PhD program in Translational and Molecular Medicine

DIMET

Studies on the mechanism of action of antiviral agents targeting the replication complex of hepatitis C virus

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“Il processo di una scoperta scientifica
è un continuo conflitto di meraviglie”
(A. Einstein)

To Andrea and my family

Table of Contents

CHAPTER I

GENERAL INTRODUCTION

A. Hepatitis C Virus

1 The discovery of Hepatitis C virus

After the development of diagnostic tests for hepatitis A (HAV) and B (HBV) viruses in mid-seventies, it became apparent that most cases of post-transfusion hepatitis were not caused by these agents or any other known hepatotropic virus, such as cytomegaloviruss (CMV) or Epstein-Barr virus (EBV) (Feinstone et al., 1975; Knodell et al.,1975). The causative agent (or agents) of the so-called non-A, non-B hepatitis (NANBH), remained frustratingly elusive despite intensive research for over a decade. Many reports of antigens, antibodies, enzyme activities and virus particles specific for NANB hepatitis were published in the following years, although none have been confirmed subsequently as being related directly to the causative agent (Shih et al., 1986, Dienstang et al., 1986, Overby et al., 1987; Hallam et al., 1985). However, in the late 1970s, the agent causing NANBH was succefully transmitted to chimpanzees (Tabor et al., 1978; Alter et al.,1978). As in humans (Alter et al., 1989), about 50 % of infected chimpanzees developed chronic hepatitis following inoculation with contaminated human serum or blood clotting concentrates.

In humans, the gradual progression of chronic hepatitis to cirrhosis of the liver in about 20 % of chronic cases of NANBH was documented thus reflecting the insidious, serious nature of this infectious agent (Bradley et al 1981, 1986; Dienstang et al., 1983; Alter et al., 1982, 1988, 1989). In fact, although most acute infections occured asymptomatically, many cases were associated with serious liver

damage. The incidence of infection following multi-unit blood transfusion in the 1970s was estimated to be as high as 7-12 % in the U.S.A. and post-transfusion NANBH (PT-NANBH) was considered a major world-wide health problem (Dienstang et al., 1986). While NANBH was frequently observed as a complication of blood transfusion, it also occurred frequently in the general community in the absence of any prior history of transfusion.

In 1989, the causative agent of NANBH was identified and characterized by molecular cloning techniques using serum from a NANBH virus infected chimpanzee, and was denominated hepatitis C virus (HCV) (Choo et al., 1989). The discovery of HCV was an important milestone in the field of viral hepatitis. It allowed screening of blood products and – several years later - the development of an antiviral treatment.

1.1 Classification of Hepatitis C Virus

Originally, Choo and his colleagues had described a positive-sense, ss RNA enveloped virus, which could be classified in the *Togaviridae* or *Flaviviridae* family of viruses (Choo et al., 1989). Analysis of derived amino acid sequences found similarities in nonstructural protein 3 between HCV and the dengue type 2 flavivirus. Flavivirus are a genus of the the *Flaviviridae* family. Furthermore, HCV contained some protein sequence similarity to pestiviruses, also members of the *Flaviviridae* family. Based on these features, HCV was classified into

the *Flaviviridae* family of viruses (Miller et al., 1990). Although some sequence similarity was present, this classification was based mainly on genome organization. Gene organization and hydrophobicity profiles suggested HCV was closer to the pestivirus genus than the flaviviruses (Choo et al., 1991). Currently, the *Flaviviridae* family contains 3 genera with HCV classified in its own genus, Hepacivirus. This was decided on the basis of sequence and phylogenetic analysis, which showed that HCV sequence was too divergent from either pestivirus or flavivirus to be assigned to either genus (Robertson et al., 1998).

2 Molecular organization of Hepatitis C Virus

2.1 Genomic heterogeneity and classification systems of HCV

Soon after the publication of the first nearly complete genome sequence of hepatitis C virus (HCV) in 1989, (Choo et al., 1989) it became apparent that isolates from different individuals or countries showed remarkable sequence variation. Such great genetic diversity is thought to be generated by the lack of proofreading activity by the RNA polymerase (see 2.2.3) and a high *in vivo* productive rate of an estimated 10^{12} virions per day (Neumann et al., 1998). To reflect this diversity, HCV has been classified in genotypes and subtypes. Typically, isolates within a given HCV genotype share from 65.7 % to 68.9 % sequence similarity, whereas a isolates of the same subtype share 76.9 % to 80.1 % sequence similarity. Even within a single sample of

infected blood, the virus is present as a relatively heterogeneous "quasispecies", with individual variants sharing 90.8 % to 99 % sequence similarity (Simmonds et al., 1994). Typically, the viral population of a "quasispecies" has an RNA sequence that is dominant (called the master sequence) and a number of variants differing from this (Forns et al., 1999).

In 2005 a new consensus classification was proposed to be used for HCV classification. Genotype and subtype assignments required: (1) one or more complete coding region sequence(s); (2) at least three epidemiologically unrelated isolates; (3) a phylogenetic group distinct from previously described sequences; (4) exclusion of intergenotypic or intersubtypic recombination, whether the components were classified or not (Simmonds et al., 2005). The application of these criteria confirmed the assignment of six distinct genotypes, comprising 18 subtypes. In addition, 58 subtypes were provisionally assigned pending the availability of a complete coding region sequence or additional isolates.

This agreement on nomenclature was mirrored by the establishment of several curated databases that organized HCV sequences as they became available and indicated which genotypes and subtypes were confirmed or provisionally assigned (Los Alamos HCV Sequence Database (Yusim et al., 2005), euHCVdb (Combet et al., 2007), Hepatitis Virus Database: http://s2as02.genes.nig.ac.jp/) (Kuiken et al., 2008). Subsequently, the available dataset of HCV sequences has vastly expanded through advancement in nucleotide sequencing technologies and an increasing focus on the role of HCV

genetic variation in disease and treatment outcomes. In May 2013 there was a major update to the previous consensus HCV classification, incorporating additional sequence information derived from over 1,300 near-complete genome sequences of HCV available on public databases. Analysis resolved several nomenclature conflicts between genotype designations and using consensus criteria created a classification of HCV into seven confirmed genotypes and 67 subtypes (Smith et al., 2014). Additionally the development of a Web resource hosted by the International Committee for Taxonomy of Viruses (ICTV) has resulted in the maintenance and regular updating of tables of reference isolates, accession numbers, and annotated alignments (http://talk.ictvonline.org/links/hcv/hcv-classification.htm).

2.2 Genome Organization

The HCV particle is about 50-60 nm in diameter. HCV possesses a genome which is a uncapped, positive-sense, single-stranded RNA molecule encoding a single (~3000 aa) open reading frame (ORF) of approximately 9.6 kb which is flanked at the 5’ and 3’ ends by small, highly structured untraslated regions (UTR). The cleavage of this polyprotein precursor occurs co-translationally and post-translationally by viral and cellular proteases at the endoplasmatic reticulum (ER) and results in 10 mature structural and non-structural (NS) proteins. The structural proteins, located in the N-terminal region, include the core (C) protein and the envelope glycoproteins, E1 and E2. A small

hydrophobic peptide p7 separates the structural proteins from nonstructural proteins, which consist of NS2, NS3, NS4A, NS4B, NS5A and NS5B (reviewed in Moradpour et al., 2007). The genetic organization of the HCV genome is depicted in Fig. 1.

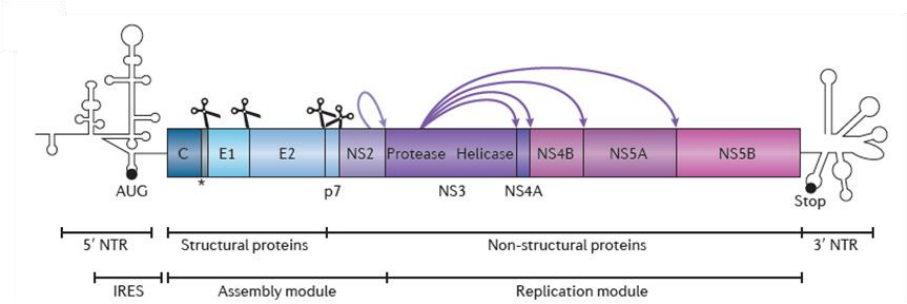

Fig. 1. Hepatitis C virus genome organization.

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. 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. *Adapted from Bartenschlager et al., 2013.*

2.2.1 Untranslated RNA Segments

The **5' UTR** region of HCV comprising the first 341 nucleotides is a highly conserved sequence involved in replication and translation of viral genome (Bukh et al., 1992; Honda et al., 1999; Friebe et al., 2001). The 5' UTR contains 4 highly structured domains (termed I-IV) consisting of numerous stem-loop motifs (Honda et al., 1996a). Domain II-IV together with 12-40 nucleotides of the core sequence forms the IRES that directly recruits the 40s ribosomal unit to the AUG

codon and initiates protein translation in a cap-independent manner, involving eukaryotic translation factor eIF3 (Reynolds et al., 1996; Honda et al., 1999). Domain IV is not required for ribosome binding. In fact the structural stability of this region is negatively correlated with the translation efficiency (Honda et al., 1996b). Domain I seems to be dispensable for overall IRES activity but may have a regulatory role in translation efficiency (Honda et al., 1996a). Apart from its importance in protein translation, the 5' UTR also serves an important role in RNA replication. Domain I and II are sufficient for viral RNA synthesis, although the efficiency of this process is enhanced by the presence of the complete 5' UTR (Friebe et al., 2001; Kim et al., 2002). Apart from translation factor eIF3, the 5'UTR is also known to interact with a number of other cellular proteins (PTB, La, hnRNP L and PCBP) that are important in regulating both replication and translation of HCV viral genome (Ali & Siddiqui, 1995, 1997; Hahm et al., 1998; Fukushi et al., 2001; Jopling et al., 2005). Moreover, interactions between the liver-specific microRNA, miR-122, and domain I and II of the 5'UTR have shown to be essential for efficient HCV RNA replication both in infected chimpanzees and during infection in cultured cells (Jopling et al, 2005; 2008; Lanford et al, 2010; Machlin et al., 2011). It has been hypothesized that miR-122 may protect HCV RNA from nucleolytic degradation or may prevent the activation of enzymes that induce innate immune response (Machlin et al, 2011). Due to its considerable importance and conservation, the 5' UTR constitutes a promising target for RNA interference based therapy (Kanda et al., 2007).

The **3' UTR** contains approximately 225 nucleotides and is organized into three domains consisting of a variable region of around 40 nucleotides, a poly (U/UC) stretch that regulates replication and a highly conserved 98-nucleotide X-tail (Kolykhalov et al., 1996). Based on biochemical and structural studies, 3' UTR is proposed to harbour two stem loops in the variable region (VSL1 and VSL2) and three stem loops in the X-tail (SL1, SL2 and SL3) (Tanaka et al., 1996; Blight & Rice, 1997). VSL1 and VSL2 are dispensable for RNA replication in cell culture and for HCV infectivity in chimpanzees as small deletions within these regions significantly reduce the efficiency of viral replication but are not lethal (Yanagi et al., 1999; Friebe & Bartenschlager, 2002). However, the U/UC region and X-tail are essential for HCV RNA replication as deletions or substitutions within any of these regions often prove lethal. At least 50 nucleotides of the U/UC stretch are required to allow viral replication (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003). Moreover, cis-acting replication elements (CREs) have been identified in the NS5B coding sequence that have shown to interacts with 3'UTR (Friebe et al., 2005). Apart from its role in replication, 3'UTR is also thought to enhance IRES dependent translation of viral RNA by increasing the efficiency of termination (Bradrick et al., 2006; Song et al., 2006)

2.2.2 Structural Proteins

The HCV **core** protein forms the viral capsid into which the viral genome is packaged (McLauchlan et al., 2009). It is located at the N-terminus of the HCV polyprotein and is cleaved by host signal peptidase (SP) as a 191 amino acid precursor of 23kDa, which remains anchored to the cytoplasmic side of the endoreticulum (ER) membrane via its C-terminal hydrophobic tail (Santolini et al., 1994; Moradpour et al., 1996; Yasui et al., 1998). Additional processing by signal peptide peptidase (SPP) gives rise to the mature 21 kDa form of the core protein (McLauchlan et al., 2002). The mature form of core is predicted to contain two distinct domains D1 and D2. D1 is highly hydrophilic and is principally involved in RNA binding (Boulant et al., 2005). The interaction with RNA motifs located in the 5' UTR probably facilitates the oligomerization of the core protein and initiates the virus packaging reaction (Majeau et al., 2004). D2 on the other hand is more hydrophobic in nature and is believed to be essential for core association with lipid droplets (LDs) and ER membranes (Moradpour et al., 1996; McLauchlan et al., 2000). Following its interaction with LDs, core directs the redistribution of LDs into the vicinity of membranes bearing genome replication complexes, a process that has shown to play a major role in the assembly of infectious virus particles (Boulant et al., 2007; Miyanari et al., 2007). In addition to being a virion component, the core protein interacts with a variety of cellular proteins and has shown to influence numerous host cell functions including apoptosis, gene transcription, cell proliferation, immune

response modulation, lipid metabolism and HCV-related steatosis (McLauchlan et al., 2000, 2009; Roingeard & Hourioux, 2008). However, all these observations are derived from heterologous overexpression experiments and still need to be confirmed in an infectious system. The translation of an alternative reading frame in the core coding sequence can also yield a small protein (~17 kDa), called ARFP or F protein. Its possible function is unclear so far, however, specific immune responses targeting the F protein have been detected in some chronically infected patients (Xu et al., 2001).

The structural proteins **E1** and **E2** are highly N-glycosylated transmembrane type I proteins located at the virus envelope and are involved in the receptor-binding and membrane fusion of the virion (Dubuisson et al., 2000). E1 and E2 contain a large N-terminal ectodomain and a C-terminal hydrophobic region harboring a transmembrane domain. During synthesis, the N-terminal domain is targeted towards the ER lumen and modified by N-glycosylation, while the transmembrane domain anchors the glycoproteins to the ER membranes. E1 and E2 contain up to respectively 6 and 11 potential glycosylation sites. The glycans on the envelope proteins are involved in the proper folding of these proteins either directly or through interaction with calnexin, an ER chaperone, and some of these glycans have been shown to be essential for the entry function of E1 and E2 (Goffard et al., 2005). Besides membrane anchoring, the transmembrane domains also function as a signal sequence, an ER retention signal and as a major regulator of the assembly of the non-

covalent E1-E2 heterodimer (Fig. 2). This heterodimer is the functional unit for productive HCV infection (Op De Beeck et al., 2004). E2 plays an important role in the interaction between HCV and its major cellular receptors CD81 and SR-BI. Since E1 and E2 are located at the surface of the HCV virion, they are targeted by neutralizing antibodies. These neutralizing antibodies often inhibit the interaction(s) between E2 and the CD81 receptor (Owsianka et al., 2001).Interestingly, a 3D model of the structure of E2 has recently been published (Krey et al.,2010). The isolated truncated E2 form reacts with a number of conformation-sensitive monoclonal antibodies and binds the soluble CD81 large external loop.

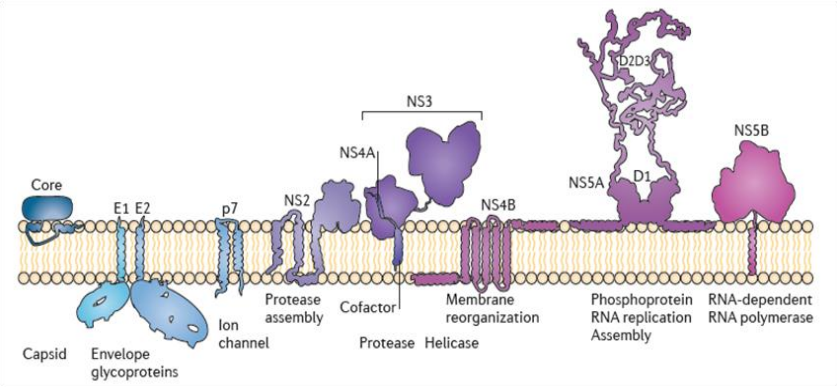

Fig. 2. Membrane topologies and major functions of the HCV polyprotein cleavage products.

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). *Adapted from Bartenschlager et al., 2013.*

p7 is a small (7 kDa) intrinsic membrane spanning protein with a cytoplasmic loop and its N- and C- termini oriented towards the ER lumen (Carrere-Kremer et al., 2002). It belongs to the viroporin family of proteins, which is able to oligomerize *in vitro* and form a hexameric cation-selective ion channel (Griffin et al., 2003; StGelais et al., 2007; Luik et al., 2009), which constitutes a potential antiviral target. Indeed, p7 has been shown to be crucial for assembly and release of infection virions, but has no role in RNA replication (Jones et al., 2007; Steinmann et al., 2007a).

2.2.3 Non-structural proteins

NS2 is a hydrophobic integral membrane protein responsible for the autoproteolytic cleavage at the NS2/NS3 junction of the polyprotein. The 23-kDa protein is believed to harbour multiple membrane spanning domains that localize NS2 to the ER membrane. However, the exact membrane topology and mechanism of translocation remains controversial (Santolini et al., 1995). The catalytic activity of the NS2-3 cysteine protease resides in the C-terminal half of NS2 and requires the N-terminal one-third of NS3, notably through the zinc binding domain (Grakoui et al., 1993; Hijikata et al., 1993a). The crystal structure of NS2/3 proposes three crucial residues (His143, Cys184 and Glu 163) forming the catalytic triad that together form an active site (Lorenz et al., 2006). It has been suggested that NS2 is dispensable for RNA replication (Lohmann et al., 1999), but it is critical for the

assembly of infectious particles (Jirasko et al., 2008; Jones et al., 2007). There is increasing evidence supporting a crucial role for NS2 in factoring the coordination of virus assembly through stable interactions with E1, E2, p7, NS2, NS3 and NS5A (Jirasko et al., 2010; Ma et al., 2011; Popescu et al., 2011; Stapleford & Lindenbach, 2011).

NS3 is a multifunctional 70-kDa protein harbouring a serine-type protease within its N-terminal, while the two-third of C-terminal features a RNase helicase/NTPase motif. The NS3 catalytic triad is formed by residues His-57, Asp-81 and Ser-139 and mutation of any of these amino acids abolishes proteolytic cleavage (Bartenschlager et al., 1993; Grakoui et al., 1993).

NS3 does not contain disulfide bridges, but a zinc ion tetra-coordinated by three cysteine residues and a histidine residue in its *C*-terminal domain (Love et al., 1996). It has been suggested that both protease activities, NS2/3 and NS3, rely on the same zinc ion bound to NS3, but with the zinc ion having a catalytic role in NS2/3 protease and a structural role in NS3 protease (Tedbury et al.,2007).In addition, the NS3 *N*-terminal domain, but not its activity, is required for NS2/3 activity, and the NS2/3 activity was shown to be more sensitive than the NS3 activity to inhibition by zinc-chelating agents.Therefore, the zinc bound to NS2/3 and NS3 proteases may be the same metal ion, bound to structurally different binding sites sharing common residues, playing a catalytic and structural role in NS2/3 and NS3 proteases, respectively.

The NS3 serine protease require NS4A as a cofactor**. NS4A** is the smallest HCV-encoded protein (6 kDa) and consists of an N-terminal hydrophobic region, a central domain and a C-terminal acidic region. The crystal structure of NS3/NS4A revealed that the central part of NS4A is essential for proper NS3 folding (Bartenschlager et al., 1995; Lin et al., 1995; Tai et al., 1996), while its N-terminal transmembrane domain allows stabilization and localization of NS3 at the ER membrane (Wolk et al., 2000). The C-terminal acidic region of NS4A influences NS5A phosphorylation and HCV RNA replication (Lindenbach et al., 2007). Upon association with the ER membrane, NS3 serine protease facilitates HCV polyprotein processing in *cis* at the NS3/NS4A and in *trans* at the NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions.

In addition to processing viral proteins, NS3/4A protease also cleaves cellular targets, in particular, Cardif and TRIF thereby blocking the antiviral innate immune response triggered by RIG-I and TLR-3 at the early stage of infection (Li et al., 2005; Meylan et al., 2005). The NS3 helicase-NTPase has several functions, including RNA-stimulated NTPase activity (Yao et al., 1997; Kim et al., 1998), RNA binding and unwinding of RNA regions of extensive secondary structure (Serebrov & Pyle, 2004). Although the structural data has been determined, the mode of action and precise role of the helicase in the virus life cycle remains unclear. Recent studies with intergenotypic chimeras have identified compensatory mutations within the NS3 helicase domain that rescue the assembly of virus particles of chimeras otherwise defective for virion production (Ma et al., 2008; Phan et al., 2009).

NS4B is a highly hydrophobic protein consisting of an N-terminal part, a central part harboring four transmembrane segments and a C-terminal part (Gouttenoire et al., 2010). It is an integral membrane protein with the N- and C-terminal parts located on the cytosolic side of the ER membrane. The N-terminal region contains two amphipathic α-helices (AH1 and AH2) of which the second helix can traverse the membrane bilayer and is essential for membrane trafficking, HCV genome replication and protein oligomerization. NS4B oligomerization is critical for replication complex formation (Gouttenoire et al., 2009, Palomares-Jerez et al., 2013). Based on sequence homology, it was proposed that the N-terminal part contains a leucine zipper motif (Welsch et al., 2007). Very recently, the role of this leucine zipper domain in the efficiency of HCV replication was demonstrated. The C-terminal part of NS4B contains two palmitoylation sites. The importance of C-terminal palmitoylation of NS4B remains to be clarified.

NS4B plays a central role in HCV RNA replication. In particular, the ectopic expression of NS4B induces the formation of a so called membranous web (MW). The MW refers to specific alterations of intracellular membranes that contains the HCV RNA replication complexes (Egger et al., 2002). However, the mechanism by which NS4B induces MW formation is unknown. NS4B also interacts with other non-structural HCV proteins and with negative-strand HCV RNA. The multi-functional NS4B is also shown to activate ER stress pathways, (Gouttenoire et al., 2010). Moreover, it has an anti-apoptosis function that might be associated with HCC development

(Einav et al., 2008). NS4B was also found to possess NTPase activity, suggested by the presence of a nucleotide-binding motif similar to the P-loop of a number of NTPases. However, this motif is not absolutely conserved across different genotypes and a mutation within this motif was identified as a cell- culture adaptive change in Con1 HCV replicons. Therefore, the enzymatic activity of NS4B remains controversial (Gouttenoire et al., 2010). Finally it has recently been found that NS4B has a role in virus particle assembly (Jones et al., 2009).

NS5B is a conserved RNA-dependent RNA polymerase (RdRp) that promotes complementary negative-strand RNA synthesis and then synthesizes positive-strand RNA using the newly synthetic negative-strand RNA as template in the absence of other viral or cellular factors in vitro (Behrens et al., 1996; Lohmann et al., 1997).

Due to the lack of proofreading of RdRp, HCV replication is error-prone (Sharma et al., 2010, Moradpour et al., 2013). A specific interaction between NS5B and the 3'UTR has been reported (Cheng et al., 1999). NS5B can interact with other viral proteins such as NS3, NS4A and NS5A, and cellular proteins like hVAP-33, which facilitates the formation of the viral RNA replication complex (Ishido et al., 1998, Tu et al., 1999). Furthermore, it can form a complex with the retinoblastoma tumor suppressor protein (pRb) and promote pRb degradation in an ubiquitin dependent manner, therefore contributing to HCC development (Munakata et al., 2007). NS5B has been shown to interact with cellular components that modulate its polymerase activity. For example, interaction with cyclophilin B seems to enhance

viral RNA synthesis through modulation of the RNA binding capacity of NS5B (Watashi et al., 2005).

Like other polymerases, the crystal structure of NS5B reveals that it resembles the configuration of a right hand. The fingers, thumb and palm domains compose a unique shape. The active site located in the palm domain has a highly conserved GDD motif. There are four allosteric sites within the thumb and palm domains which serve as targets for antiviral development (Bealieu et al., 2009; Membreo et al., 2011;Mayhoub et al., 2012). Besides, NS5B is a tail-anchored protein with its C-terminal hydrophobic tail associated to the ER membrane (Sharma et al., 2010).

The function of many cellular enzymes for DNA and RNA metabolism as weel as viral RdRps is often regulated by phosphorylation (Kim et al., 2004). Hwang et al., 1997 demonstrated that NS5B is a phosphoprotein in insect cells. Kim et al., 2007 discovered that the protein kinase C-related kinase 2 (PRK2) is the specific enzyme for NS5B phosphorylation within the N-terminal finger domain. Knock-down and overexpression of PRK2 demonstrated this protein able to up-regulate HCV RNA replication in HCV subgenomic replicon cells, suggesting that NS5B phosphorylation can enhance HCV replication.

Gao et al., 2003 identified an interaction between ubiquitin-like protein hPLIC1 (human homolog 1 of protein linking integrin-associated protein and cytoskeleton) and NS5B. Since hPLIC1 interacts with both proteasome and E3 ubiquitin protein ligases E6AP and βTrCP, the ubiquitination modification of NS5B through hPLIC1 binding

promotes ubiquitin-dependent proteasome degradation, resulting in decreased level of NS5B. NS5B mainly functions in RNA replication, so decreased NS5B leads to HCV genome RNA reduction (Gao et al., 2003, Kleijnen et al., 2000). Although the ubiquitination sites within NS5B and the detailed mechanism of hPLIC1-induced NS5B degradation are still not clear, up-regulating NS5B ubiquitination may represent a target for anti-viral development.

The **NS5A** protein is of particular relevance for this dissertation and therefore an in-depth discussion is provided.

2.2.3.1 NS5A: structure, phosphorylation and interactions

Structure

NS5A is one of the most enigmatic HCV proteins. It is a ~450 amino acid phosphorylated zinc-metalloprotein that plays an essential role in HCV viral lifecycle, both by affecting directly the viral RNA replication as well as by modulating the physiology of the host cell to favor viral replication. It has no known intrinsic enzymatic activity, but likely exerts its functions through interactions with viral and cellular factors. NS5A is composed of an N-terminal amphipathic α-helix and three domains (Domain I, II and III) that are separated by so-called low-complexity sequences (LCS1 and LCS2), a term that reflects the sensitivity of these sequences to limited proteolysis (Fig. 3)

(Tellinghuisen et al., 2004). The N-terminal amphipathic α-helix is monotopic and tethers NS5A to the cytosolic leaflet of intracellular membrane bilayers (Penin et al., 2004). Domain I contains a zinc-binding motif and is a nucleic acid-binding domain that binds to the 3' G/C rich sequence in HCV RNA. Domain I is primarily required for RNA replication, whereas Domain III plays a major role in the assembly of infectious virus particles, probably by interacting with the core protein at lipid droplets (Tellinghuisen et al., 2008; Masaki et al., 2008). In fact, Domain III can be deleted without affecting RNA replication and tolerates the in-frame insertion of heterologous proteins such as GFP. Although Domain II is also required for RNA replication, a large segment can be deleted with no significant effect on viral replication and virus production in cultured cell lines (Appel et al., 2008). It may also play a role in evading innate immune response, since it contains the so-called "interferon sensitivity determining region" (ISDR). The ISDR has been correlated with the efficacy of IFN treatment, but this finding remains controversial (Brillet et al., 2007).

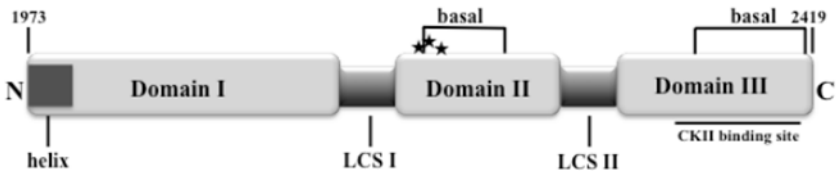

Fig. 3. Structure of NS5A.

The NS5A protein is comprised of amino acids 1973-2419 of the polyprotein (based on JFH-1 sequence) as indicated in the figure, and is characterized by 3 domains, which are separated by low complexity sequences (LCS) of repetitive amino acid residues. Amino acids 2200-2250 and 2350-C-terminus contain serine residues that

are basal phosphorylation sites (basal). The characterized hyperphosphorylation sites are denoted by ★ (serine residues 2197, 2201, and 2204). The N-terminus helix membrane anchor (helix) and the C-terminus CKII binding site (residues 2352-2419) are indicated. *Adapted from Lemmon et al., 2010.*

Two crystal structures of domain I (Fig. 4) without the amphipathic helix reveal a novel fold with charged surface patches (Tellinghuisen et al., 2005; Love et al., 2009). A zinc ion likely stabilizes NS5A domain I for its function. An intramolecular disulfide bond was found in one structure although it is not required for replicase function (Tellenghuisen et al., 2005). Interestingly, NS5A domain I can adopt different packing contacts for possible dimerization under different crystallization conditions, suggesting that this protein may be able to form alternate dimeric states that could contribute to its multifunctional roles in the virus life cycle. One type of dimer forms a positively charged groove which is believed to serve as a railway for viral RNA, tethering the RNA onto intracellular membranes and eventually shield it from degradation as well as from being perceived by pattern recognition receptors (Appel et al., 2006).

On the other hand, an alternative arrangement of the domain I homodimer was reported in which the proposed RNA binding cleft is absent (Love et al., 2009). It is conceivable that the two dimeric conformations observed thus far in crystallized domain I reflect two different functional states of membrane-tethered NS5A and that other configurations (possibly even monomeric) might exist. Biochemical studies suggest that the Domain I, LCS1 and Domain II

bind RNA, and that this binding promotes NS5A dimerization (Hwang et al., 2010).

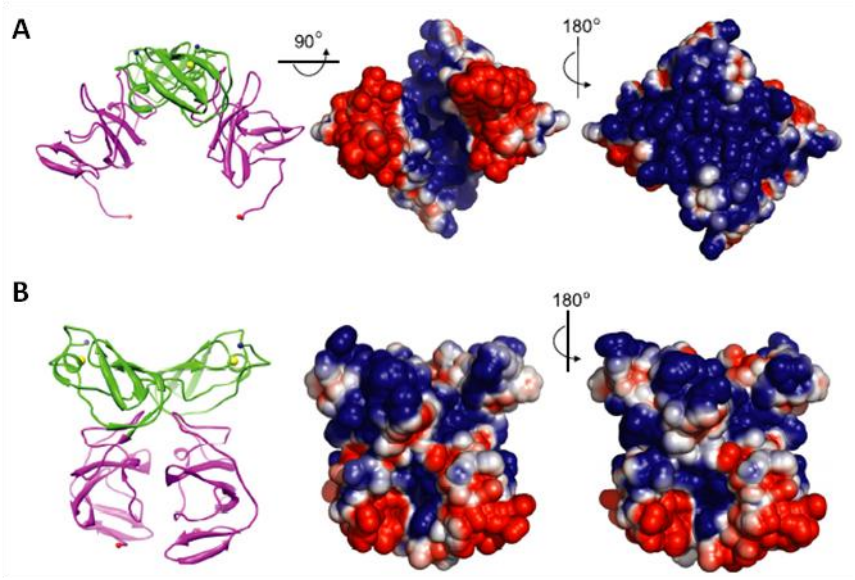

Fig. 4 NS5A domain I crystal structure with surface potential.

(A) Left, ribbon diagram of the first domain I dimer; the two subdomains (1, aa 2008 to 2072; 2, aa 2073 to 2170) are colored in green and purple, respectively. The two zinc ions are displayed as yellow spheres; the N and C termini of each monomer are depicted as small spheres colored in blue and red, respectively. Right, the surface electrostatic potential of the dimer (rotated 90°) calculated by the APBS program contoured at _1 KT (red), 0 (white), _1 KT (blue). Blue and red colors indicate positively and negatively charged amino acids, respectively. (B) Left, alternate ribbon diagram of the domain I dimer using the same color scheme as in panel A. Right, the surface electrostatic potential of the alternate dimer as calculated based on the same parameters as for panel A.

Domain II and III of NS5A are intrinsically unfolded monomers (Hanoulle et al., 2009; Verdegem et al., 2011) and as such are not true

structural domains. This structural flexibility might explain the promiscuous interaction properties of NS5A, which has been reported to bind to numerous cellular proteins (de Chassey et al., 2008).

Phosphorylation

NS5A is a phosphoprotein that exists in two forms, a basally phosphorylated form (56 kDa) and a hyperphosphorylated form (58 kDa), which is conserved among HCV genotypes (Huang et al., 2007). The basally phosphorylated sites are mainly serine residues (amino acids 228, 278 and 378-C-terminus) and the minority are threonine residues located in the central and C-terminal region (Pawlotsky et al., 1999). The p56 phosphoform is thought to exist as a heterogenous population of proteins phosphorylated on different serine residues but migrating at similar molecular weights (Tanji et al., 1995). NS5A p56 is required for several replicative functions including formation of the replicase complex on intracellular membranes (Miyanari et al., 2007); core-NS5A colocalization on lipid droplets (Appel et al., 2008); and interactions with NS5B and RNA (Blight et al., 2000; Shirota et al., 2002; Evans et al., 2004; Shimakami et al., 2004; Huang et al., 2005; Appel et al., 2005 Lemons et al., 2010), which are mediated by associations with hVAP-A (Gao et al., 2004). The hyperphosphorylated p58 phosphoform is generated by phosphorylation of p56 (Neddermann, Clementi, De, Molecolare, & Roma, 1999)in a serine-rich region in the central portion of NS5A (Macdonald et al., 2004).

The phosphorylation status is directly or indirectly modulated by NS3, NS4A, NS4B and NS5B (Koch et al., 1999; Neddermann et al.,

1999; McCormick et al., 2006). However, cellular kinases and mechanistic details regulating NS5A phosphorylation are still poorly defined. Basal phosphorylation seems to depend on kinases of the CMGC family (e.g. casein kinase (CK) II (Kim et al., 1999; Tellinghuisen et al., 2005)) and involves mainly sequences in NS5A D2 and D3 (reviewed in Huang et al., 2007). In contrast, p58 synthesis is mediated by the CKI protein kinase family, particularly CKIα (Quintavalle et al., 2006a and 2006b) and recently an additional role of Polo-like kinase 1 has been identified (Chen et al., 2010). A cluster of serine residues encompassing amino acids (aa) 222–235 at the C-terminus of Domain I and in LCS I has been shown to be involved in hyperphosphorylation of NS5A and in the regulation of RNA replication by adaptive mutations (Blight et al., 2000; Katze et al., 2000 and Appel et al., 2005). Mutations in this region typically decrease p58/p56 ratio and increase RNA replication of HCV genotype 1 isolates, raising the concept that p56 is mainly involved in RNA replication (Reiss et al., 2013). This is corroborated by the finding that kinase inhibitors reducing NS5A hyperphosphorylation stimulate HCV replication (Neddermann et al., 2004). However, a complete loss of hyperphosphorylation typically abrogates RNA replication indicating that low amounts of p58 are essential for RNA replication ((Neddermann et al., 2004); Appel et al., 2005). Replication analysis of phosphomimetic mutations of Ser 222 in the linker between domain I and II suggests that hyperphosphorylation could be a negative regulator of RNA replication and/or is required for assembly (Lemay et al., 2013; Gu and Rice, 2013). This indicates that

the phosphorylation state of NS5A modulates the efficiency of HCV RNA replication (Tellinghuisen 2007 and 2008; Appel et al 2008).

Recently a complex pattern of phosphorylation within LCS I has been revealed providing evidence for a sequential cascade of phosphorylation across this region, and regulation by a distal phosphorylation event within domain I. It has been proposed that this regulation could drive a conformational switch between the dimeric structures of NS5A, and could also explain the different functions of the protein in the virus lifecycle (Douglas Ross-Thriepland and Mark Harris 2013).

