the amplification of the viral RNA genome.

Core

It is a multifunctional protein. One of its main functions is to form the viral capsid with the genomic RNA, thereby contributing to particle assembly. The monomeric, mature form of HCV core is a 21-kDa protein with lipid and RNA binding activities (Santolini et al., 1994). The three-dimensional structure of core is still unknown, but biophysical studies revealed that the protein forms dimers. The monomers are organized in two domains, D1 and D2. A large part of the D1 domain is intrinsically disordered. This

feature might contribute to core RNA chaperoning functions, critical for the structural remodeling and packaging of the RNA genome into the viral particle (Cristofari et al., 2004). As other intrinsically unstructured proteins, core is expected to adopt different conformations depending on the presence of specific cellular partners, involved in gene transcription, lipid metabolism, apoptosis, and cell signaling. Through its mainly α -helical D2 domain, core associates to the membranes of endoplasmic reticulum (ER) and lipid droplets (LDs) (Boulant et al., 2006) (Fig 1), where efficient viral replication, assembly and infectious particle production occur. Comprehensive mutagenesis studies revealed residues critical for HCV infectivity, but not for viral RNA replication, evidencing the key role of core in HCV particle formation (Murray et al., 2007).

Core recruits nonstructural proteins, HCV RNA and the replication complex to LD-associated membranes (Miyanari et al., 2007). In particular, core recruits NS5A to LDs through direct protein/protein interactions, at regions enriched in HCV glycoproteins, E1 and E2. This spatial vicinity between structural proteins involved in particle formation and nonstructural proteins of the replication complex closely links particle assembly to virus replication. It was therefore suggested that LDs can serve as platforms for virion formation (Miyanari et al., 2007). Core also interacts with host cell factors that play a role in the viral assembly process. Diacylglycerol acetyltransferase-1 (DGAT1), a key enzyme of LD biogenesis, binds core and targets it to LDs; the inhibition of this trafficking impairs HCV particle production (Herker et al., 2010). DGAT1 was also shown to facilitate the binding of NS5A to core, and guide both proteins onto the surface of LDs, aiding infection (Camus et al., 2013). Nucleoporin-98 is a structural component of the nuclear pore complex, which interacts with HCV core and relocalizes to LDs (Lussignol et al., 2016); it is a key factor involved in late steps of viral particle biogenesis, whose functions are most likely hijacked by HCV to efficiently transport its proteins from various compartments. The tail-interacting protein of 47 kDa (TIP47) also contributes to the targeting of NS5A to core-enriched HCV assembly sites on the surface of LDs, by direct TIP47/NS5A interactions (Vogt et al., 2013).

E1 and E2

HCV virions harbor at their surface two transmembrane type I proteins, E1 and E2, in the form of a covalent heterodimer stabilized by disulfide bridges (Vieyres et al., 2010). The transmembrane domains of E1 and E2, located at their C-termini, are involved in heterodimerization and have ER retention sequences (Moradpour et al., 2007). E1 and E2 are heavily glycosylated, with respectively 5 and 11 conserved N-linked glycans. This N-linked glycosylation plays key roles in : (i) HCV assembly by regulating the folding of E1 and E2 and the formation of the E1/E2 heterodimer; (ii) HCV entry into target cells by modulating the affinity of viral particles for cell surface receptors, in particular CD81 (see below); (iii) HCV susceptibility to neutralizing antibodies by contributing to the evasion of HCV from the humoral immune response (Helle et al., 2010). Both proteins are involved in the steps of viral entry and fusion, unlike other members of the *Flaviviridae* family such as alphaviruses or flaviviruses where E1 or E trimers, respectively, are sufficient. Due to their high glycan content, HCV E1 and E2 long resisted attempts to produce and purify them at high yields and to determine their 3D-structure.

E1 is a 192aa protein. In the absence of high resolution structure, it was modeled *in silico* as a truncated class II fusion protein, mainly composed of β -sheet folds (Garry and Dash, 2003). The 3D-structure of its N-terminal domain has recently been solved by X-ray crystallography (El Omari et al., 2014) and revealed an unusual and totally unexpected architecture for a protein (domain) presumably involved in processes of viral entry and fusion. It forms a covalent homodimer, and its closest structural homologue is a human phosphatidylcholine transfer protein in complex with a phospholipid. Although it is still unclear if this structure corresponds to the structure on the virion, a post-attachment structure, or a post-fusion fold, this opens novel perspectives in terms of lipid binding capacity of the viral particles. Indeed, the N-terminal domain of HCV E1 has been suggested to be responsible for the binding to ApoB and ApoE, which are components of LVPs. This same domain was also suggested to facilitate virus entry through either low-density lipoprotein receptor or heparan sulfate (Mazumdar et al., 2011, Jiang et al., 2012). At the surface of HCV virions, E1 forms a trimer, most likely assembled through interactions in the transmembrane domains (Falson et al., 2015). The formation of this trimer depends on the presence of E2, and this trimeric form is essential for virus infectivity. This configuration was recently confirmed by computational analyses, supporting a trimeric arrangement of E1/E2 further assembled into a pentamer, with 12 pentamers comprising a single HCV virion (Freedman et al., 2017).

E2 is a 363aa glycoprotein, composed of three hypervariable regions called HVR1, HVR2 and the intergenotypic variable region (igVR), a stem region and the transmembrane domain. E2 is the receptor-binding protein, shown to interact with the tetraspanin CD81 and the scavenger receptor BI (SRB1), two HCV co-receptors. The HVR1 region plays an instrumental role in HCV entry into hepatocytes, by contributing to E2-SRB1 interactions (Scarselli et al., 2002, Bartosch et al., 2003b). E2 is also the major target of neutralizing antibodies, and the first neutralizing epitopes described on HCV E2 were found within HVR1 (Farci et al., 1996). Anti-HVR1 antibodies neutralize HCV by interfering with E2-SRB1 interactions (Bankwitz et al., 2014, Bartosch et al., 2005). HCV can continuously escape the host neutralizing response by mutations, resulting in loss of recognition of HCV envelope glycoproteins by antibodies; the genetic evolution of the envelope is therefore shaped by neutralizing antibody pressure, in particular in the HVR1 region (Liu et al., 2012). HVR1 conceals the CD81 binding site on E2, thereby decreasing exposure of conserved epitopes (Bankwitz et al., 2010). HVR1 may thus act as an immunological decoy, diverting the immune system.

Since E2 is twice the size of E1, it was hypothesized to be the fusion protein of HCV, by analogy to the E protein of the Dengue (Modis et al., 2003, Modis et al., 2004) and tick-borne encephalitis flaviviruses (Bressanelli et al., 2004), or to the E1 protein of the Semliki Forest alphavirus (Lescar et al., 2001). Therefore, it has long been considered as a class II fusion protein rich in β -strands, containing putative fusion peptides (Krey et al., 2010, Lavillette et al., 2006) (chapter on Viral Entry/Fusion for more details). A truncated version of E2 was recently generated and complexed with a neutralizing antibody, which was amenable to X-ray diffraction. As for E1, the high resolution structure of E2 revealed an unexpected fold. Indeed, most of the protein residues are either in loops or intrinsically disordered (Kong et al., 2013, Khan et al., 2014). Only one β -sandwich region is shared with presumed structural homologues from flaviviruses and alphaviruses. This truncated HCV E2 protein, which maximally measures ~ 50 Å, does not adopt the extended class II fusion protein fold (100–120 Å) (Vaney and Rey, 2011).

Taken together, the recent structural data for HCV E1 and E2 do not support the prediction that they are class II fusion proteins. Moreover, one predicted putative fusion peptide of E2 is contained within the hydrophobic core, ruling out membrane fusion activity. The E2 ectodomain also does not undergo major rearrangements upon exposure to low pH (Khan et al., 2014). This strongly suggests that E2 is not a fusion protein. The N-terminus of E1 lacks any structural resemblance to other viral fusion proteins. Therefore HCV membrane fusion might be a completely different mechanism from that described for other *Flaviviridae* and related viruses such as alphaviruses.

In the ER of infected hepatocytes, HCV E1/E2 glycoproteins, ApoB and ApoE colocalize and form a ternary physical complex (Boyer et al., 2014). This complex then readily associates with intracellular infectious viral particles and is found conserved in the secreted infectious viral particles. This strongly suggests that the complex formed by HCV E1/E2, ApoB and ApoE may initiate LVP morphogenesis.

P7

P7 is a member of the family of viral viroporins, such as Vpu of the human immunodeficiency virus (HIV-1), E5 of the human papillomavirus 16 (HPV16), and the M2 protein of the influenza A virus. It is classified as a viroporin because it oligomerizes to form hydrophilic pore/ion channels. It is a 63aa membrane-spanning protein, essential for efficient virus particle assembly and release (Gentzsch et al., 2013). The structure of p7 is still a matter of debate mainly due to its sensitivity to the solvent or lipid environment generally used for purification. The oligomerization state of p7 has been described as a hexamer (Griffin et al., 2003), and recent computer simulations yield hexameric and heptameric channels (Chandler et al., 2012). P7 has non-selective pore properties, and like several other viroporins, displays a low ion selectivity, with minor preferences for cations vs anions. It has proton conductance properties and may act to prevent acidification in otherwise acidic intracellular compartments. This action is required for productive HCV infection (Wozniak et al., 2010). Therefore, p7 might be regarded as a modulator of the pH-mediated maturation process of HCV particles as a first stage, and as a key factor to render secreted virions pH-resistant as a second stage.

Regardless of its ion channel activity, p7 interacts with the protein NS2. This serves to recruit the envelope glycoproteins E1 and E2 to the ER membrane sites of HCV assembly, and regulates core localization to these sites (Boson et al., 2011). Direct interactions between p7, core and HCV glycoproteins have been reported in infectious cell systems (Hagen et al., 2014). P7 is also necessary for the final steps of capsid

assembly and for capsid envelopment, linking capsid assembly to membrane envelopment of nascent RNA-containing core protein multimers (Gentzsch et al., 2013). As the HCV glycoproteins interact with ApoB and ApoE (Boyer et al., 2014), these data suggest that p7 may influence apolipoprotein incorporation into HCV particles or particle maturation, thus contributing to virus production and infectivity.

NS2

The 217aa long NS2 protein is membrane-associated via three putative transmembrane segments with a perinuclear ER localization in its N-terminus (Tomei et al., 1993, Jirasko et al., 2010). The C-terminal protease domain (aa 94–217) resides on the cytoplasmic face of the ER membrane (Tomei et al., 1993) and its activity is strongly enhanced by the N-terminal domain of NS3 (Lorenz et al., 2006, Reed et al., 1995). NS2/NS3 catalyze the cleavage at the NS2/NS3 site (Grakoui et al., 1993b, Welbourn et al., 2009) which frees fully functional NS3 protein. The NS2 protease domain is highly conserved and requires dimerization for the formation of a composite active site with a catalytic triad analogous to those of cysteine proteases (Grakoui et al., 1993a). NS2 expressed alone has been shown to exhibit only low-level intrinsic protease activity and as mentioned, its protease activity is stimulated by the NS3 serine protease domain (residues 1–180) (Hijikata et al., 1993). Mutational analysis revealed a hydrophobic NS3 surface patch that mediates NS2 protease stimulation as well as NS5A hyperphosphorylation and viral RNA replication (Grakoui et al., 1993b, Yi et al., 2009, Grakoui et al., 1993a). The finding that NS2 dimerization is required for proteolysis suggests that certain concentrations of the protein have to be accumulated before NS2-3 processing and thus initiation of RNA replication can occur. Within this time window, the virus may generate sufficient amounts of NS3/4A to antagonize the activation of the interferon pathway, which is necessary to allow the onset of viral replication (see below)(Foy et al., 2003).

NS2 protein, independently of its proteolytic activity, is required for viral assembly (Jones et al., 2007, Kolykhalov et al., 2000, Schregel et al., 2009, Lorenz et al., 2006, Reed et al., 1995). Several laboratories have shown that full-length NS2 is required for particle production (Jirasko et al., 2008). Construction of chimeric HCV genomes derived from different genotypes revealed that interactions of the N-terminal transmembrane segment of NS2 with upstream structural proteins as well as interactions between the C-terminus of NS2 with downstream nonstructural proteins are likely required for virion assembly (Pietschmann et al., 2006). Moreover, NS2 was shown to co-localize with several other HCV proteins, and in particular with E2 and NS5A, raising the question whether NS2 may induce membrane alterations and act as bridge to mediate interactions between structural virion components and the viral RNA replication complex that are required for assembly, encapsidation and release. NS2 has in addition been reported to interact with various cellular proteins resulting in interference with apoptosis, inhibition of cell proliferation, inflammatory processes, hepatic lipid metabolism and DNA repair (Erdtmann et al., 2003, Yang et al., 2006, Kim et al., 2007, Oem et al., 2008a, Oem et al., 2008b, Bittar et al., 2013), but most of these data remain to be validated in physiologically relevant replication systems.

NS3/4A

NS3 is a 70kDa protein with a serine protease domain located in the N-terminus and NTPase/RNA helicase activity in the C-terminal two thirds of the protein (Yao et al., 1999). It forms a chymotrypsin-like fold with two beta-barrel subdomains. NS3 associates non-covalently with the 54aa long cofactor NS4A. The central residues 21-32 of NS4A form a beta-strand that is integrated into the N-terminal beta-barrel of NS3, while the N-terminus forms a transmembrane helix that mediates membrane association of the NS3/4A complex. The C-terminus of NS4A (aa 40-54) forms an alpha helix that interacts with replicase components and is required for viral RNA replication and assembly (Lindenbach et al., 2007). The substrate specificity of NS3/4A remains ill defined, but is thought to be determined by a rather common consensus cleavage sequence as well as positioning of the NS3/4A complex in respect to the membrane (Morikawa et al., 2011). Indeed, membrane association of NS3/4A is determined in a sequential manner

by the amphipathic alpha helix in the N-terminus (aa 12-23) of NS3 and the N-terminal alpha helix (aa 1-21) of NS4A. Importantly, NS3/4A is not only located on ER membranes but also at mitochondria and mitochondria-associated membranes (Wolk et al., 2000, Horner et al., 2011), where the NS3/4A complex cleaves and thus inactivates the innate immune sensor MAVS (Meylan et al., 2005). An additional NS3/4A substrate important for viral immune evasion is importin beta1, which is required for IRF3 and NF-kB signaling (Gagne et al., 2017). The NTPase/RNA helicase activity of NS3 couples ATP hydrolysis to double-stranded RNA unwinding and is required for viral RNA replication and assembly (Morikawa et al., 2011).

NS4B

NS4B is the least characterized of the HCV proteins due to its hydrophobicity, strong membrane association and lack of well-defined enzymatic functions. NS4B is an oligomeric membrane protein of 261 aa located on the cytoplasmic side of the ER membrane (Yu et al., 2006). The N-terminus of NS4B is thought to be at least partially translocated into the ER lumen (Lundin et al., 2003) and consists of two amphipathic alpha helices AH1 (aa 3-35) and AH2 (aa 42-66). Oligomerization of the AH2 helix is thought to mediate transition of the membrane bilayer (Gouttenoire et al., 2010b, Yu et al., 2006). Furthermore, site directed mutagenesis of AH1 and AH2 have implied these helices in virion production (Gouttenoire et al., 2014) and assembly of the viral replication complex (Gouttenoire et al., 2009). The central part of NS4B (aa 191-261) comprises four predicted transmembrane domains, the C-terminus consists of two strongly conserved amphipathic alpha-helices (aa 201-213; aa 229-253). The central transmembrane domains of both, N- and C-terminal helices play important roles in membrane association. NS4B induces the formation of particular membranous vesicles in ER- or ER-derived membranes, within which viral replication takes place (Egger et al., 2002, Gosert et al., 2003). Amongst the factors that drive the vesicle formation process are the oligomerization of NS4B and certain host cell factors known to interact directly with NS4B (Ashworth Briggs et al., 2015, Kong et al., 2016). The induction of membranous vesicles by NS4B may be due to its capacity to induce membrane curvature which in turn maintains nonstructural proteins and viral RNA inside newly formed replication vesicles (Quinkert et al., 2005, Biswas et al., 2016, Romero-Brey et al., 2015). Indeed, NS4B interacts with other viral nonstructural proteins and binds viral RNA (Einav et al., 2008). In addition, NS4B displays NTPase activity and is required for viral assembly (Jones et al., 2009). While NS4B is considered the main driver of membranous vesicle formation, other viral proteins participate. Recent data show that also the sole expression of NS3/4A, NS5A, and even NS5B can trigger vesicle formation (Romero-Brey et al., 2012). Interestingly, membrane vesicles triggered by the expression of individual viral proteins are different from those observed upon expression of the entire NS3–5B cassette, which results in formation of double membrane vesicles (Romero-Brey et al., 2012).