Since the subcellular distributions of both NS5A forms are similar, the degree of phosphorylation does not affect NS5A localization to the ER membrane. However, the degradation of NS5A is enhanced by increased degree of phosphorylation (Hundt et al., 2013).

Interactions

NS5A exerts multiple functions by interacting with viral and cellular proteins. It was shown that NS5A interacts with NS5B and that this interaction is essential for viral replication., NS5A was also found to interact with Core protein onto lipid droplets (Masaki et al., 2008). This interaction seems to be required for viral assembly. NS5A also interacts with several cellular signal cascades (Macdonald et al., 2004). Furthermore an interaction between NS5A and the IFN signaling pathway has been provided. This interaction includes the binding and inactivation of PKR, an antiviral effector of interferon-alpha (Gale et

al., 1998). The binding to dsRNA, an intermediate formed during viral RNA replication, activates PKR, than in turn shuts down protein translation. Furthermore, NS5A is also thought to interact with pathways of cell proliferation, apoptosis, stress-response, calcium/reactive oxygen, which may be related to HCV pathogenesis. (Macdonald et al., 2003; 2005).

Cyclophilin A (CypA) has been shown to bind via its active site to the domain II of NS5A protein (Yang et al., 2010; Foster et al., 2011). CypA is a peptidyl-prolyl isomerase (PPIase) identified as a critical host factor for the successful infection of many viruses, including human immunodeficiency virus, influenza and recently HCV (Fischer et al., 2010). Disruption of the isomerase activity of Cyclophilin A through chemical inhibitors such as cyclosporin (CsA), active-site mutations or small interfering RNA (siRNA) knockdown has been shown to be deleterious to HCV replication (ChatterIi et al., 2009; Foster et al., 2011) and, importantly, specific residues within NS5A are the target for this isomerase activity (Hanoulle et al., 2009). The disruption of specific residues for binding NS5A-CypA by mutation/deletion in domain II is inhibitory to HCV replication.

NS5A interacts with FK506-binding protein 8 (FKBP8) and vesicle-associated membrane protein associated protein A (VAP-A) (He et al., 2006), also called VAP-33, which is an integral membrane protein with a coiled-coil domain, is widely expressed in human tissues (Christopher et al., 2005) and might be involved in the HCV replication machinery (Evans et al. 2004). Previous studies have shown that VAP-A binds to HCV NS5A and NS5B proteins and is important for the assembly of the

HCV replication complex (Tu et al., 1999; Gao et al., 2004). Recently, G protein pathway suppressor 2 (GPS2) has been identified to interact with HCV NS5A (de Chassey et al., 2008) and this interaction is required for the association of NS5A with VAP-A and Hepatitis C virus replication (Xu et al., 2013).

Another interaction has been identified between NS5A and apoliprotein (ApoE), and their binding is required for the assembly and export of infectious virions (Berga et al., 2010).

Lately, a number of groups have used small interfering RNA (siRNA) screens to identify cellular cofactors of HCV infection. Although there has been notable divergence in the array of host cofactors of HCV infection identified in these screens, a remarkable constant has been the identification of phosphatidylinositol (PI) 4-kinase III alpha (PI4KIIIα) as an essential cofactor for HCV RNA replication (Berger et al., 2009; Borawski et al., 2009; Tai et al., 2009; Vaillancourt et al., 2009). Subsequently, many groups demonstrated the physical and functional interaction between NS5A and the phosphatidylinositol 4-kinase PI4KIIIα (Berger et al., 2011; Lim and Hwang, 2011) and that the lipid kinase activity was stimulated by this binding in i*n vitro* assays (Reiss et al., 2011). In HCV-infected cells, PI4KIIIα colocalizes with HCV NS5A and viral double-stranded RNA, the HCV replication intermediate, suggesting a role in replication complex formation or function (Berger et al., 2009). Very recently, Lohmann's group identified a site in NS5A Domain I that is involved in functional PI4KIIIα interaction and RNA replication. Mutations in this site phenocopied PI4KIIIα silencing. Importantly the same mutations resulted in

increased NS5A p58 levels showing that enzymatic activity of PI4KIIIα plays a role in the modulation of NS5A phosphorylation (Reiss et al., 2013).

3 The viral life cycle

Insights in the HCV lifecycle, that could reveal potential antiviral targets, have long been hampered by the lack of efficient cell culture systems. However the generation of subgenomic HCV replicons, the HCV pseudoparticle model and more recently infectious HCV culture models were landmark of developments that helped the understanding of the lifecycle of HCV and drug development (Fig. 5).

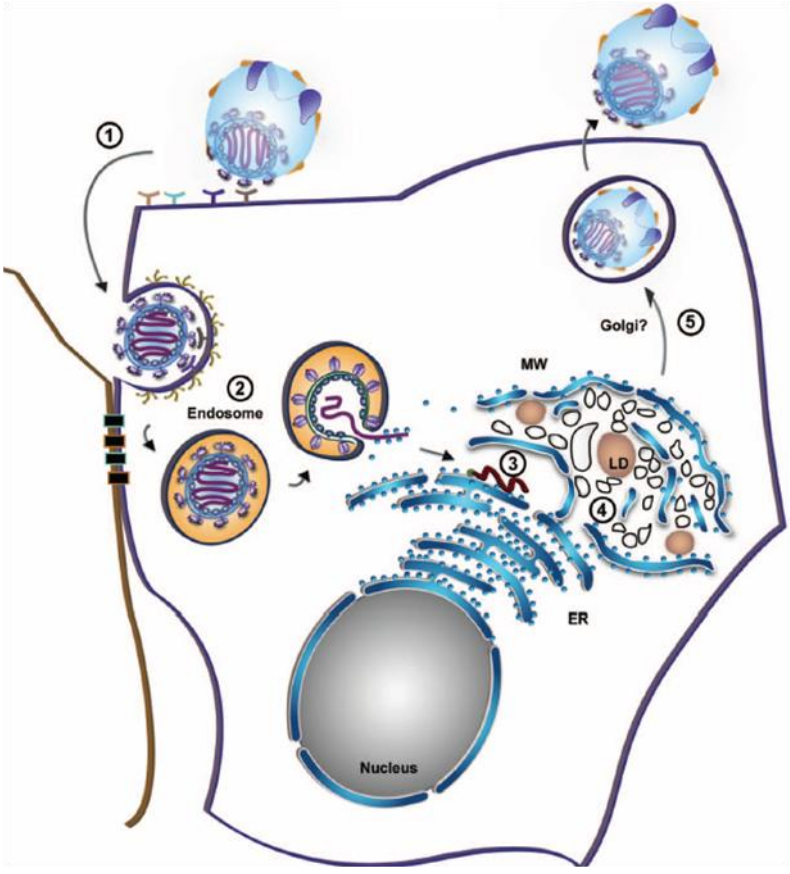

Fig. 5 The HCV life cycle.

(1) Attachment of HCV to the cell surface. The HCV particle decorated with the glycoproteins E1 and E2 and cellular apolipoproteins, binds to a set of entry molecules at the surface of the hepatocyte. (2) Receptor-mediated endocytosis of HCV leads to internalization in a clathrin-dependent manner. The low pH (indicated in orange) in the endosome might trigger the fusion between the endosomal and the viral membrane, thus releasing the RNA genome (red waved line) into the cytoplasm. (3) Translation of the viral genome occurs at the rough ER giving rise to a single polyprotein precursor that is cleaved by cellular and viral proteases. (4) HCV RNA replication takes place at the membranous web (MW), consisting of clusters of virus-induced vesicles, ER membranes and lipid droplets (LDs), which are the presumed sites of HCV assembly. (5) Release of HCV is thought to be linked to the VLDL secretion pathway. *Adapted from Alvisi et al., 2011.*

3.1 The Viral Particle

Much remains to be understood on the composition and structure of infectious HCV particles. Enveloped HCV virions are 50–80 nm in diameter, with E1 and E2 glycoprotein heterodimers embedded in the lipid bilayer surrounding a nucleocapsid composed of core protein and the single-stranded RNA genome (Gastaminza et al., 2010; Bassendine et al., 2011). HCV virions, existing as lipoviroparticles (LVPs), are not icosahedral, and because of their association with low-density and very-low-density lipoproteins (LDL and VLDL) in the infected host (Andrè et al., 2002; Merz et al., 2011), in fact they are pleomorphic with heterogeneous and low buoyant density, which varies depending upon growth conditions (Lindenbach et al., 2006). This Trojan horse strategy may help shield the virus from neutralization (Bartenschlager et al., 2011). Apolipoprotein E (apoE) and C (apoC) are associated with both *in vivo*– and cell

culture–derived particles, whereas association with apoB is less pronounced in cell culture (Chang et al., 2007).

3.2 Entry and uncoating

HCV entry is a complex and multi-step process requiring a set of entry proteins (Zeisel et al, 2011). In the early stage of HCV entry, HCV virions are concentrated at the cell surface by non-specific attachment factors. Glycosaminoglycans seem to be the initial attachment sites for HCV. Also the LDL receptor is thought to be involved in early attachment of HCV, although this finding remains somehow controversial (Burlone and Bukowska, 2009). Following initial attachment, HCV virions bind virus-specific receptors. Currently, a number of receptors essential for HCV entry are identified: the scavenger receptor class B type I (SR-BI) (Scarselli et al., 2002), the tetraspanin CD81 (Pileri et al., 1998), and tight junction proteins claudin-1 (CLDN1) (Evans et al., 2007) and Occludin (Ploss et al., 2009). Very recently, new player in HCV entry has been uncovered: receptor tyrosine kinases (RTKs) (Lupberger et al., 2011) As discussed in the previous paragraph,HCV particles associate with very-low-density and low-density lipoproteins (LPs) in the bloodstream. Once arrived in the liver, the particles t bind the basolateral surface of hepatocytes through glycosaminoglycans (GAGs) and the low-density lipoprotein receptor (LDLR). Interaction of glycoprotein E2 with both SR-BI and CD81 are necessary but not sufficient for HCV entry. SR-BI is a

lipoprotein receptor that is responsible for the selective uptake of cholesterol esters from HDL (high density lipoprotein) particles. In addition, SR-BI plays an important role in VLDL catabolism. As regards HCV, SR-BI acts as a post-binding receptor, since SR-BI antibodies inhibit HCV infection when added 60 min following virus binding. CD81 belongs to the family of tetraspanins which are ubiquitously expressed. Similar to SR-BI, it acts as a post-binding entry factor, since anti-CD81 antibodies inhibit HCV infection only after virus attachment. Furthermore, CD81 actively promotes infection upon HCV binding by triggering signaling cascades that are important for virus entry (Zeisel et al., 2011). Very recently, RTKs were identified as cofactors required for HCV entry, more in details, it has been shown how pharmacological blockage of these molecules can prevent viral infection *in vitro* and *in vivo*. Clinically approved inhibitors exist for two of these kinases: epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2). EGFR activation promotes CD81–CLDN1 interactions and membrane fusion, thereby enhancing productive HCV uptake. RTKs, upon dimerization and autophosphorylation, provide signals inducing proliferation, migration, morphogenesis and other fundamental cellular processes (Lupberger et al., 2011). CLDN1 is predominantly expressed in the liver, where it forms networks at tight junctions. Silencing of CLDN1 inhibits HCV infection in susceptible cells (Evans et al., 2007), nevertheless, there is still no evidence for a direct interaction between the virus and CLDN1. On the other hand CLDN1 has been shown to play a role in the post-binding phase subsequent to HCV binding to CD81 and SR-BI. Analogous to other viruses like

coxsackievirus B, it is speculated that the interaction with CLDN1 takes place after migration of the virus-CD81/SR-BI complex to tight junctions (Coyne et al., 2006). Occludin is expressed in tight junctions of polarized cells and is also involved in the post-binding phase of HCV entry (Ploss et al., 2009). Engagement by the virus of SR-BI, the tetraspanin CD81 and the tight junction proteins claudin-1 and occludin leads to clathrin-mediated endocytosis of virions (Blanchard et al., 2006), delivering HCV-receptor complexes to early endosomes. The low pH in the endosome triggers the fusion of the virion envelope with the membrane of the early endosome particle. Membrane fusion is catalyzed by fusion peptides embedded in the viral glycoproteins. For HCV related viruses like flaviviruses, the structural basis for low pH-induced membrane fusion has been elucidated (Modis et al., 2004). The envelope proteins of these viruses contain an internal fusion peptide, which is exposed during membrane reorganization mediated by low pH (class II fusion). The scaffolds of class II fusion proteins are remarkably similar. Therefore it has been suggested that the entry of all *Flaviviridae*, including HCV, may include a class II fusion step. The identity of the fusion protein of HCV is still debated, since. some evidence indicates E2, but also E1 has been suggested as possible fusion protein. Following membrane fusion, the viral RNA is released into the cytosol (Meertens et al., 2006). How the viral RNA is exactly released from the nucleocapsid is not known. It was shown for other positive strand RNA viruses (alphaviruses) that binding of ribosomes to the viral genome supports RNA release (Singh et al., 1992).

3.3 Translation and Polyprotein Processing

Once release in the cytoplasm, the HCV RNAgenome is immediately translated into the viral polyprotein by the host ribosomal machinery via the HCV IRES located within the 5' UTR, thus demonstrating that the viral genome can directlyserve as a messenger RNA for the synthesis of the HCV polyprotein precursor. IRES-mediated translation is initiated by direct binding of a vacant 40S ribosomal subunit to the IRES and positioning of the polyprotein start codon into the 40S P site (Kieft et al., 2001; Otto & Puglisi, 2004). The IRES can capture the 40S ribosomal subunit in the absence of other initiation factors known to be required for ribosome binding to a cellular mRNA. Following its formation, the IRES-40S complex then binds to the eukaryotic initiation factor 3 (eIF3), followed by recruitment of the eIF2:Met-tRNAi: GTP complex to generate a 48S-like complex (Pestova et al., 1998; Ji et al., 2004). Upon hydrolysis of GTP, the initiator factors are released and the 60S ribosomal subunit can then attach to the 48S complex to form a functional IRES-80S complex, which can initiate viral protein synthesis. Translation occurs in close association with ER membrane where the resulting HCV polyprotein is co- and post-translationally cleaved by cellular proteases and the viral NS2-NS3 and NS3-NS4A proteases to release ten HCV protein. In particular, the structural proteins are generated by proteolytic activity of host signal peptidases at the core/E1, E1/E2, and E2/p7 junctions (Okamoto et al., 2004). The C-terminus of core protein is additionally processed by a host signal peptide peptidase (Hussy et al., 1996). The NS2 cysteine protease

cleaves the NS2/NS3 junction of the polyprotein, releasing the NS3-5B portion of the polypeptide. The NS3/4A serin protease cleaves the remaining junctions, sequentially releasing all other NS proteins (Grakoui et al., 1993). The NS proteins NS3/4A, 4B, 5A, and 5B are required for subsequent formation of the replicase complex and viral RNA replication (El-Hage et al., 2003; Miyanari et al., 2003).

3.4 HCV RNA replication

HCV RNA replication occurs in detergent-resistant membranous vesicles formed from ER membranes and lipid rafts (Aizaki et al., 2004; Egger et al., 2002; Miyanari et al., 2007; Shi et al., 2003). These HCV replicative membrane form peculiar three-dimensional structures that have been termed “membranous web” (see also above). The membranous web contains the viral replicase complex consisting of NS proteins and viral RNA (El-Hage and Luo, 2003; Moradpour et al., 2003). Membrane alterations are induced by NS4B, which independently associates with lipid rafts (Egger et al., 2002). NS5A p56 associates with the viral RNA (Huang et al., 2005), and both the NS5A-RNA complex and NS5B bind human vesicle-associated protein A (hVAP-A) independently (Tu et al., 1999), thus localizing NS5A, NS5B, and the viral RNA to the membranous web lipid rafts. NS3 is recruited to the replicase complex either independently (Miyanari et al., 2007) or by complexing with NS4A prior to joining the replicase complex (Lin and Rice, 1995). Besides its role as a critical cofactor for NS3 activity, NS4A is also necessary for later formation of NS5A p58 (Kaneko et al.,

1994). The membranous web is stabilized by the presence of NS5A, as NS5A recruits the cellular kinase PI4KIIIα to the replicase complex, thereby stimulating its kinase activity (Berger et al., 2011; Lim and Hwang, 2011; Reiss et al., 2011). Increased PI4KIIIα activity induces membrane accumulation of its product, phosphatidylinositol 4-phospate (PI4P), which is critically implicated in the maintenance and integrity of the membranous web (Berger et al., 2009; 2011; Lim and Hwang, 2011; Reiss et al., 2011; Tai et al., 2011). Other cellular factors have been identified with potential roles in HCV RNA replication (Ploss and Rice, 2010). The best characterized of these include the DEAD-box RNA helicase DDX3 and GBF1 - a guanine nucleotide exchange factor for small GTPases of the ARF family. DDX3 is crucial for virus RNA replication and is believed to incorporate into replication complexes to facilitate genome translation (Ariumi et al., 2007; Geissler et al., 2012). The presence of GBF1 in the cells is critical for replication complex activity, although its exact mechanism of action remains to be determined (Gouslain et al., 2010).

Once the replicase complex is formed in the membranous web, RNA synthesis is initiated *de novo* by highly structured RNA elements in the 3'-NTR of the template RNA strand and the polymerase activity of NS5B (Luo et al., 2000; Zhong et al., 2000). Multiple positive strand RNA products are synthesized from each negative strand replicative intermediate (Behrens et al., 1996; Lohmann et al., 1997; Lindenbach and Rice,2005). The new RNA genomes are used for translation, further RNA replication, or are recruited for inclusion in infectious virions.

3.4.1 The HCV RNA replication complex

After the establishment of the replicon system (see 5.2) it became clear that the proteins NS3-to-NS5B are necessary and sufficient for RNA replication in Huh-7 (hepatoma) cells harboring autonomously replicating subgenomic HCV RNAs (Lohmann et al., 1999). All NS proteins are associated with ER-membranes by one or several transmembrane domains or an amphipathic α-helix. Several groups demonstrated that the membrane-interactions of the NS proteins NS3, NS4B, NS5A, and NS5B are critical for viral RNA replication (Elazar et al.,2004, 2004; Penin et al., 2004). Likewise, the protein-protein interaction among different NS proteins has also been extensively studied and seems to be essential for replication, too (Dimitrova et al., 2003; Ishido et al., 1998; Lin et al., 1997; Qin et al., 2001; Shimakami et al., 2004). Together with the viral RNA, the NS proteins NS3 to NS5B form the so-called replication complex (RC) in which the replication of HCV RNA occurs (Fig. 6). Gosert and coworkers showed that the NS proteins colocalized with newly synthesized RNA (Gosert et al., 2002, 2003).

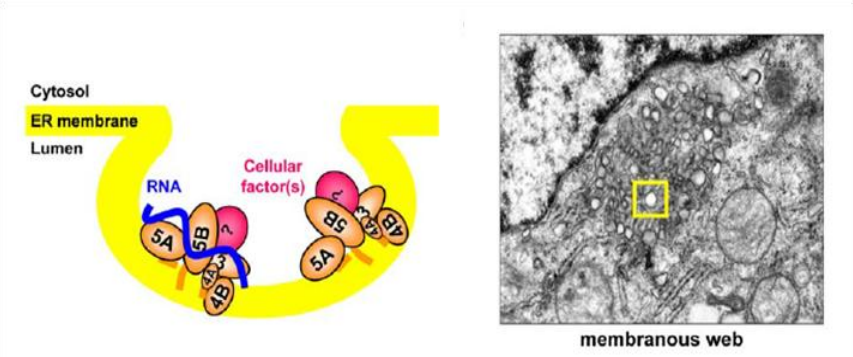

Fig. 6 Hypothetical model of the HCV replication complex.

(A) HCV NS proteins are indicated by orange ellipses, potential cellular factors by pink ellipses, and viral RNA by a blue wavy line. Individual NS proteins and RNA are not drawn to scale. (B) Electron micrograph of the membranous web, membrane alterations in Huh-7 cells containing subgenomic HCV replicons. The web is composed of small vesicles embedded in a membrane matrix and closely associated to the rough ER. These vesicular structures (one highlighted by a yellow rectangle) harbor the HCV replication complexes containing viral NS proteins and RNA and represent the site of viral RNA replication. *Adapted from Gosert.*

3.4.2 The HCV induced-membranous web

A common feature of all positive-strand RNA viruses is the remodeling of intracellular membranes creating mini-organelles or 'replication factories' where RNA amplification and eventually also virion assembly take place (reviewed in Miller and Krijnse-Locker, 2008). Formation of such sites facilitates coordination of the different steps of the replication cycle, but might also shield viral RNA, especially double strand (ds) RNA replication intermediates, from recognition by innate sensors such as RIG-I (retinoic acid-inducible gene I, also known as DDX58) or MDA5 (melanoma differentiation-associated gene 5, also known as IFIH1 or Helicard). In the case of flaviviruses such as Dengue virus or West Nile virus, it has been shown that RNA replication occurs most likely within membrane invaginations originating from the endoplasmic reticulum (ER) (Welsch et al., 2009; Gillespie et al., 2010). Similar invaginations have been described e.g. for Flock House virus or Semliki Forest virus, although in these cases membrane alterations occur at other sites: the outer mitochondrial membrane or the plasma

membrane, respectively (Kopek et al., 2007; Spuul etal., 2010). In contrast, in case of the poliovirus, the prototype member of the picornaviruses, complex membrane rearrangements have been described that are formed most likely as protrusions originating from *cis*-Golgi membranes and transforming in a time-dependent manner from single membrane tubular compartments into double-membrane structures (Belov et al., 2012). Likewise, coronaviruses (Gosert et al., 2002; Knoops et al., 2008) and arteriviruses (Pedersen et al., 1999; Knoops et al., 2012) induce double membrane vesicles (DMVs) that resemble exvaginations of ER-derived membranes.

In case of HCV, membrane rearrangements with a 'membranous web' (MW)-like appearance were originally detected in cells over-expressing the viral polyprotein, or only NS4B Egger et al., 2002; Gosert et al., 2003). Morphologically the MW is a cytoplasmic accumulation of highly heterogeneous membranous vesicles that are embedded into an amorphous matrix. A recent study suggests that the predominant structures within the MW are Double Membrane Vescicules (DMVs) and less frequently, multivesicular membranes (Ferraris et al., 2010). Expression of NS4B triggers formation of membranous vesicles by an as yet unknown mechanism. Nevertheless, several hypotheses can be put forward to explain the origin of the MW and how NS4B alters cellular membranes.

(1) NS4B may promote membrane curvature (a requisite for formation of membrane vesicles) by oligomerization (McMahon et al., 2005). In this case membrane bending might be induced by amphipathic α-helices located in the N-terminal region. Alternatively,

by analogy to the E viroporin of the mouse hepatitis coronavirus, another oligomeric protein which possesses an amphipathic α-helix, NS4B might form membrane integral pores inducing membrane curvature (Raamsman et al., 2000; Wilson et al., 2006).

(2) The recruitment of cell factors such as Arfs (ADPribosylation factors), which contain an N-terminal amphipathic helix or proteins containing BAR (Bin-Amphiphysin-Rvs) domains with a characteristic banana-like structure, might deform membranes (Gallop et al., 2005).

(3) The lipid composition of the membrane itself might directly trigger membrane curvature (McMahon et al., 2005). As previously described, the membranous web is highly enriched in specific phosphatidylinositol (PI) species (Hsu et al.,2010), which can induce membrane curvature by different mechanisms (Ishiyama et al., 2002) Moreover, the fact that NS proteins are recruited to cholesterol-rich lipid rafts, likely by interacting with NS4B, and that replication complexes remain active after treatment with detergents, indicates that enrichment of cholesterol-rich lipid rafts might also contribute to HCV replicative vesicle formation.

(4) DMVs might be induced, at least in part, by autophagy. Autophagosomes, which engulf long-lived proteins and damaged organelles, transport them to the lysosomes for degradation (He et al., 2009). The origin of the autophagosomal membrane is still under debate and various sources including mitochondria, the Golgi apparatus and the ER have been proposed (Juhasz et al., 2006). Autophagy can be activated in response to infection, starvation and different stress conditions (Kim et al., 2000). Importantly, proteins of

the autophagy pathway have recently been implicated in the HCV replication cycle. However, the lack of colocalization between HCV proteins and autophagosomal markers suggests that the viral replication complex does not assemble on autophagosomes (Dreux et al., 2009). Nevertheless, HCV might exploit certain components of the autophagy machinery to generate DMVs with a specific protein composition. In fact, it has been suggested that HCV prevents the degradation of long-lived proteins, by blocking the fusion between autophagosomes and lysosomes, thus leading to the accumulation of DMVs required for HCV RNA replication (Sir et al., 2008).

Taking advantage of a combination of confocal microscopy, electron microscopy (EM) and electron tomography (ET), Bartenschlager's group dissected the composition, 3D architecture and biogenesis of the various HCV-induced membrane alterations (Fig. 7). Surprisingly, the 3D architecture of these membrane rearrangements is similar to those induced by the unrelated picorna- and coronaviruses, but in striking contrast to the closely related flaviviruses. Early in infection HCV induces double membrane vesicles (DMVs) that emerge as protrusions of the ER; later on, HCV induces in addition multi-membrane vesicles that are probably the result of a cellular stress reaction and that are reminiscent to an autophagic response. These profound membrane rearrangements are induced by the concerted action of HCV-encoded nonstructural proteins of which NS5A is the only one capable to induce DMVs. These results provide important insights into the 3D architecture of the membrane alterations induced by HCV and reveal unexpected similarities between HCV and the very

distantly related picorna- and coronaviruses (Romero-Brey et al., 2012).

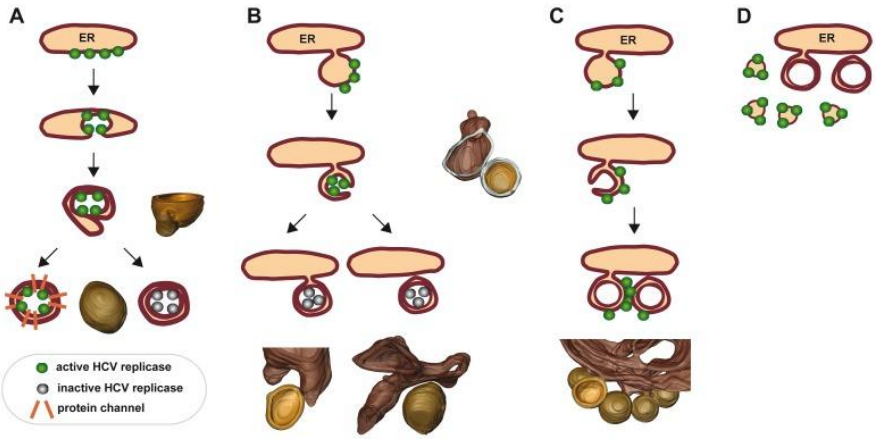

Fig. 7. Hypothetical models describing the formation of double membrane vesicles and their possible role in viral RNA replication.
Enzymatically active HCV replicase (green dots). Upon closure of the DMV, the replicase would become inactive (grey dots). The resulting DMV might stay connected to the ER via a stalk or be released as a 'free' DMV (left or right drawing, respectively). *For description, see Romero-Brey et al., 2012*.

3.4.3 Known cellular factors of HCV replication: the important role of PI-4 kinase type-IIIα

Viruses are obligatory intracellular parasites always exploiting host cell resources: besides using general cellular mechanisms like translation, energy supply, secretion, etc., many viruses recruit specific host cell factors for particular purposes. These host cell factors may serve as targets of antiviral therapeutics and are therefore extensively studied. A number of cellular proteins important for HCV RNA replication have already been identified by proteomic approaches, for

example Yeast-two Hybrid (Y2H) assays. One class of those host factors is represented by RNA-binding proteins. Besides the ribosomal proteins, other host cell factors are also involved in the viral translation process, e.g. the La antigen and the polypyrimidine tract-binding protein (PTB). It was shown that PTB interacts with HCV NS3 and NS5B (Domitrovich et al., 2005), whereas NS5A cooperates with the RNA binding protein La (Houshmand et al., 2003). The important role of La in the HCV replication was proven by siRNA silencing (Domitrovich et al., 2005). Other cellular proteins acting as cofactors of viral replication include the F-box and leucine rich protein 2 (FBL2) (Wang et al., 2005), amphiphysin II (Masumi et al., 2005), Vap-A, Vap-B (Hoofnagle et al., 2002), and cyclophilin B (CyPB) (Watashi et al., 2005).

In 2009, six different research groups conducted siRNA library screenings in order to find host cellular factors required for HCV life cycle (Berger et al., 2009; Borawski et al., 2009; Tai et al., 2009; Trotard et al., 2009; Vaillancourt et al., 2009; Reiss et al., 2011). Interestingly, two phosphatidylinositols (PIs)-specific kinases, PI-4 kinases type III alpha (PI4KIIIα) and beta (PI4KIIIβ) were consistently identified as key host factors essential for HCV replication. Several following studies further confirmed their crucial role in viral genome replication (Berger et al., 2011; Lim and Hwang, 2011). In addition, specific PIs including phosphatidylinositol 4-phosphate (PI(4)P) (Hsu et al., 2010; Berger et al., 2011; Reiss et al., 2011; Tai and Salloum, 2011; Zhang et al., 2012) as well as phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) (Lee et al., 2008) were also found to associate with HCV non-structural viral

proteins and implicated in HCV replication. All these data strongly suggest that HCV takes advantage of normal physiological roles of different kinds of PIs as well as PI-4 kinases in vesicular trafficking, membrane transport, and cytoskeleton remodeling to facilitate its RNA genome replication.

This finding is further supported by data showing that the kinase inhibitors wortmannin or LY294002, used at doses known to favor inhibition of type III PI4Ks, significantly decreased HCV replication (Berger et al., 2009; Tai et al., 2009). PI kinases and the lipids they phosphorylate are essential regulators of membrane trafficking and protein sorting.

PI4KIIIα is one of four cellular PI 4-kinases that all function to phosphorylate phosphatidylinositol at the 4 position of an inositol head group, which then serves as a beacon to recruit proteins containing lipid-binding motifs with affinity for PI4P (reviewed in references Balla et al., 2006; 'Angelo et al., 2008; Vicinanza et al.,2008). Different PI 4-kinases have different subcellular localization, thus generating pools of PI4P at specific membrane compartments. PI4KIIIα is the predominant endoplasmic reticulum (ER) resident PI 4-kinase (Balla et al., 2008), although it critically contributes to maintenance of the plasma membrane PI4P pool (reference). In HCV-infected cells, PI4KIIIα co-localizes with HCV NS5A and viral double-stranded RNA, the HCV replication intermediate, suggesting a role in replication complex formation or function (Berger et al., 2009). Treatment with siRNAs and pharmacological inhibition of PI4KIIIα dramatically inhibit HCV replication. PI4KIIIα is not required for HCV

entry or initial translation of the viral genomic RNA. It co-localizes with markers of the HCV replicase, and most importantly, membranous webs fail to accumulate in cells that have been silenced for PI4KIIIα expression (Berger et al., 2009). A role for PI4KIIIα in forming replication complexes is also supported by elegant studies from Tai et al., 2009who show that the HCV replicase-associated NS5A protein has aberrant localization in cells that have inducible expression of the full HCV polyprotein and have been silenced for PI4KIIIα expression (Tai et al., 2009). On the basis of these results, it was proposed a model in which infecting RNA genomes are translated at the rough ER, giving rise to high amounts of polyprotein (Quinkert et al., 2005). NS5A generated by polyprotein cleavage binds to the kinase in a domain I-dependent manner and recruits the enzyme to ER-derived membranes. Binding of NS5A to the kinase stimulates its activity, resulting in high levels of PI4P at these membrane sites (Fig. 8).

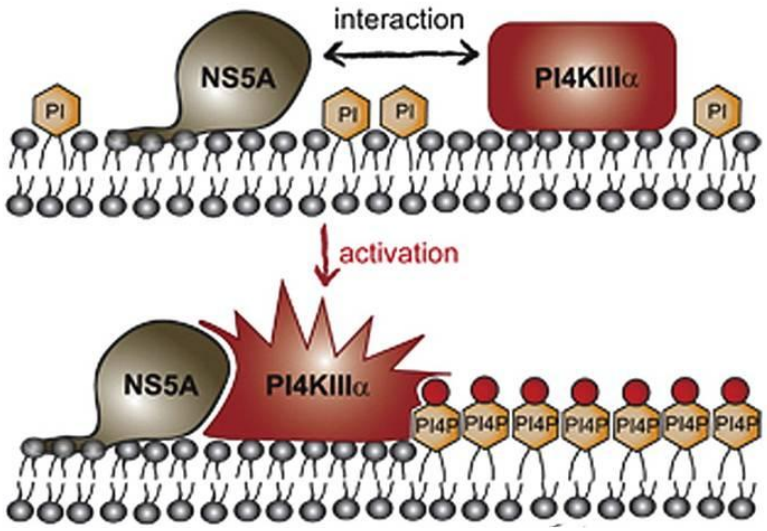

Fig. 8. Formation of PI4P-enriched membranous environment.

Cartoon from Reiss et al., 2011

What could be the role of elevated PI(4)P levels in the HCV replication compartment? In one scenario the specific PIP composition might directly influence membrane bending and thus contribute to membrane curvature necessary for MW formation (Ishiyama et al., 2002). In a second scenario PIPs might regulate intracellular processes including vesicle fusion, budding and sorting required for MW formation (Vicinanza et al., 2008). In fact, different PIPs are recognized by distinct effector proteins via highly conserved, specialized domains, including the pleckstrin homology (PH), the PHOX homology as well as the FYVE, ENTH and ANTH domains (De Matteis et al., 2004).

Two proteins recently implicated in the HCV replication cycle can specifically recognize PIPs: first, Annexin AII, which is important for virus assembly and specifically binds PI(4,5)P2 (Saxena et al., 2012) second, oxysteroyl binding protein (OSBP), which appears to be essential for both HCV RNA replication and production of infectious virus, and which specifically recognizes PI(4)P via a PH domain (Amako et al., 2009). OSBP and its related proteins have been implicated in regulating numerous cellular processes, which play a central role in HCV life cycle, such as sphingomyelin synthesis (Perry et al., 2006), autophagy (Leblanc et al., 2009) and LD biogenesis (Hynynen et al., 2009). Very recently it has been demonstrated that OSBP is a downstream PI4P effector required for HCV replication and membranous web integrity. OSBP is recruited to membranous webs in

a PI4KIIIα -kinase-dependent manner, and both these factors were found to regulate cholesterol trafficking to the web. (Tai et al., 2014).

In addition to the interest in the biology of PI4KIIIα in HCV infection, it is possible that PI4KIIIα may be a legitimate drug target for treating HCV infection. Pharmacological inhibitors of PI kinase activity prevent HCV replication *in vitro* (Berger et al., 2009, Tai et al., 2009) and PI-3 kinase inhibitors have been successful therapies against certain cancer (Ma et al., 2009). We speculate that inhibitors specific to PI4KIIIα may be successful therapeutics for HCV with fewer issues of resistance. The main question remaining is the mechanism of HCV replication complex formation.

RNAi analysis has identified a number of cellular candidates that may be involved in constructing membrane-associated sites of replication.