NS5A

NS5A is a 447 amino acids long zinc-binding and proline-rich hydrophilic phosphoprotein with pleiotropic roles in the HCV life cycle. NS5A alters NS5B polymerase activity *in vitro*, is required for viral RNA replication and assembly and modulates cell signaling pathways, interferon responses and apoptosis. NS5A is composed of an N-terminal amphipathic helix of 30 aa that mediates membrane association (Elazar et al., 2003) followed by three structural domains separated by two low-complexity sequences (Moradpour et al., 2005). Domain I (aa 28 to 213) is known to bind RNA and lipid droplets and is essential for viral RNA replication (Huang et al., 2005, Foster et al., 2010, Romero-Brey et al., 2015, Tellinghuisen et al., 2004). It has been crystallized as a dimer in two different configurations (Love et al., 2009, Tellinghuisen et al., 2005) pointing to distinct functional conformations and/or multimerization of NS5A. Domains II (aa 250 to 342) and III (aa 356 to 447) are naturally unstructured and known to interact with a number of different viral and cellular proteins (Hanouille et al., 2009, Liang et al., 2007). Roles of these domains in RNA replication and viral assembly have recently been described (Appel et al., 2008, Ross-Thriepfand et al., 2013, Tellinghuisen et al., 2008). Phosphorylation has been implicated as regulatory switch that modulates several functions of NS5A depending on the ratio of phosphorylated versus hyperphosphorylated forms (Appel et al., 2005, Huang et al., 2007b). While basal phosphorylation has

been observed at central and C-terminal residues, highly conserved serine residues located between aa 214 to 249 have been shown to be hyperphosphorylated (Tanji et al., 1995, Appel et al., 2005, Tellinghuisen et al., 2008).

The IFN-sensitivity determining region (ISDR) at the C-terminus of NS5A has been reported to contain strong *trans*-activating capacities, suggesting that NS5A likely functions as a transcriptional activator (Macdonald and Harris, 2004). In addition, NS5A has been shown to interact with a number of cellular factors (Ahn et al., 2004, de Chasseay et al., 2008). In particular the activation of phosphatidylinositol-4-kinase alpha (PI4KA) by NS5A is critical for the creation of the viral replication compartment. PI4P, the product of PI4KA activity, recruits e.g. cellular lipid transfer proteins to this replication compartment, such as the cholesterol transporter oxysterol binding protein (Wang et al., 2014). In addition, PI4KA modulates the phosphorylation status of NS5A (Reiss et al., 2013). NS5A is also known to interact with other cellular factors and pathways that either impact the HCV life cycle as well as associated pathogenic processes. Amongst these are e.g. pro-proliferative targets such as p53/p21 (Majumder et al., 2001), the cell cycle kinases cdc2 and cdk2 (Arima et al., 2001) and Grb2 (Tan et al., 1999). Interactions between NS5A and apoptosis regulating proteins such as Bax (Chung et al., 2003), members of the Src family of tyrosine kinases (Macdonald and Harris, 2004), modulation of epidermal growth factor receptor (EGFR) trafficking and signaling (Macdonald et al., 2005) (Mankouri et al., 2008), possibly via interactions with cellular SH3-domain-containing are also likely to have pro-viral as well as pro-oncogenic effects. Finally, NS5A has been shown to co-localize with core protein on lipid droplets and to interact with different lipoproteins (Shi et al., 2002). In particular, NS5A may cause inhibition of ApoB100 secretion (Domitrovich et al., 2005).

NS5B

During HCV replication, the positive-strand RNA genome is used as the template for an RNA-dependent RNA polymerase (RdRp), which synthesizes a complementary negative-strand RNA, and subsequently genomic positive-strand RNA. Both of these steps are catalyzed by the RdRp NS5B (Lesburg et al., 1999), which has rapidly emerged as a major target for antiviral intervention (see below, chapter Direct-acting antivirals and their mode of action). This protein contains motifs shared by all viral RdRps, and has the classical fingers, palm and thumb subdomains, based on the similarity of the enzyme structure with the shape of a right hand. The palm domain is the most highly conserved domain across different polymerases, and the location of the active site. In contrast, the thumb domain is the most variable. Fingers and thumb domains vary significantly in both size and secondary structure depending on the specific requirements for replication in a given virus (Sesmero and Thorpe, 2015). A special feature of the HCV RdRp is that extensive interactions between the fingers and thumb subdomains result in a completely enclosed active site. HCV NS5B functions as an oligomer, which was reported to be important for cooperative RNA synthesis activity (Wang et al., 2002). It is associated to the ER membrane through its C-terminus (tail-anchored protein) (Moradpour et al., 2004) and membrane targeting occurs by a post-translational mechanism. NS5B has been recently identified as a cofactor of HCV assembly through a genetic association with the p7 viroporin contributing to virion specific infectivity (Aligeti et al., 2015).

Life cycle

Entry

Viral entry is defined as the stages by which a virus penetrates into and delivers its genetic material into the host cell. This does not necessarily lead to productive infection and frequently the process of entry is being studied regardless of the capacity of the virus to replicate or not in infected target cells. General features of viral entry comprise the attachment of viral particles to cell surface molecules, the recognition of specific receptors at this surface, followed by internalization of virions in intracellular compartments by mechanisms that depend or not on pH. This leads to viral fusion, which triggers the release of the viral genetic material into the cytoplasm of the target cell. Studies of HCV entry have long been hampered by the absence of relevant virus/cell culture models. Major advances were made with the advent of HCVpp as a surrogate model of HCV particles (Bartosch et al., 2003a). HCVpp consist of a retroviral nucleocapsid and harbour the HCV glycoproteins E1 and E2 at their surface in the form of a noncovalent E1/E2

heterodimer (Op De Beeck et al., 2004, Sandrin et al., 2005). Many key observations on HCV entry obtained with the HCVpp model have retrospectively been validated using infectious cell-culture grown HCV virus, HCVcc.

The process of HCV entry is initiated by physical interactions between structural elements of the virions (E1/E2 heterodimer, apolipoproteins) and host cell surface molecules. The heparan sulfate proteoglycan (HSPG) syndecan-1 is an element of the extracellular matrix, reported to bring about viral attachment to the surface of hepatocytes (Grigorov et al., 2016, Shi et al., 2013b) through the involvement of virion-associated apoE (Jiang et al., 2012). The HSPGs syndecan-2 and -4 were also reported to play a role in viral attachment, but to a lesser extent than syndecan-1 (Fan et al., 2017). Syndecan-1 forms a complex with the tetraspanin CD81 (Grigorov et al., 2016), a key HCV receptor (Pileri et al., 1998, Bartosch et al., 2003b), thereby linking the process of attachment to that of receptor recognition. Through its numerous glycosaminoglycan moieties and their high level of sulfation specific to liver cells, as well as through its engagement in a complex with CD81, syndecan-1 may contribute to HCV hepatotropism.

Initially described as an HCV receptor (Dreux et al., 2009, Scarselli et al., 2002), the lipoprotein-binding molecule scavenger receptor BI (SRBI) has also been described as an attachment factor, involving the apoE moiety of HCVcc particles for initial interactions (Dao Thi et al., 2012). HCV entry mediated by SRBI also relies on the lipid transfer function of this molecule, and on interactions between the hypervariable region 1 (HVR1) region of E2 and SRBI. This receptor is also a key entry factor for virions produced in mice with humanized livers (Calattini et al., 2015). However SRBI engagement in the entry of such particles seems to solely rely on its lipid transfer function, and to be independent of E2 (Calattini et al., 2015).

Since HCV associates with serum lipoproteins, the involvement of the receptor for low density lipoproteins (LDL-R) in HCV entry has been suggested (Agnello et al., 1999, Molina et al., 2007). However, engagement of this receptor seems to lead to non-productive infection as virions end up in degradation compartments such as lysosomes (Albecka et al., 2012). Its implication in HCV entry deserves further *in vivo* investigations.

CD81 is a member of the tetraspanin web, which coordinates extracellular and intracellular processes, in particular through its link with the cytoskeleton (Levy and Shoham, 2005). Tetraspanins have four transmembrane passages, and two extracellular loops. HCV virions recognize a patch of amino-acids in CD81, present in the large extracellular loop (Petracca et al., 2000, Pileri et al., 1998), through specific residues of E2 contained in the recently published three-dimensional structures (Khan et al., 2014, Kong et al., 2013). The HVR1 region of E2 is thought to conceal CD81 binding sites (Bankwitz et al., 2010), suggesting that structural rearrangements are necessary for E2-CD81 interactions. As described above, syndecan-1 and CD81 form a complex, involved in HCV internalization in intracellular acidic compartments (Grigorov et al., 2016).

Claudin-1 and occludin are integral components of the tight junctions that delineate the basolateral from the apical membranes of hepatocytes. These molecules were reported to play a key role in HCV entry (Benedicto et al., 2009, Evans et al., 2007, Liu et al., 2009a, Ploss et al., 2009), and to compose the minimal quartet of receptors required for efficient HCV entry, together with SRBI and CD81. Claudin-1 forms a complex with CD81, instrumental to viral particle internalization (Harris et al., 2010). The component Sec24C of the coat protein complex II (COPII) machinery of intracellular protein transport was found crucial for proper claudin-1 transport to and exposure at the hepatocyte cell surface, which identifies this molecule as a key cofactor of HCV entry (Yin et al., 2017). After infection, the expression of claudin-1 and occludin is down-regulated (Liu et al., 2009a); this downregulation serves to prevent secondary infection of already infected hepatocytes, a phenomenon known as exclusion of superinfection (Tscherne et al., 2007). Similar observations have been described for syndecan-1 (Grigorov et al., 2016).

The receptor of the epidermal growth factor (EGFR) and ephrin receptor A2 (EphA2) have been identified as host cofactors of HCV entry (Lupberger et al., 2011). Entry involves the receptor tyrosine kinase (RTK) activity of both receptors, as shown in *in vitro* and *in vivo* models of HCV infection. Engagement of these molecules in HCV entry is achieved by regulating the association of CD81 and claudin-1, thereby activating the GTPase Hras (Zona et al., 2013). Indeed, the current view is that HCV binding to CD81 induces RTK phosphorylation, thereby leading to HRas activation. HRas may then promote lateral diffusion of CD81 within the plasma membrane and its stable clustering with Claudin-1, prior to subsequent virus internalization.

In the search for the involvement of lipid receptor molecules in HCV/LVP entry, the cholesterol transporter Niemann Pick C1-like1 (NPC1L1) and the receptor for very low density lipoproteins (VLDLR) were identified as entry factors. NPC1L1 is a 13-transmembrane domain protein of the apical membrane of hepatocytes. It is involved in cellular cholesterol absorption and contributes to cholesterol homeostasis. The implication of this molecule in HCV entry expands the view that virion-associated cholesterol plays a key role in viral entry (Sainz et al., 2012). However the exact nature of HCV/NPC1L1 interactions remains unclear. NPC1L1 expression is down-regulated during HCV infection, which might contribute to the exclusion of superinfection, as suggested for claudin-1, occludin and syndecan-1.

Most recently, the VLDLR was identified as a new molecule of the (complex) pathway of HCV access to hepatocytes (Ujino et al., 2016). Surprisingly, HCV infection using VLDLR does not require any of the above-described HCV receptors; therefore VLDLR-mediated HCV infection may be different from previously reported entry mechanisms. The interaction between HCV and VLDLR requires E2 and the apoE part of the LVP.

Our current model of HCV entry implies that multiple interactions take place at the surface of hepatocytes for subsequent efficient entry. Virion binding to attachment factors may lead to structural rearrangements of protein components of the viral particle, with the exposure of previously masked regions in the E1/E2 heterodimer. SRBI is the first receptor molecule to be recognized in the cascade of HCV entry events (Zeisel et al., 2007). This triggers the binding to CD81, induces EGFR phosphorylation and activation of the GTPase HRas, drives the lateral diffusion of CD81 molecules in the membrane plane and their clustering at claudin-1-enriched sites, where virions also likely interact with occludin. Virions are then internalized through a clathrin-dependent endocytosis process involving the small GTPase Rab5 (Blanchard et al., 2006, Blaising et al., 2013), together with syndecan-1 complexed with CD81 (Grigorov et al., 2016). At this stage, NPC1L1 might alter the lipid composition of the viral envelope, to facilitate subsequent viral fusion.

HCV infection can be achieved by two modes : (i) a cell-free transmission, where virions come from the blood circulation and infect naïve hepatocytes, or transit outside an infected cell to reach adjacent targets; (ii) via cell-to-cell transmission, where the virus propagates between adjacent hepatocytes without being exposed to the extracellular medium. This latter mode of viral transmission is particularly efficient to escape the immune system, and presumably facilitates the establishment of chronic infection. The cell-to-cell transmission route was found dependent on SRBI, claudin-1 and occludin (Brimacombe et al., 2011, Timpe et al., 2008), and on the E1/E2 heterodimer of HCV (Witteveldt et al., 2009). The NPC1L1 molecule also plays a role in this mode of transmission, in a manner involving its cholesterol transporter properties, and maybe in conjunction with the tight junction components claudin-1 and occludin, located in the vicinity of NPC1L1 at the hepatocyte apical membrane (Barretto et al., 2014). ApoE incorporated onto the viral particle was found to play a key role in HCV cell-to-cell spread, thereby linking viral assembly to viral propagation (Hueging et al., 2014). In another study, apoE was found dispensable to this mode of HCV transmission (Barretto et al., 2014); these conflicting data might evolve from the use of different cellular models. The involvement of CD81 in HCV cell-cell propagation is still a matter of debate: initial findings suggested that CD81 is dispensable (Witteveldt et al., 2009). However, cumulating pieces of evidence now suggest that CD81 plays at least a partial role in cell-to-cell transmission (Grigorov et al., 2016, Brimacombe et al., 2011, Catanese et al., 2013).

The process of viral fusion is the stage where the viral envelope merges with cellular membranes through the action of viral fusion proteins. This leads to the release of the viral genetic material in the cytosol. After their internalization in intracellular compartments through clathrin-dependent endocytosis, HCV virions are exposed to low pH in the endosomes, where conformational rearrangements of their envelope glycoproteins are likely to occur. At present and in spite of the recent publication of the 3D-structures of both E1 and E2, it is impossible to determine which protein triggers membrane fusion. Experimental evidence using HCVpp and HCVcc in combination with artificial membranes (liposomes) or cellular membranes show that HCV-induced fusion of liposomes is pH-dependent, over a large range of pH of 6 to 4 which would correspond to early to late endosomal compartments *in cellulo* (Lavillette et al., 2006, Haid et al., 2009, Blaising et al., 2013). CD81 and claudin-1 are involved in HCV fusion in endosomal compartments positive for the small GTPase Rab5 (Blaising et al., 2013). CD81 was found to prime HCV for low pH-dependent fusion, likely through conformational rearrangements in the E1/E2 heterodimer (Sharma et al., 2011). Fusion is also strongly dependent upon cholesterol and sphingomyelin present in

the target membranes (Haid et al., 2009, Lavillette et al., 2006), and in the viral envelope (Sainz et al., 2012).