These include early endosomes (EEA1, RAB5 and PIK3C2G)159, late endosomes (RAB7A, RABEPK) , vesicles associated with the Golgi apparatus (COPI components and RAB7L1159), and the ER (PIK4CA, TBC1D20 and RAB1B250). Components of vesicular trafficking and membrane fusion (VAP-A and VAPB) and regulators of actin reorganization (CDC42 and ROCK2) may also be involved (Randall et al., 2007). A role in replication complex formation for EEA1, RAB5, RAB7, COPI subunits, PIK4CA, TBC1D20, VAP-A, VAP-B and CDC42 is further supported by microscopy, proteomic and/or protein biochemistry studies (Berger and Randall, 2009). These cofactors, in addition to others, may work in combination to establish the membrane-associated replication complexes in infected cells.

3.5 HCV Assembly and Release

Newly synthesized HCV RNA genomes can be assembled into new virions (Bartenschlager et al., 2011). Viral proteins involved in this assembly process are the core protein, the envelope proteins E1 and E2, viroporin p7, and nonstructural proteins NS2 and NS5A (Appel et al., 2008; Masaki et al., 2008; Jones et al., 2007). Lipid droplets and the VLDL assembly pathway are major contributors of the host cell to virion assembly. The mature HCV virion consists of a nucleocapsid, composed of core proteins and the viral genome, and an outer envelope, containing a lipid membrane and the envelope proteins. The early stage of assembly, i.e. nucleocapsid formation, involves oligomerization of the core and encapsidation of the viral genome. Currently it is not clear where the nucleocapsid formation initiates. In a first model, nucleocapsid formation is initiated at the surface of cytosolic LDs. Viral RNA is delivered to the core protein via NS5A that is also mobilized onto LDs or remains bound to the ER. In a second model, assembly initiates at the ER membrane. Core protein is initially transferred to LDs but is re-recruited to ER membranes at the assembly sites where it interacts with NS5A. Recently, it was demonstrated that a concerted action of HCV p7 and NS2 regulates Core localization at the ER and virus assembly. The genotype 2a JFH1 HCV strain replicates and assembles in association with LD associated membranes, around which viral core protein is predominantly detected. In contrast, despite its intrinsic capacity to localize to LDs when expressed individually, they found that the Core protein of the

high-titer Jc1 recombinant virus was hardly detected on LDs of cell culture-grown HCV (HCVcc)-infected cells (see section 5.4), but was mainly localized at ER membranes where it colocalized with the HCV envelope glycoproteins. Furthermore, high-titer cell culture-adapted JFH-1 virus, obtained after long-term culture in Huh 7.5 cells, exhibited an ER localized core in contrast to non-adapted JFH-1 virus, strengthening the hypothesis that ER localization of core is required for efficient HCV assembly. They also demonstrated that p7 and NS2 are HCV strain specific factors that govern the recruitment of core protein from LDs to ER assembly sites. Indeed, using expression constructs and HCVcc recombinant genomes, they found that p7 is sufficient to induce Core localization at the ER, independently of its ion-channel activity. Importantly, the combined expression of JFH1 or Jc1 p7 and NS2 induced the same differential core subcellular localization detected in JFH1- *vs* Jc1 infected cells. Finally, results obtained by expressing p7-NS2 chimeras between either virus type indicated that compatibilities between the p7 and the first NS2 trans-membrane domains is required to induce core-ER localization and assembly of extra- and intra-cellular infectious viral particles (Boson et al., 2011). Another group studied the recruitment of core from LD into nascent virus particles. To investigate the kinetics of core trafficking, they developed methods to image functional core protein in live, virus producing cells. During the peak of virus assembly, core formed polarized caps on large, immotile LDs, adjacent to putative sites of assembly. In addition, LD-independent, motile puncta of core were found to traffic along microtubules. Importantly, core was recruited from LDs into these

puncta, and interaction between the viral NS2 and NS3-4A proteins was essential for this recruitment process (Counihan et al., 2011). The late stage of assembly, i.e. the maturation and release of HCV virions, is closely associated with the VLDL pathway (Huang et al., 2007; Gastaminza et al., 2008). The nucleocapsid can be incorporated in the precursor of VLDL, the luminal LDs. How the envelope proteins are incorporated into virions is not clear. Recently it was reported that NS2 might “crosstalk” between envelope glycoproteins and non-structural proteins by forming multiple protein-protein interactions (Jirasko et al., 2010). Mature HCV virions contain apoB, apoE and eventually other apolipoproteins. After maturation, HCV virions are transported and secreted along the VLDL secretory pathway. Therefore, the structure of HCV virions resembles that of triglyceride-rich lipoproteins containing a core of neutral lipids surrounded by a phospholipid monolayer and stabilized by apolipoproteins (Olofsson et al., 2009).

4 Lipid metabolism and HCV Replication

A particular hallmark of HCV is the intimate link with the host lipid metabolism. HCV virions were found to circulate in the blood in complex with lipoprotein (Thomssen et al., 1992). These lipoviroparticles (LVP) are highly enriched in triglycerides and contain apoB and apoE (Andrè et al., 2002). The infectivity of HCV-positive

inocula is inversely correlated with the buoyant density of LVPs (Hijikata et al., 1993).

Since HCV virions are associated with host lipoproteins, it seems logical that HCV entry is connected to the host lipid metabolism. Lipoprotein receptors (LDL receptor, SR-BI receptor) have been reported to be involved in HCV entry. Furthermore, ligands of SR-BI can modulate HCV entry. For instance, HDL enhance HCV entry, whereas oxidized LDL inhibits HCV entry (Von Hahn et al., 2006). Also the lipid composition of the plasma membrane influences HCV entry. Cholesterol depletion from the plasma membrane decreases HCV entry by reducing the expression of CD81 receptors at the cell surface (Kapadia et al., 2007).

HCV exploits the VLDL assembly and secretion pathway to be released from hepatocytes. Furthermore, it has been shown that lipid droplets (LDs) play an important role in HCV virion assembly. LDs are intracellular storage vesicles that contain triacylglycerols and cholesteryl esters surrounded by a phospholipid monolayer. HCV core protein accumulates around these LDs. This association is a peculiarity of the Hepacivirus genus in the Flaviviridae family and is essential for the recruitment of other viral proteins like NS5A and NS3 and for virus production (Hope et al., 2000).

Apart from HCV entry and HCV virion assembly, lipids including cholesterol play critical roles in HCV replication. Fatty acids can stimulate or inhibit HCV replication depending on their degree of saturation. Polyunsatured fatty acids inhibit HCV replication, whereas saturated and mono-unsaturated fatty acids stimulate it (Kapadia et

al., 2005). Fatty acids can be used as prosthetic groups that may facilitate the anchoring of viral non-structural proteins to membranes. For example, NS4B undergoes palmitoylation at two cysteine residues in the C-terminal region. These lipid modifications were shown to be necessary for protein-protein interactions (Yu et al., 2006). Thus, palmitoylation can be a post-translational modification required for the life cycle of HCV. Studies revealed that HCV replication occurs on cholesterol-rich domains within the cell as part of the HCV replication complex (Aizaki et al., 2004). Inhibition of the cholesterol synthetic pathway by inhibiting the rate-limiting step 3-hydroxy-methylglutaryl CoA reductase completely disrupted HCV replication (Ye et al., 2003). Aside from cholesterol synthesis, this could also be due to an earlier branch point of this synthetic pathway that functions to add prenyl groups to proteins, namely geranylgeranylation. It was demonstrated that the geranylgeranylation of host protein FBL2 binds to HCV nonstructural protein 5A (NS5A), playing a critical role in HCV replication (Wang et al., 2005). The presence of HCV replication complexes in turn functions to elevate the levels of enzymes involved in cholesterol and fatty acid synthesis by activation of SREBPs (Waris et al., 2007; Kapadia et al., 2005). Another transcriptional regulator of cholesterol metabolism that is activated by the bile acid catabolic products of cholesterol is the farnesoid X receptor (FXR). Bile acids were interestingly found to increase HCV replication through FXR activity (Scholtes et al., 2008), athough this may be genotype specific (Chhatwal et al., 2012). Statins, which are widely used for the treatment of hypercholesterolemia, have also been reported to inhibit

HCV replication. Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis in the liver. The HMG-CoA reductase catalyzes the conversion of HMG-CoA into mevalonic acid. Besides their cholesterol-lowering effect, statins were shown to inhibit the replication of HCV subgenomic genotype 1b replicons and to suppress RNA replication of infectious JFH-1 HCV (Ikeda et al., 2006; Amemiya et al., 2008). The precise mechanism of the anti-HCV activity of statins has not yet been unraveled. The anti-HCV activity of statins may result from inhibition ofgeranylgeranylation of cellular proteins rather than the inhibition of cholesterol synthesis (Kapadia et al., 2005).

Recent evidence suggests that not only cholesterol synthesis, but also sphingolipid synthesis contributes to HCV replication. HCV nonstructural proteins function to modify the endoplasmic reticulum and cytosolic lipid droplets to form double membrane vesicles containing HCV replication complexes where viral RNA is protected from host RNAses and sensors of the innate immunity that would respond to double-stranded RNA (Romero-Brey et al., 2012; Miyanari et al., 2007). The lipid composition of replication complexes is not completely established, but it is known that HCV replication occurs on detergent resistant membranes, indicating that they are likely rich in both cholesterol and sphingolipids (Shi et al., 2003). HCV infection induces the synthesis of sphingomyelin by increasing the expression of the genes encoding sphingomyelin synthases 1 and 2 (Hirata et al., 2012). Indeed, inhibition of sphingolipid synthesis dramatically modulates HCV replication (Romero-Brey et al., 2012; Sakamoto et al.,

2005) . While it is not completely understood how the lipid composition of these specialized compartments are formed, recent evidence indicates a key role for phosphotidylinositol 4-kinase, subtype III alpha (PI4KIIIα) (Reiss et al.,2011).

An important element of both HCV replication and cholesterol metabolism is the liver-specific miR-122, which is the predominant miRNA expressed in the liver and plays a key role in hepatic fatty acid and cholesterol metabolism (Esau et al., 2006). Deletion of miR-122 results in hepatosteatosis and diminished circulating cholesterol and lipoprotein levels, a phenotype also reflected in HCV infected patients (Hsu et al., 2012). Also as in HCV infection, this phenotype may in part be mediated through transcriptional modulation of microsomal triglyceride transferase, an enzyme required for lipoprotein production (Mirandola et al., 2006;Tsai et al., 2012). Furthermore, the absence of miR-122 leads to the development of hepatocellular carcinoma in mice (Tsai et al., 2012). This miRNA is also unique in that it directly binds to the HCV genome and acts to boost its replication (Jopling et al., 2005). In fact, this is one of a few factors that confer permissibility of non-hepatic cells for robust HCV infection (Da Costa et al., 2012). The mechanism of miR-122 enhancement of HCV infection is at least in part due to the stabilization of the genome by forming a protein complex with host Argonaute 2, protecting the genome from exonucleases (Shimakami et al., 2012). The therapeutic possibility of miR-122 as a target has been explored in chimpanzees with HCV infection, yielding promising results (Lanford et al., 2010). It is an intriguing hypothesis that the pathology of HCV relating to lipids could

be due to the sequestration of miR-122 into these complexes, but the physiological levels of HCV RNA during infection are not likely sufficient to exert such an effect and there is no *in vivo* correlation with HCV RNA and miR-122 levels (Sarasin-Filipowicz et al., 2009). The interplay between this important regulator of cholesterol and lipid metabolism and the pathologic effects of HCV infection remain an interesting aspect of HCV research.

5 Model to study HCV

5.1 Animal models

Many features of the HCV infection are similar between human and chimpanzees and for more than a decade the chimpanzee was the only animal model available to study the course of HCV infection (reviewed in Bukh, 2004). Due to the silent nature of HCV disease, acute infections in human usually go unnoticed and the chimpanzee model provided the advantage of monitoring the progression of HCV disease from beginning to end. Studies in the chimpanzee shed further light on different aspects of the cellular immune responses and their role in disease outcome (Jo *et al.*, 2011). Although chimpanzees provide a clinically relevant model for the study of HCV, it is extremely limited in its availability, is highly expensive and due the ethical issues has been used for anti-HCV drug discovery only in a very limited way.

Many attempts have been made to establish a small animal model for HCV that is widely available, affordable and reproducible and one that represents most aspects of human HCV infection and disease. Chimeric (xenograft) mice harbouring human hepatocytes uPA/SCID (urokinase plaminogen activator/ severe combined immunodeficiency) is probably the most relevant small animal model set up so far (Meuleman & Leroux-Roels, 2008). Inoculation of SCID mice with HCV derived from human serum has shown to upport prolonged HCV infections with clinically relevant titers (Mercer et al., 2001; Meuleman et al., 2005). Most importantly, SCID mice have proven useful in the

evaluation of efficacy and toxicity of antiviral compounds (Kremsdorf and Brezillon, 2007). More recently an immunocompetent humanized mouse model for HCV was described. This model was developed by inoculating mice with a recombinant adenoviral construct that induced the expression of the four HCV human receptors in the mouse hepatocytes that are sufficient for HCV infection (Dorner et al., 2011). Infection of these mice was shown using a bicistronic HCV genome expressing CRE recombinase (Bi-nlsCre-Jc1FLAG2, abbreviated HCV-CRE), which activates a loxP-flanked luciferase reporter in Rosa26-Fluc mice. The mice were infected with the HCV-CRE and displayed high luciferase values that peaked at 72 hours postinfection. This system represents the first immunocompetent small animal model for studying HCV coreceptor biology in vivo an evaluation of passive immunization strategies.

5.2 The replicon system

Detailed studies on the HCV life cycle were hampered, primarily due to the incapacity of HCV serum particles to establish infection in cell culture. Nevertheless a major breakthrough came in 1999 with the development of a selectable, subgenomic replicon (SGR) system that allowed HCV RNA replication in cell culture (Lohmann *et al.*, 1999).

The first functional SGR, derived from a genotype 1b strain called Con1, was a bicistronic construct. In this system the first cistron encoded neomycin phophotransferase (neo^r) gene, translated under the control of the HCV IRES, resulting in G418 resistance. The second

cistron encoded non-structural proteins (NS3-NS5B) directed by a heterologous EMCV IRES. Upon electroporation of Huh-7 cells with *in vitro* synthesized SGR RNA and selection with G418 resulted in low numbers of surviving cell colonies. The low frequency of cells supporting SGR RNA replication was partly because replicon RNAs had to acquire adaptive mutations for efficient replication in the Huh-7 cells and secondly due to limited host cell permissiveness. Adaptive mutations tend to cluster within the nonstructural proteins NS3, NS4B, NS5A and NS5B. Several replication-enhancing mutations resulted in loss of NS5A hyperphophorylation (Blight et al., 2000). Introducing adaptive mutations into the parental replicon significantly enhanced RNA replication level to various extents (Blight et al., 2000; Krieger et al., 2001; Lohmann et al., 2001). Furthermore, curing Huh-7 cells supporting viral replication by IFN treatment identified cell clones that supported greater levels of RNA replication. For the Huh-7.5 cell clone, this phenotype was thought to be due to a defective IFN response in these cells caused by a mutation in the RIG-I gene (Sumpter et al., 2005). The aforementioned advancements in the understanding of the SGR system through viral adaptive mutations and highly permissive cell clones eventually led to the development of autonomously replicating full length replicons harbouring the entire HCV ORF (Blight et al., 2003; Ikeda et al., 2002; Pietschmann et al., 2002).

5.3 HCV pseudoparticles (HCVpp)

Availability of stable cell lines harbouring autonomously replicating SGR RNAs helped define the functional replication unit, as well as the viral and cellular determinants involved in replication complex and their subcellular localization. It was however, the development of HCV pseudoparticle system (HCVpp) that enabled the study of viral entry (Bartosch et al., 2003; Drummer et al., 2003; Hsu et al., 2003).

HCVpp are engineered viral particles consisting of a retroviral or lentiviral core enveloped by a lipid bilayer. Present within the envelope are HCV E1 and E2 gene products, which confer entry of the pseudoparticle into target cells. To quantify the number of infected cells, a reporter gene, such as luciferase or green fluorescent protein (GFP), is also included. HCVpp harvested from transfected 293T cell supernatant can be used to infect naive Huh-7 cells, where entry is mediated by HCV glycoproteins and can be blocked by anti-E1 or anti-E2 specific antibodies (Bartosch et al., 2003; Hsu et al., 2003; Lavillette et al., 2005b). Since HCVpp are replication deficient and support only a single infection event, the quantification directly reflects the HCVpp capacity to enter the cells. Studies have shown HCVpp and HCVcc virions share the same pH-dependent internalisation and fusion steps of the entry process (Tscherne et al., 2006; Sharma et al., 2011). More importantly, HCVpp representing glycoproteins of all major genotypes have been successfully generated (Lavillette et al., 2005b; Owsianka et al., 2005) and have allowed in depth studies of virus binding,

attachment and internalization and also helped in identifing novel HCV receptors.

5.4 Cell-culture derived HCV (HCVcc)

In 2005, three research groups published reports on the development of a cell culture system capable of producing infectious HCV particles (HCVcc) (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Each system relied on the properties of a unique genotype 2a isolate, known as JFH1. SGRs derived from JFH1 were previously shown to be capable of efficient RNA replication in multiple liver and non-liver specific cell lines without the need for adaptive mutations (Kato et al., 2003; Kato et al., 2005). Later, it was demonstrated that RNA transcripts from the full-length JFH1 genome transfected into Huh-7 cells produced infectious virus (Wakita et al., 2005) (Figure 1.6). However, virus titers released from transfected or infected cells were moderate at best. Higher virus titers were achieved when JFH1 was propagated in the Huh-7.5.1 cell-line (Zhong et al., 2005). Also, a chimeric JFH1 clone containing the core to NS2 region of HCV strain J6 fused to the JFH1 NS3-NS5B was found to be more infectious than full-length JFH1 (Lindenbach et al., 2005). The virus release was further improved by altering the fusion junction from NS2/NS3 to a crossover point that resides after the first TM segment of NS2 (Pietschmann et al., 2006). This cross-over point also improved the infectivity of other intergenotypic chimeras. Thus, virus release is more efficient when the first TM NS2 segment is from the same isolate as

the core-to-p7 region and the remainder of NS3 is homologous to the replicase. It is believed that this enhancement may be related to interactions between the N-terminal NS2 region and the structura protein(s) or p7, alterations of cleavage at the p7–NS2 site that is processed with delayed kinetics, or effects on cleavage at the NS2–NS3 site. Since the development of the J6/JFH1 chimera, viable JFH1 chimeras representing all genotypes have been generated (Gottwei et al., 2009).

B. Hepatitis C Virus infection: clinical aspects

1 Epidemiology, Geographical Distribution and Transmission

HCV infection is responsible for a major global health hazard. There are around 170 million people worldwide who are chronically infected by HCV and more than one million new infection cases are reported annually (Cooreman et al., 1996). In the United States alone, nearly four million persons are infected and 30,000 acute new infections are estimated to occur each year (Armstrong et al., 2006). In Europe and Japan, the disease is already more important numerically than is either hepatitis B virus (HBV) or human immunodeficiency virus (HIV) infection (Hatzakis et al., 2011)) and due to the availability of the HBV vaccine the relative impact of hepatitis C infections will increase further.

HCV infection causes a substantial portion of chronic liver disease mortality due to the induction of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) (Marcellin, 1999). About 4 to 20% of patients with chronic hepatitis C will develop liver cirrhosis within 20 years and HCC may develop after about 20 to 35 years. There is a significant rise in the incidence of HCC in many developed countries including Japan, Spain, France, and Italy, where the proportion of HCC attributable to HCV ranges from 50% to 70% (Michielsen et al., 2005). HCC induced by HCV accounts for 60%- 70% of all primary liver cancers in Europe, 50%-60% in North America and 20% in Asia and Africa (Hatzakis et al., 2011). In Japan, HCV-related HCC incidence has more than tripled over the past four decades and accounts in the 60–70 year

age group for as much as 90% (Kato et al., 1999). Apart from HCC, co-infections with other viruses, especially HIV-1 and other hepatitis viruses, have gained more attention. These are of clinical importance, since the course of HCV infection is accelerated by co-infection with HIV (Soto et al., 1997), hepatitis A virus (HAV) or HBV (Zarski et al., 1998).

HCV has a heterogenous geographical distribution (Fig. 9). Countries with the highest reported prevalence rates are located in Africa and Asia; areas with lower prevalence include the developed nations in North America (Alter et al., 2007). In Europe, chronically infected patients are around 9 million in comparison with 1.5 million infected by HIV (Hatzaski et al., 2011).

The seroprevalence rate is about 1% in Western Europe and North America, 3-4% in some Mediterranean and Asian countries and up to 10-20% in parts of Central Africa and Egypt (Wasley et al.,2000). Populous nations in the developed countries with relatively low rates of HCV seroprevalence include Germany (0.6%), Canada (0.8%), France (1.1%), and Australia (1.1%). Low, but slightly higher seroprevalence rates have been reported in the USA (1.8%), Japan (1.5–2.3%), and Italy (2.2%).

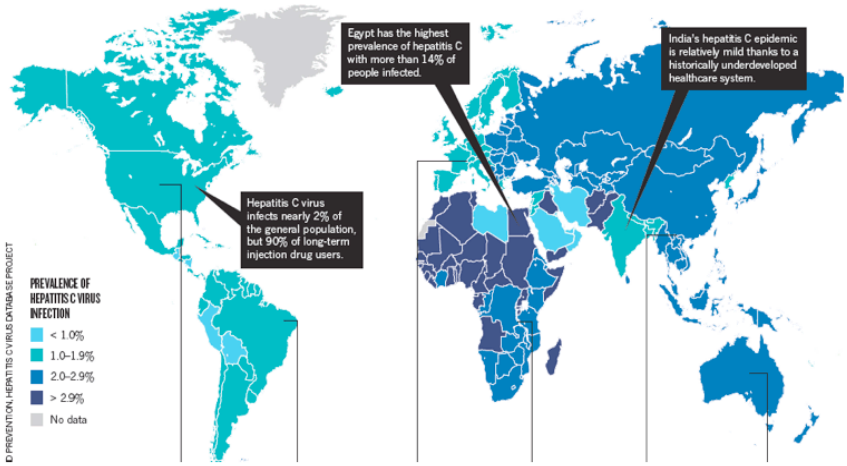

Fig. 9 Geographical distribution of Hepatitis C virus. (Source, WHO 2011) (www.natap.org/2011/newsUpdates/062911_04.htm)

HCV is transmitted mainly through parental route. Blood transfusion was one of the major threats for HCV infection before the launching of improved blood screening measures in 1990 and 1992 (Laurer and Walker, 2001). Similarly to other parenterally-transmitted infections such as HIV and HBV, other various modalities of HCV transmission have been documented:

- *Intravenous drug use*. Since the most efficient transmission route of hepatitis C virus is percutaneous exposure, it is not surprising that intravenous needle sharing drug users show high infection rates, that may be as high as 90% when HIV co-infected drug addicts are considered (Taylor et al., 2012). In the United Kingdom 90% of infectious cases are due to injecting drug abuse (Martin et al., 2012).

- *Non-intravenous recreational drug exposure*. Increasing evidence is accumulating that HCV may also cross the nasal mucosa and infect subjects chronically using inhalatory recreational drugs, such as cocaine, by the sharing of inhalatory instrumentation, favored by the frequent bleeding of the nasal mucosa occurring in these individuals (Allison et al., 2012). All persons who use or have used illicit injection drugs in the present or past, even if only once, as well as intranasal drug users who share paraphernalia, should be tested for HCV infection. Individuals who have received a blood or blood component transfusion or an organ transplant before 1992 should also be tested. With the introduction of sensitive tests to screen blood donors for HCV antibodies in 1992, transfusion-transmission of HCV has become rare (Alter et al., 2002).

- *Accidental exposure*. The risk of HCV infection after accidental needle stick exposure has been reported to range between 0.2% to 10%, depending on various factors including hollow-bore needles, percutaneous exposure, high HCV viral load or HIV co-infection of the index case (Corey et al., 2009).

- *Healthcare procedures*. Exposure to unsafe healthcare practice, including hemodyalisis, has been reported to be one of the most important risk factors associated with HCV infection, even in western countries (Perz et al., 2012).

- *Mother to child vertical transmission*. Mother-to-child vertical transmission of HCV is reported to occur in 3-10% of cases, mostly in

the late intrauterine period, at delivery or in the perinatal period. Many factors have been reported to influence the transmission rate, including maternal high viral load, labour duration, newborn gender, premature membrane rupture and genotype (Murakami et al., 2012). The role of elective cesarean section to reduce mother-to-child transmission rates is debated and controversial and the guidelines of the European Association for the Study of the Liver (EASL) does not recommend cesarean section to prevent HCV vertical transmission (EASL, 2011).

- *Sexual exposure*. The efficiency of the sexual transmission of HCV has been the subject of extensive debate and it is generally considered to be very low (Tohme et al., 2010). However, among male intravenous drug users, the rate of HCV infection was found to be one third higher in those who had sex with men (MSM) than in heterosexuals (Marongiu et al., 2012). Recent evidence points to the increasing incidence of HCV infections in MSM, probably facilitated by rectal mucosa traumatisms, especially when HIV-infection coexists.

Folk medicine practices, including acupuncture and ritual scarification, as well as body piercing, tattooing and commercial barbering are potential modes for transmission of HCV infection when performed without appropriate infection control measures(Hwang et al., 2006). Transmission of HCV infection by body piercing is, however, rare. Because symptoms are generally absent in individuals with chronic HCV infection, recognition of infection requires risk factor

screening, which should be done whenever it is possible to link with appropriate HCV testing and counseling (Alter et al., 2004).

However, for a large share of cases, estimated at around 30%, no definite exposure source may be identified. Studies of age-specific prevalence rates of anti-CV in the population show that HCV infection is rare among children while it increases with age, suggesting a possible cohort effect in anti-HCV positive elderly individuals who acquired the infection several years ago, before the introduction of effective preventive measures such as screening of donors and use of disposable needles and syringes in medical practice. During the last 10 years, the rate of transfusion-associated hepatitis C has significantly dropped as a consequence of the introduction of increasing restrictions on donor eligibility and the implementation of effective anti-HCV or HCV-RNA screening. Consequently, most of the millions who are chronically infected with HCV are now in the fourth or fifth decade of life (“baby boomers”). As they move into their 60s and 70s, these individuals constitute a wave of asymptomatic HCV infection that may move toward clinical disease.

2 Natural history of Infection

HCV is a significant cause of both acute and chronic hepatitis. The clinical course of hepatitis C is variable with no single typical course or natural history of disease, but instead a broad clinical spectrum of disease presentations and outcomes (Hoofnagle et al., 1997).

2.1 Acute Hepatitis C

The majority of HCV infections are asymptomatic during the acute phase and therefore remain undiagnosed. Data available of acute phase infection comes from studies conducted on patients who acquired infection via needle stick injury or following transfusion. In very few cases patients develop non-specific symptoms including decreased appetite, fatigue, malaise, jaundice, anorexia and fever. The acute phase is considered the first 6 months after infection (Alter & Seeff, 2000; McCaughan et al., 1992; Thimme et al., 2001). The infection is marked by viral RNA detection within 1 to 2 weeks following exposure and elevated serum alanine aminotransferase (ALT) levels (Farci et al., 1991; Thimme et al., 2001). Resolution of acute infection occurs spontaneously in around 25% of the cases, which is marked by absence of HCV RNA and normal ALT levels (Hoofnagle, 1997) and is more common among infected infants and young women than among persons who are older when they develop acute hepatitis.

Cases of fulminant HCV causing liver failure during acute infection have been documented but are very rare (Farci et al., 1996a). Fulminant hepatic failure is a dramatic clinical syndrome characterized by the massive necrosis of liver cells, which in the HCV associated cases arises shortly following infection (2-8 weeks) (Farci et al., 1996; Kato et al., 2001). Such cases are thought to be caused by highly virulent strains that circulate in the patients' blood at high titers (105-108/ml) during the peak stages of infection (Farci et al., 1996; Kato et

al., 2001). Interestingly, the only HCV strain (JFH1) to date that is capable of completing the full viral lifecycle in cell culture was isolated from a Japanese fulminant hepatitis patient (Wakita et al., 2005).

2.2 Chronic Hepatitis C

Chronic hepatitis C infection is diagnosed by the detection of HCV RNA in the blood for at least 6 months after infection. In general, prospective studies have shown that 60-85 % of acutely infected patients will develop chronic infection (NIH Consensus, 2002). Once chronic, HCV infection usually persists for decades, causing insidious and progressive liver damage in most patients. Most patients (80 %) remain asymptomatic for 10-30 years despite having moderate t severe chronic liver disease (Hoofnagle et al., 1997; Sharara et al., 1997). There are many risk factors involved in the development of chronic HC infection, including the age at time of infection, gender, ethnicity, and the development of jaundice during the acute infection.

2.3 Cirrhosis and Hepatocellular Carcinoma

Structural liver damage, known as fibrosis implies possible progression to extensive tissue scarring called cirrhosis. The time from HCV infection to cirrhosis is dependent on multiple internal host factors, and cannot be predicted in an individual patient. In a large cohort study by Poynard (et al. 1997) the median duration of infection for progression to cirrhosis was estimated to be 30 years, with host

factors including ageing, alcohol consumption and male sex having a strong association with fibrosis progression. Also, 10-20 year follow-up studies show that cirrhosis develops in 20-30 % of patients (Di Bisceglie et al., 1991; Haber et al., 1995; Seeff et al., 1992; Takahashi et al., 1993; Vaquer et al., 1994). The risk of developing cirrhosis ranges from 5% to 25% over periods of 25 to 30 years (Seeff et al., 2002). Progression to cirrhosis may be accelerated in persons who are of older age, who are obese, who are immunosuppressed (e.g., HIV co-infected (Benhamou et al., 1999), and who consume more than 50g of alcohol per day, although the precise quantity of alcohol associated with fibrosis progression is unknown (Harris et al., 2001). The progression to cirrhosis is often clinically silent, and some patients are not known to have hepatitis C until they present with the complications of end-stage liver disease or hepatocellular carcinoma (HCC). Virtually all HCV related HCC occurs among patients with cirrhosis. Once cirrhosis is established, HCC develops at an annual rate of 1- 4 % (NIH Consensus, 2002). Infection with HCV can also cause extrahepatic diseases including mixed cryoglobulinemia, types II and III. Indeed, symptomatic cryoglobulinemia is an indication for HCV antiviral therapy regardless of the stage of liver disease.

3 Current management of Hepatitis C: diagnostic tools and treatment responses

The optimal approach to detecting HCV infection is to screen persons for a history of risk of exposure to the virus, and to test selected individuals who have an identifiable risk factor. Three HCV markers are useful in clinical practice, including (1) total antibodies to HCV, (2) HCV RNA and (3) HCV genotype (Chevaliez and Pawlotsky, 2009).

(1) Serologic assays that detect specific antibody to hepatitis C virus (anti-HCV) are used both to screen for and to diagnose HCV infection.

Anti-HCV can be detected in the serum or plasma using two enzyme immunoassays (EIAs) approved by the U.S. Food and Drug Administration (FDA) for clinical use as well as one enhanced chemiluminescence immunoassay (CIA).

(2) Many commercial assays for the detection (qualitative assays) or quantification (quantitative assays) of HCV RNA are available. Historically, qualitative assays have been more sensitive than quantitative assays. With the recent availability of real time polymerase chain reaction (PCR)-based assays and transcription-mediated amplification (TMA) assays, with sensitivities of 10-50 IU/mL, there is no longer need for qualitative assays (Scott et al., 2007).

HCV RNA testing should be also performed in patients with unexplained liver disease whose anti-HCV test is negative and who are immunocompromised or suspected of having acute HCV infection. A sensitive quantitative HCV RNA assay is recommended for diagnosis

because it also provides information on the level of virus which is helpful in management.

One pattern is the identification of both anti-HCV and HCV RNA in a person with recent elevation of the ALT value. This scenario is consistent with either acute HCV infection when there is a recent known risk exposure, with exacerbation of chronic HCV infection, or with an acute hepatitis of another etiology in a patient with chronic HCV infection. Another pattern is the detection of anti-HCV but with a negative test for HCV RNA. This may represent acute HCV infection during a period of transient clearance of HCV RNA, a false positive or negative result or, more commonly, recovery from HCV infection.

(3) Genotyping is useful in epidemiological studies and in clinical management for predicting the likelihood of response and determining the optimal duration of therapy. HCV isolates have been grouped into seven major genotypes (1–7, ~30% sequence divergence) and a number of subtypes (a, b, and so on, ~20% sequence divergence) (Gottwein et al., 2008). Several commercial assays are available to determine HCV genotypes using direct sequence analysis of the 5' non-coding region, that include reverse hybridization analysis using genotype specific oligonucleotide probes located in the 5' non-coding region. Incorrect typing among the major genotypes is rare (<3%) and mixed genotypes occur but are uncommon. Occasionally (<5%), tested samples cannot be genotyped. This usually results from low viral levels, issues with the PCR amplification step of the assay, or extreme nucleotide variability within the HCV genome (Germer et al., 1999).

HCV genotyping should be performed in all HCV-infected persons prior to interferon-based treatment in order to plan for the dose and duration of therapy and to estimate the likelihood of response (Ghany et al., 2009).

The liver biopsy is another diagnostic tool. There are three primary reasons for performing a liver biopsy: it provides helpful information on the current status of the liver injury, it identifies features useful in the decision to embark on therapy, and it may reveal advanced fibrosis or cirrhosis that necessitates surveillance for hepatocellular carcinoma (HCC). The biopsy is assessed for grade (the extent of necroinflammatory activity) and stage (the extent of fibrosis or the presence of cirrhosis) of the liver injury, but also provides information on other histological features that might have a bearing on liver disease progression (Kleiner et al., 2005).

(4) The liver biopsy has been widely regarded as the "gold standard" for defining the liver disease status, but the procedure is not without risks (including pain, bleeding and perforation of other organs), is subject to sampling error, requires special expertise for interpreting the histopathology and adds cost to medical care (Crockett et al., 2006). Thus, efforts are underway to seek alternative means of establishing information on the extent of fibrosis by focusing on noninvasive tests of fibrosis (Rockey et al., 2006).

Accordingly, treatment responses are defined by a surrogate virological parameter rather than a clinical endpoint.

Short-term outcomes can be measured biochemically (normalization of serum ALT levels), virologically (absence of HCV RNA

from serum by a sensitive PCR-based assay), and histologically (>2 point improvement in necroinflammatory score with no worsening in fibrosis score) (Fried et al., 2002).

Several types of virological responses may occur, labeled according to their timing relative to treatment. The most important is the sustained virological response (SVR), defined as the absence of HCV RNA from serum by a sensitive PCR assay 24 weeks following discontinuation of therapy. This is generally regarded as a "virological cure", although liver cancer has been identified years later, especially if cirrhosis existed at the time of achieving an SVR (Kobayashi et al., 2007). Undetectable virus at the end of either a 24-week or 48-week course of therapy is referred to as an end-of-treatment response (ETR). An ETR does not accurately predict that an SVR will be achieved but is necessary for it to occur. A rapid virological response (RVR), defined as undetectable HCV RNA at week 4 of treatment, using a sensitive test with a lower limit of detection of 50 IU/mL, predicts a high likelihood of achieving an SVR (Yu et al., 2007). An early virological response (EVR) is defined as a ≥2 log reduction or complete absence of serum HCV RNA at week 12 of therapy compared with the baseline level.

4 Treatment of HCV Infection.

The goal of the therapy is to prevent complications and death from HCV infection.

For more than a decade, the standard treatment of chronic hepatitis C has been based on the combination of **polyethylene glycol (PEG-)** conjugated **interferon-α** (PEG-IFNα) and **ribavirin** (RBV), administered for 24 or 48 weeks (Patel et al., 2006). The interferons are a group of naturally occurring cytokines that exhibit a variety of immunomodulatory, antiproliferative, and antiviral effects. Ribavirin is a purine nucleoside that has antiviral effects against hepatitis C virus only when combined with IFN-α. Depending on the viral genotypes, these regimens yielded eradication of the infection in a fraction of the treated individuals that could vary between 40-50% in genotype 1 (HCV-1) and 80% in genotype 2 and 3 HCV infections (HCV-2 and HCV-3, respectively).

PEG-IFN/RBV regimens not only are poorly tolerated and contraindicated in a high number of patients, but their effectiveness in eradicating the infection is limited to a fraction of the individuals who are eligible for therapy. Therefore, there has been a tremendous pressure to develop more effective and tolerated treatments. The development of efficient cell culture systems for HCV (Lohmann et al., 1999; Wakita et al., 2005) has led to the identification of several novel therapeutic targets (Fig. 10.) and the subsequent development of hepatitis C virus specific antiviral compounds.

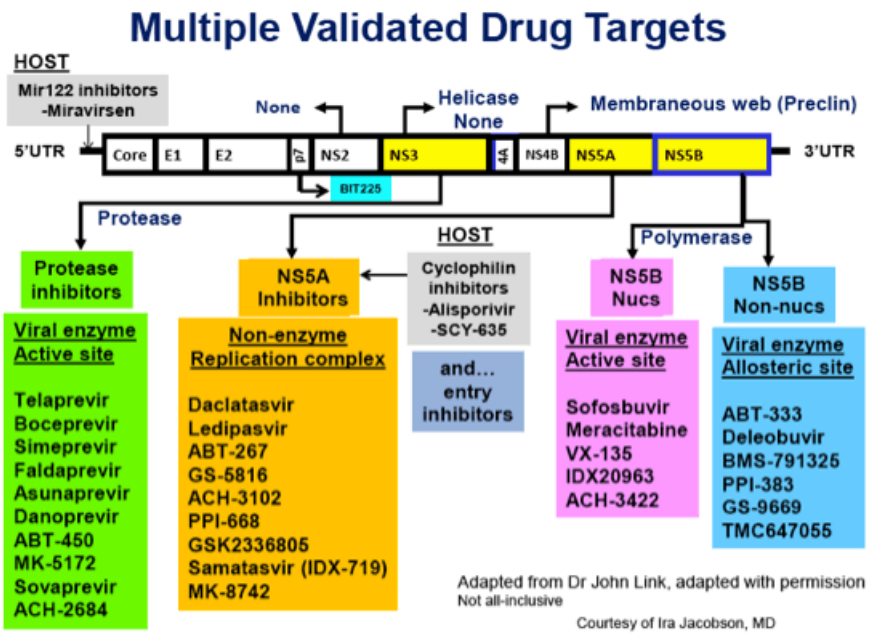

Fig. 10 Hepatitis C virus (HCV) genome and potential drug discovery targets. All the HCV proteins are potential drug discovery targets.

4.1 The era of Direct-Acting Antivirals

More recently, new direct-acting antiviral agents (DAAs) have been developed that target essential components of the HCV life cycle. A new standard-of-care treatment has been approved in 2011 for patients infected with HCV genotype 1, based on a triple combination of pegylated IFN-α, ribavirin, and either **telaprevir** (Forestier and Zeuzem 2012) or **boceprevir (**Chang et al. 2012), two NS3/4A oral protease inhibitors (Pawtosky et al., 2013).

The triple combination increase the chance of cure for naïve patients by 30% (Poordad et al., 2011; Jacobson et al., 2011). The

benefit is even greater in treatment-experienced patients: the chance of cure increases by 50%–60% for relapsers, 40%–45% for partial responders, and 25% for null responders (Bacon et al., 2011; Zeuzem et al., 2011). Therefore the ribavirin dose can be reduced for noncirrhotic patients without impairing SVR, even at early stages of treatment when HCV RNA is still detectable (Sulkowski et al., 2012; Poordad et al., 2012). However, these DAA-containing regimens still exhibit severe limitations: (1) they increase the spectrum of serious side effects associated with anti-HCV therapy; (2) they are associated with a huge pill-burden and complex dosing schedule; (3) they are limited to genotype 1 infections and for the 50–70 million people infected with other HCV genotypes, the combination of pegylated IFN-α with ribavirin (PR) remains the current standard-of-care (SOC) treatment (Wartelle et al.., 2012); (4) selection of DAA-resistant viral variants does occur in patients who respond poorly to the PEGIFN/RBV component of the triple therapy (Pawlorsky et al., 2013). The development of more effective treatments for non-GT-1 patients is essential, especially for GT-4 patients for whom SVR rates are relatively low with PR (see Wendt et al., 2014 for a review).

In order to overcome these limitations, a number of other DAAs are being developed with the objective to develop therapeutic regimens that are more efficacious and convenient, better tolerated, active on all viral genotypes, and with a negligible likelihood to develop viral resistance. These are primarily targeted at the NS3/4A protease, NS5A protein, or NS5B RNA-dependent RNA polymerase. Additionally, other, less-studied viral proteins, such as the ion channel formed by p7 or the

replication complex protein NS4B have been recently demonstrated too be “druggable”, providing potential new targets for pharmacological intervention (Moradpour and Penin, 2013 and Delang et al., 2013).

Second wave and Second-generation PIs The main weaknesses of the first-generation PIs are their low genetic barrier to resistance and the fact that their effectiveness is limited to GT-1 patients. A number of so-called second-wave NS3/4A protease inhibitors are currently being studied in Phase II or III clinical trials. They include: linear noncovalent inhibitors **faldaprevir/BI 201335** (White et al. 2010), **asunaprevir/BMS-650032** (McPhee et al. 2012b), **sovaprevir/ACH-1625** (Agarwal et al. 2012), and **GS-9451** (Sheng et al. 2012a); P3-P1 macrocyclic inhibitors **simeprevir/TMC435** (Lin et al. 2009), **danoprevir/RG7227/ITMN-191** (Seiwert et al. 2008), **ABT-450** (Wagaw et al. 2009), and **GS-9256** (Sheng et al. 2012b); and P4-P2 macrocyclic inhibitor **vaniprevir/ MK-7009** (Liverton et al. 2010). These agents are characterized by very potent activity on HCV genotype 1 (typically, low-nM EC_{50} in the replicon system) as well as antiviral efficacy on HCV genotype 1 patients similar to that of boceprevir or telaprevir (Lee et al. 2012; Sarrazin et al. 2012; Schaefer and Chung 2012). They differ from their first-wave counterpart in that they do not have the chemical reactivity required to make a covalent bond to their target and are therefore expected to display fewer- and less severe side effects. In addition, these agents have substantially improved pharmacokinetic profiles, which allows for less frequent dosing, typically once a day. Although second wave-first generation NS3/4A HCV protease

inhibitors tend to have a significantly broader spectrum of action on the different HCV genotypes compared to the inhibitor of the first wave, these agents cannot be considered pangenotypic antivirals since they do not exhibit the same potency across all viral genotypes Second-generation NS3/4A protease inhibitors are defined as agents that (1) pose a high barrier to the development of viral resistance, (2) retain activity against the viral variants that are resistant to first-generation compounds, and (3) are active across the different genotypes, including HCV genotype 3. Besides, the safety profile seems to be good. The pan-genotypic activity of this new treatment provides new therapeutic options for a greater number of patients, in particular for those infected with GT-4 (Bronowicki et al., 2012; Moreno et al., 2010; Hezode et al., 2012). Second-generation PIs are in early stage of development. **MK-5172** in monotherapy demonstrates pan-genotypic activity (Brainard et al., 2010); other molecules such as **ACH-2684**, **AVL-181**, and **AVL-192** show promising results in vitro (Bourliere et al., 2011).

Of note, Simeprevir and Faldaprevir have been very recently approved to the market for the treatment of genotype 1 or genotype 1b chronic hepatitis C, respectively, in combination with PEG-IFN and ribavirin.

NS5B polymerase-inhibitors (NS5B.I) This class of inhibitors classified in NS5B nucleoside/nucleotide inhibitors (NIs) and Non-nucleotide inhibitors (NNIs). HCV-specific nucleoside inhibitors (NI)mimic natural polymerase substrates and bind to NS5B active sites, thereby causing chain termination and increasing the number of errors

when incorporated into a growing RNA chain. As these NS5B sites are well conserved among genotypes, NIs tend to have activity across all genotypes (Membreno et al., 2011). They also have the highest barrier to resistance of all DAAs so far.

The first NI developed was the nucleoside analog **Mericitabine** (Stuyver et al. 2006), which shows less antiviral activity for GT-1 patients in triple regimen with PR compared to triple regimen with PIs (Wedemeyer et al., 2012; Pockros et al., 2012). However, it has high antiviral activity for GT-2 and GT-3 treatment-experienced patients and for GT-4 patients (Gane et al., 2010).

Nucleotide polymerase inhibitors are liver-targeted prodrugs designed to enhance formation of its active triphosphate in the liver, while minimizing systemic exposure of the nucleotide drug and its nucleoside metabolite. Such prodrugs of nucleotide analogs (i.e., nucleoside 5'-monophosphates) are preferentially cleaved by hepatic enzymes, thereby efficiently releasing their nucleoside monophosphate in liver cells. The rate-limiting step for metabolic activation of nucleoside analogs, the initial phosphorylation to a nucleoside monophosphate, is bypassed, resulting in higher levels of nucleoside triphosphates in the cell.

Nucleotide polymerase inhibitors, **Sofosbuvir/GS-7977** (Murakami et al., 2008), **BMS-986094** (McGUigan et al., 2010), **IDX184** (Zhou et al., 2011), **GS-938** (Lam et al., 2011b) are liver-targeted prodrugs designed to enhance formation of its active triphosphate in the liver, while minimizing systemic exposure of the nucleotide drug and its nucleoside metabolite. The pyrimidine nucleotide analog Sofosbuvir is

currently the most advanced NS5B polymerase inhibitor in clinical development (Phase III). It has highly potent pan-genotypic antiviral activity and a high genetic barrier to resistance. Phase II clinical studies revealed that addition of sofosbuvir to PEG-IFN and RBV for 12 weeks resulted in SVR rates in excess of 90 % for naïve genotype 1 HCV infected patients (Kowdley et al. 2012b). Furthermore, IFN-free, all-oral sofosbuvir/RBV demonstrated consistent antiviral suppression across HCV genotypes: sofosbuvir achieves SVR rates from 84% to 100% for GT-1, GT-2, and GT-3 naïve patients (Gane et al. 2012b). Viral resistance was hardly observed in any clinical studies using sofosbuvir, confirming the high genetic barrier to resistance

Importantly, Sofosbuvir was very recently approved in combination with ribavirin for the treatment of chronic infection with genotype 3 HCV, and in triple combination with PEG-IFN and ribavirin for patients with genotype 1 HCV infection. Moreover, Sofosbuvir is currently studied in IFN-free combinations with a number of other DAAs, including NS3/4A protease inhibitors (GS-938, simeprevir) and NS5A inhibitor (daclatasvir, GS-5885), with or without ribavirin, in order to identify an all-oral therapy suitable for all viral genotypes.

Non-nucleoside inhibitors (NNIs), such as **Lomibuvir/VX-222** (Rodriguez-Torres et al. 2010), **Setrobuvir/RG7790** (Thompson et al., 2009), **HCV-796** (Kneteman et al. 2009), **Tegobuvir/GS-9190** (Zeuzem et al. 2012b) are noncompetitive with NTP substrates and inhibit the polymerase at a stage preceding the elongation reaction (Gu et al. 2003; Tomei et al. 2003; Tomei et al. 2004). They have a restricted spectrum of activity against the various genotypes, and a low barrier to

resistance. They are active against GT-1, with less antiviral activity for GT-1a infected patients than for GT-1b infected patients. They appear to have less antiviral activity compared to triple regimen with PIs or NS5A.I. Nevertheless, the fact that they target five different sites of the NS5B-polyprotein suggests that combinations with other NNIs or with different DAAs (PIs or NS5A.I) could be effective.

HCV p7 inhibitors (p7.I). A number of HCV p7 inhibitors have been identified, such as **amantadine**, **rimantadine, long-alkylated iminosugasr** and amiloride derivatives. The *in vitro* sensitivity to HCV to these drugs is highly genotype dependent, presumably because of the high sequence variability associated with the p7 genetic region (Aghemo and De Francesco, 2013).

NS4B Inhibitors (NS4B.I). Several classes of NS4B inhibitors have been recently identified (Rai and Deval, 2011). **Clemizole**, a first generation antihistamine, inhibits NS4B RNA-binding thereby preventing HCV RNA replication (Einav et al., 2008). Preliminary data reveal that clemizole, while inactive as a single agent, when combined with PEG-IFN and RBV may result in a more efficacious reduction in viral load than PEG-IFN/RBV alone (Choong et al., 2010). Unexpectedly, very recent data point to NS4B as a candidate target for the anti-HCV action of silibinin (SIL) (Esser-Nobis et al., 2013). SIL is an intravenous drug that has recently been administered to HCV(+) liver transplant recipients, leading in some instances to eradication of the infection. In cell culture, SIL potently inhibits HCV RNA replication for genotype 1a and genotype 1b, but not for genotype 2a. These new exciting data point to the possibility that an already approved agent

may be added to the growing armamentarium of HCV DAAs. Unfortunately, the intravenous mode of administration will ultimately limit the use of SIL in all-oral DAA combinations.

NS5A-replication complex inhibitors (NS5A.I) Not being associated to any measurable enzymatic activity, NS5A has been considered "not druggable" for a very long time. In recent years, however, compound acting on NS5A have emerged as efficacious inhibitors of HCV replication, with specific examples displaying in vitro anti-HCV activity in the low pM range. The first NS5A inhibitors were discovered by replicon-based high-throughput screening (Conte et al., 2009; Lemm et al., 2010). The initial lead compounds had moderate potency and narrow anti-HCV activity, mainly on genotype 1b. Subsequent medicinal chemistry efforts (Lemm et al., 2011) resulted in the discovery of extremely potent compounds characterized by a very peculiar, highly symmetrical dimeric structure. The most studied of this "palindromic" NS5A inhibitor class is **Daclatasvir (DCV)/BMS-790052** (Gao et al., 2010), a highly optimized NS5A inhibitor. Other NS5A inhibitors in clinical development include **GS-5885**, **ABT-267**, **PPI-461**, **ACH-3102** and **MK-8742**. The latter two are early-stage agents with a higher barrier to resistance and which retain substantial levels of potency against resistance mutations selected by early NS5A inhibitors. These novel agents can thus be viewed as "second-generation" NS5A inhibitors (Belda and Targett-Adams, 2012). Although no NS5A inhibitor has yet been approved for therapeutic use, these agents are viewed with optimism due to their favourable characteristics, including the requirement for low dosing to inhibit HCV

replication; pan-genotypic activity; once-daily dosing; resistance profiles that do not overlap with those of other DAAs in development; and successful suppression of HCV replication with an acceptable safety profile in early clinical trials (Targett-Adams, 2012).

A less characterized series of compounds, belonging to a different chemical class, was also initially indicated to target NS5A on the basis of the mutation pattern observed in resistant replicons (Schmitz et al., 2008). The common structural element of this class of inhibitors is a 4-anilino quinazoline core. However, for these agents, the mutations reported to be associated with resistance were found to be different from those expected for the canonical NS5A inhibitors and reverse genetics studies did not recapitulate the resistant phenotype observed in the original cellular clones (Najarro et al., 2011). Surprisingly, I contribuited to demonstrate that AL-9, a member of this class of compounds, clearly targets the phosphatidylinositol 4-kinase IIIα (PI4KIIIα) (See Chapter II).

4.2 Daclatasvir: the first in class of NS5A Replication complex inhibitors

Daclatasvir (DCV) was initially discovered by means of a chemical genetic strategy (Gao et al., 2010; Polet al., 2012). In this strategy, a large number of chemically diverse compounds are first screened based on their effect on HCV replication, without any knowledge of their mechanism of action. Then, an error-prone HCV RNA-dependent

RNA polymerase (RDRP) generates mutant HCV genomes, which are resistant to identified compounds. Analysis of these resistant mutant genomes leads to the identification of their potential viral targets. Following successful screening, a number of compounds with a thiazolidinone core were identified as inhibitors of HCV replication. Among them, BMS-824 demonstrated a half-maximum effective concentration (EC50) of 5 nM and a half-maximum cytotoxic concentration of more than 50 µM, with a therapeutic index of more than 10,000 in the GT 1b replicon (Lemm et al., 2010). However, BMS-824 showed a relatively poor activity against the GT 1a replicon (10 µM). After realizing symmetry as an important contributor to their antiviral activity in GT 1a, BMS-790052 (a previous name of DCV), with a symmetric structure (Fig. 11) and greater potency against both the GT 1a and 1b replicons, was identified and developed as a candidate for advancement into clinical trial (Lemm et al., 2011). DCV has been the most potent HCV replication inhibitor reported so far, with a picomolar EC50 towards the GT 1 replicons (50 pM against GT 1a, 9 pM against GT 1b), which represent the majority of HCV patients. In addition, DCV also turned out to be an inhibitor of the GT 2a JFH1 replicon and cell culture infectious J6/JFH1 virus, with EC_{50} values of 46.8 pM and 16.1 pM, respectively. A study of hybrid HCV replicons harboring either GT 3a or 4 NS5A genes also confirmed their susceptibilities to DCV, with EC50 values ranging from 120−870 pM for GT 3a and from 7−13 pM for GT 4 (Wang et al., 2012, 2013).

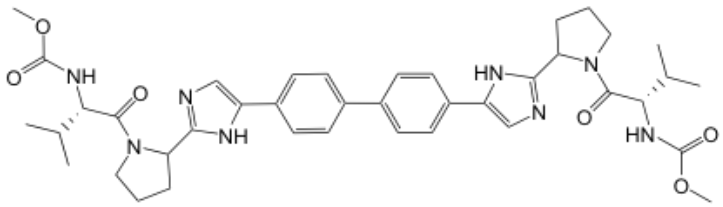

Fig. 11 Chemical structure of Daclatasvir. *From Lemm et al., 2011.*

Clinically,Daclatasvir is an oral, once-daily, highly selective NS5A inhibitor, currently in Phase III clinical trials. Inhibition of NS5A has been associated with steep reductions in HCV RNA in monotherapy (Gao et al., 2010) and enhanced SVR rates when combined with PEG-IFN and RBV (Pol et al., 2012). When patients with chronic HCV genotype 1 infection were administered single doses of 1, 10 and 100 mg of Daclatasvir, viral load dropped by up to 3.6 $\log_{10}$ IU/ml. Strikingly, the drop in viremia observed at the highest dose was sustained for 144 hours post-dose in two patients infected with genotype 1b virus (Gao et al., 2010). In spite of the strikingly antiviral activity, the genetic barrier to resistance for this drug is low, and resistant variants are selected readily *in vitro* as well as in patients, with the more relevant substitutions found at NS5A residues 28, 30, 31, and 93 for genotype 1a and residues 31 and 93 for genotype 1b (Fridell et al., 2011). A recent phase II trial highlighted the efficacy of Daclatasvir in combination with PEG-IFN/RBV. All subjects received 48 weeks of triple-therapy. SVR rates for 10 and 60 mg doses were 83%, whereas the standard therapy only achieved an SVR of 25% (Pol et al.,

2012). Quadruple therapy with Daclatasvir in association with the PI asunaprevir and PR for 24 weeks results in SVR rates of more than 90% for GT-1 null responders (who are the most difficult to treat) (Lok et al., 2012a, 2012b). However, this approach is feasible only for patients who can tolerate PEG-INF and ribavirin. For cirrhotic patients, response rates are likely to be lower, while adverse event rates are probably higher. Several INF-free regimens with Daclatasvir have been studied. Dual therapy with Daclatasvir and Asunaprevir for 24 weeks achieves SVR for 65%–78% of GT-1b patients who are previous null responders (Lok et al., 2012b). Dual or triple therapy with daclatasvir or sofosbuvir (with or without ribavirin) for 24 weeks achieves SVR for 93%–100% of naïve GT-1 patients and for 83%–100% of naïve GT-2/3 patients (Everson et al., 2012; Sulkowski et al., 2012).

It should be pointed out that the precise mechanism of action of DCV and in general of NS5A-inhibitors is not completely understood. They were initially claimed to be NS5A inhibitors mainly based on the selection of specific resistant mutations that mapped in NS5A Domain I (Conte et al. 2009; Lemm et al. 2010). In particular, changes corresponding to variants of NS5A Tyr93 (Y93H/C/N) were found by different groups to be most common mutations conferring broad resistance to this class of antivirals. Interestingly, Tyr93 is found near the protein dimer interface, leading to speculate that NS5A inhibitors might act by modulating NS5A monomer/dimer equilibrium (Conte et al. 2009). However, the palindromic topology of Daclatasvir and related compounds rather suggests a binding interaction with NS5A in which the inhibitor interacts across the dimer interface, making

simultaneous contacts to both protein monomers (Belda and Targett-Adams 2012). This could at least partly explain the extraordinary potency observed for the palindromic inhibitor series. Among the reported properties of NS5A inhibitors are the ability to inhibit NS5A hyperphosphorylation (Lemm et al. 2010). Phosphorylation of NS5A seems required for viral production (Masaki et al., 2008), but the relative roles of the phosphorylated and hyperphosphorylated forms are unclear, and conflicting results have been reported suggesting that reduced hyperphosphorylation may either enhance or reduce replication (Evans et al., 2004; Quintavalle et al., 2007). It is thought that a tightly regulated control of phosphorylation *vs.* hyperphosphorylation is required for efficient viral function. It was also shown that NS5A acts in two different pathways in RNA replication, and one of them likely requires hyperphosphorylation (Fridell et al., 2011). However, other mechanisms may also play a role. For instance, NS5A inhibitors alter the subcellular localization of NS5A, which may cause faulty viral assembly and to cause the redistribution of NS5A from the HCV membranous web to different subcellular localizations, thus impeding the formation of new replication complexes (Qiu et al. 2011; Lee et al., 2011; Targett-Adams et al. 2011). For this effect, NS5A inhibitors are classified as replication complex inhibitors (Belda and Targett-Adamns, 2012).

4.3 Host targeting agents (HTA) and other antiviral treatments

The viral replication cycle has three stages: entry, replication, and assembly/release (Moradpour et al., 2007). All stages depend on various host encoded factors (Bode et al., 2009). Targeting these host factors is an attractive option for future HCV therapies. The intention is to minimize development of HCV resistance and to enable efficient treatment of all HCV genotypes. This approach has been pursued successfully in the HIV field, where Maraviroc (Selzentry, Celsentri), a small molecule compound that binds to the essential HIV co-receptor chemokine receptor type 5 (CCR5) and prevents its interaction with HIV, is now in clinical use (Gilliamo et al., 2011).

By the use of siRNA-based and other screening approaches, several dozens of host factors involved in or, in some cases, even essential for HCV RNA replication have been identified in recent years.

A representative factor that generated a lot of interest is cyclophilin A (CypA), a cellular cis-trans-prolyl isomerase that is required for HCV RNA replication and probably also assembly (Watashi et al., 2005; Yang et al., 2008). It has been shown *in vitro* that CypA binds to HCV NS5A and can facilitate replication through an unknown mechanism. Moreover, it has been shown that HCV is much more sensitive to CypA inhibitors in the presence of an intact NS2/3 junction, indicating that important interactions between HCV and CypA may occur outside NS5A (Kaul et al., 2009). Importantly, CypA seems to be important for all HCV genotypes. Interestingly, the first compound exerting an inhibitory effect on HCV replication by targeting CypA was

the immunosuppressant cyclosporine A (Watashi et al., 2005). Subsequently, cyclosporine A derivatives without immunosuppressive properties were developed as potential antivirals. The CypA inhibitor Alisporivir (ALV), formerly known as Debio-025259, is a nonimmunsuppressive form of cyclophilin inhibitor that shows a potent antiviral activity for many HCV genotypes, low resistance rates without cross-resistance to PI, NS5B.I, and NS5A.I. Phase II studies show increased SVR rates compared to PR for naïve G1 patients receiving a combination of ALV and PR for at least 24 weeks. For GT-1 treatment-experienced patients, ALV + PR demonstrated higher complete early virological response compared to PR in relapsers, partial responders, and null responders (Alberti et al., 2012). Moreover, for GT-2/3 naïve patients, the possibility of an INF-free regimen has been studied (Pawlostsky et al., 2012). Among patients treated with only ALV or with RBV in combination, 22%–34% reach rapid virological response (RVR) and undergo 24 weeks of INF-free treatment. SVR in this subgroup varies from 82% to 91%. These results demonstrate the ability of such a regimen to cure these patients.57 In the Phase II program, ALV appears to have a good safety profile, with rare cases of hyperbilirubinemia. However, during the Phase III program in April 2012, the US Food and Drug Administration (FDA) placed the ALV program on a full clinical hold and, all patients discontinued therapy. This was due to a cluster of three cases of acute pancreatitis, one of them fatal, in the treatment group receiving ALV and PR. Two other cyclophilin inhibitors are currently in development (SCY-635, NIM-811). Cyclophilin inhibitors could be an important

addition to DAAs in treatment combinations; they may be able to shorten and simplify therapy. They have the potential to be a cornerstone drug for an IFN-free regimen in combination with DAAs, because they have a high barrier to resistance, no cross-resistance with DAAs, and a pan-genotypic activity. ALV development is likely to move forward, but will not be used in association with PEG-INF because of the potential risk of acute pancreatitis.

Another host factor critical for HCV genome replication has recently been described by several groups: the enzyme phosphatidyinositol-4-kinase III alpha (PI4KIIIα) binds to HCV NS5A and its enzymatic activity is required for efficient HCV RNA replication (Berger et al., 2009; Tai et al., 2009; Trotard et al., 2009). Recent data suggest that the direct activation of this lipid kinase by HCV NS5A contributes critically to the integrity of the membranous viral replication complex. Whether this kinase is also a suitable antiviral target for the treatment of HCV and if it is possible to design a potent inhibitor with tolerable side effects *in vivo* remain to be determined. Anyway, we described AL-9 as a HCV inhibitor which targets PI4KIIIα (Chapter 2). Moreover , Leivers et al., 2013 identified, in preliminary studies, a quinazolinone that displays high selectivity for PI4KIIIα and potently inhibits HCV replication *in vitro* .

An unusual and scientifically intriguing host encoded antiviral target is the microRNA 122 (miR-122) (Jopling et al., 2005). miR-122 is an abundant liver-specific miRNA which is crucial for efficient HCV RNA replication in cultured Huh7 cells stably expressing HCV replicons. It stimulates HCV RNA replication and translation through interaction

with two adjacent sites downstream of stem loop I within the HCV 5' untranslated region. Moreover, a recent study found that among chronically HCV-infected individuals pre-treatment intrahepatic miR-122 levels were significantly lower among patients who responded poorly to interferon therapy. Santaris Pharma has developed a locked nucleic acid-modified oligonucleotide (miravirsen or SPC3649) complementary to the 5'-end of miR-122 that resulted in functional inactivation of miRNA-122. Miravirsen was shown to be active in HCV positive chimpanzees, markedly reducing HCV RNA replication and showing no significant side effects except for a profound decrease in serum cholesterol levels (Lanford et al., 2011). Moreover, miravirsen-induced miR-122 antagonism had a potent antiviral effect against HCV genotypes 1-6 *in vitro* (Li et al., 2011).Thus miravirsen holds promise as a new antiviral therapy with a high barrier to resistance and a tolerable side effect profile. Moreover, functional inactivation of a miRNA to treat an infectious disease represents a truly novel therapeutic paradigm far beyond the HCV or virology field. A phase II trial of miravirsen in chronically HCV infected individuals is currently recruiting patients.

Additionally, HCV RNA replication seems to be intricately linked to the cholesterol and fatty acid biosynthesis pathways. Statins, a widely used group of drugs that target cholesterol metabolism by inhibition of 3-hydroxyl-3-methylglutary coenzyme A (HMG CoA) reductase, have been reported to inhibit HCV RNA replication *in vitro*, albeit the exact mechanism is still under investigation (Ikeda et al., 2006). Interestingly, the addition of fluvastatin to PEG-IFN /RBV has been reported to

improve rapid viral response rates (RVR) but not SVR in HIV/HCV genotype 1 co-infected patients and SVR rates in diabetic patients with chronic hepatitis C261. However, in another study atorvastatin showed no effect on HCV RNA and statins are currently not considered part of the standard of care (O'Leary et al., 2007).

Therapeutic vaccines fail to show any significant positive results yet. GI-5005 is not able to increase SVR compared to PR alone, except in a subgroup of difficult-to-treat patients. Other vaccines, such as ChronVac-C, IC41, and TG-4040, demonstrate limited antiviral activity (Wedemeyer et al., 2012). Polyclonal and monoclonal antibodies do not reach any endpoint in trials evaluating recurrence after liver transplantation. Toll-like receptors may enhance the host immune system against HCV; they are currently in the early development phase.

Silibinin is a flavonoid compound targeting the host cell. It is a direct inhibitor of HCV RNA-dependent RNA polymerase and a blocker of virus entry and transmission. It was recently proved to be successful in the treatment of previous PR nonresponders (Biermer et al., 2012).

Peg interferon lambda has the same antiviral efficacy as PEG-INF alpha, but significantly reduces side effects for GT-1 and GT-4 patients (even if they are cirrhotic) (Muir et al., 2012). It may become a new alternative in combination with DAAs.

5 Still unmet clinical needs in Hepatitis C: diagnostic tools, biomarkers of disease progression, new therapies

Liver cirrhosis and its sequelae are the most unwanted consequences of chronic HCV infection. Apart from virus eradication, a very major clinical goal for HCV is to prevent or to follow closely the transition from fibrosis to cirrhosis. The progression rate from fibrosis to cirrhosis, varies widely among HCV patients going from few years to several decades and it is often unpredictable (Missiha et al., 2008). While in some cases the rate of HCV disease progression is clearly influenced by a number of external factors such as age at infection, gender, immune status and alcohol consumption, obesity, and liver co-infections, in most cases, there are no clinical markers available to assess the risk of developing progressive liver disease and liver cancer in individual subjects (Feld et al., 2006). Moreover, the liver is an organ that can often compensate very well for the lost of a sizeable fraction of hepatic tissue. Indeed, it is not infrequent that cirrhosis is diagnosed only at very advanced stages as patients live acceptably well with a liver that has only 20-30% of its functionality. Understanding what are the patients at risk of developing a faster progressive liver disease would have great advantages. From the clinical point of view, antiviral therapy would be the most beneficial in patients at higher risk of developing progressive liver disease and would therefore be given as soon as possible. Furthermore, the gold standard for assessing hepatic fibrosis, that is liver biopsy, is invasive, subject to sampling errors, and has rare but occurring potentially life threatening complications, thus limiting its acceptability in patients with mild or moderate disease.

There is therefore the medical need to develop non-invasive and reliable serum markers that accurately reflect hepatic fibrotic and cirrhotic disease. Moreover, effective surveillance of patients at high risk of developing HCC (i.e., patients with cirrhosis) could potentially decrease HCC-related mortality rate, and the availability of early and reliable serological markers of HCC remains a huge unmet medical need. Finally, from the basic research point of view, the study of how host and viral factors could affect disease progression may shed light on fundamental pathogenic mechanisms in the path from fibrosis to cirrhosis to cancer, which still remain poorly defined.

Another important need in the Hepatitis C field regards the development of an IFN-free method of achieving SVR. Currently, for the 50–70 million people infected with other HCV genotypes, the combination of pegylated IFN-α with ribavirin (PR) remains the current standard-of-care treatment. Almost all patients treated with PEG-IFNα and RBV experience one or more adverse events during the course of therapy. Adverse events are a major reason that patients decline or stop therapy altogether. The most common adverse events in the registration trials of PEG-IFNα alfa-2a and 2b plus ribavirin were influenza-like side effects such as fatigue, headache, fever and rigors, which occurred in more than half of the patients, and psychiatric side effects (depression, irritability, and insomnia), which occurred in 22% to 31% of patients (Ghany et al., 2009). Currently, several companies are exploring different anti-HCV all-oral, interferon-free regimens by combining different classes of direct-acting antivirals. Some of these combinations are proving highly effective in treating chronic hepatitis

C and they promise to revolutionize the treatment of chronic infection with hepatitis C virus.

C. SCOPE OF THE THESIS

At the moment, several companies are studying the clinical potential of different all-oral combinations of direct-acting antivirals in ongoing studies. The most promising interferon-free combination therapies that are on the horizon include linear or cyclic NS3/4A protease inhibitors, nucleoside as well as non-nucleoside NS5B polymerase inhibitors , and NS5A inhibitors. DAAs that target NS3/4A (protease) and NS5B (RNA-dependent RNA polymerase) inhibit the enzymatic activity of these proteins. NS5A replication complex inhibitors will likely form a component of future interferon-free drug regimens but despite their remarkable potential to treat chronic hepatitis C, the detailed mechanism of action for this class of drug remains unclear.

The goal of my work was to investigate the mechanism of action of different classes of antiviral agents believed to target the NS5A protein in the replication complex in order to improve the possibility to translate basic knowledge to a more meaningful clinical application. More specifically I focused my research on two classes of compounds, characterized by distinct resistance patterns in NS5A: a first class – with examples at the final stages of clinical development, represented by Daclatasvir (Lemm et al., 2011), and a second class - at earlier stages of development - represented by anilino-quinazolines such as A-831/AZD-2836 (Quinkert et al., 2008).

I contributed to demonstrate that both of these inhibitor classes, by binding respectively to either HCV NS5A or to an NS5A-associated protein, PI4KIIIα, eventually interfere with the accumulation of PI4P

and cholesterol in the HCV replication membranous compartment, thus abrogating the ability of the virus form to replicate its RNA genome.

CHAPTER II: **Metabolism of Phosphatidylinositol 4-Kinase IIIα-Dependent PI4P is Subverted by HCV and Is Targeted by a 4-Amino Quinazoline with Antiviral Activity.**

A class of compounds with a 4-anilino quinazoline core structure, having a potent *in vitro* anti-HCV activity in the replicon system and good pharmacokinetic properties, was initially proposed to target NS5A on the basis of the mutation pattern observed in resistant replicons (Schmitz et al., 2008). Reverse genetics studies however, did not recapitulate the resistant phenotype observed in the original cellular clones (Quinkert et al.,2008), leaving thus the possibility open that these compounds act through a different viral or cellular target. Several small-interfering RNA (siRNA) screening campaigns have identified type III α phosphatidylinositol 4-kinase (PI4IIIKα) as crucial host factor for HCV replication. In particular it was shown that its catalytic activity is required to rescue HCV replication in cells with a stable knock-down of PI4KIIIα. In addition, it has been proposed that NS5A stimulates PI4KIIIα activity by direct interaction via domain I (Berger et al., 2011; Lim et al., 2011; Reiss et al., 2011). For all these observations we considered this kinase a potential alternative candidate target for 4-anilino quinazoline inhibitors of HCV replication. We clearly demonstrated that AL-9, a member of this

class of compounds, directly inhibits PI4IIIKα. Therefore, PI4IIIKα is a suitable antiviral target for the treatment of HCV. Our work paved the way to the search of potent and selective PI4KIIIα inhibitors as anti-HCV agents (Leivers et al., 2013).