Replication

Soon after fusion and release of HCV positive-strand RNA into the cytosol, replication begins in a peculiar compartment derived from the ER, called membranous web (Egger et al., 2002). At the ultrastructural level, this web is composed of single and double membrane vesicles, and contains markers of rough ER, early and late endosomes, transport COP vesicles, mitochondria and lipid droplets (LDs) (Romero-Brey et al., 2012). Double membrane vesicles are induced by NS5A, while NS4B induces single membrane vesicles. Within this membranous compartment, active replication and assembly of *de novo* synthesized viral machineries take place (see Assembly chapter). NS3 to NS5B are minimal viral proteins components required for RNA replication. NS5B is the RNA dependent RNA polymerase and constitutes the catalytic core of the replication machinery. Purified NS5B is not very efficient at replication suggesting cellular cofactors are required in addition. NS5B can be phosphorylated by the interacting cellular protein kinase C related kinase 2 (PRK2) and depletion of PRK2 inhibits HCV replication, suggesting that the PKR2-mediated phosphorylation is important for function (Kim et al., 2004). Interaction with the cellular chaperonin TRiC/CCT is also important for NS5B function (Inoue et al., 2011), but the underlying mechanism is not elucidated.

NS4B drives the scaffold formation required for HCV RNA replication, which takes place in membranous vesicles. The viral replication complex contains besides NS4B also NS3/4A, NS5A and NS5B. Physical interactions between these viral proteins lead to formation of the membrane-associated multiprotein complex that coordinates RNA replication (Dimitrova et al., 2003, Paredes and Blight, 2008, Piccininni et al., 2002, Gouttenoire et al., 2010a). NS5B is the key enzyme of RNA synthesis and uses the positive incoming RNA genome to generate a first negative-strand genome, which in turn serves as template for positive-strand RNA synthesis. These steps produce double stranded RNA intermediates. The newly replicated positive-strand RNA can then be re-used for replication or translation or be packaged into virions. The 3' end of the HCV negative strand is an excellent template for *de novo* initiation, whereas the 3' end of the positive-strand hardly gives rise to terminal *de novo* initiation, probably because it is buried within a stable stem structure (Binder et al., 2007, Reigadas et al., 2001). Indeed, auxiliary factors are required for and control initiation of negative-strand synthesis by NS5B (Harrus et al., 2010). After binding, NS5B synthesizes a dinucleotide primer (Ferrari et al., 2008) and a conformational change in the polymerase structure then allows egress of the template-primer duplex (Biswal et al., 2005, Mosley et al., 2012). RNA synthesis then proceeds at 100–400 nts per minute and is highly error prone, resulting in the high genetic variability of HCV isolates (Powdrill et al., 2011). The 3' non-translated region (NTR) is essential for initiation and regulation of negative strand RNA synthesis (Binder et al., 2007, Friebe and Bartenschlager, 2002). It is composed of a variable region, which forms two stem-loop structures, a polyU/UC tract and a conserved 98 nucleotide long X-tail, which also comprises three stem-loop structures at the end of the viral genome (Blight and Rice, 1997). Additional stem loops have been identified within the NS5B coding region that are implied in the formation of a kissing-loop interaction with the second stem loop in the X-tail. This interaction, which forms a pseudoknot structure at the 3' end of the genome, is essential for RNA replication (Diviney et al., 2008, Tuplin et al., 2012, Friebe et al., 2005). NS3, NS5A as well as NS5B have all been shown to bind to the polyU (Friebe and Bartenschlager, 2002, You and Rice, 2008, Gwack et al., 1996, Huang et al., 2005, Lohmann et al., 1997). However, the distinct functional role of the polyU/UC region in viral RNA synthesis has not been clarified yet. The 5'NTR has a dual function in genome replication. It contains an IRES sequence and thus drives translation of the positive RNA strand. The 5'NTR and a complementary stretch at the 3' end of the negative-strand RNA have been shown to adopt particular secondary structures. In particular, two adjacent miR-122 seed sequences located in the 5'NTR point to a primordial role of this miR in the proper formation of RNA secondary structures. The two adjacent sites as well as the intermittent, strongly conserved 8 nt spacer are all important for HCV replication and translation (Jopling et al., 2005, Roberts et al., 2011). Different lines of evidence suggest roles of NS3/4A and NS5A in RNA replication, but the underlying mechanisms have not been fully elucidated. NS3 helicase activity is thought to resolve the stem-loop structures in the 3'NTR to facilitate *de novo* replication initiation by NS5B. Structure determination of NS5A suggests that NS5A forms dimers that bind RNA (Tellinghuisen et al., 2005, Foster et al., 2010). Thus

NS5A may play a role in RNA transport. In addition, NS5A may interact with cellular proteins that impact viral replication. Indeed, NS5A and NS5B-stimulated PI4KA activity is required for membranous vesicle formation and HCV RNA replication (Lohmann, 2013). In addition, HCV replication depends on altered membrane structure, for which the expression of genes that regulate the hepatic lipid metabolism is necessary (Yamane et al., 2014, Diamond et al., 2010, Blackham et al., 2010). In addition, post-translational modifications such as e.g. phosphorylation, geranyl geranylation or palmitoylation of cellular and viral factors are required for viral replication (Narayanan et al., 2016). Geranyl geranylated FBL2, for example, interacts with NS5A and this is critical for HCV RNA replication (Wang et al., 2005). Oligomerization of NS4B depends on palmitoylation of C-terminal cysteine residues (Yu et al., 2006). HCV core (Barba et al., 1997, Moradpour et al., 1996, McLauchlan et al., 2002) and NS5A (Shi et al., 2002, Brass et al., 2002) are located on lipid droplets, which are frequently found in vicinity of membranous vesicles induced by HCV. LDs, or rather core protein on the surface of LDs is thought to coordinate replication with assembly, possibly via a direct interaction with NS5A (Appel et al., 2008, Tellinghuisen et al., 2008, Miyanari et al., 2007, Masaki et al., 2008). In addition, LDs may also play an important role in RNA replication (Targett-Adams et al., 2008).

Amongst the long list of host factors involved in RNA synthesis are e.g. the human VAMP-associated protein A (hVAP-A) and its isoform hVAP-B (Gao et al., 2004, Hamamoto et al., 2005), which play a role in cellular vesicle transport and are thought to impact HCV-induced membrane rearrangements (reviewed in (Moriishi and Matsuura, 2007)). hVAP-A e.g. binds to hypophosphorylated NS5A and is thought to regulate viral replicase activity in an NS5A phosphorylation-dependent manner (Evans et al., 2004). Prolactin regulatory element binding protein, which regulates anterograde ER-Golgi transport, interacts with NS4B, relocates to the viral replication complex and is also required for membranous vesicle formation (Kong et al., 2016). Within the family of the peptidyl-prolyl cis/trans isomerases, cyclophilin B was initially found to interact with NS5B and to regulate template binding of the polymerase (Watashi et al., 2005). More recent data point however to a role of cyclophilin A (CyPA) in HCV replication (Kaul et al., 2009, Liu et al., 2009b) by modulating conformation of NS5A (Kaul et al., 2009) and biogenesis of the membranous web (Chatterji et al., 2015). Binding of the liver specific, highly abundant microRNA miR-122 to two seed sequences in the 5'NTR probably prevents degradation by RNases, stabilizes the viral genome (Shimakami et al., 2012), stimulates translation of the viral RNA and prevents activation of innate immune responses (Machlin et al., 2011).