CHAPTER III: **NS5A inhibitors interfere with NS5A- PI4KIIIα complex formation and cause a concomitant decrease of PI4P and cholesterol in the HCV membranous web.**

The HCV NS5A protein is an established and clinically validated antiviral target (Nettles et al., 2008; Gao et al., 2008, 2010). Without having an enzymatic function, NS5A is essential for replication and assembly of HCV. It associates with PI4KIIIα, and stimulates its enzymatic activity in order to generate PI4P at the site of replication (Reiss et al., 2011; Berger et al., 2011; Tai et al., 2011).

NS5A inhibitors are currently the most potent DAA that target HCV. Different chemotypes are classified as NS5A inhibitors due to their profile of resistance mutations and sensibility of the various HCV genotypes (Gao et al., 2013), however their mode of action is still enigmatic.

My PhD work demonstrated for the first time that NS5A inhibitors both destabilize the NS5A-PI4KIIIα complex and cause a concomitant reduction of the product of PI4KIIIα, PI4P, and cholesterol, two major required components of the HCV replication structures. I proposed that destabilization of the NS5A - PI4KIIIα complex can be considered a mechanism of action of this class of HCV inhibitors and that the

decrease of PI4P together with cholesterol are consequences of the inhibition of NS5A - PI4KIIIα complex function.

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CHAPTER II

Metabolism of Phosphatidylinositol 4-Kinase IIIα-Dependent PI4P is Subverted by HCV and Is Targeted by a 4-Anilino Quinazoline with Antiviral Activity

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A. Abstract

4-anilino quinazolines have been identified as inhibitors of HCV replication. The target of this class of compounds was proposed to be the viral protein NS5A, although unequivocal proof has never been presented. A 4-anilino quinazoline moiety is often found in kinase inhibitors, leading us to formulate the hypothesis that the anti-HCV activity displayed by these compounds might be due to inhibition of a cellular kinase. Type III phosphatidylinositol 4-kinase α (PI4KIIIα) has recently been identified as a host factor for HCV replication. We therefore evaluated AL-9, a compound prototypical of the 4-anilino quinazoline class, on selected phosphatidylinositol kinases. AL-9 inhibited purified PI4KIIIα and, to a lesser extent, PI4KIIIβ. In Huh7.5 cells, PI4KIIIα is responsible for the phosphatidylinositol-4 phosphate (PI4P) pool present in the plasma membrane. Accordingly, we observed a gradual decrease of PI4P in the plasma membrane upon incubation with AL-9, indicating that this agent inhibits PI4KIIIα also in living cells. Conversely, AL-9 did not affect the level of PI4P in the Golgi membrane, suggesting that the PI4KIIIβ isoform was not significantly inhibited under our experimental conditions. Incubation of cells expressing HCV proteins with AL9 induced abnormally large clusters of NS5A, a phenomenon previously observed upon silencing PI4KIIIα by RNA interference. In light of our findings, we propose that the antiviral effect of 4-anilino quinazoline compounds is mediated by the inhibition of PI4KIIIα and the consequent depletion of PI4P required for the HCV membranous web. In addition, we noted that HCV has a

profound effect on cellular PI4P distribution, causing significant enrichment of PI4P in the HCV-membranous web and a concomitant depletion of PI4P in the plasma membrane. This observation implies that HCV – by recruiting PI4KIIIα in the RNA replication complex – hijacks PI4P metabolism, ultimately resulting in a markedly altered subcellular distribution of the PI4KIIIα product.

B. Introduction

Hepatitis C virus (HCV) is an enveloped, single-stranded RNA virus classified as member of the Hepacivirus genus within the Flaviviridae family. The 9.6 kb positive-sense RNA genome contains a single open reading frame encoding a polyprotein of about 3,000 amino acids, flanked by highly structured 5' and 3' untranslated (UTR) regions. Following its release into the cytoplasm of the host cell, viral RNA is translated via an internal ribosome entry site (IRES), giving rise to a single polypeptide that is cleaved into 10 different mature protein products: Core, gpE1, gpE2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. HCV RNA replication takes place in the cytoplasm, in association with a virus-induced intracellular membrane structure termed "membranous web", onto which NS proteins assemble to form the so-called RNA replication complexes.

It is estimated that 3% of the world's population are chronically infected by the hepatitis C virus (HCV). Most infections become chronic and over time evolve into chronic hepatitis. The most unwanted

complication of chronic hepatitis is cirrhosis, a massive liver fibrosis, which can lead to liver failure and hepatocellular carcinoma.

Since the discovery of hepatitis C virus (HCV) in the late 1980's much progress has been made in the understanding of the viral life cycle of HCV. Nonetheless, to date no vaccines are available and the current standard of care, involving lengthy treatment with a combination of ribavirin and pegylated interferon-α (PEG-IFN-α), eradicates the infection in half of treated patients. A large effort has been made in the past two decades in order to develop novel anti-HCV therapies with greater efficacy. Two oral direct-acting antiviral agents (DAA) targeting the HCV NS3/4 protease, boceprevir and telaprevir, have recently reached the market and more are being developed {}. While the initial efforts to the discovery of DAA focused almost exclusively on the best characterized HCV enzymes required for viral replication – the NS3/4A protease and the NS5B polymerase – in the past few years the NS5A viral protein has been attracting more and more attention as a target for drug development[1, 2]. NS5A possesses no known enzymatic activity. It is a multifunctional non-structural protein important for viral replication[3-6] as well as viral assembly[7-9]. It is a phosphoprotein consisting of three domains[10]. Domain I is highly conserved and forms a dimeric structure[11, 12], whereas domain II and III are believed to adopt a "natively unfolded" conformation[13, 14].

In recent years, several anti-HCV compounds identified using cell-based replicon screens were indicated to target NS5A based on the analysis of the mutations associated with emergence of resistance in the replicon system[15, 16].

The most studied series of these “NS5A inhibitors” is represented by BMS-790052, an agent that is leading the field, having demonstrated potent antiviral activity in clinical studies. Compounds in this class are characterized by a complex, dimeric or pseudo-dimeric structure and a high molecular weight, when compared with conventional “drug-like” small molecules. Resistance mutations against these compounds emerge readily in Domain I of NS5A[17], with the most recurrent of these changes corresponding to variant of tyrosine at position 93[17]. Although direct interaction with purified NS5A has not been demonstrated, compelling reverse genetic experiments[17] as well as molecular models[15, 18] strongly support the notion that NS5A is the direct target of these compounds.

A less characterized series of compounds, belonging to a different chemical class, was also initially indicated to target NS5A on the basis of the mutation pattern observed in resistant replicons[18]. The common structural element of this latter class of inhibitors is a 4-anilino quinazoline core. A representative member of this class of compounds is A-831/AZD-2836, an experimental antiviral agent that entered clinical trials but was later discontinued due to the lack of adequate exposure. For these agents, the mutations reported to be associated with resistance were found to be different from those expected for the NS5A inhibitor described above, pointing to a different mechanism of action: a few mutations were found at the C-terminal end of NS5A Domain I (E212D, L199F and T200P), whereas most mutations occurred in NS5A Domains II and III (P299L, S370P, V388D, V362A, S390G and S370P). Additional mutations were also found in NS4B (S258T) and

NS5B (S76A)[18]. Reverse genetics studies in which these mutations were reintroduced in the replicon, however, did not recapitulate the resistant phenotype observed in the original cellular clones, leaving thus the possibility open that these compounds act through a different viral or cellular target.

Interestingly, many kinase inhibitors, including some approved antitumoral drugs (gefitinib, lapatinib, erlotinib) are 4-anilino quinazoline derivatives[19-21]. Altogether, these considerations led us to investigate whether the anti-HCV activity displayed by these compounds might be due to inhibition of a cellular kinase.

Recently, several small-interfering RNA (siRNA) screening campaigns have identified type III phosphatidylinositol 4-kinases (PI4K) as crucial host factors for HCV replication. In particular, PI4KIIIα was found to be required for HCV RNA replication in a cell line- and genotype-independent manner, whereas the requirement for the β isoform was observed to be less dramatic and limited to Con-1 (genotype 1b) replicons[22-25]. It was shown that the catalytic activity of PI4KIIIα is required to rescue HCV replication in cells with a stable knock-down of PI4KIIIα. In addition, it has been proposed that NS5A stimulates PI4KIIIα activity by direct interaction via Domain I[26-28]. All these observations taken together made us consider the phosphatidylinositol 4-kinases a potential alternative candidate target for 4-anilino quinazoline inhibitors of HCV replication.

In this paper, we present evidence that AL-9, a member of this class of compounds previously reported to target NS5A, inhibits PI4P formation by direct inhibition of phosphatidylinositol 4-kinase IIIα

(PI4KIIIα). In addition, we provide evidence that pharmacological inhibition of PI4KIIIα with AL-9 results in altered subcellular distribution of NS5A similar to that observed after RNAi knock-down of the PI4KIIIα mRNA, strongly supporting a mechanism of HCV inhibition mediated by the inhibition of PI4KIIIα. Moreover, we show that HCV subverts components of the phosphatidylinositol-4 phosphate (PI4P) pathway to function in favor of its own life cycle, thereby enriching the PI4P concentration in the membranous web while depleting the plasma membrane PI4P pool.

C. Results

1 Compound AL-9 inhibits HCV replication in vitro

AL-9 is a member of 4-anilino quinazoline-containing HCV replication inhibitors described previously ([18]; Fig. 1). In order to confirm its anti-HCV activity, we tested the effect of this compound on HCV replication in Huh7.5 cells stably expressing genotype 1b or 2a subgenomic replicons (Con1-SR and JFH-A4, respectively). The EC50 values, calculated by measuring viral RNA after incubation with AL-9 for three days, are reported in Table 1. Replicon EC50 values for AL-9 were found to be 0.29 µM and 0.75 µM for genotype 1b and 2a, respectively. In order to prove that AL-9 inhibits HCV replication not only in the context of a HCV subgenomic replicon, but also in the context of the complete viral life-cycle, we determined the inhibitory

activity using the J6/JFH-1 HCV virus. In this case, the EC50 value was found to be 1.2 µM, a figure comparable with the result obtained with genotype 2a subgenomic replicon. CC50 values are shown for Con1-SR, JFH-A4 and Huh7.5 cells, respectively. In summary, AL-9 inhibits HCV across different genotypes with activity in the sub-micromolar to low micromolar range in the absence of significant cytotoxic effects.

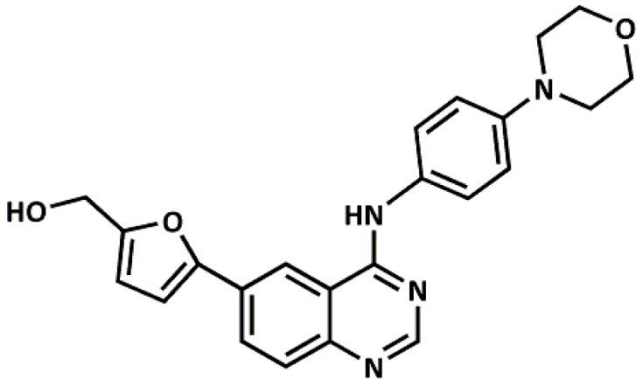

Fig. 1. Chemical structure of AL-9

For the synthetic pathway and procedure see Supporting Information.

	genotype	EC_{50} (µM)	CC_{50} (µM)
Con1-SR	**1b**	**0.29 (+/- 0.09)**	**29.3 (+/- 2.8)**
JFH-A4	**2a**	**0.75 (+/- 0.15)**	**18.9 (+/- 3.2)**
Huh7.5 + J6/JFH-1 HCV	**2a**	**1.2 (+/- 0.37)**	**25.1 (+/- 4.6)***

Table 1. List of EC50 values of AL-9 for different HCV genotypes

Huh7.5 cells replicating subgenomic replicons of genotype 1b or 2a (Con1-SR and JFH-A4, respectively) or Huh7.5 cells infected with the chimeric virus J6/JFH were treated with AL-9 for three days and intracellular viral RNA was measured by real time PCR. The data are representative of at least three independent experiments, and the standard deviations are shown. *CC50 measured in uninfected Huh7.5 cells.

2 AL-9 is an inhibitor of PI4KIIIα

In the following experiment, we investigated whether AL-9 inhibits the purified type III phosphatidylinositol 4-kinases PI4KIIIα and PI4KIIIβ (Fig. 2). Both enzymes were inhibited by AL-9 with a five-fold preference for PI4KIIIα (IC50 of 0.57 µM and 3.08 µM, respectively). This result demonstrates that AL-9 inhibits type III PI4 kinases in vitro at concentrations similar to those required for its anti-HCV activity, displaying a moderate selectivity for the α over the β isoform. We also tested the activity of AL-9 on two class I PI3-kinases (p110α and p110β). While PI3-kinase p110α was inhibited with an IC50 of 1.1 µM, the potency of AL-9 for PI3-kinase p110β was significantly lower (40% inhibition @10 µM, data not shown).

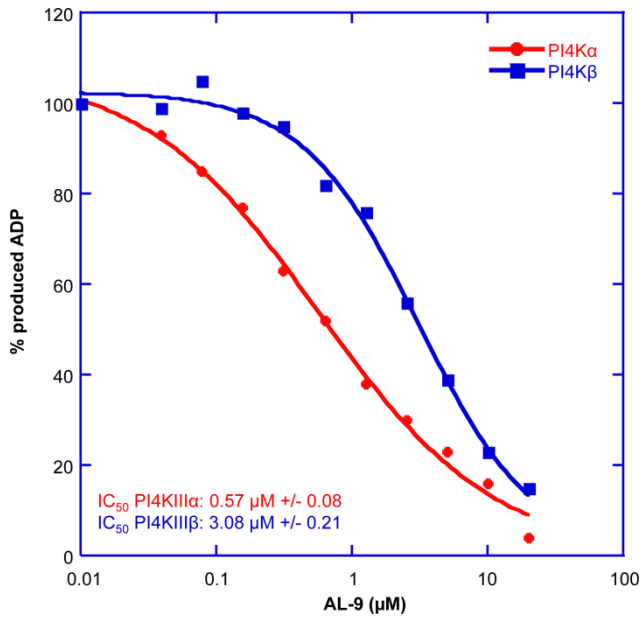

Fig. 2. Inhibitory dose-response curve of AL-9 for purified PI4KIIIα and PI4KIIIβ

The enzymes were preincubated for 10 min with the indicated concentrations of AL-9 or DMSO and the reaction was started by addition of 100 µM ATP and 150 µM PI:PS substrate as described in Materials and Methods. Activity, measured as conversion of ATP to ADP, is expressed as percent of the DMSO control. Shown is a representative experiment of three independent experiments performed in duplicate. IC50 and SD of PI4KIIIα and PI4KIIIβ are indicated.

Our hypothesis is that AL-9 inhibits HCV replication via inhibition of PI4KIIIα. Thus, we wanted to assess whether AL-9 also inhibited PI4KIIIα in living cells. To this aim, we needed to set up an assay that allowed us to monitor the activity of this kinase in intact cells. PI4KIIIα is primarily localized to the ER, whereas PI4KIIIβ is localized to the Golgi membranes[29]. It was shown that PI4KIIIβ contributes to the synthesis of PI4P at the Golgi membranes[30, 31]. Subcellular localization of the enzymes, however, does not always coincide with their function. Thus, PI4KIIIα, considered to be an ER-resident enzyme, has previously been shown to be critical for the generation and maintenance of the plasma membrane PI4P pool during phospholipase C activation and Ca_2 signaling in HEK-293 or Cos-7 cells[31, 32] as well as in resting Cos-7 cells[33]. Whether PI4KIIIα is responsible for the maintenance of the plasma membrane PI4P pool under normal cell culture conditions in hepatoma cells is currently not known. Hammond et al[33] have developed immunocytochemical techniques that enable selective staining of the PI4P pool present in the plasma membrane (plasma membrane staining protocol) or in the intracellular membranes (Golgi staining protocol), respectively. We used this technique, in combination with RNA gene silencing or pharmacological inhibition, to

decipher which of the type III enzymes participates in the synthesis of the Golgi- or plasma membrane PI4P -pools in Huh7.5 hepatoma cells.

To address which type III PI4 kinase is responsible for the synthesis of the different cellular PI4P pools, HuH7.5 cells were treated with siRNAs targeting PI4KIIIα, PI4KIIIβ or an unrelated siRNA (mock-siRNA) as described in the Materials and Methods section. Immunoblots assays show specific knockdown of PI4KIIIα or PI4KIIIβ by their corresponding siRNAs (Fig. 3C). Three days after siRNA treatment, PI4P was revealed either by the plasma membrane staining protocol (Fig 3A, upper panel) or by the Golgi membrane staining protocol (Fig. 3A, lower panel). In cells treated with the unrelated siRNA (mock-siRNA), PI4P was detected both in the plasma membrane and in intracellular membranes. Intracellular PI4P was localized primarily in the Golgi membranes, as judged by the colocalization with the Golgi marker giantin. Silencing of PI4KIIIα resulted in a significant decrease of the PI4P level in the plasma membrane. Concomitantly with the decrease in the plasma membrane PI4P levels, we consistently observed a pronounced increase of PI4P level in the Golgi membrane following PI4KIIIα knockdown. In the case of PI4KIIIβ knockdown, we observed a ~30% decrease of Golgi membrane PI4P level, whereas the PI4P levels of the plasma membrane remained substantially unaffected (Fig. 3B).

These results are in line with the previously reported role for PI4KIIIα in maintaining the PI4P plasma membrane pool[31-33] and confirm the importance of PI4KIIIβ for the synthesis of at least part of the Golgi membrane PI4P[30, 31]. We also observed that decreased expression of PI4KIIIα resulted in an unexpected increase in the level

of the Golgi membrane pool (Fig. 3A), suggesting a complex level of cross-talk between the cellular type III PI4 kinases in maintaining the physiological PI4P levels at the Golgi membrane, at least in our experimental model.

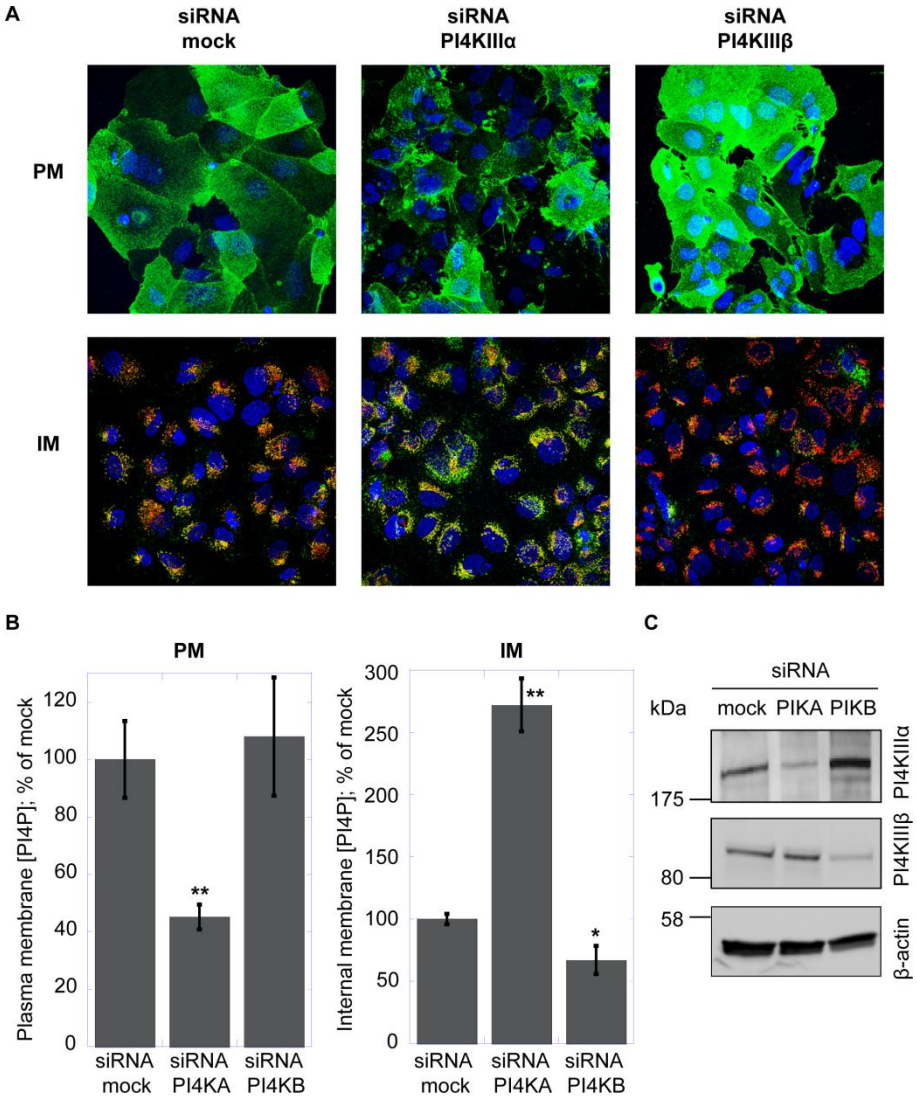

Fig. 3. RNA interference analysis of PI4P production in Huh7.5 cells.

Huh7.5 cells were treated with irrelevant (mock) siRNA or siRNA targeting PI4KIIIα or PI4KIIIβ as detailed in Materials and Methods. The data were collected three days after initial siRNA transfection.

(A) Confocal microscopy images of Huh7.5 cells treated with PI4KIIIα siRNA, PI4KIIIβ siRNA or mock siRNA. Cells were fixed and stained as described in Materials and Methods. PI4P (green) localized to the plasma membrane (PM) was detected using the plasma membrane staining protocol (upper panel) [33]. Nuclei were stained by the Hoechst dye (blue). PI4P in the intracellular membranes (IM) was revealed using the Golgi staining protocol (lower panel). Together with PI4P, Golgi membranes were stained with the Golgi marker giantin (red). Colocalization of PI4P with Golgi membranes results in yellow color.
(B) Quantification of PI4P levels by immunofluorescence analysis. Changes in mean fluorescence intensity relative to the control (mock siRNA) are shown. Four randomly picked fields were analyzed per each condition, as described in Materials and Methods. Data are presented as averages ± SEM. *, $P < 0.05$; **, $P < 0.01$.
(C) Immunoblot analysis of protein expression after RNAi silencing. Lysates prepared from Huh 7.5 cells transfected with irrelevant siRNA (mock), PI4KIIIα siRNA (PIKA) or PI4KIIIβ siRNA (PIKB) were analyzed by immunoblotting with PI4KIIIα, PI4KIIIβ or β-actin antibodies as indicated in the figure. Positions of the protein molecular weight markers are shown on the left side.

In order to confirm and extend the results described above, we utilized a known pharmacological inhibitor of the type III PI4 kinases. PIK93 was previously exploited to distinguish between the roles of the two PI4KIII isoforms[34, 35]. In particular, a concentration of 0.5 μM PIK93 is expected to affect only PI4KIIIβ, whereas 30 μM PIK93 should inhibit both PI4KIIIβ and PI4KIIIα. Thus, Huh7.5 cells were treated with 0.5 μM or 30 μM PIK93 or with DMSO as control. After two hours of incubation, PI4P was revealed either by the plasma membrane staining protocol (Fig 4A, upper panel) or by the Golgi staining protocol (Fig. 4A, lower panel). PI4P levels associated with the Golgi membranes decreased by ~25% after incubation with 0.5 μM PIK93 (Fig. 4B). This is in line with PI4KIIIβ contributing to the production of PI4P present in the Golgi membranes (PI4KIIα, another contributor of Golgi-localized PI4P is not inhibited by PIK93[34, 35]). Increasing PIK93 concentration to 30 μM further increased the inhibition of the intracellular membrane

PI4P pool, to ~65% (Fig. 4B). This could be due to a more complete inhibition of PI4KIIIβ; however, based on this experiment, we cannot rule out a contribution of PI4KIIIα activity to the maintenance of the Golgi membrane PI4P pool. In contrast to what observed in the Golgi-associated membranes, the plasma membrane PI4P level was not significantly affected upon incubation with 0.5 μM PIK93, but decreased by nearly 50% after incubation with 30 μM of PIK93 (Fig. 4B). Combined with the RNAi experiments described above, these results support the notion that, in Huh7.5 cells, PI4KIIIα is involved in the maintenance of the plasma membrane PI4P pool, whereas PI4KIIIβ is at least partly responsible for the maintenance of the Golgi membrane PI4P pool.

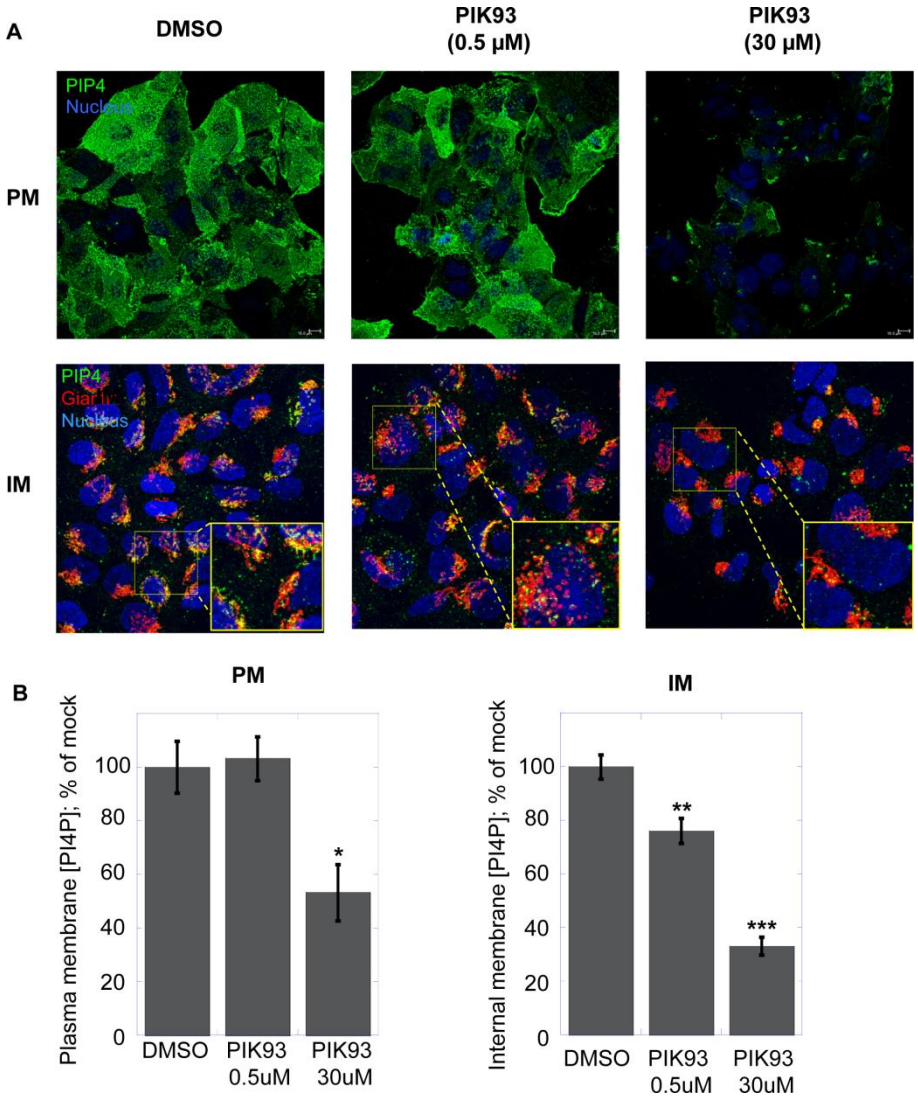

Fig. 4. Effect of PIK93 on Golgi or plasma membrane PI4P in Huh7.5 cells

(A) Confocal microscopy images of Huh7.5 cells incubated with DMSO (left column), 0.5 µM PIK93 (central column) or with 30 µM PIK93 (right column) for 2 hours prior to fixation and staining as described in Materials and Methods. PI4P (green) localized to the plasma membrane (PM) was detected using the plasma membrane staining protocol (upper panel) [33]. Nuclei were stained by the Hoechst dye (blue). PI4P in the intracellular membranes (IM) was revealed using the Golgi staining protocol (lower panel). Together with PI4P, Golgi membranes were stained with the Golgi marker giantin (red). Colocalization of PI4P with Golgi membranes results in yellow color (zoomed sections are indicated by a yellow square).
(B) Quantification of PI4P levels by immunofluorescence analysis. Changes in mean fluorescence intensity relative to the control (DMSO) are shown. Four randomly

picked fields were analyzed per each condition. Normalization was performed as detailed in Materials and Methods. Data are presented as averages ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

We then evaluated the PI4K inhibitory activity of AL-9 in Huh7.5 cells using the same methodology. Briefly, Huh7.5 cells were incubated either with DMSO or with increasing concentration of AL-9 (1, 2, 4 or 8 μM) for two hours (Fig. 5A). Treatment with AL-9 gradually reduced the amount of PI4P in the plasma membrane (Fig. 5B). Conversely, the concentration of PI4P in the Golgi-associated membranes remained substantially unaltered up to the highest AL-9 concentration used (Fig. 5B).

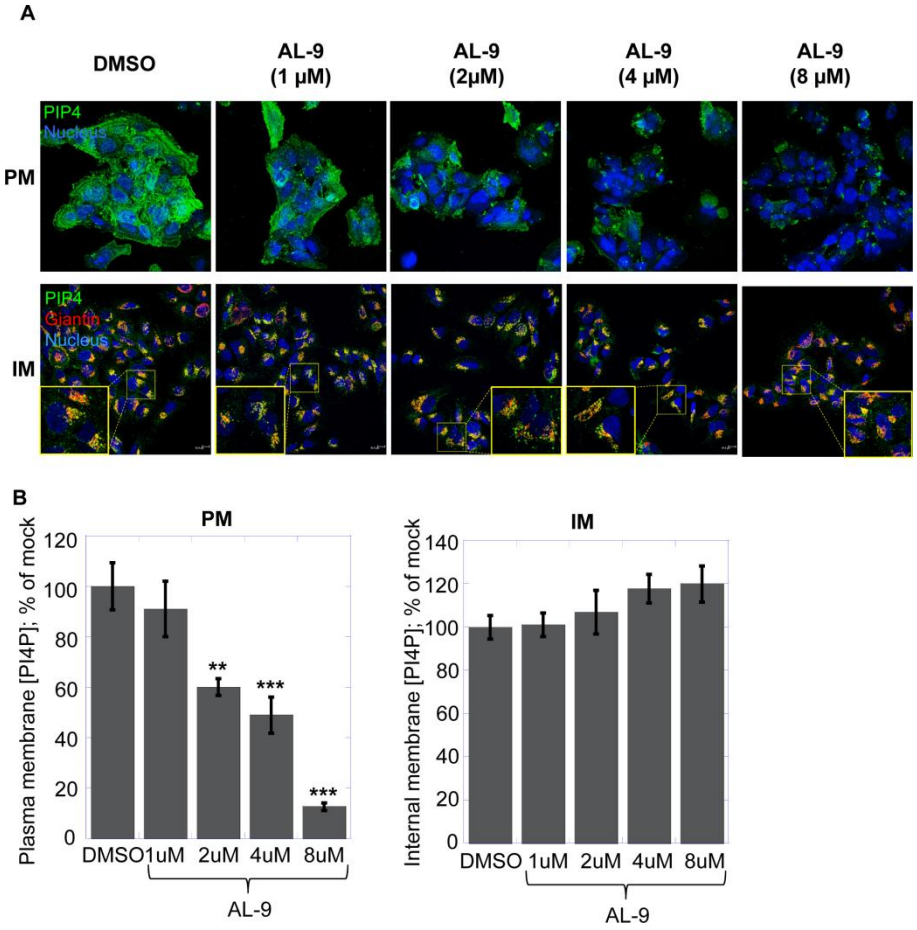

Fig. 5. AL-9 inhibits PI4KIIIα in Huh7.5 cells

(A) Confocal microscopy images of Huh7.5 cells treated for 2 hours with DMSO (left column) or with 1, 2, 4 or 8 µM of AL-9 (columns 2 to 5) . PI4P (green) localized to the plasma membrane (PM) was detected using the plasma membrane staining protocol (upper panel)[33]. Nuclei were stained by the Hoechst dye (blue). PI4P in the intracellular membranes (IM) was revealed using the Golgi staining protocol (lower panel). Together with PI4P, Golgi membranes were stained with the Golgi marker giantin (red). Colocalization of PI4P with Golgi membranes results in yellow color (zoomed sections are indicated by a yellow square).
(B) Quantification of PI4P levels by immunofluorescence analysis. Changes in mean fluorescence intensity relative to the control (DMSO) are shown. Four randomly picked fields were analyzed per each condition. Normalization was performed as detailed in the Materials and Methods. Data are presented as averages ± SEM. **, $P < 0.01$; ***, $P < 0.001$.

In all, the results described above suggest that AL-9 inhibits PI4KIIIα also in living cells, while not appreciably affecting the activity of PI4KIIIβ. This is in line with the selectivity for PI4KIIIα over PI4KIIIβ observed in the biochemical assays.

3 HCV alters the intracellular and plasma membrane distribution of PI4P

Viral infection induces modification of intracellular membrane structures[36] and, for some RNA viruses including HCV, it has been shown that these induced membranous structures are highly enriched for PI4P[28, 37]. Before testing the activity of AL-9 in HCV-infected cells, we wanted to know what the impact of HCV on cellular membrane structures was, with special regard to the subcellular membrane distribution of PI4P.

Naïve Huh7.5 cells or cells actively replicating the genotype 2a or 1b HCV subgenomic replicon were investigated for their PI4P concentration in internal membranes or plasma membranes, respectively (Fig. 6A). As previously shown, cells expressing the HCV replicon form a membranous web that is highly enriched for PI4P (Fig. 6A, lower panel). The level of PI4P in these virus-specific membrane structures is markedly higher in JFH-A4 cells, containing the very efficient genotype 2a JFH-1 replicon, compared to the Con1-SR cells, which are based on the genotype 1b Con1 replicon, possibly mirroring the different RNA replication efficiency. It is well established that the kinase responsible for the production of the PI4P pool present in these

structures is PI4KIIIα. In the current model, PI4KIIIα interacts with the viral protein NS5A, leading to up-regulation of the kinase activity and accumulation of PI4P in the virus-specific membranous web[26-28]. Conversely, the results shown in Figures 3-5 suggest that – in absence of viral replication – a major function of PI4KIIIα is the synthesis of the PI4P pool in the plasma membrane. We therefore asked ourselves whether the presence of HCV could not only influence distribution and enrichment of PI4P in internal membranes, but also alter the PI4P plasma membrane pool. In Fig. 6A, we show that, concomitantly with the increase of PI4P in the internal membranes (lower panel), HCV replication promotes a marked decrease of PI4P concentration in the plasma membrane (upper panel). Relative quantification of the PI4P levels in the different experimental conditions is shown in Fig. 6B. This experiment demonstrates that the presence of HCV causes a dramatic change of PI4P localization in cellular membranes, whereby the increase of PI4P concentration in the virus-specific membranous structures appears to be accompanied by a depletion of the PI4P pool normally present in the plasma membrane.

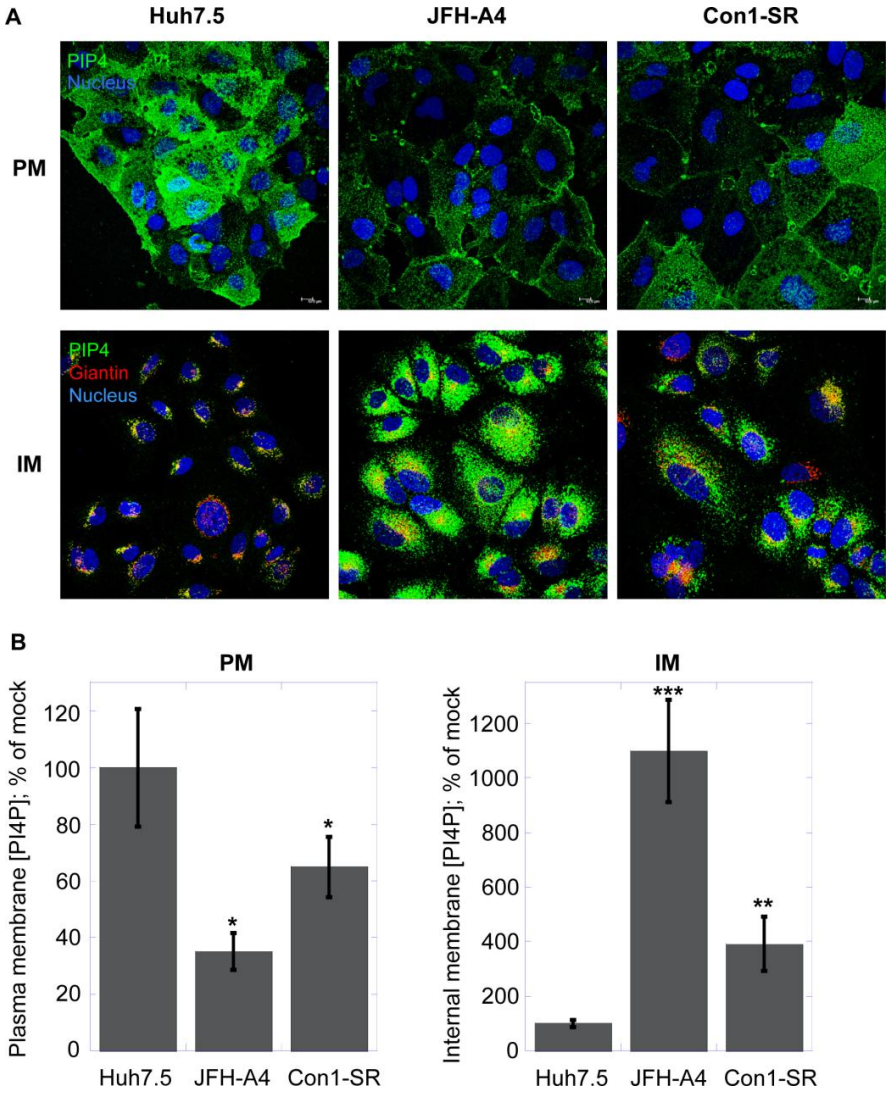

Fig. 6. HCV impacts subcellular PI4P distribution

(A) Huh7.5 cells, JFH-A4 and Con1-SR cells were analyzed by confocal microscopy for the presence of PI4P (green) in the plasma membranes (PM, upper panel) or in the intracellular membrane (IM, lower panel) using the protocols described in Materials and Methods. Nuclei were stained by the Hoechst dye (blue). For internal membrane staining, giantin (red) was used as a specific marker for Golgi membranes.

(B) Quantification of PI4P levels by immunofluorescence analysis. Changes in mean fluorescence intensity relative to the control (Huh7.5 cells) are shown. Four randomly picked fields were analyzed per each condition. Normalization was performed as detailed in Materials and Methods. Data are presented as averages ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

We next investigated whether HCV-associated changes in PI4P distribution could be reverted upon cure of the HCV replicon by specific inhibitors. We treated JFH-A4 cells for two weeks either with the HCV RdRp inhibitor HCV-796 or with the HCV NS3/4A protease inhibitor MK-5172 and followed PI4P localization in internal membranes and in the plasma membrane (Fig. 7). Independent of the type of inhibitor used, the result shows that the HCV-induced PI4P – enriched membranous web in JFH-A4 cells disappeared upon suppression of HCV replication and that the intracellular PI4P localization returned to the Golgi-localization as observed in the naïve Huh7.5 cells (left column). In parallel, the plasma membrane concentration of PI4P increases to the levels observed in naïve cells (middle column). NS5A staining (right column) as well as real-time RT-PCR (not shown) indicated that the prolonged treatment with HCV-inhibitor led to complete and stable suppression of viral protein expression and undetectable level of HCV RNA. Thus, removal of HCV RNA brings PI4P synthesis and distribution back to a level comparable to naïve Huh7.5 cells. It is worth of note, however, that the previous presence of HCV replicons in the cured cells induced some irreversible morphological changes of unknown nature, such as a smaller cell size.

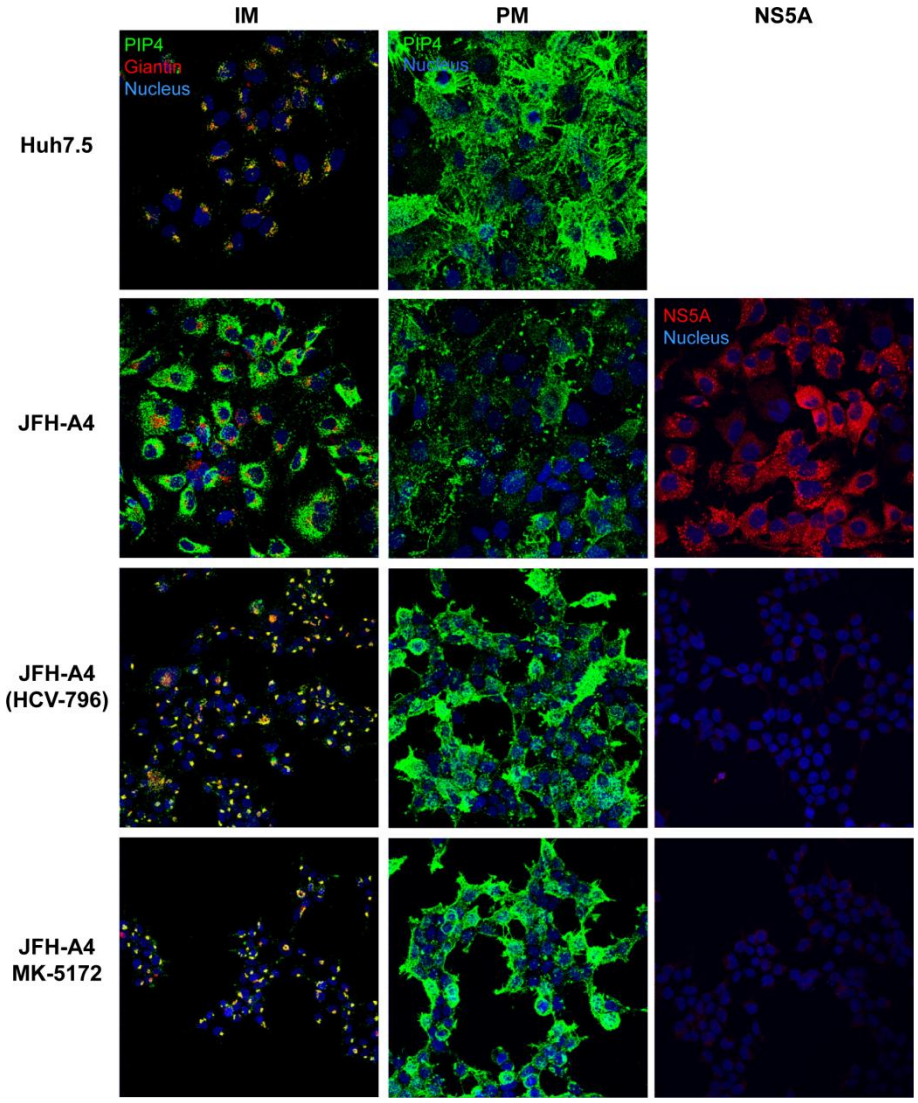

Fig. 7. Reversibility of HCV-induced changes in PI4P subcellular distribution

JFH-A4 cells were incubated for 14 days with the HCV RdRp inhibitor HCV-796 (2 µM) or the HCV NS3/4A protease inhibitor MK-5172 (0.2 µM). Cure from HCV was controlled by detection of NS5A with a specific NS5A antibody (red, right column). As control, untreated Huh7.5 cells or JFH-A4 cells were used. Cells were fixed and PI4P (green) was detected in the internal membranes (IM, left column) or in the plasma membrane (PM, central column). For internal membrane staining giantin (red) was used as a specific marker for Golgi membranes. Nuclei were stained by the Hoechst dye (blue).

4 AL-9 inhibits PI4KIIIα in HCV-replicating cells

We have shown that PI4KIIIα is inhibited by AL-9 in naïve Huh7.5 cells. As discussed above, in HCV-replicating cells, the kinase activity of PI4KIIIα is up-regulated by a direct protein-protein interaction with the viral protein NS5A. In the following experiment (Fig. 8), we explored whether AL-9 is able to inhibit PI4KIIIα also in this context. JFH-A4 cells were incubated with increasing concentration of AL-9 for 4 hours and PI4P concentration in the HCV membranous web was followed by immunostaining (Golgi staining protocol). Treatment of cells with AL-9 lead to clear inhibition of PI4P accumulation in the HCV membranous web. Incubation with 8 µM AL-9 depleted as much as 70% of the PI4P present in the intracellular membranes of replicon-containing cells. This result confirms that AL-9 inhibits PI4KIIIα independent of its membranous localization and suggests that this inhibition could be responsible for the observed antiviral effect. Since AL-9 has anti-HCV activity in the concentration range used here, longer incubation of HCV replicons with AL-9 results in inhibition of HCV- RNA and -protein synthesis. As a consequence the PI4P –enriched HCV membranous web would disintegrate. In this case loss of PI4P in the internal membranes could be not a direct consequence of PI4KIIIα inhibition, but a consequence of disintegration of the HCV membranous web.

In order to rule out this possibility, we checked localization of NS5A, a presumed marker for HCV replication sites, after 4 hours of incubation with AL-9. Localization of NS5A does not change, suggesting that AL-9 does not significantly change the structure of the HCV

membranous web upon 4 hours of treatment. Moreover, incubating the same replicon cells for 4 hours with HCV-796, an HCV polymerase inhibitor, did not lead to appreciable depletion of the membranous web PI4P pool indicating that the loss of PI4P in the HCV-induced intracellular membranes is the direct consequence of inhibition of PI4KIIIα, and not the consequence of inhibition of HCV replication. Additional evidence is provided in the experiment below, in which expression of the HCV polyprotein, and consequently formation of a membranous web, was driven by cDNA plasmid rather than by autonomously replicating HCV RNA.

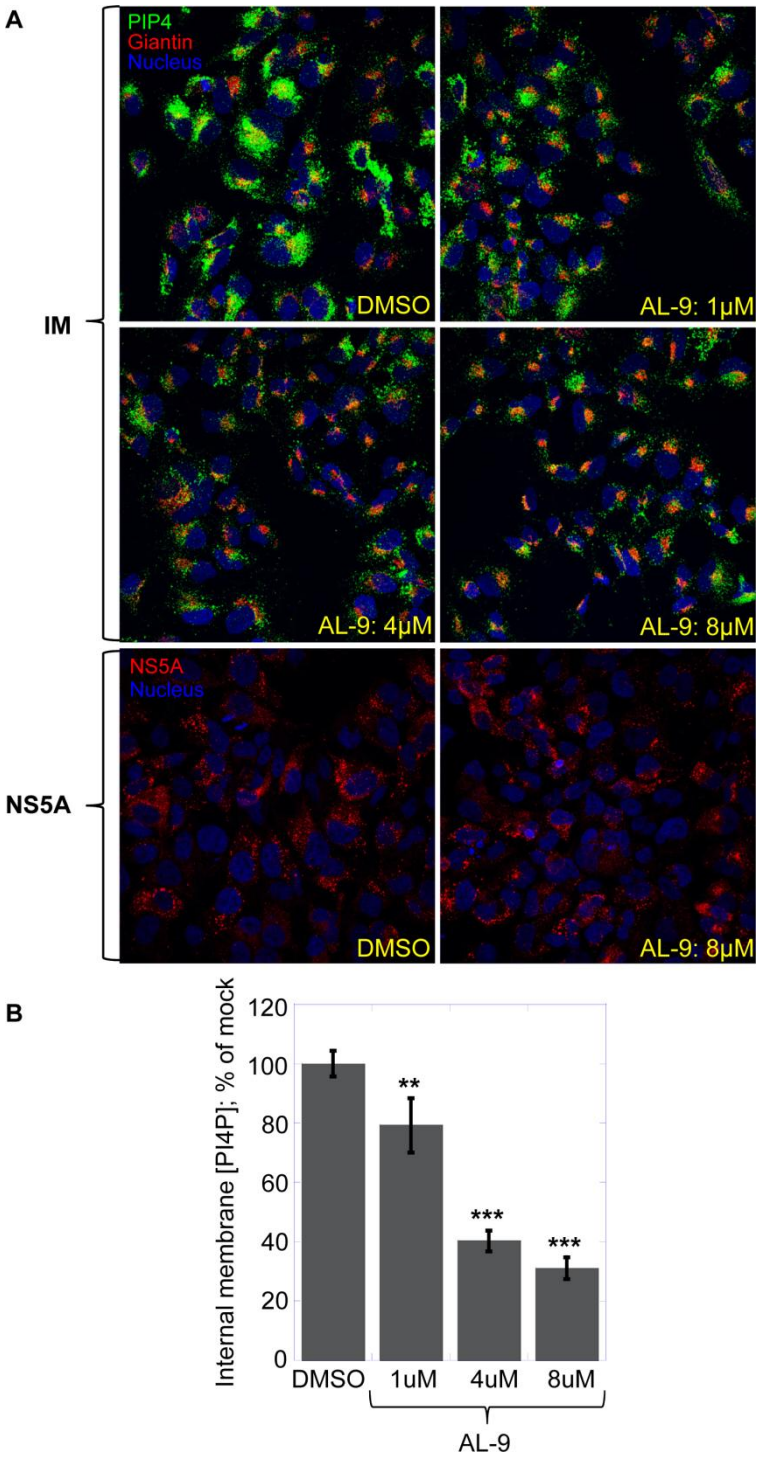

Fig. 8. AL-9 inhibits PI4KIIIα in HCV-replicating cells

(A) JFH-A4 cells were treated with DMSO or AL-9 for 4 hours and internal membranes were stained for PI4P (green) and the Golgi marker giantin (red) using the Golgi staining protocol, as described in Materials and Methods. DMSO or AL-9 concentrations are indicated within the image. Alternatively, cells were stained for NS5A as described in Materials and Methods (indicated as NS5A). Nuclear DNA was stained with Hoechst dye (blue). PI4KIIIα, associated with the HCV-associated membranous web is inhibited by AL-9. The decrease of PI4P is not due to inhibition of the HCV replication indicated by unchanged NS5A expression and localization (lower panel).
(B) Quantification of PI4P levels by immunofluorescence analysis. Changes in mean fluorescence intensity relative to the control (DMSO) are shown. Four randomly picked fields were analyzed per each condition. Normalization was performed as detailed in Materials and Methods. Data are presented as averages ± SEM. **, $P < 0.01$; ***, $P < 0.001$.

5 PI4KIIIα inhibition by AL-9 alters sub-cellular distribution of NS5A

It was previously shown that knock-down of PI4KIIIα expression by RNAi resulted in the production of large NS5A clusters. This was achieved in an experimental setting where the HCV polyprotein was expressed from DNA constructs, thus avoiding potential confounding effects due to inhibition of HCV RNA replication[23, 28]. We wanted to assess whether pharmacological inhibition of PI4KIIIα kinase activity would lead to similar effects on NS5A subcellular localization. Thus we followed the effect of AL-9 on NS5A localization after transient DNA transfection in Lunet-T7 cells with a plasmid expressing genotype 2a nonstructural proteins NS3-NS5B under the control of a T7 promoter[38]. Cells were treated either with DMSO (upper panels) or with 8 μM AL-9 (lower panels) for 2, 8 or 16 hours and localization of NS5A as well as PI4P were followed by indirect fluorescence microscopy (Fig. 9). Cells successfully transfected with the HCV polyprotein expressed NS5A and

induced the PI4P -enriched membranous web. After 8 hours of treatment with AL-9, changes in NS5A localization in form of larger clusters become visible. At the same time, PI4P concentration in the membranous web started to decrease. After 16 hours of incubation with AL-9, NS5A was concentrated almost exclusively in large clusters. At this time-point, PI4P in the internal membranes had completely vanished.

In summary, this experiment shows that, in cells expressing the HCV polyprotein from cDNA, prolonged treatment with AL-9 results in a redistribution of NS5A into large clustered structures with high resemblance to the structures previously observed after silencing of the PI4KIIIα gene by RNAi[23, 28]. Concomitantly, we observed a depletion of the PI4P pool present in the HCV-induced membranous structures. These results indicate that the catalytic activity of PI4KIIIα is directly or indirectly required for the proper localization of HCV NS5A protein into the membranous web. Furthermore, the experiment just described lands additional support to the notion that the antiviral effect of AL-9 is mediated by the inhibition of PI4KIIIα.

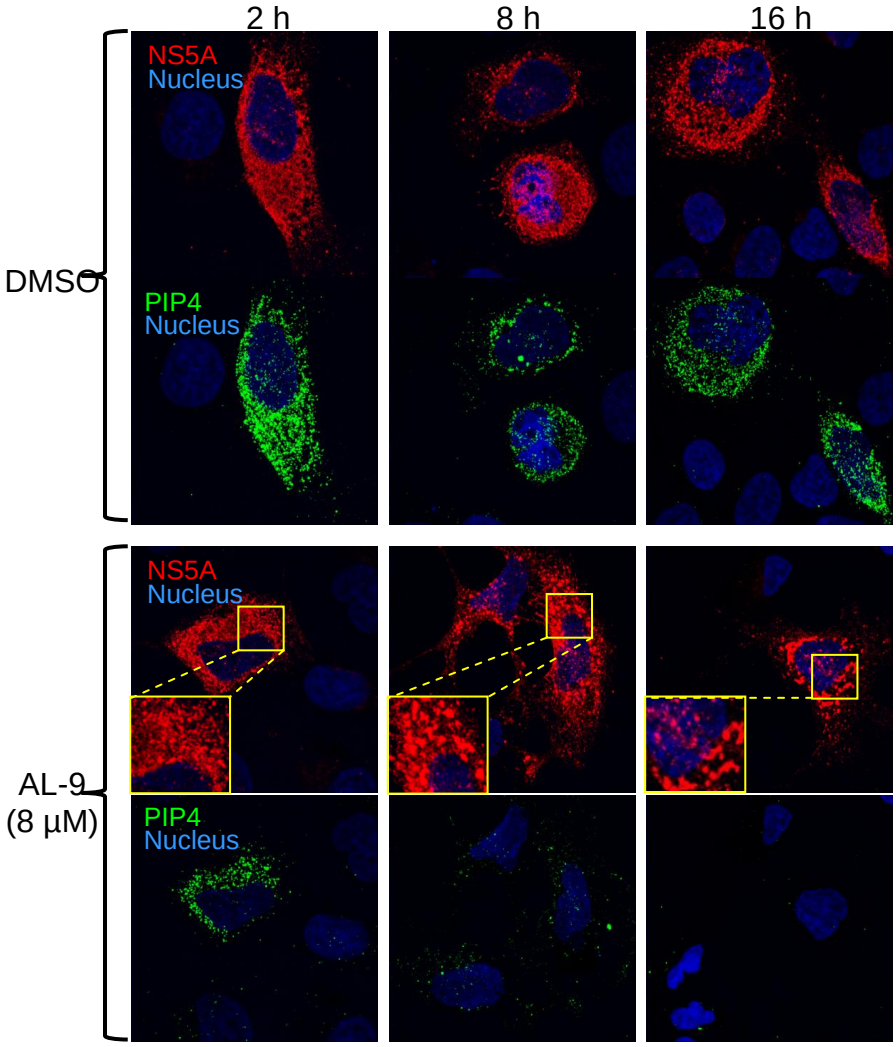

Fig. 9. Inhibition of PI4KIIIα by AL-9 induces the formation of large NS5A clusters

Huh7-Lunet/T7 cells were transiently transfected with the plasmid pTM-NS3-5B which expresses the HCV nonstructural proteins under the control of the T7 RNA polymerase promoter. Cells were treated with DMSO (upper panels) or 8 µM AL-9 (lower panels) for 2, 8 or 16 hours and were then stained for NS5A (red) and PI4P (green) using the Golgi staining protocol as described in Materials and Methods. Nuclear DNA was stained with the Hoechst dye (blue). Zoomed sections are indicated by a yellow square. Long incubation with AL-9 (8-16 hours) results in increased NS5A clustering and concomitantly a decrease of PI4P in the internal membranes.

D. Discussion

In the present paper, we show that a compound belonging to the class of 4-anilino quinazoline inhibitors of HCV replication is an inhibitor of PI4KIIIα, a cellular lipid kinase required for viral replication.

PI4KIIIα belongs to the family of type III phosphatidylinositol 4-kinases, enzymes that catalyze the conversion of phosphatidylinositol to phosphatidylinositol 4-phosphate (PI4P). PI4P is the most abundant monophosphorylated inositol phospholipid in mammalian cells and the importance of this phospholipid is just started to be unraveled[39]. In addition to playing important roles in intracellular signaling and membrane trafficking, phosphatidylinositol lipids and their metabolizing enzymes are also exploited by many different viruses in order to transform cellular membranes in structures supporting their replication[36, 40, 41]. PI4KIIIβ was shown to be a host factor required for enterovirus replication[37], whereas several reports have demonstrated that PI4KIIIα is crucial for HCV replication[22-25]. Owing to the importance of this pathway, the need for specific inhibitors of PI4III kinases is increasing. Only recently, some enviroxime-like compounds with anti-viral activity against enterovirus have been demonstrated to target PI4KIIIβ. One of these agents is a very specific inhibitor of the β-isoform of the type III PI4-kinases[42]. So far, no such compound exists for the PI4KIII-α isoform. A commonly used inhibitor for type III phosphatidylinositol 4-kinases is PIK93, which has originally been designed to inhibit class I PI3-kinases[34]. This compound allows differential inhibition of PI4KIIIβ alone or PI4KIIIα and PI4KIIIβ together

depending on the concentration used. In this paper, we show that a 4-anilino quinazoline derivative, termed AL-9 (Fig. 1 and S1), is able to inhibit PI4KIIIα in a test tube as well as in living cells. AL-9 inhibited purified PI4KIIIα, with a moderate (~5-fold) selectivity over the β isoform (Fig. 2). In cell culture, we observed that treatment with AL-9 efficiently inhibits the maintenance of the plasma membrane PI4P pool in Huh7.5 cells while not significantly affecting the Golgi membrane pool at the highest concentration used (Fig. 5). This finding is in line with the moderate selectivity observed in the biochemical assay. Thus, AL-9 represents a lead candidate for the development of more potent and more specific inhibitors of PI4KIIIα.

Anti-HCV compounds of the 4-anilino quinazoline class were previously assumed to exert their antiviral effect via inhibition of the viral protein NS5A. This conclusion rested on analysis of the mutations found in the HCV replicon in association with resistance to these agents[18]. Mutations generated against 4-anilino quinazolines where localized mainly in NS5A, in triplets that occurred all in NS5A or appeared concomitant with changed in NS4B or NS5B (see also Introduction). Reverse genetic experiments, in which these mutations were reintroduced in the replicon (single, double and triple combinations), however, did not support a role for these mutations in conferring resistance to 4-anilino quinazolines. In order to assess whether the reported mutations conferred any level of resistance to AL-9, we independently performed reverse genetics studies in which selected mutations triplets, reported to be associated with the higher level of resistance, were reintroduced in a genotype 1b replicon with

the same genetic background as the one reported in the original resistance study (Fig. S2). These mutation triplets are: FAG: L199F+V362A+S390G (NS5A), DLD: E212D+P299L+V388D (NS5A), and PPA: T200P+S370P(NS5A)+S76A(NS5B). We observed that the replicon containing the first triplet lost the ability to replicate at significant level. For replicons containing the latter two combinations of mutations, RNA replication could be measured, although at a lower level compared to the parental construct (35% and 20%, respectively). These replicons, however, remained equally sensitive to AL-9 as the parental replicon (Fig. S2), opening the question as to which really is the target of this compound class. We are currently trying to select HCV replicons resistant to AL-9. So far we were unable to identify mutations that confer resistance to AL-9.

Our new data on AL-9 suggest that inhibition of HCV replication by 4-anilino quinazoline compounds is a consequence of PI4KIIIα inhibition. Our conclusion rests on a number of experimental findings. First of all, we showed that AL-9 is an inhibitor of purified type III PI4 kinases. Furthermore, we clearly demonstrated that AL-9 inhibits PI4KIIIα both in naïve Huh7.5 cells (Fig. 5, discussed above) as well in cells harboring actively replicating HCV RNA (Fig. 8). In cells where HCV replication occurs, PI4KIIIα interacts physically with HCV NS5A. This interaction, in turn, leads to the stimulation of PI4P synthesis at the HCV replication sites[28]. Treatment of replicon-harboring cells with AL-9 leads to efficient suppression of the PI4P pool at the HCV replication sites and does so independently of inhibition of HCV replication. This indicates that – although the enzymatic activity of PI4KIIIα is

modulated by the interaction with an HCV protein NS5A – it remains sensitive to the action of the 4-anilino quinazoline inhibitor.

We also investigated whether the dramatic changes observed in PI4P membrane levels by treatment with AL-9 could be associated with alteration in the subcellular distribution of type III PI4 kinases. To this aim, we analyzed the subcellular distribution of the type III PI4 kinases in Huh7.5 or Luc-A4 cells following incubation with AL-9 (Fig. S3). We observed no major effect of AL-9 on the localization of either PI4KIIIα or PI4KIIIβ, in line with the notion that the observed effects are primarily due to the inhibition of the kinase activity rather than to an altered protein subcellular distribution.

In cells that express the HCV polyprotein from a trans-gene, knock-down of PI4KIIIα by RNAi was previously shown to cause a dramatic change in NS5A subcellular distribution, from a pattern consistent with localization in the membranous web replication complexes to abnormally large cytoplasmic clusters[23, 26, 28]. In Fig. 9, we show that AL-9 treatment of cells ectopically expressing the HCV nonstructural proteins results in a time-dependent depletion of PI4P and a concomitant change of NS5A localization to the large-clustered structures discussed above, reinforcing the notion that the anti-HCV effect of AL-9 and related compounds are likely to be mediated by the inhibition of PI4KIIIα.

We also found that PI3K p110α is inhibited by AL-9 in vitro at concentration similar to those needed to inhibit type III PI4-kinases. However, no Class I PI3-kinase has been shown to influence HCV replication thus inhibition of HCV replication by AL-9 is not due to

inhibition of Class I PI3-kinases. So far, the only PI3-kinase that resulted as positive hit for HCV replication inhibition in siRNA screens is PI3-kinase C2 gamma[24]. Future work will have to address whether AL-9 inhibits PI3KC2G in addition to Type III PI4-kinases.

During the characterization of AL-9 we focused our attention on various aspects of PI4P metabolism in Huh7.5 cells with and without replicating HCV. We observed a typical Golgi localization of PI4P in intracellular membranes of naïve Huh7.5 cells and confirmed a role for PI4KIIIβ in maintaining at least part of this pool. In order to get the complete picture we also investigated the PI4P pool present in the plasma membrane. In yeast, Stt4p, the ortholog to the mammalian PI4KIIIα, is localized at the plasma membrane and it is the major contributor for the synthesis of the plasma membrane-localized PI4P[39]. In mammalian cells, the role of PI4KIIIα for the maintenance of the plasma membrane PI4P pool has been demonstrated in HEK-293 and Cos-7 cells[31-33]. Here we demonstrate that liver-derived Huh7.5 cells are endowed with a rich PI4P pool in the plasma membrane and that the enzyme responsible for its maintenance is PI4KIIIα. In HCV-replicating cells, the subcellular PI4P distribution is profoundly altered. As already reported previously, the presence of HCV causes the induction of a membranous web highly enriched for PI4KIIIα-syntesized PI4P. In accordance, several reports demonstrate that NS5A recruits PI4KIIIα to the membranous web by direct protein-protein interaction, thereby stimulating its enzymatic activity[26-28]. Concomitantly with the induction of highly PI4P -enriched internal membranes, we observe a marked decrease of PI4P in the plasma

membrane. One possible explanation could be that – by hijacking PI4KIIIα - HCV might be able to enrich PI4P in the virus-induce membranous web not only by directly activating the enzymatic activity of PI4KIIIα recruited into the HCV RNA replication compartment, but also by preventing transport of the PI4KIIIα-synthesized PI4P from the synthesis site to the plasma membrane. How PI4KIIIα, localized at the ER, synthesizes the PI4P pool present in the plasma membrane it is still an enigma. This topological discrepancy can partially be resolved assuming that PI4KIIIα-dependent PI4P production occurs on ER-PM contact sites, that is, sites of close apposition between ER and PM. In yeast it has been demonstrated that a complex interplay between different proteins regulate the PI4P metabolism at the plasma membrane[43]. Among these proteins are Osh, the yeast ortholog of the human OSBP and the ER membrane VAP proteins Scs2 and Scs22, the yeast orthologs of human VAP proteins. Interestingly, h-VAP-33 and OSBP have been shown to be important for HCV replication[44-46]. It may be possible that recruitment of PI4KIIIα to the HCV membranous web through NS5A prevents interaction of PI4KIIIα with its cellular protein partners required to direct PI4P to the plasma membrane. Upon withdrawal of HCV from the cells (Fig. 7) PI4KIIIα is again free for interaction with the adequate partners. A possible role of PI4KIIIα in PI4P trafficking between the plasma- and intracellular membranes is suggested by our finding that RNAi silencing of this PI4 kinase results in decreased concentration of PI4P in the plasma membrane with a concomitant increase in the level of PI4P in the endomembranes (Fig. 3). Such a function of PI4KIIIα would have to be independent of the

kinase activity, since pharmacological inhibition (with PIK93 or AL-9) does not recapitulate this phenomenon observed by knocking down the protein expression.

In summary, the presence of HCV may change PI4P metabolism not only by activating the catalytic activity of PI4KIIIα by NS5A but also by modulating the PI4P distribution between different membrane compartments. The net result is an enrichment of the PI4P pool in the HCV-induced membranous web with a concomitant depletion of the plasma membrane PI4P pool.

Concluding, in this paper we demonstrate that a class of HCV inhibitors originally proposed to target NS5A does in fact target the host factor PI4KIIIα. Compounds targeting host factors may have the general advantage of imposing a higher genetic barrier to the development of resistance. AL-9, a member of this class of compounds, inhibits PI4KIIIα and to our knowledge, it is the first compound with a clear preference for PI4KIIIα over PI4KIIIβ. For this reason, AL-9 offers a good candidate as lead compound for the development of more potent and specific pharmacological inhibitors of PI4KIIIα to be used both as important research tools as well as leads for initial drug discovery.

E. Materials and Methods

1 Reagents and plasmids

The HCV RNA polymerase inhibitor HCV-796 and the PI kinase inhibitor PIK93 were a gift from Arrow Pharmaceuticals. The HCV protease inhibitor MK-5172 was purchased from Selleck Chemicals. Nucleic acids were manipulated according to standard protocols. Plasmid FBac-His-CD-PI4KA was constructed as follows: the catalytic domain of PI4KIIIα was amplified by PCR using the oligonucleotides 5'-cactgcggatccataatggggatgatgcagtgtgtgattg-3' (sense), 5'-cctgcgaattctcagtaggggatgtcattc-3' (antisense) and the plasmid pEF1A-PIK4CA untagged (a kind gift from G. Randall, Department of Microbiology, University of Chicago) as template. The resulting PCR fragment was subcloned into the vector pCR-Blunt II-Topo (Invitrogen) and finally cloned into the BamH1–XhoI cloning sites of the plasmid vector pFastBac THT-B. The resulting protein expressed from this plasmid contains an N-terminal hexa-histidine tag and starts at PI4KIIIα amino acid G873 (reference sequence NM_058004). pTM-NS3-5B expression vector expressing the HCV genotype 2a nonstructural proteins under the control of the T7 promoter was a generous gift from V. Lohmann (Department of Molecular Virology, University of Heidelberg)[38]. Synthesis of compound AL-9 is described in Supporting Information.

2 Cell lines and culture conditions

The human hepatoma-derived cell line Huh7.5[47] were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml Streptomycin and 2 mM L-glutamine; G418 (0.8 mg/ml) was added to cell lines containing the HCV replicon. Stable cell lines expressing HCV genotype 1b or 2a subgenomic replicons were generated by electroporation of in vitro-transcribed RNA into Huh7.5 cells[48] and following selection with G418 (0.8 mg/ml) for three weeks. Con1-SR: Huh7.5 cells replicating the Con1 subgenomic replicon with the adaptive mutations E1202G in NS3 and S2204R in NS5A. JFH-A4: Huh7.5 cells replicating the JFH-1 subgenomic replicon together with the luciferase reporter gene constructed as described previously[49]. JFH-A4 cells were cured from the HCV replicon by two weeks of treatment with the protease inhibitor MK-5172 (0.2 µM) or the HCV RNA polymerase inhibitor HCV-796 (2 µM), respectively. Huh-LUNET/T7 cells were a kind gift from V. Lohmann (Department of Molecular Virology, University of Heidelberg, Germany).

3 Replication and infection assays

For replication assays, JFH-A4 or Con1-SR cells were plated at the density of $3x10^4$ or $6x10^4$ cells/well, respectively, in 24-well dishes the day before the experiment. Cells were treated with AL-9 resulting in a final concentration of 1 % DMSO in the cell medium. After three days

of treatment, RNA was extracted using the RNeasy Mini Kit (Qiagen) and HCV RNA was quantified by real time PCR using the following oligonucleotide and probe set designed for the HCV IRES as described previously [48]: sense (5'-GCGAAAGGCCTTGTGGTACT-3'), antisense (5'-CACGGTCTACGAGACCTCCC-3'), and probe (5'-CCTGATAGGGTGCTTGCGAGTGCC-3', 5' 6-carboxyfluorescein [FAM]/3' 6-carboxytetramethylrhodamine [TAMRA]). GAPDH mRNA was used as internal control for data normalization.

Production of infectious virus was performed as follows: J6/JFH-1 chimeric RNA (1-846(J6CF)/847-3034(JFH1) was electroporated into Huh7.5 cells using the protocol described previously[48]. Briefly, $2x10^6$ cells were electroporated with 10 µg of RNA in a final volume of 200 µl and $4x10^6$ cells were plated in a T-75 flask. Three days post electroporation, medium was harvested and stored at -20°C in small aliquots. Calculation of EC50 of AL-9 using the infectious HCV virus was performed as follows: Huh7.5 cells were plated at $4x10^4$ cells/well in 24-well plates the day before infection. Infection was started by addition of 10 µl of cell medium containing infectious virus (see above) at an MOI of 50 in a final volume of 400 µl. After 6 hours of incubation, medium was removed and replaced with 400 µl of fresh medium containing serial dilutions of AL-9. RNA was collected after 72 hours of incubation and quantified by real time PCR. Cell cytotoxicity (CC50) of AL-9 was calculated using the cell viability assay CellTiter-Blue (Promega). Huh7.5, JFH-A4, or Con1-SR cells ($5x10^3$ cells/well in 96-well dishes) were plated the day before treatment. AL-9 was added and cell viability was measured after four days of treatment.