As mentioned above, NS5B has an intrinsically high error rate estimated at 2.5×10^{-5} mutations per nucleotide per genome replication (Ribeiro et al., 2012). In addition, selective pressure exerted by antiviral immune responses drives evolution of the genetic diversity of HCV. HCV is today classified into seven genotypes, characterized by differences in over 30-33% of nucleotide positions. In addition HCV genotypes 1 to 6 have been classified into sub-types that differ in at least 15% of nucleotide positions (Simmonds, 1995). HCV genetic variability is not evenly distributed across the viral genome. The 5'-NTR region and viral capsid are the most conserved regions of the genome while the most variable region codes for the envelope glycoproteins glycoproteins E1 and E2. In particular, the sequences encoding HVR1 and HVR2 of E2 display the least sequence homology between different isolates (Dusheiko and Simmonds, 1994, Simmonds, 2001).

Assembly

Fractionation studies have shown that membranes composing the membranous web contain NS5B, viral RNA, phosphatidylinositol 4-kinase III- α (PI4KIII α), ApoE, core protein, and infectious HCV (Nielsen et al., 2008, Berger et al., 2009). At early stages of assembly, core associates with lipid droplets (LDs) followed by the recruitment of the replication complex (Miyanari et al., 2007), in an NS2-dependent manner (Boson et al., 2011). LDs therefore play a central role in the coordination of viral RNA synthesis and virion assembly, by physically associating replication and assembly sites. Recently, the heterogeneous nuclear ribonucleoprotein K (HNRNPK) was found recruited close to LDs, where it co-localized with core and with the HCV plus-strand RNA (Poensch et al., 2015). HNRNPK might limit the availability of viral RNA for incorporation into virions, thereby determining efficiency of HCV particle production. The recruitment of NS5A to either LDs or assembly sites in the ER (through interaction with core protein) is also required for proper viral particle assembly (Eyre et al., 2014). The correct distribution of HCV NS5A to replication and assembly sites is under the regulation of the ATPase p97/VCP (Valosin-

containing protein) (Yi et al., 2016), a cytosolic enzyme involved in particular in the fusion of Golgi membranes (Otter-Nilsson et al., 1999). NS5A recruitment to LDs is also promoted by the tail-interacting protein 47 (TIP47), through direct protein interactions (Vogt et al., 2013). The interactions between NS5A and core are stabilized by the host molecular chaperone ApoJ, thus facilitating infectious HCV particle production (Lin et al., 2014).

HCV virions are LVPs and HCV production depends on VLDL assembly and secretion (Huang et al., 2007a). Thus the roles of VLDL-associated proteins such as apoB, apoE, apoA1, apoC1 and microsomal triglyceride transfer protein (MTP) in the formation of infectious HCV particles have been addressed. ApoB and MTP were found dispensable for infectious particle production (Hueging et al., 2014). NS5A and the E1/E2 heterodimer interact with apoE (Benga et al., 2010, Cun et al., 2010, Boyer et al., 2014, Lee et al., 2014). ApoE is a key component of HCV virions, required for virion infectivity and production (Chang et al., 2007). While budding, the viral particle interacts with nascent or immature lipoproteins, which indicates that the lipoprotein secretion machinery is involved in the formation of HCV viral particle (Icard et al., 2009). Mechanistically, the current evidence suggests that: (i) viral capsids bud into the ER lumen, followed by the incorporation of large amounts of cholesteryl esters and triglycerides into these nascent HCV particles; (ii) apoB and exchangeable apolipoproteins (A1, C1, E) bind to these lipid-rich particles as if they were lipoproteins; (iv) these HCV enveloped particles fuse with lipoproteins to generate LVPs (Bartenschlager et al., 2011).

HCV assembly is modulated by several host factors, of which most are related to lipid metabolism and LDs. Nucleoporin-98, a structural element of the nuclear pore complex, has been recently described as a key factor of HCV morphogenesis, whose nuclear functions are diverted by HCV for the transport of viral proteins to guide assembly (Lussignol et al., 2016). Diacylglycerol acyltransferase 1 (DGAT1) performs the last step in triacylglycerol synthesis pathway and is involved in LD biogenesis, together with DGAT2. DGAT1 plays a direct role in the recruitment of core and NS5A to LDs, through direct interactions with core (Herker et al., 2010). The small GTPase Rab18 is a LD-associated protein that binds NS5A, thereby physically linking LDs to ER membranes. Its GTP-bound (active) form is prominent over the GDP-bound (inactive) one for this activity (Salloum et al., 2013). Rab32, another small GTPase of the Rab family, was recently described to play a role in core recruitment to ER-derived membranes, likely at virion assembly sites (Pham et al., 2017). Unlike Rab18, Rab32 displayed this activity under its GDP-bound form. The cytosolic group IVA phospholipase A2 gamma (PLA2G4A), involved in the release of arachidonic acid, was shown to affect the amount of core on LDs, and the efficiency of core envelopment (Menzel et al., 2012). The phosphatidylinositol 3,5-bisphosphate 5-phosphatase FIG4 is implicated in HCV morphogenesis and infectivity through its capacity to modulate the amounts of cholesteryl esters (Cottarel et al., 2016). ABHD5 (α/β hydrolase domain containing protein 5) was recently identified as a host factor promoting both virus assembly and release (Vieyres et al., 2016). ABHD5 associates with LDs and triggers their hydrolysis, and HCV usurps ABHD5 lipase cofactor function. HCV also recruits the lipid-binding protein annexin A3 to LDs to achieve proper morphogenesis (Rosch et al., 2016). Cell-death-inducing DFFA-like effector B (CIDEB), a regulator of the VLDL pathway associated with LDs, was shown to be required for HCV assembly (Cai et al., 2016). A kinase involved in the innate pathway, I κ B kinase- α (IKK- α), has been described as a crucial host factor for HCV assembly: HCV, through its 3' untranslated region, interacts with DEAD box polypeptide 3, X-linked (DDX3X) to activate IKK- α , which translocates to the nucleus and induces a transcriptional program involving sterol regulatory element-binding proteins (SREBPs) (Li et al., 2013). Consequently, this innate pathway induces lipogenic genes and enhances core-associated LD formation to facilitate viral assembly.

Maturation/Release

According to the current model of HCV assembly, E1/E2-enriched ER membranes encompass luminal lipid droplets, into which hydrophobic nucleocapsids become enclosed. Similar to the production of VLDL, where lipid-poor VLDL precursors fuse with luminal LDs, these immature virion particles are thought to fuse with luminal lipid droplets or VLDL precursors to form properly lipidated "virus like particles" (LVP). Amongst the VLDL assembly machinery, in particular ApoE is thought to play a primordial role in this intracellular lipidation by mediating fusion between nascent VLDL and viral precursors (Chang et al., 2007, Benga et al., 2010, Jiang and Luo, 2009, Rosch et al., 2016, Hueging et al., 2014, Collier et al., 2012); however ApoE does not affect formation of HCV nucleocapsids and their envelopment per se (Lee et al.,

2014). Indeed, particles can efficiently be produced in sh-apoE cells, but have increased density and low specific infectivity (Lee et al., 2014). While association with ApoE during the assembly process renders particles infectious (Gastaminza et al., 2008, Collier et al., 2012), only particles containing sufficient ApoE amounts are secreted. Mechanistically the role of ApoE in HCV lipidation and secretion remains unclear, even though direct interactions between ApoE and the glycoproteins E1/E2 have been implied (Hueging et al., 2014, Lee et al., 2014). Recently annexin A3 (ANXA3), a protein recruited to lipid-rich fractions in HCV-infected cells has been shown to be essential for interactions between ApoE and the E2 glycoprotein. Curiously, ANXA3 silencing affected trafficking, but not lipidation of HCV virions (Rosch et al., 2016). Reconstitution of HCV particle assembly in non-hepatic cell lines has shown that besides ApoE members of the ApoA and ApoC apolipoprotein families can also sustain HCV particle lipidation and secretion, and the required conserved structural domains in these apolipoproteins have been identified as amphipathic alpha-helical repeats (Fukuhara et al., 2014, Hueging et al., 2014).

HCV virions then bud from the ER and traffic to the Golgi with COPII-vesicles (Neveu et al., 2012, Collier et al., 2012). It has also been shown that localization of ARF GTPases such as CYTH3 and ARF3 to the trans Golgi network (TGN), where they interact with coat proteins to regulate vesicle budding and sorting, is necessary for HCV secretion. Release of HCV from the TGN requires furthermore PRKD1. Secretion is then thought to occur via a clathrin-dependent transendosomal secretory route and depends on components of the endosomal-sorting complex required for transport pathway (Collier et al., 2012, Mankouri et al., 2016, Benedicto et al., 2015, Lai et al., 2010, Corless et al., 2010). Both viral glycoproteins, E1 and E2, possess high mannose and complex N-linked glycans and essential disulfide bonds that are thought to be rearranged in the Golgi during secretion (Falson et al., 2015, Vieyres et al., 2010). To prevent premature fusion of the glycoprotein complex, nascent virions may be protected from acidification and consequent conformational changes via the ion channel activity of p7 (Wozniak et al., 2010). Interestingly, autophagy has been shown to impact HCV assembly and secretion via exosomes (Ploen et al., 2013, Shrivastava et al., 2015). Interactions of HCV particles with the TIP47-GTP-Rab9 complex are thought to protect the nascent virus from autophagosomal-mediated degradation during release (Ploen et al., 2013). Finally, secreted ApoE can modify infectivity of already released, circulating HCV virions, and thus protect virions from antibody-mediated neutralization and enhance particle interactions with cellular HSPGs and thus potentially facilitate cell entry (Bankwitz et al., 2017).

Direct-acting antivirals (DAA) and their mode of action

Thanks to the knowledge of the molecular biology of HCV and the development of cell culture assays, direct acting antivirals (DAAs) were discovered and developed. It was known from early clinical and experimental observations that infected hepatocytes could be cured by IFN administration. With the discovery of novel DAAs, it was shown that the cure of infected cells could occur in an IFN independent manner. The first generation of DAAs was mainly directed against HCV genotype 1. It was rapidly observed that a combination of these drugs with IFN or other antiviral agents would be necessary to prevent the emergence of drug resistance. The second generation of drugs was developed to be pangenotypic and to confer a high barrier to resistance when administered in combination. Pangenotypic direct acting antivirals have been developed for several viral proteins. The predominantly used DAA combinations that are currently in clinical use target the NS3/4A protease, NS5A or the NS5B RNA-dependent RNA polymerase. Due to extremely high efficacies of these DAA regimens of close to 100% independently of genotype and fibrosis stage, the concomitant use of interferon has been stopped over the last few years and current therapies are predominantly based on DAAs only. We will focus predominantly on the currently used classes of DAAs, and only briefly mention DAAs targeting additional viral proteins.

In respect to the HCV proteases NS2 and NS3, only compounds that target the NS3 protease have reached the clinic. Based on the crystal structure of NS3 in complex with candidate compounds, the two structurally related HCV protease inhibitors telaprevir and boceprevir were developed and the first protease inhibitors (PIs) to be approved for clinical use in 2011. They are linear peptidomimetic structures that react reversibly with the catalytic serine of NS3 to form a covalent bond (Kwong et al., 2011, Hazuda et al., 2013). To avoid the emergence of drug resistance, these antiviral agents had to be combined with IFN and ribavirin. The major limitations of these first generation macrocyclic PIs were strong side effects, inconvenient dosing regimens, narrow genotype specificity and low barrier to resistance. Resistance-

conferring mutations that emerged during therapy were predominant at positions R155, 156 and 168 and affected binding to the inhibitor; in contrast changes in binding to natural NS3 substrates were minimal (Romano et al., 2012). The second wave of first-generation macrocyclic PIs, such as asunaprevir and simeprevir, had similar limitations (Pawlotsky, 2015). In contrast, the second-generation pan-genotypic PIs, derived from the peptide substrate of NS3/4A protease, were modified with various tailor-made amino acids in order to achieve high sustained virologic response (SVR) against HCV (Wang et al., 2017). Amongst these 2nd generation PIs glecaprevir showed potent activity against all genotypes with equal and even superior results compared to grazoprevir and paritaprevir in replicon assays but was found clinically effective only in combination therapies. Grazoprevir retains activity against the common R155K mutant virions, as it does not interact with this residue (Summa et al., 2012). In an interferon free context, grazoprevir in combination with ribavirin (RBV) resulted in 90% sustained viral response (SVR) in patients with undetectable HCV RNA 4 weeks into treatment, however, in patients with detectable RNA at week 4 the SVR rate was only 58% (Burstow et al., 2017). Resistance to treatment with the acylsulfonamide paritaprevir is uncommon, because it targets the protease-binding site, but requires surveillance.

Two classes of clinically relevant NS5B inhibitors have been described: non-nucleotide inhibitors (NNIs) and nucleotide inhibitors (NIs) (Deore and Chern, 2010). They act at distinct stages of RNA synthesis. Initiation of RNA synthesis by NS5B is slow, does not need a primer and is accompanied by frequent dissociation events. Formation of the first phosphodiester bond then stabilizes the complex and triggers the elongation phase. NS5B is known to be present in functionally distinct complexes in these two phases. Crystal structures of NS5B have indeed shown that NS5B undergoes drastic conformational changes to accommodate the newly synthesized RNA strand in the elongation phase (Appleby et al., 2015). NNI are chemically diverse and generally interfere with the conformational dynamics at the transition from the initiation to the elongation phase. NNI binding pockets are allosteric sites, which mediate a non-competitive mechanisms of action and are located in distinct regions in the thumb or palm regions. Examples for thumb-targeting NNIs are benzimidazole, indole scaffolds such as Beclabuvir or thiophene-based inhibitors. In contrast to inhibitors targeting the thumb region, palm site inhibitors such as benzothiadiazine scaffolds are thought to inhibit initiation of RNA synthesis through interference with nucleotide incorporation. Importantly, the antiviral activity of NNIs is very limited to predominantly genotype 1. Dasabuvir, the first approved NNI has a low barrier to resistance and was there therefore part of complex combinations with NS5A and NS3A inhibitors. In contrast to NNIs, NIs compete with the incoming nucleoside triphosphate for binding and incorporation but modifications of their sugar moiety mediates the inhibitory, chain-terminating effect. Because NIs are administered as prodrugs they need to be processed into the active triphosphate form that can then access the nucleotide-binding site of NS5B (Sofia et al., 2012). The only currently approved NI drug sofosbuvir is a phosphoramidate prodrug that is intracellularly hydrolyzed and further modified into its active triphosphate form, a uridine analogue containing a 2'-fluoro-C-methyl motif. This motif is thought to cause chain termination via a steric conflict with the incoming nucleotide substrate (Appleby et al., 2015). Sofosbuvir has pan-genotypic antiviral activity and although it was originally approved with PEG-IFN and ribavirin, it is highly effective when combined with other DAA classes, either as dual or triple DAA combinations in an interferon-free context. Generally, sofosbuvir is well tolerated with a good safety profile, but reports of hepatotoxicity have emerged in the patients with decompensated cirrhosis (Dyson et al., 2016). Other NIs are currently at early stages of clinical development.

Unlike NS3 and NS5B, NS5A does not possess any enzymatic activities. Furthermore, the limited structural information that is currently available for NS5A is based on non-phosphorylated proteins. Hence, the development of DAAs for NS5A has been difficult. Inhibitors were identified in cell-based replicon assays that screened for the emergence of resistance-conferring mutations in the NS5A coding region. NS5A inhibitors show exceptional potencies, with EC₅₀ values in the pM range. Daclatasvir, the first NS5A inhibitor and the related compounds ledipasvir and ombitasvir are in clinical use (Berger et al., 2014). Resistance-conferring mutations such as e.g. Leu31 and Tyr93 in genotype 1 have been used to identify the putative binding site of the inhibitors. Indeed, several models for complexes of NS5A bound to daclatasvir or related compounds have been proposed. Recent quantitative binding approaches suggest that daclatasvir competes with RNA such that a dynamic equilibrium occurs between NS5A dimers that bind either RNA or daclatasvir (Ascher et al., 2014). The high potency of these NS5A inhibitors

has also been shown to be due to the inhibition of DMV formation. NS5A inhibitors are amongst the most potent DAAs developed to date, display an excellent safety and pan-genotypic efficacy profile and are therefore present in most combination treatments that are currently being applied in the clinic (Gotte and Feld, 2016). However, resistance mutations have been observed that persist on the long term (Krishnan et al., 2015). The NS5A inhibitor pibrentasvir, currently being evaluated as pangenotypic regimen in combination with the 2nd generation protease inhibitor glecaprevir, shows high barrier to resistance and potency against common NS3 and NS5A polymorphisms and seems efficient even in the setting of compensated cirrhosis (Forns et al., 2017).