4 Expression and purification of the catalytic domain of PI4KIIIα

Recombinant baculovirus was generated with the plasmid FBac-His-CD-PI4KA using the Bac-to-Bac system following the instructions of the manufacturer (Invitrogen). For protein expression, Sf9 cells were infected with recombinant baculovirus at a density of $2x10^6$ Sf9 cells/ml for 3 days at 20°C. To prepare cell extract ($1.5x10^8$ cells), cells were incubated in hypotonic buffer (10 mM HEPES (pH 7.5), 10 mM NaCl, 1 mM Tris(2-carboxyethyl)phosphine (TCEP) and EDTA-free protease inhibitor cocktail (Complete, Roche) for 30 min in ice and mechanically broken by 20 strokes of a Dounce homogenizer. After homogenizing, cells were incubated in lysis buffer (50 mM HEPES (pH 7.5), 500 mM NaCl, 10 % glycerol, 1 % Triton-X100, 1 mM TCEP and EDTA-free protease inhibitor cocktail (Complete, Roche) for further 30 min in ice and cell extract was cleared by centrifugation for 45 min at 20.000g. The cleared supernatant was incubated in batch with Ni-Sepharose High Performance (GE Healthcare) for 2 hours at 4°C with continuous shaking. The resin was first washed with 10 resin-volumes of wash buffer (50 mM HEPES (pH 7.5), 10 % glycerol, 0.4 % Triton X-100, 150 mM NaCl and 20 mM imidazol) followed by elution with wash buffer containing 250 mM imidazole. Active fraction (0.5 ml) were dialyzed against 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM DTT, 0.4 % Triton X-100 and 10% glycerol and stored at -80°C in small aliquots.

5 In vitro kinase assay

PI4K kinase activity was assayed with the ADP-Glo Kinase Assay (Promega), according to the manufacturer's instructions. Briefly, 0.5 µl of PI4KIIIα-CD or 0.05 µl PI4KIIIβ (32 ng, Invitrogen) were preincubated with DMSO or AL-9 in reaction buffer (20 mM Tris (pH 7.5), 5 mM MgCl2, 2 mM DTT, 0.5 mM EGTA, 0.4% Triton X-100) for 10 min at room temperature in a final volume of 8 µl. The reaction was started by addition of 2 µl of ATP and PI:PS Lipid Kinase Substrate (Invitrogen) to give a final concentration of 100 µM and 150 µM, respectively. After 1 hour of incubation at room temperature the reaction was stopped and further processed as described by the manufacturer. In parallel the reaction was performed without PI:PS substrate in order to detect contaminating ATPase activity present in the protein fractions. This activity was subtracted from the measured kinase activity. Kinase activity of PI3Kα (p110α/p85α) and PI3Kβ (p110β/p85α) was assayed as above using 5 ng or 20 ng, respectively (Millipore). Reaction buffer was changed to 50 mM HEPES pH7.5, 10 mM MgCl2 and 1 mM DTT.

6 Indirect Immunofluorescence

Cells were plated one day before the experiment in 24-well plates ($5x10^4$ cells/well for Huh7.5 and JFH-A4 cells, $7x10^4$ cells/well for Con1-SR and $1x10^5$ cells/well for cured JFH-A4 cells). Cells were either untreated or treated with compounds for the time as indicated in the figure legend. PI4P staining of the plasma membrane or internal

membranes was performed exactly as described previously[33]. Primary antibodies used were: anti- PI4P IgM (Cat. No. Z-P004, 1:300, Echelon), anti-Giantin antibody (Cat. No. PRB-114C-200 1:1000, Covance), affinity-purified rabbit anti NS5A antibody (1:2000), anti-PI4KIIIα kinase (Cat. No. 4902, 1:50, Cell Signaling), anti-PI4KIIIβ kinase (Cat. No, 611817, 1:500, BD Transduction). Secondary antibodies used were goat anti-mouse IgM Alexa Fluor 488 (Cat. No. A-21042, 1:600, Invitrogen) and goat anti-rabbit Alexa Fluor 568 (Cat. No. A-11011, 1:600, Invitrogen). For type III PI4K kinases or NS5A staining, all incubations were performed at room temperature. Cells were washed once with PBS and fixed with 300 µl of 4% PFA for 15 min. Cells were washed three times with PBS and permeabilized with 500 µl of 0.1 % Triton X-100 (or 0.5% for PI4KIIIα kinase staining) in PBS for 10 min. Unspecific binding was blocked by incubation with 3 % BSA in PBS (for PI4KIIIα staining no blocking was performed). After incubation with the primary antibody in blocking buffer, cell were washed with PBS and subsequently incubated with goat secondary antibodies conjugated to Alexa-Fluor 568, or Alexa-Fluor 488 at a dilution of 1:600. Nuclei were stained with Hoechst dye 33342 (Sigma; 1:4000). Slides were then mounted with 5 µl ProLong Gold Antifade (Invitrogen) and analyzed by using an inverted Leica TCS SP5 scanning laser confocal microscope. Digital images were taken using LAS AF software (Leica) and processed using Volocity software (Perkin Elmer). Quantification of fluorescence intensity was determined from multiple images using Volocity. Relative changes in fluorescence intensity mean values where obtained from four randomly picked fields for each condition (150 ~ 300 cells). For

plasma membrane staining, total PI4P fluorescence intensity obtained in each condition was normalized to the number of cells present in each field. For the quantification of relative PI4P levels in internal membranes, PI4P fluorescence intensity was normalized using the fluorescence intensity of the Golgi marker giantin. Quantitative immunofluorescence data are presented as means ± the standard error of the mean (SEM). For the calculation of statistical significance, a two-tailed, unpaired t-test was performed.

7 siRNA silencing

$3x10^4$ Huh7.5 cells/well were seeded in 24-well plates on microscope cover glasses and transfected with 50 nM of siRNAs in serum-free Opti-MEM (Invitrogen) using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's protocol. For western blot analysis, the transfection reaction was proportionally scaled up to 6-well plates. In order to maximize the silencing efficiency, 24 hours after the first transfection, the cells were subjected to a second round of siRNA transfection. siRNA sequences were the following (5'->3' sense strand): mock siRNA, GUA UGA CCG ACU ACG CGU ATT (custom, Sigma-Aldrich); PI4KIIIα siRNA, CCG CCA UGU UCU CAG AUA ATT (custom, Sigma-Aldrich); and PI4KIIIβ siRNA, GCA CUG UGC CCA ACU AUG ATT (Silencer Validated siRNA s10543; Ambion). Three days after the initial transfection, cells were stained for PI4P as described previously[33], or subjected to western blot analysis. For immunoblot

analysis of protein expression, cells were harvested with TEN buffer (10mM Tris/HCl pH 8.0, 1 mM EDTA, 100 mM NaCl), washed once with PBS and lysed with 2X protein sample buffer (125 mM Tris-HCl pH 6.8, 10 mM EDTA, 0,003 gr bromophenol blue, 20% glycerol, 4% SDS and 10% β-mercaptoethanol; 200µl). The samples were then sonicated, heated at 95°C and loaded onto 7.5% polyacrylamide-SDS page (Criterion, Biorad). After electrophoresis proteins were transferred to a nitrocellulose membrane and unspecific binding was blocked by PBS supplemented with 0.5% Tween (PBS-T) and 5% milk. Membranes were then incubated overnight at 4°C with primary antibodies (anti-PI4KIIIα, cat no. 4902, 1:250 Cell Signaling, anti-PI4KIIIβ, cat. No. 611817, 1:3000 BD Transduction Laboratories, mαβ-actin, cat. No. A1978, 1:5000, Sigma). HRP-conjugated secondary antibodies (donkey anti-rabbit, Cat. No. 9341 and sheep anti-mouse, Cat. No. 9311, GE Healthcare) were incubated for 1 hour at room temperature and detection was performed using SuperSignal-Femto chemiluminescent substrate (Pierce-Thermo Scientific).

8 T7-driven HCV polyprotein expression

1.5 x 106 Huh7-Lunet/T7 cells/100 mm dish were transfected with 20 µg pTM-NS3-5B using the transfection reagent Lipofectamine 2000 (Invitrogen). Six hours after transfection, cells were seeded in 24-well plates on microscope cover glasses for indirect immunofluorescence. After 5 hours, cells were treated either with DMSO or with 8 µM AL-9

for 2, 8 or 16 hours and co-staining of NS5A and PI4P was performed using the Golgi staining protocol, as described previously[33].

F. Supporting Information

1. Figure S1 (see p. 155). Synthetic pathway for compound AL-9
2. Figure S2 (see p. 156). HCV replicons harboring putative 4-anilino quinazoline resistance mutations retain sensitivity to inhibition by AL-9
3. Figure S3 (see p. 157). Effect of AL-9 on subcellular distribution of type III PI kinases

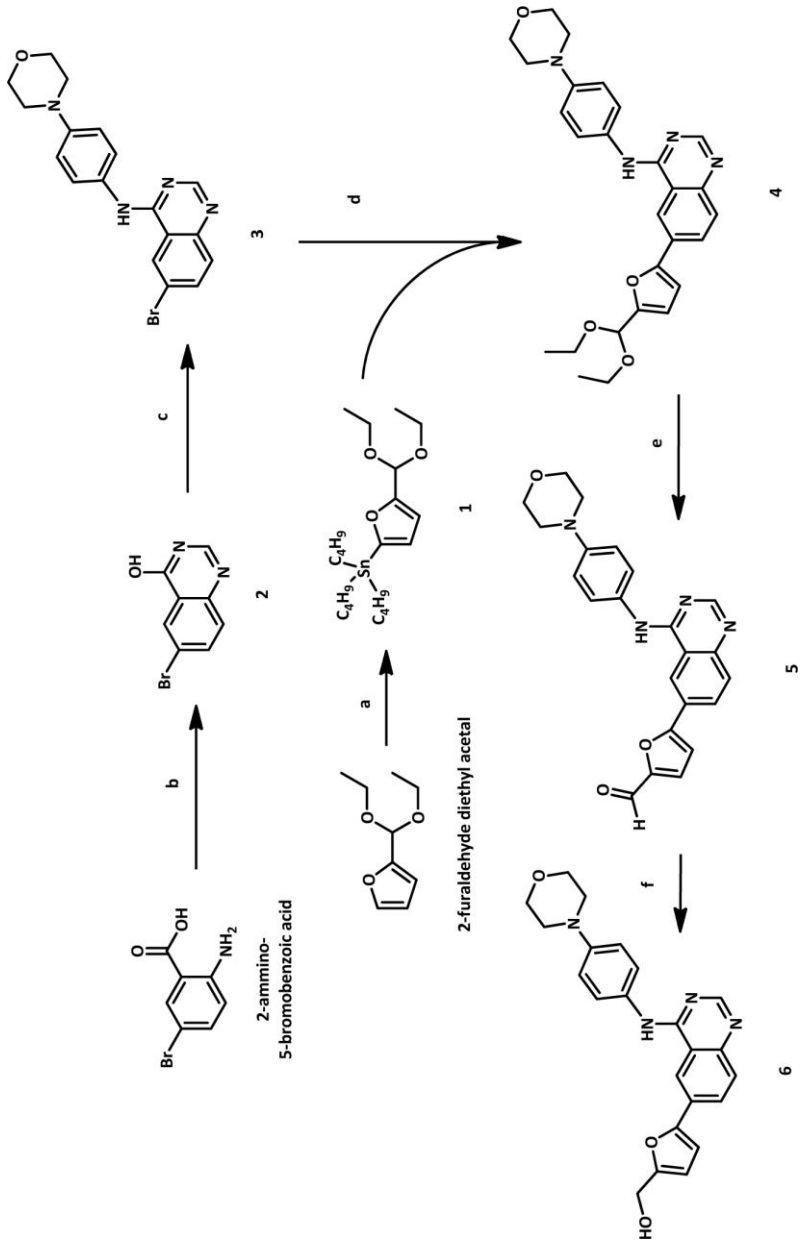

Fig. S1. Synthetic pathway for compound AL-9

Reagents and conditions: (a) n-BuLi, dry THF, 278uC 1 h, 20uC 3 h, Bu3SnCl, 278uC 2 h, RT overnight; (b) Formamide, 155uC, 16 h; (c) SOCl2, dry DMF, reflux, 5 h, 4-morpholinoaniline, dry CH3CN, reflux, 16 h; (d) compound 1, bis(triphenylphosphine) palladiumdichloride, dry THF, reflux; (e)HCl 2M, THF/H2O 1 : 1, RT; (f) NaBH(OAc)3, CH2Cl2/AcOH (15:1), RT.

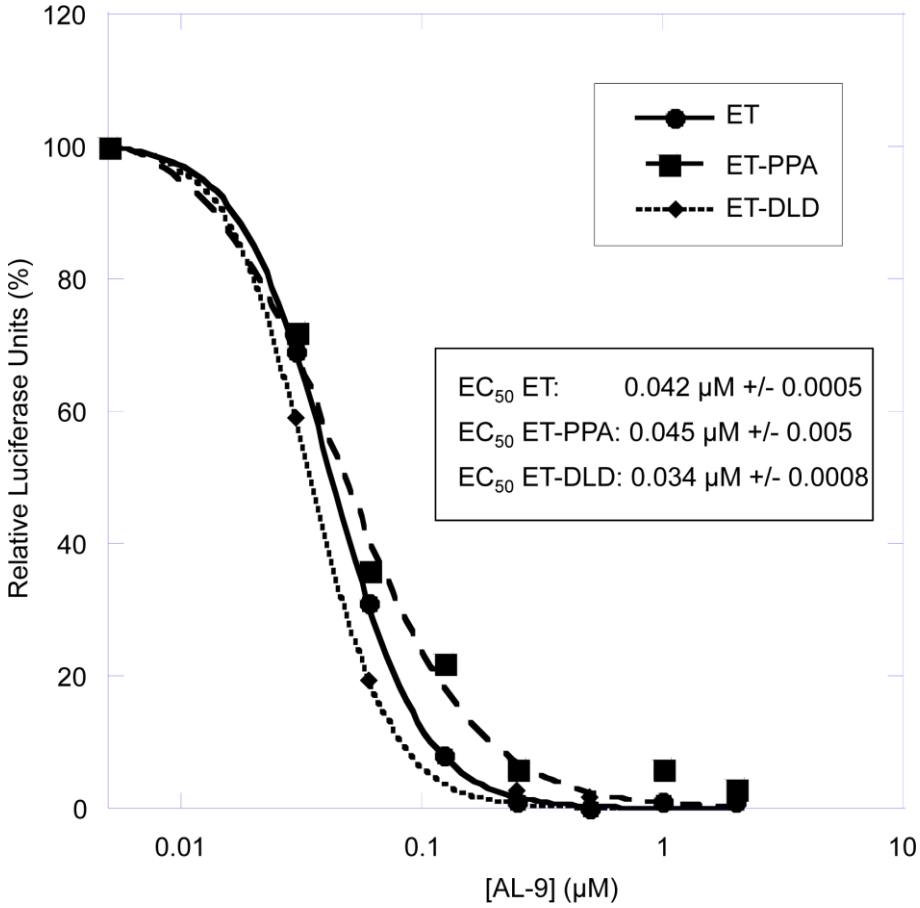

Fig. S2. HCV replicons harboring putative 4-anilino quinazoline resistance mutations retain sensitivity to inhibition by AL-9.

Huh7.5 cells where transiently transfected with genotype 1b subgenomic replicons carrying mutation triplets reported to be associated to resistance to 4-anilino quinazolines (ET-FAG, ET-PPA or ET-DLD) or with the parental replicon (ET). The ET replicon is a derivative of the Con-1 replicon that contains adaptive mutations at positions E1202G, T1280I, and K1846T, i.e, the same genetic background used in the original resistance study. The putative resistance mutations triplets engineered in this replicon were as follows: ET-FAG (L199F, V362A, S390G in NS5A); ET-PPA (T200P, S370P in NS5A and S76A in NS5B); ET-DLD (E212D, P299L, V388D in NS5A). Transfected cells were treated with AL-9 for three days. Inhibitory dose-response curve of AL-9 are shown. Transient HCV replication was measured by Luciferase activity and is expressed as % of the DMSO control. The data are averages from of three experimental replicates. EC50 values +/21 SD are shown in the figure inset. Replicon ET-FAG did not replicate at appreciable levels.

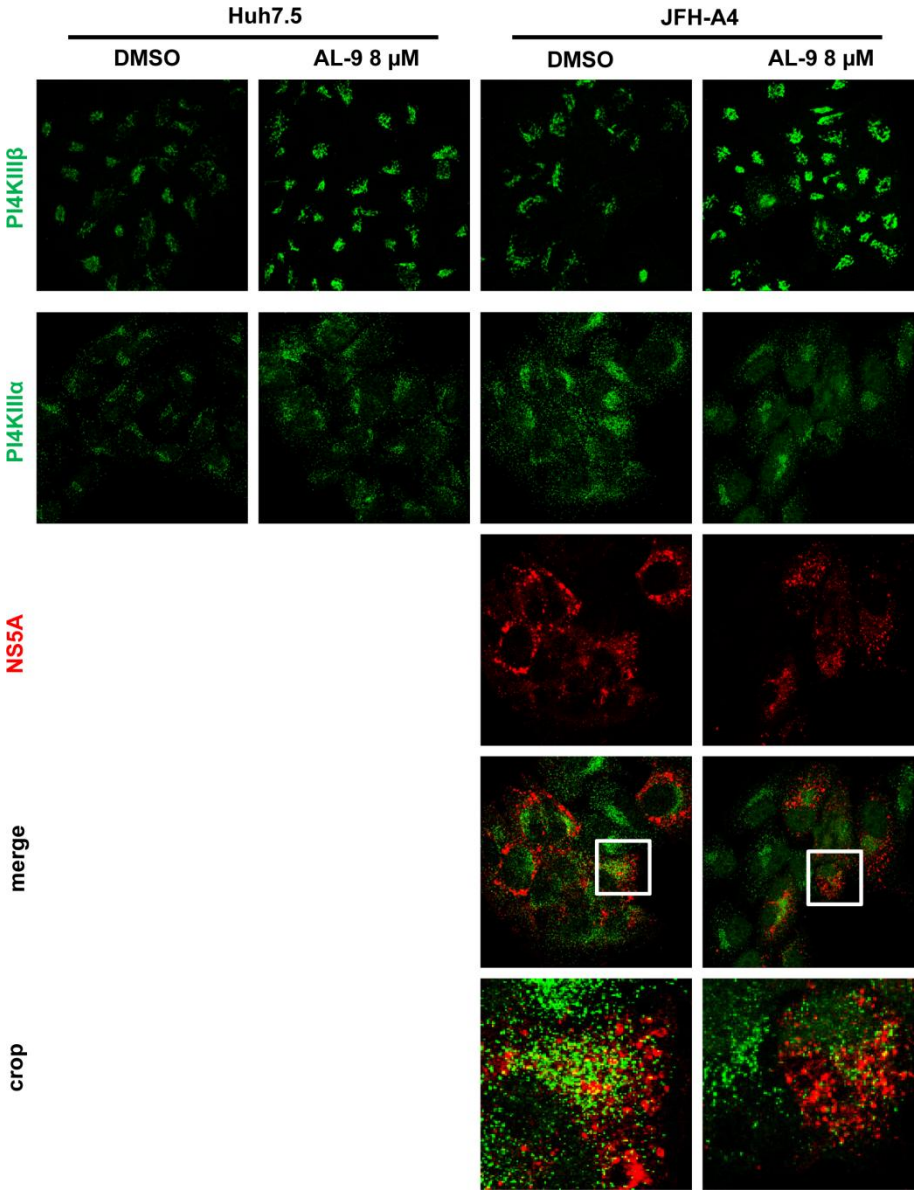

Fig. S3. Effect of AL-9 on subcellular distribution of type III PI kinases

Cellular localization of PI4KIIIa (green), PI4KIIIb (green) or NS5A (red) was analyzed by immunofluorescence in Huh7.5 or JFH-4A cells incubated for 4 hrs with 8 mM AL-9 or DMSO (control). Zoomed sections are indicated by a white square. No major effect of AL-9 on the localization of either PI4KIIIa or PI4KIIIb was observed. Under our experimental conditions, we observe very limited colocalization of PI4KIIIa with NS5A (yellow) independent of the treatment with AL-9.

4. Protocol S1. Chemical synthesis of compound AL-9

All the reagents were commercially available and used without further purification unless indicated otherwise. All solvents were anhydrous grade unless indicated otherwise. When dry conditions were required, the reactions were carried out in oven-dried glassware under a slight pressure of argon. Reaction were magnetically stirred and monitored by thin-layer chromatography (TLC) on silica gel. TLC was performed on Silica Gel 60 F254 plates (Merck) with UV detection, or using a developing solution of 0.5% orcinol in EtOH/H_2SO_4 (3%), followed by heating at 180°C. Flash column chromatography was performed on silica gel 230–400 mesh (Merck). The petroleum ether used as eluent in chromatography has boiling range of 40–60°C. ^{1}H and ^{13}C-NMR spectra were recorded on a Varian 400 MHz MERCURY instrument at 300 K. Chemical shifts are reported in ppm downfield from TMS as internal standard. Mass spectra were recorded on ESI-MS triple quadrupole (model API2000 QTrap™, Applied Biosystems).

tributyl-(5-(diethoxymethyl)furan-2-yl)stannane (1)

To a solution of 2-furaldehyde diethyl acetal (500µL, 2.9 mmol) in diethyl ether (3 mL) cooled to -78°C, *n*-butyllitium (3.9 mmol) was added under Ar atmosphere and the solution was stirred at -78°C for 1h and then for 3 h at 20°C. The solution was cooled again to -78°C and tributyltin chloride (1 mL, 3.9 mmol) was added dropwise; stirring for 2 h at -78°C and 12 h at room temperature.

The reaction was quenched by adding aqueous NH_4Cl and product extracted with diethyl ether. The organic layer was dried over Na_2SO_4

and concentrated *in vacuo*. The crude product was used without any further purification (brown oil, R_f = 0.74, AcOEt/hexane 0.2:9.8).

6-bromoquinazolin-4-ol (2)

To a solution of 2-ammino-5-bromobenzoic acid (5 g, 23.1 mmol), formamide (15 mL, 377.3 mmol) was added and the mixture was refluxed under Ar atmosphere for 16 h. Then 6 mL of water were carefully added (warning! boiling solution) and product immediately precipitated as a white solid. The mixture was then cooled at RT and 12 mL of water were added slowly. The mixture was stirred 30 min and the resulting precipitate was filtered and re-crystallized from cold ethanol obtaining product (white solid, yield: 53%, R_f = 0.51, AcOEt/ petroleum ether 9.4:0.6)

^{1}H-NMR (400 MHz; $CDCl_3$/CD_3OD 8:1) δ = 8.34 (d, 1H, J = 2 Hz), 7.97 (s, 1H), 7.83 (dd, 1H, J = 8.7 Hz, J = 2.3 Hz), 7.55 (d, 1H, J = 8.7 Hz).

6-bromo-N-(4-morpholinophenyl)quinazolin-4-amine (3)

To a solution of 6-bromoquinazolin-4-ol (2.84 g, 12.6 mmol) in DMF (0.84 mL) thionyl chloride (28 mL) was added and the mixture was refluxed for 5 h. The solvent was evaporated *in vacuo* by adding some toluene (3 x 28 mL) to completely remove thionyl chloride. The residue was dissolved in CH_3CN (38 mL), 4-morphoaniline (2.48 mg, 13.9 mmol) was added and the mixture was refluxed for 72 h. Crude product precipitated upon cooling the solution and the solid precipitate was recrystallized from diethyl ether obtaining pure product (orange solid, yield: 79%, R_f = 0.37, AcOEt).

^{1}H-NMR (400 Mhz, DMSO-d_6) δ = 11.30 (bs, 1H, NH), 9.04 (bs, 1H), 8.87 (s, 1H), 8.19 (bd, 1H, J = 8.8 Hz), 7.79 (d, 1H, J = 8.8 Hz), 7.57-7.04 (AA'XX' system, 4H, J = 9.0 Hz), 3.74 (m, 4H), 3.14 (m, 4H).

6-(5-(diethoxymethyl)furan-2-yl)-N(4-morpholinophenyl)quinazolin-4-amine (4)

To a solution of 6-bromo-N-(4-morpholinophenyl)quinazolin-4-amine (**3**) (300 mg, 0.78 mmol) in dry THF (7 mL), tributyl-(5-(diethoxymethyl)furan-2-yl)stannane (**1**) (1.4 g, 3.1 mmol) and bis(triphentlphosphine) palladium dichloride (44 mg, 0.06 mmol) were added. The reaction mixture was heated to reflux for 25 h under Ar atmosphere. The solvent was evaporated *in vacuo*, the crude product was dissolved in AcOEt and washed with water and brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The crude product obtained was purified with flash chromatography on silica gel (AcOEt/exane 8:2) obtaining pure product (yellow solid, yield 74%, R_f = 0.24, AcOEt /petroleum ether 9.4:0.6).

^{1}H-NMR (400 MHz, $CDCl_3$) δ= 8.65 (s, 1H), 8.20 (d, 1H, J = 1.5 Hz), 7.97 (dd, 1H, J = 8.8 Hz, J=1.5 Hz), 7.84 (d, 1H, J = 8.8 Hz), 7.56-6.93 (AA'XX' system, 4H, J = 8.5 Hz), 6.70 (d, 1H, J = 2.7 Hz), 6.53 (d, 1H, J = 2.7 Hz), 5.57 (s, 1H), 3.87 (m, 4H), 3.60 (m,4H), 3.13 (m, 4H), 1.25 (m, 6H).

^{13}C-NMR (400 MHz, $CDCl_3$) δ = 158.02, 155.05, 152.70, 151.97, 149.30, 148.73, 130.24, 129.03, 128.89, 128.48, 124.21, 116.19, 115.31, 114.91, 110.71, 106.86, 96.27, 66.88, 61.54, 49.51, 15.15.

ESI-MS: calculated 474.34, found: 475.21 [M+H^+]; 497.21 [M+Na^+].

5-(4-((4-morpholinophenyl)amino)quinazolin-6-yl)furan-2-carbaldehyde (5)

To a solution of 6-(5-(diethoxymethyl)furan-2-yl)-*N*-(4-morpholinophenyl)quinazolin-4-amine (**4**) (87 mg, 0.18 mmol) in THF (8 mL), HCl 2M (1.6 mL) was added and the mixture was reacted for 2 h at room temperature. The reaction mixture was basified to pH 8 with NaOH 2M. Organic solvent was evaporated *in vacuo* and the aqueous residue was extracted with AcOEt. The organic layer was dried over Na_2SO_4 and concentrated *in vacu*o. Product (orange solid, yield 98%, R_f = 0.61, AcOEt /MeOH 9:1) was used without any further purification.

^{1}H-NMR (400 MHz, CDCl3) δ = 9.60 (s, 1H), 8.65 (1H, s), 8.41 (1H, d, J =1.3 Hz), 8.01 (dd, 1H, J= 8.8 Hz, 1.5 Hz), 7.85-7.57 (AA'XX' system, 4H, J = 8.8 Hz), 7.32 (d, 1H, J = 3.7 Hz), 6.92 (m, 3H), 3.82 (t, 4H, J = 4.8 Hz), 3.11 (t, 4H, J = 4.8 Hz).

^{13}C-NMR (400 MHz, $CDCl_3$) δ = 177.21, 158.30, 156.05, 152.12, 150.55, 148.81, 130.03, 129.35, 129.31, 126.35, 124.28, 117.92, 116.03, 115.47, 108.67, 66.84, 49.39.

ESI-MS: calculated 400.15, found: 401.13 [$M+H^+$].

(5-(4-((morpholinophenyl)amino)quinazolin-6-yl)furan-2-yl)methanol (6)

To a solution of 5-(4-((4-morpholinophenyl)amino)quinazolin-6-yl)furan-2-carbaldehyde (**5**) (140 mg, 0.35 mmol) in dry CH_2Cl_2 (5mL) and AcOH glacial (0.3 mL), sodium triacetoxyborohydride (148 mg, 0.70 mmol) was added. The solution was reacted for 3 h at room temperature. Fresh CH_2Cl_2 was added and washed with water and brine. Aqueous layer was washed again with fresh AcOEt. The

combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. The crude product obtained was purified with flash chromatography on silica gel (AcOEt) obtaining pure product (yellow solid, yield 35%, R_f = 0.53 AcOEt/MeOH 9:1).

^{1}H-NMR (400 MHz, DMSO-d_6) δ = 9.84 (s, 1H), 8.74 (d, 1H, J = 1.4 Hz), 8.44 (s, 1H), 8.11 (dd, 1H, J = 8.8 Hz, J = 1.4 Hz), 7.74-7.61 (AA'XX' system, 4H, J = 8.8 Hz), 7.03 (d, 1H, J = 3.2 Hz), 6.98 (d, 2H, J = 8.8 Hz), 6.48 (d, 1H, J = 3.2 Hz), 5.32 (t, 1H, J = 5.7 Hz), 4.50 (d, 2H, J = 5.7 Hz), 3.74 (t, 4H, J = 4.7 Hz), 3.09 (t, 4H, J = 4.7 Hz).

^{13}C-NMR (100 MHz, DMSO-d_6) δ= 157.80, 156.04, 154.53, 151.79, 148.88, 147.90, 130.79, 128.36, 128.09, 124.14, 116.59, 115.43, 115.07, 109.59, 107.65, 66.13, 55.84, 48.81.

ESI-MS: calculated, 402.17, found: 403.19 [$M+H^+$].

5. Protocol S2. Construction and assays of HCV replicons harboring putative resistance mutations

The mutants pFKi341-PiLuc-NS3-3'/ET-DLD (E212D, P299L, V388D in NS5A) and pFKi341-PiLuc-NS3-3'/ET-FAG (L199F, V362A, S390G in NS5A) were generated starting from pFKi341-PiLuc-NS3-3'/ET (provided by V. Lohman). Two synthetic fragments (737 or 767 bp, respectively) containing the indicated triple mutations were provided from MWG-Eurofins. Using Crossover PCR-based method SalI-BamHI fragments carrying mutations were obtained and transferred into pFKi341-PiLuc-NS3-3'-ET backbone. The mutant pFKi341-PiLuc-NS3-3'-ET-PPA (T200P, S370P in NS5A and S76A in NS5B) was obtained using Multi Site-Directed Mutagenesis Kit (Agilent Technologies). In

particular, the MluI-SpeI fragment of pFKi341-PiLuc-NS3-3'-ET was subcloned into pCR2.1 vector, mutated according to manifacturer's protocol and transferred back into the pFKi341-PiLuc-NS3-3'-ET backbone. All numbers refer to aminoacid position of HCV proteins (HCV Con-1; EMBL database accession number AJ238799).

The pFKi341-PiLuc-NS3-3'/ET construct and the mutants pFKi341-PiLuc-NS3-3'/ET-DLD, pFKi341-PiLuc-NS3-3'/ET-FAG and pFKi341-PiLuc-NS3-3'/ET-PPA were linearized at the 3' end of the viral genome by ScaI digestion and purified by Sodium Acetate precipitation. RNA transcripts were generated and purified by MEGAscript T7 kit (Ambion) according to the manufacturer's protocol. 2x106 Huh7.5 cells were electroporated with 10 µg of each RNA construct in a final volume of 200 µl as described previously[48]. Electroporated cells were plated at the density of 7.5x103 cells/well in 96-well plates. 6 hours after electroporation, cells were treated with serial dilutions of AL-9. After three days of treatment, cell viability was measured by CellTiter-Blue® (Promega) and luciferase activity was measured by Bright-Glo™ Luciferase Assay System (Promega), according to the manufacturer's protocols.

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CHAPTER III

NS5A Inhibitors Interfere with NS5A-PI4KIIIα Complex Formation and Cause a Concomitant Decrease of PI4P and Cholesterol in the HCV Membranous Web

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A. Abstract

The hepatitis C virus (HCV) nonstructural (NS) protein 5A is a multi-functional protein that plays a central role in viral replication and assembly. Antiviral agents directly targeting NS5A are currently among the most potent antivirals in clinical development. Although the elucidation of the mode of action (MOA) of these HCV inhibitors has been the focus of intensive research, the precise MOA is still enigmatic. In this present work we confirm that downregulation of NS5A hyperphosphorylation is a phenotypic marker associated with the action of NS5A inhibitors belonging to different chemotypes. NS5A is known to recruit the type III phosphatidylinositol 4-kinase α (PI4KIIIα) to the HCV-induced membranous web in order to generate the phosphatidylinositol-4 phosphate (PI4P) at the site of replication. We demonstrate that NS5A inhibitors interfere with the NS5A-PI4KIIIα complex formation, resulting in a significant reduction of PI4P and cholesterol within the HCV-induced intracellular membranous structures. The same decrease of PI4P- and cholesterol level was also obtained upon treatment with a PI4KIIIα-targeting inhibitor, in this case not by interfering with NS5A- PI4KIIIα complex formation but by directly inhibiting the PI4KIIIα enzymatic activity. In addition, both classes of inhibitors, NS5A- as well as PI4KIIIα inhibitors, cause the formation of large NS5A-containing clusters. Based on our findings we propose that interference with the NS5A-PI4KIIIα complex formation can be considered an important component of the MOA of NS5A

inhibitors and that the decrease of PI4P together with cholesterol and the formation of NS5A-containing clusters are consequences of the inhibition of NS5A- PI4KIIIα complex function.

B. Introduction

The hepatitis C virus (HCV) is an important human pathogen that is believed to infect as many as 2% of the world's population [1] and currently represents a major cause of end-stage liver disease and liver cancer [2]. Traditionally, the standard treatments of chronic hepatitis C have been based on regimens including combinations of interferon-α (IFN) and ribavirin (RBV). While regimens based on the IFN/RBV backbone have been gradually improved in terms of patients cure rates, they remain poorly tolerated and contraindicated in a high number of patients. The recent advent of direct acting antivirals (DAAs) is radically transforming the treatment scenario for patients with chronic hepatitis C. These new drugs, several of which have reached regulatory approval, offer the promise of well-tolerated, interferon-free, oral regimens that are able to cure the majority of infected patients [3].

For the last two decades, the effort to identify DAAs has focused primarily on inhibitors of the NS3/4A protease and the NS5B polymerase. This is because these enzymes are readily recapitulated in biochemical assays that facilitate screening and evaluation of lead

candidates. More recently, however, the clinical validation of NS5A inhibitors [4] has generated increasing interest in this target class, as reflected by the increasing number of NS5A-targeting compounds currently undergoing clinical evaluation [5]. Strikingly, while it appears very likely that NS5A inhibitors will form a component of future interferon-free drug regimens, the exact mechanism of antiviral action of NS5A inhibitors is unknown [6].

HCV NS5A is a zinc-containing phosphoprotein with pleiotropic functions, including roles in viral replication and assembly, as well as complex interactions with cellular factors. In HCV infected cells, NS5A and the other HCV non-structural proteins are invariably found in association with cholesterol-enriched virus-induced intracellular membranes termed the "membranous web" (MW). The MW is a specialized membranous compartment, derived from the endoplasmic reticulum, that harbors the viral replication sites [7]. NS5A is expressed as basally- and hyper-phosphorylated forms (p56 and p58, respectively) [8]. The differentially phosphorylated forms have distinct functions in the regulation of HCV RNA replication versus particle production [9]. The NS5A protein structure consists of three domains: Domain I (aa 1-213), Domain II (aa 250-342) and Domain III (aa 356-447). While Domains II and III have not been structurally characterized, the crystal structure of Domain I has been crystallized in alternative dimer forms [10, 11] both containing zinc- and RNA-binding motifs.