Further clinical results based on these DAAs will be treated in subsequent chapters.

Host-targeting agents (HTA) and their mechanisms of action

Given the high amount of host factors involved in HCV life cycle, we will only focus on those factors suggested to represent potential targets for HTAs during distinct steps of HCV infection. Basically each step of HCV life cycle is amenable to antiviral therapy, with several molecules already licensed for their anti-HCV potential. Moreover, several molecules targeting different steps could be combined to achieve a better efficiency and to lower the doses of each individual molecule, thereby increasing the therapeutic index of the medication. Finally, HTAs could be successfully associated to DAAs to gain in antiviral potency.

Entry inhibitors have long been designed to block the virus before it causes damages to its host, and to protect naïve cells from infection. A vast panel of molecules, from fully synthetic ones to extracts of natural compounds have been proposed and assayed. However only a little number could be reasonably proposed for antiviral strategies, since most of these molecules raise safety concerns and/or display insufficient purity to identify the compound endowed with antiviral activity. In the following we will therefore only focus on molecules currently under *in vivo* investigations. Monoclonal antibodies targeting cellular proteins/receptors have long been proposed as antiviral strategies; however most of these proteins are essential to many cellular functions, so their targeting cannot be applied *in vivo* for safety reasons. Nevertheless, successful attempts have led to the development of monoclonal antibodies directed toward CD81 (Meuleman et al., 2008), claudin-1 (Mailly et al., 2015) and SRBI (Vercauteren et al., 2014), that showed potent antiviral activity in mice with humanized livers. The compound ITX-5061 inhibits SRBI-mediated lipid transfer from high density lipoproteins (HDL), but not the binding of HDL to SRBI (Syder et al., 2011); this promotes HDL levels, and since SRBI activity is targeted, HCV entry is inhibited. ITX-5061 is currently under phase I clinical investigation, in chronically infected patients receiving a liver transplantation (NCT01292824 and NCT01560468) (Rowe et al., 2016). The involvement of the EGFR and of NPC1L1 in HCV entry allowed the use against hepatitis C of specific inhibitors of these receptor molecules, already licensed with other therapeutic indications. In this context, erlotinib, an anticancer agent used in lung cancer and inhibiting the tyrosine kinase activity of EGFR, was assessed in a clinical trial of chronic hepatitis C (NCT01835938), with no results posted yet. The inhibitor of the NPC1L1 cholesterol transporter ezetimibe is an already approved molecule against hypercholesterolemia; it showed potent anti-HCV activity both *in vitro* and *in vivo* in mice with human livers (Sainz et al., 2012), and has now entered 2 clinical trials (NCT02126137 and NCT02971033). Silymarin is a natural extract from milk thistle (*Silybum marianum* sp.), shown to display anti-HCV activity in clinical settings (Fried et al., 2012); however its efficacy was questioned since it comprised several molecules. Silibinin is the main active compound of silymarin, that showed potent antiviral activity, *in vitro* (Wagoner et al., 2010, Blaising et al., 2013) and in patients (Ferenci et al., 2008). It acts by slowing the clathrin-dependent endocytosis of HCV particles and by altering the recruitment of Rab5 to endosomes, which leads viral particles to degradation compartments (Blaising et al., 2013). It has also an inhibitory effect on HCV replication (Esser-Nobis et al., 2013) and assembly (Wagoner et al., 2010). A recent clinical trial showed favorable treatment outcomes with silibinin in difficult-to-treat patients co-infected with HCV and HIV (Braun et al., 2015). The antimalarial chloroquine is a chemical analogue of ferroquine, shown to exhibit an inhibitory activity on HCV infection, at the stages of cell-to-cell transmission and fusion (Vausselin et al., 2013). Chloroquine is an affordable molecule for emerging countries, and has recently entered a phase IV clinical in Iran (NCT02058173), with favorable but modest antiviral outcomes (Peymani et al., 2016).

Inhibitors of host cell factors involved in HCV replication have been designed, and some used with success in clinical settings. Miravirsen, an inhibitor of miR-122, an hepatocyte-specific host factor involved in HCV RNA translation and replication, has proven its efficacy in reducing viral loads in chronic hepatitis C patients in phase 1-2 clinical trials (Janssen et al., 2013, van der Ree et al., 2016). Miravirsen may act by two complementary mechanisms: it might hybridize to mature miR-122, thereby blocking its interaction with HCV RNA, and bind to the stem-loop structure of miR-122 precursors, leading to impaired processing of these molecules. Importantly, miR-122 may play an important role in the suppression of pro-fibrogenic actions of stellate cells, indeed its downregulation is associated with fibrogenesis (Bartosch, 2015, Zeng et al., 2015). Cyclosporins are inhibitors of specific signal transduction pathways that lead to T lymphocyte activation. These immunosuppressive agents bind with high affinity to the cytoplasmic receptors cyclophilins. Several drugs devoid of immuno-suppressive properties were designed and displayed *in vitro* anti-HCV activity (Chatterji et al., 2016). Alisporivir has been successfully used in clinical settings (NCT02094443, NCT01215643, NCT02753699), with a pan-genotypic anti-HCV activity and a high barrier to viral resistance; in combination with ribavirin, it displayed a favorable safety profile, and a good antiviral efficiency (Pawlotsky, 2015). As already mentioned, cyclophilin inhibitors block HCV replication by preventing the formation of double membrane vesicles, thereby reorganizing the ER-derived membranous web where viral replication occurs and by preventing the formation of complexes between cyclophilin A and NS5A, thereby inhibiting HCV replication (Chatterji et al., 2016).

The list of assembly inhibitors in the clinical process is currently very limited. A most promising host target protein is DGAT-1, shown to play a key role in HCV morphogenesis and secretion (see above). However, the specific inhibitor LCQ908 that exhibited antiviral potency *in vitro* proved disappointing *in vivo* (Gane et al., 2017), and the corresponding clinical trial was terminated (NCT01387958).

The only HAT extensively used in the clinic is ribavirin. Ribavirin was part of the standard of care treatment in combination with IFN. Although it did not have a direct antiviral effect, it enhanced the antiviral activity of IFN α . It was maintained in the triple combination regimen with the first protease inhibitors and IFN. With the development of more potent DAA, ribavirin was still used in the IFN-free regimen in combination with other DAAs in difficult-to-treat patients to increase the rate of sustained virologic response. Ribavirin was shown to modify the epigenetic environment of interferon-stimulated genes and thus to restore IFN responsiveness (Testoni et al., 2016). More recently, with the development of even more potent DAAs, the role of ribavirin in the treatment of CHC patients has vanished.

Conclusion

The last two decades of research allowed to understand the molecular biology of HCV replication, characterize the crystal structure of most viral proteins, and develop experimental models to study HCV infection. This knowledge assisted the drug discovery efforts that led to the development of potent antiviral drugs. The clinical development of these agents led to a medical revolution as it is now possible to nearly cure all patients with IFN-free regimens.

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Figure 1

A simplified scheme of lipoviral particle assembly: assembly occurs at the ER at the interface between the HCV-induced membranous web containing the HCV replication complex and lipid droplets where core and NS5A are localized. LVP production depends on cellular VLDL assembly and secretion, which is mediated by lipidation of nascent ApoB via mitochondrial triglyceride transfer protein (MTP). See text for further details.