NS5A has been shown to interact with a variety of host proteins (Macdonald and Harris, 2004), including a critical interaction with phosphatidylinositol 4-kinase IIIα (PI4KIIIα), a cellular lipid kinase that

is absolutely required for HCV replication [12-15]. This lipid kinase is normally localized to the ER and to the plasma membrane, and is responsible for phosphorylating the 4-position of the inositol ring in phosphatidylinositol to generate phosphatidylinositol 4-phosphate (PI4P). In HCV infected cells, PI4KIIIα is recruited to the HCV MW by a direct interaction with NS5A [16, 17]. By means of PI4KIIIα recruitment, HCV replication is associated with generally increased intracellular PI4P levels and – specifically – with enrichment of PI4P at the MW [18]. Very recently, Wang et al [19] demonstrated that PI4KIIIα and its PI4P product are implicated in recruiting oxysterol-binding protein (OSBP) to the MW. OSPB, in turn, appears to be critically implicated in regulating the transport of cholesterol to the membranous web. In summary, PI4KIIIα and PIP4 appear to be indirectly coupled to cholesterol enrichment of the MW.

Not being associated to any measurable enzymatic activity, NS5A was considered "not druggable" for a very long time. In recent years, however, compounds acting on NS5A have emerged as efficacious inhibitors of HCV replication. The first NS5A inhibitors were discovered by a phenotypic screen based on the genotype 1b replicon system [20, 21]. The initial lead compounds had moderate potency and narrow anti-HCV activity, mainly on genotype 1b. Subsequent medicinal chemistry efforts [21] resulted in the design of extremely potent compounds characterized by a very peculiar, highly symmetrical dimeric structure (reviewed [22]). The most studied of these "palindromic" NS5A inhibitor class is daclatasvir (DCV, formerly BMS-790052) [23], a highly optimized biphenyl derivative inhibitor for which regulatory approval is currently

being seeked (http://news.bms.com/press-release/daclatasvir-marketing-authorization-application-treatment-chronic-hepatitis-c-validate).

As of today, the precise mechanism of action of NS5A-inhibitors is not fully understood. They were initially claimed to be NS5A inhibitors mainly based on the selection of specific resistant mutations that mapped in NS5A Domain I [20, 21]. In particular, changes corresponding to variants of NS5A Tyr93 (Y93H/C/N) were found by different groups to be the most common mutations conferring broad resistance to this class of antivirals. Interestingly, Tyr93 is found near the protein dimer interface, leading to the speculation that NS5A inhibitors might act by modulating NS5A monomer/dimer equilibrium [20]. However, the palindromic topology of DCV and related compounds rather suggests a binding interaction with NS5A in which the inhibitor interacts across the dimer interface, making simultaneous contacts to both protein monomers [22]. This could at least partly explain the extraordinary potency observed for the palindromic inhibitor series. Among the reported properties of NS5A inhibitors are the ability to inhibit NS5A hyperphosphorylation [21] and to cause the redistribution of NS5A from the HCV membranous web to different sub-cellular localizations, thus impeding the formation of new replication complexes [24, 25].

We show in this report that NS5A inhibitors, regardless of the chemotype, interfere with the accumulation of phosphatidylinositol 4-phosphate (PI4P) and cholesterol in HCV-induced intracellular membranes. This end-effect is achieved by interfering with the formation of a protein complex between HCV NS5A and PI4KIIIα. Our

data provide novel insight into the molecular mechanisms underlying the clinical success of this new class of DAAs.

C. Results

1 Chemically diverse NS5A inhibitors block NS5A hyperphosphorylation

In order to investigate the mechanism of action (MOA) of NS5A inhibitors, we used the compounds summarized in Fig 1. These were selected to include Daclatasvir (BMS-790052) as prototype of the highly symmetrical, palindromic bis-phenylimidazole chemotype, and piperazinyl-N-phenylbenzamides as examples of early" leads unrelated to Daclatasvir.

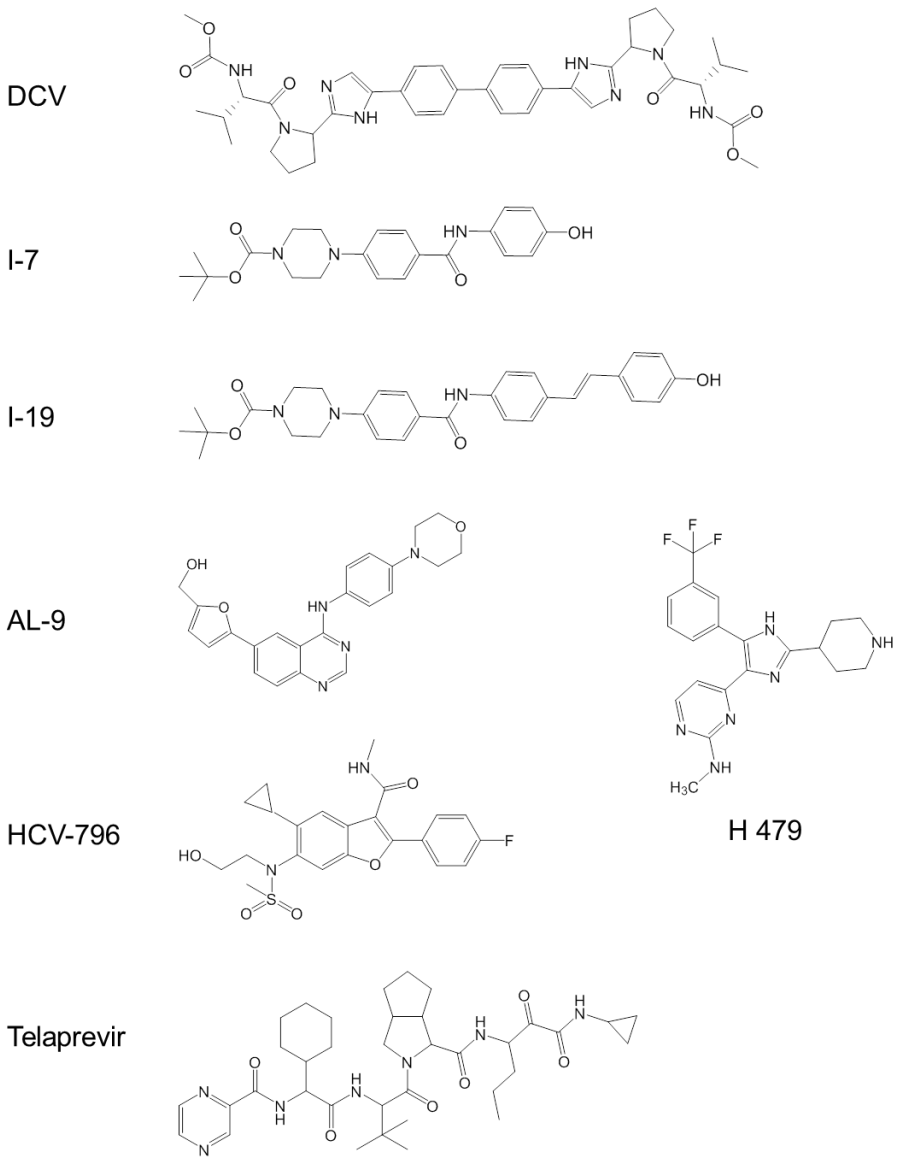


Fig. 1 Chemical structures of compounds used in this study.

Piperazinyl-N-phenylbenzamides were among the first HCV replication inhibitors proposed to target the HCV NS5A protein [20]. This chemical class of HCV inhibitors was identified in a screening effort using the Con1 HCV subgenomic replicon system. Key-mutations conferring resistance to this inhibitor class are A92V, Y93H and R157W [20, 34]. The Y93H mutation was identified to be crucial for resistance to another class of HCV inhibitors with a thiazolidinone core structure [35]. The fact that the mutation Y93H confers cross-resistance to both, piperazinyl-N-phenylbenzamides as well as DCV, suggests that both classes of HCV inhibitors, even though chemically distinct, act via a common mechanism of action. Table 1 summarizes the potency of two representative members of the piperazinyl-N-phenylbenzamides class of HCV inhibitors, namely I-7 and I-19 on Con1-replicon (Huh7.5-10A cell line) containing the adaptive mutations E176G in NS3 and insertion of lysine after V67 (K@67) in NS5A [26] and their respective fold-resistance in the presence of the mutation Y93H**.** These compounds are inactive on genotype 1a and 2a (data not shown). In addition, we studied the effect of the Y93H mutation on HCV-796 [36], an HCV RNA polymerase inhibitor, as an unrelated control, and compound AL-9, which targets an essential NS5A-interacting protein, the cellular kinase PI4KIIIα [29]. Mutation Y93H confers a 40-fold resistance to DCV, whereas a 15-fold and a 90-fold resistance to compounds I-7 and I-19, respectively. As expected, potency of HCV-796 as well as AL-9 does not change in the presence of the Y93H mutation.

HCV inhibitors	Huh7.5-10A EC_{50} (μM)	Huh7.5-10A-Y93H EC_{50}(μM)	Fold resistance
Daclatasvir (BMS-790052)	0.0000123± 0.000001	0.0005 ± 0.00003	40
I-7	0.19 ± 0.007	3 ± 0.36	15
I-19	0.04 ± 0.03	3.6 ± 3.1	90
HCV-796	0.015± 0.007	0.014 ± 0.002	0.8
AL-9	0.8 ± 0.23	1.48 ± 0.24	1.8

Table 1. List of EC50 values and fold-resistance of Y93H mutation for different HCV inhibitors in HCV genotype 1b.

Huh7.5-10A or Huh7.5-10A-Y93H cells were treated with the indicated compounds for three days and intracellular viral RNA was measured by real time PCR. The data are representative for at least three independent experiments and are presented as averages ± SD.

One of the first described effects of NS5A inhibitors, including early thiazolidinone leads as well as the related drug Daclatasvir, is the inhibition of NS5A hyperphosphorylation [21, 24]. In order to find out whether the chemically unrelated piperazinyl-N-pheylbenzamide NS5A inhibitors also affected NS5A hyperphosphorylation, we set up experiments that allows detection of the different phosphorylated forms of NS5A (Fig. 2). To avoid confounding effects of replication inhibition by the compounds we expressed the Con1 nonstructural proteins from NS3 to NS5B using the Vaccinia T7 infection/transfection system. Cells were treated for 8 hours in the presence of 20 x EC_{50} of the indicated compounds (Fig. 2A). DCV was added as positive control,

whereas the HCV polymerase inhibitor HCV-796 was added as a non NS5A-targeted inhibitor of HCV replication. In addition, we also used AL-9, which has previously been shown to positively influence NS5A phosphorylation [37]. Fig. 2A shows that DCV as well as I-7 and I-19 – but not AL-9 or HCV-796 - inhibit the formation of hyperphosphorylated form of NS5A (NS5A-p58).

In order to assess whether the inhibition of NS5A hyperphosphorylation correlates with ability of NS5A inhibitors to inhibit HCV replication, the effect of the compounds on NS5A phosphorylation was tested on a polyprotein containing the Y93H NS5A-inhibitor resistance mutation (Fig. 2B). The cDNA constructs used for this experiment harbored an adaptive mutation represented by the insertion of a lysine at position of NS5A (K@67, [26]), The K@67 HCV polyprotein was expressed with the Vaccinia T7 infection/transfection system as described above. Cells were incubated with DCV or I-7 at 20 x EC_{50}. Both compounds inhibited the production of NS5A-p58 (compare DMSO control lane 1 with lanes 2 or 3), as already demonstrated for the wt-sequence of NS5A (Fig. 1A). Conversely, the Y93H mutations in NS5A conferred resistance against inhibition of hyperphosphorylation by these compounds (lanes 6-8). Interestingly, the ratio between p58/p56 decreases in the presence of Y93H (compare lanes 1 and 6). Compound H-479 inhibits CKIα, a cellular kinase known to be important for NS5A hyperphosphorylation [27]. This agent was used in this experiment as positive control for inhibition of NS5A hyperphosphorylation. As expected, the inhibition NS5A hyperphosphorylation observed with H-479 was maintained in the

presence of the Y93H resistance mutation (compare lane 4 and 9). We also tested the effect of AL-9 on NS5A phosphorylation with or without Y93H mutation. A clear increase of the p58/p56 ratio upon incubation with AL-9 could be observed, as already described (compare lane 1 with lane 5, [37]). This increase however, was not maintained in the presence of the mutation Y93H (compare lanes 5 and 10). All together, inhibition of NS5A hyperphosphorylation is observed using chemically diverse NS5A inhibitors, such as DCV or the piperazinyl-N-pheylbenzamide class of inhibitors. Such inhibition is absent in the presence of the Y93H resistance mutation, indicating that it presumably is a direct consequence of the inhibitor-NS5A interaction.

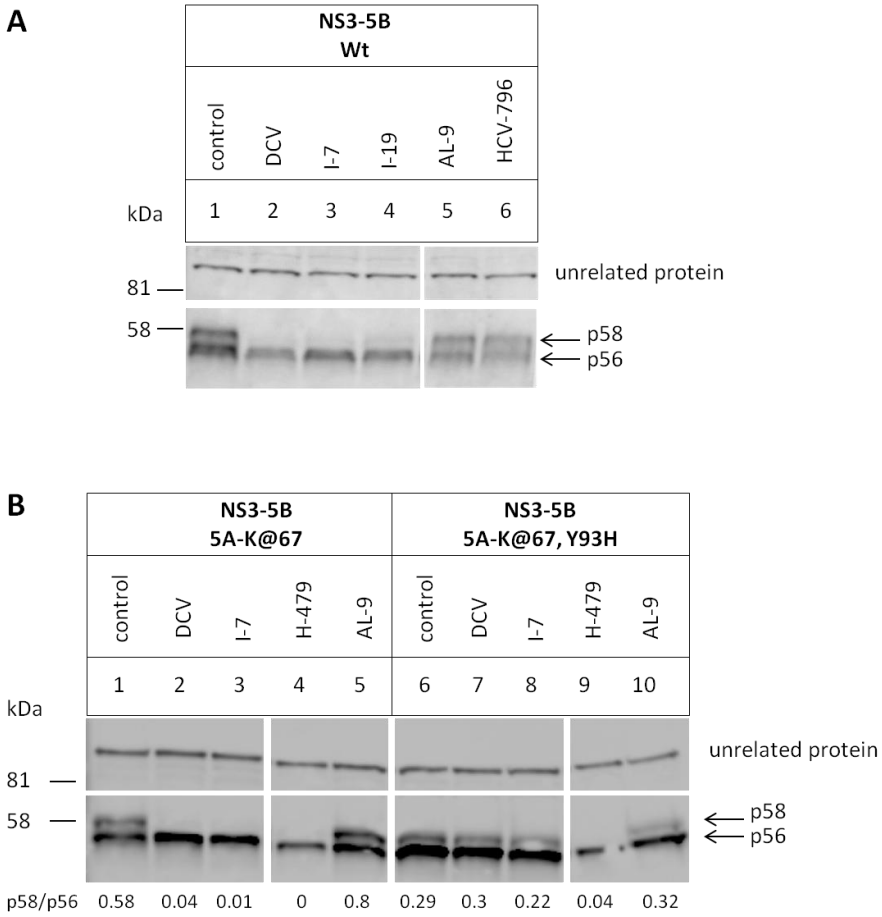


Fig. 2 Chemically diverse NS5A inhibitors block NS5A hyperphosphorylation.

10A-IFN cells were transfected with the construct pCD-BlaRep-Wt (NS3-5B Wt) (A) or pCD-BlaRep-K@67 (NS3-5B 5A-K@67) or pCD-BlaRep-K@67-Y93H (NS3-5B 5A-K@67, Y93H) (B) and Con1 HCV polyprotein from NS3 to NS5B was expressed using the Vaccinia T7-infection/transfection system. K@67 indicates the insertion of lysine at position 67 in NS5A as adaptive mutation. Cells were incubated for 8 hours with 0.2% of DMSO as control or the indicated compounds at 20 x EC_{50}: DCV: 250 pM, I-7: 4 µM, I-19: 1 µM, AL-9: 8 µM, HCV-796: 0.2 µM, H-479: 8 µM. Total cell lysates were prepared and an equal amount of protein was separated by SDS-PAGE. NS5A was detected by Western blot analysis using an anti-NS5A rabbit antibody. The molecular weight markers are indicated on the left, the positions of NS5A-p58 and -p56 are indicated on the right.
Numbers at the bottom of (B) indicate the ratio between NS5A-p58 and NS5A-p56 quantified by densitometric analysis with MultiGauge software.

2 NS5A inhibitors promote formation of “large cluster” NS5A phenotype

NS5A is part of the viral RNA replication complex and localizes to the HCV-induced membranous web (MW), which derives from the ER [38-40]. Immunofluorescence microscopy revealed NS5A localization in small punctuate structures distributed throughout the cytoplasm [25]. NS5A inhibitors have previously been shown to alter subcellular distribution of NS5A, although different investigators reported different findings (see discussion and [25, 41]). In order to assess whether chemically diverse NS5A inhibitors induce similar sub-cellular redistribution of NS5A, we performed immunofluorescence microscopy using Huh7.5 cells containing the 1b replicon described above (Huh7.5-10A). We treated the cells with DCV or piperazinyl-N-phenylbenzamide I-7. As control compounds we added AL-9, NS5B inhibitor HCV-796 and the NS3 inhibitor telaprevir (TLV, data not shown) [42] (Fig. 3). Cells were fixed after 8 hours of treatment and NS5A was detected as described in “Materials and Methods”. Incubation with DMSO, HCV-796 or TLV did not change NS5A localization, independent of the resistance mutation at position 93 (compare left and right panels). Both NS5A inhibitors, DCV as well as compound I-7, resulted in the accretion of NS5A into large, cytoplasmic aggregates, which did not appear in the presence of the resistance mutation Y93H.

Interestingly, the formation of similar large NS5A-containing clusters has previously been reported to be one of the hallmarks of

inhibiting the action of PI4KIIIα, either by gene silencing or by pharmacological inhibition [17, 19, 29]. We confirm this finding here with the PI4KIIIα inhibitor AL-9. Importantly, while the mutation Y93H confers resistance to the NS5A inhibitors and prevents NS5A-cluster formation, it does not change significantly cluster formation of NS5A (AL-9, right panels). We can therefore conclude that the formation of large NS5A clusters, a hallmark of decreased PI4KIIIα in cells replicating HCV RNA, is also observed with NS5A-targeting agents, but not with NS3- or NS5B-targeting agents.

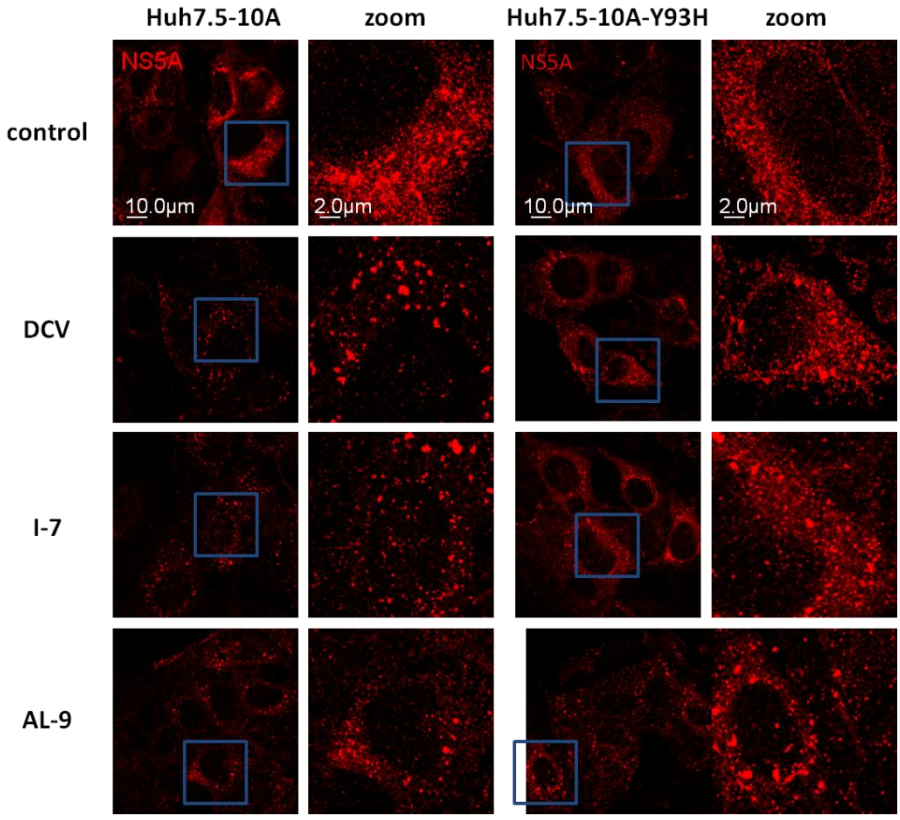
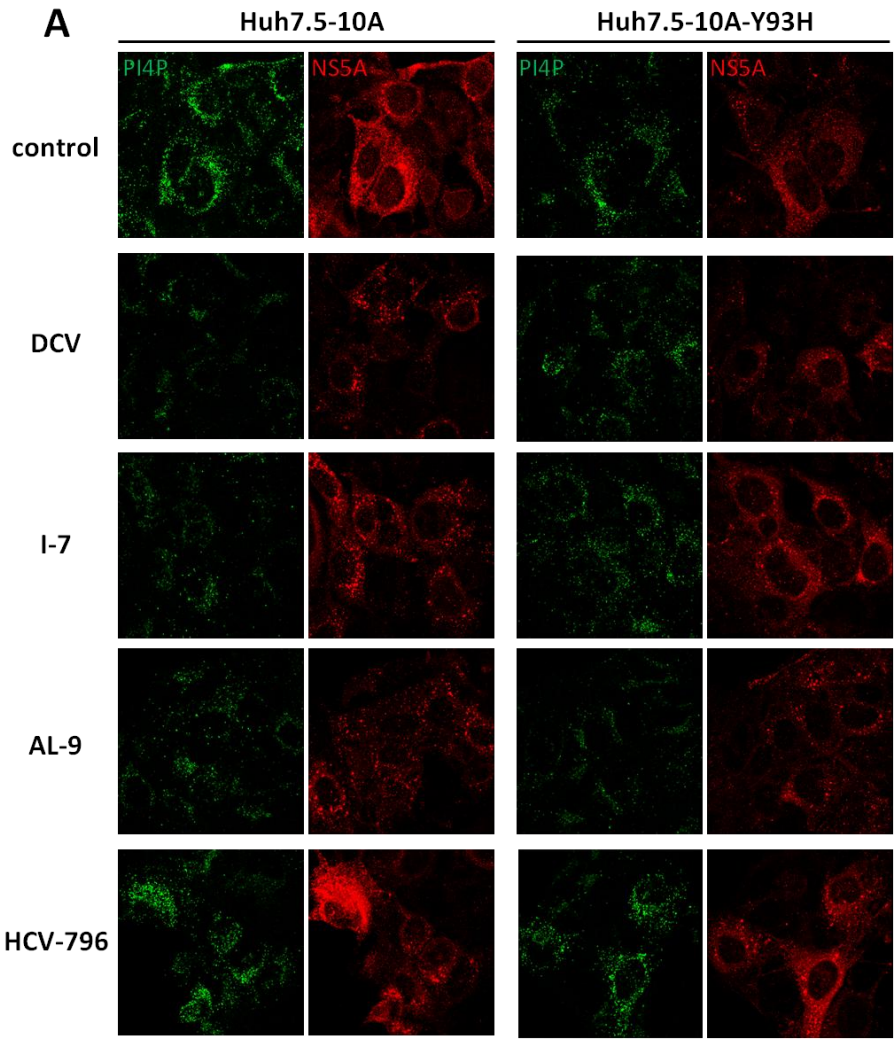


Fig. 3 NS5A inhibitors promote formation of "large cluster" NS5A phenotype. Huh7.5-10A and Huh7.5-10A-Y93H cells were treated for 8 hours with 0.2% DMSO as control or with 20 x EC_{50} of the compounds DCV, I-7, AL-9 or HCV-796 at concentrations indicated in Fig. 2. Cells were fixed and NS5A distribution (red) was assessed by immunofluorescence microscopy as reported in Materials and Methods. Boxed regions in blue are magnified 5-fold in the corresponding zoom-panel on the right.

3 NS5A inhibitors interfere with the enrichment of PI4P in HCV replication membranes

HCV replication is associated with increased total levels of intracellular PI4P and with PI4P enrichment at the MW. Acute treatment of HCV replicon cells with PI4KIIIα inhibitors causes a marked reduction of MW PI4P levels [8] eventually leading to a block viral replication. As summarized in the previous paragraph, concomitant with the reduction of PI4P, we and others observed a change of NS5A localization into large, cytoplasmic aggregates [17, 19, 29]. It is thus tempting to speculate that the formation of NS5A-positive large cytoplasmic clusters observed in the presence of NS5A inhibitors might correlate with diminished PI4P levels in the HCV replication membranes. In order to address this possibility, we set up an experiment in which Huh7.5-10A cells were treated with DCV, I-7, HCV-796 or AL-9, for 8 hours at a concentration of 20 X EC_{50}. DMSO was used as negative control. In parallel, the same type of experiment was performed using Huh7.5-10A containing the Y93H resistance mutation in NS5A (Huh7.5-10A-Y93H) (Fig. 4). After incubation, cells were fixed and NS5A as well as PI4P detected as described in "Materials and Methods". The DMSO control shows a typical staining of PI4P and NS5A in the Huh7.5-10A replicon cells (Fig.4A, left panels). This staining pattern was not altered upon incubation with the NS5B inhibitor HCV-796. As reported previously, treatment with AL-9 sharply reduced PI4P concentration associated with HCV membranous compartment (16% residual, Fig. 4B). Interestingly, a similar decrease of PI4P was also

detected in the presence of the NS5A inhibitors DCV and I-7 (26 % and 30 % for DCV and I-7, respectively). The same type of experiment was performed with Huh7.5-10A-Y93H (Fig. 4A, right panels). Baseline PI4P concentration in the Huh7.5-10A-Y93H cells is lower than in the Huh7.5-10A cells (compare DMSO left with right 58%). This might be explained, at least in part, with a lower replication fitness in the presence of the Y93H mutation (data not shown) and/or reduced NS5A - PI4KIIIα complex formation (see below). However, incubation of the resistant replicon with the NS5A inhibitors did not significantly affect PI4P concentration when compared to the own DMSO control (101% and 116% for DCV and I-7, respectively, Fig. 4B). As expected, PI4P levels remained unchanged in the presence of the control compound HCV-796 (104 %). Importantly, however, AL-9 continued to inhibit PI4P-enriched membrane formation also in the presence of the Y93H mutation (41 %).


A
Huh7.5-10A
Huh7.5-10A-Y93H
PI4P
NS5A
PI4P
NS5A
control
DCV
I-7
AL-9
HCV-796

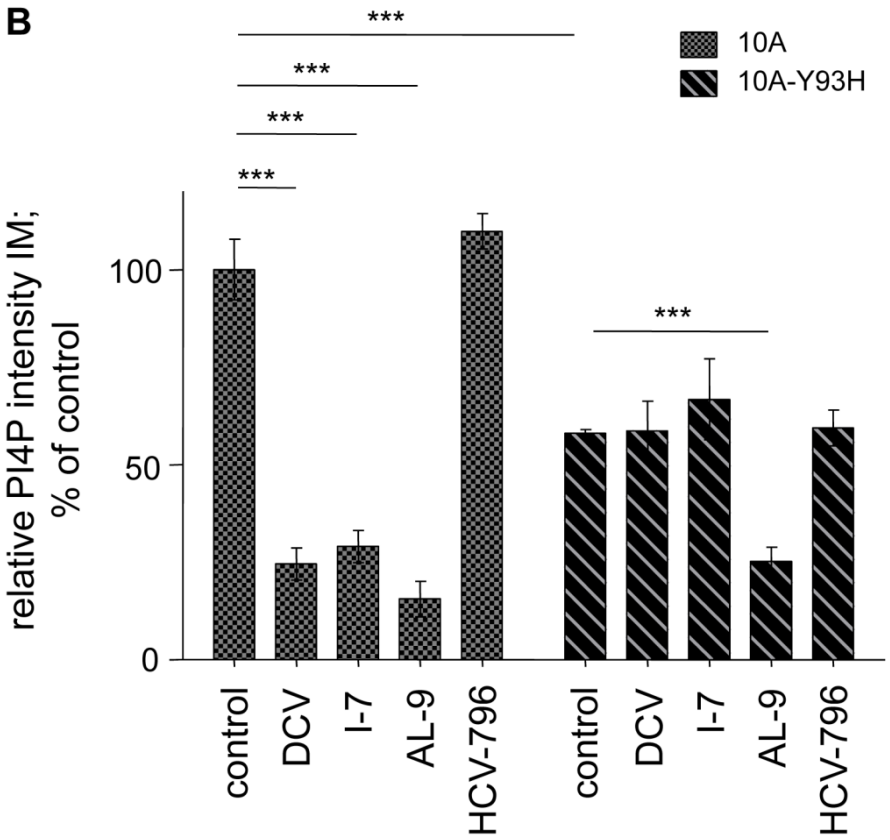


Fig. 4 NS5A inhibitors interfere with the enrichment of PI4P in HCV replication membranes.

(A) Huh7.5-10A and Huh7.5-10A-Y93H cells were treated for 8 hours with 0.2% DMSO as control or with 20 x EC_{50} of the compounds DCV, I-7, AL-9 or HCV-796 as described in Fig. 3. After treatment, PI4P present in the intracellular membranes (green) and NS5A (red) were stained for immunofluorescence microscopy as described in Materials and Methods. (B) Quantification of PI4P levels in internal membranes (IM) by immunofluorescence analysis. Changes in mean fluorescence intensity relative to the DMSO control of Huh7.5-10A are shown. 10A: Huh7.5-10A cells; 10A-Y93H: Huh7.5-10A-Y93H cells. Six randomly picked fields were analyzed for each condition. Normalization was performed as detailed in Materials and Methods. Data are presented as averages ± SD. ***, $p < 0.001$.

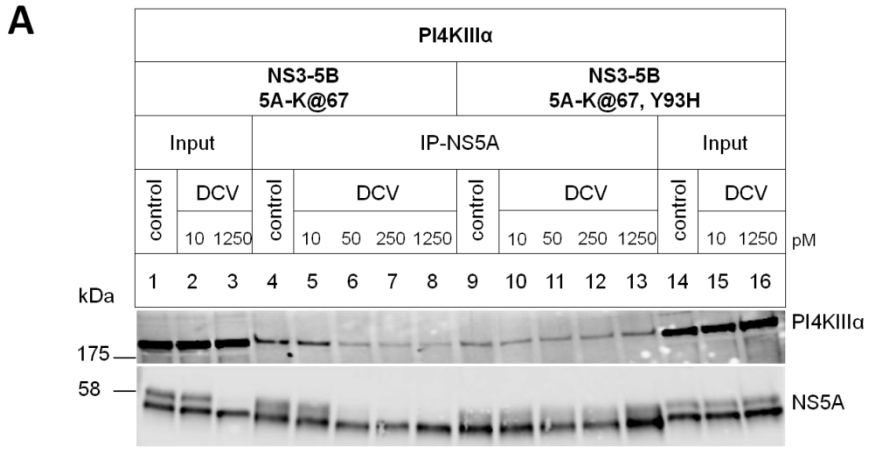
4 NS5A inhibitors interfere with the NS5A-PI4KIIIα protein complex formation

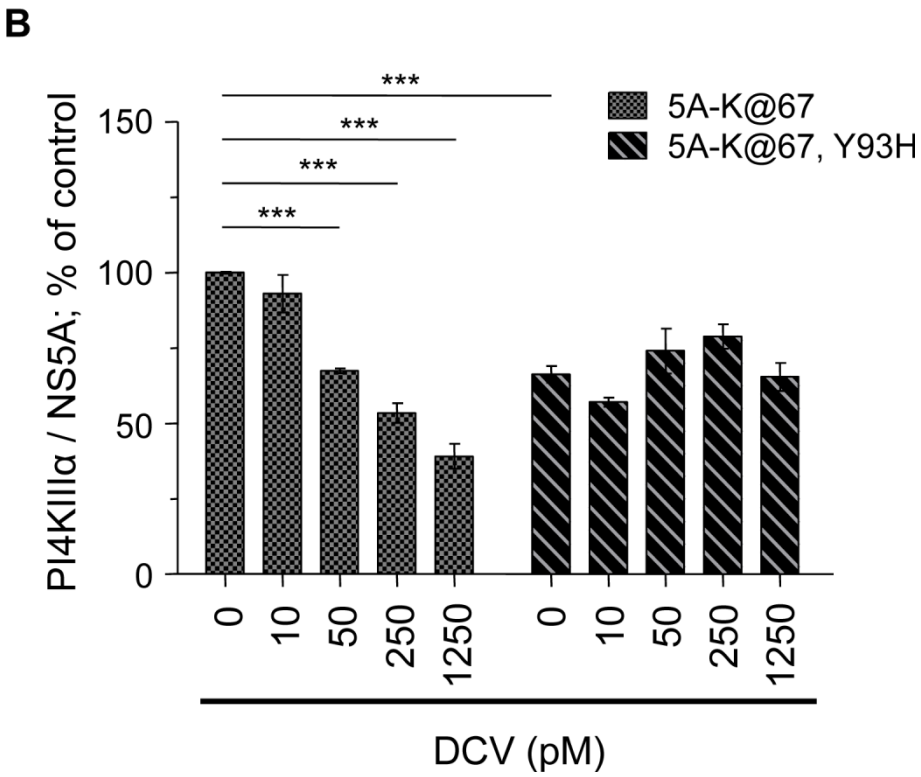
It has previously been demonstrated that NS5A recruits PI4KIIIα to the HCV MW to increase local PI4P concentration required for MW integrity and HCV RNA replication [16, 17]. One possibility to explain the observed reduction of membrane PI4P upon acute treatment with NS5A inhibitors is that these agents interfere with the NS5A-PI4KIIIα interaction, thus leading to impaired recruitment of PI4KIIIα to the MW and to decreased PI4P levels. In order to test this hypothesis, we co-expressed the Con1 polyprotein with or without the resistance mutation Y93H together with PI4KIIIα using the Vaccinia T7 infection/transfection system and treated the cells with NS5A inhibitors as described in Fig. 2. The results are shown in Fig. 5. In a first experiment, the HCV non structural proteins were expressed in the presence of increasing amount of DCV for 8 hours (Fig. 5A). Western blot analysis of total cell extracts shows that treatment with DCV did not affect HCV protein synthesis (lanes 1-3 and 14-16). At the same time, NS5A hyperphosphorylation was inhibited (compare lane 1 with lane 3) and this effect was completely abolished in the presence of the resistance mutation Y93H (lanes 14 and 16). Total protein extracts from cells treated with only DMSO (control) or with increasing concentration of DCV (lanes 4-13) were used to performed co-immunoprecipitation experiments in order to detect the NS5A-PI4KIIIα complex. NS5A was immunoprecipitated with a NS5A-specific antibody and NS5A as well as PI4KIIIα were then detected by Western blotting

with the specific antibodies indicated on the right. At a concentration of 10 pM DCV, no significant effect of DCV treatment was detectable. However, 50 pM DCV clearly reduced the level of co-immunoprecipitated PI4KIIIα (67%, lane 6). Increasing concentrations of DCV further decreased PI4KIIIα present in the immunoprecipate (39% at 1250 pM concentration DCV, lanes 5-8). The relative amounts of PI4KIIIα in the immunoprecipitate were quantified by densitometry and are shown in Fig. 5B as normalized PI4KIIIα/NS5A ratios.

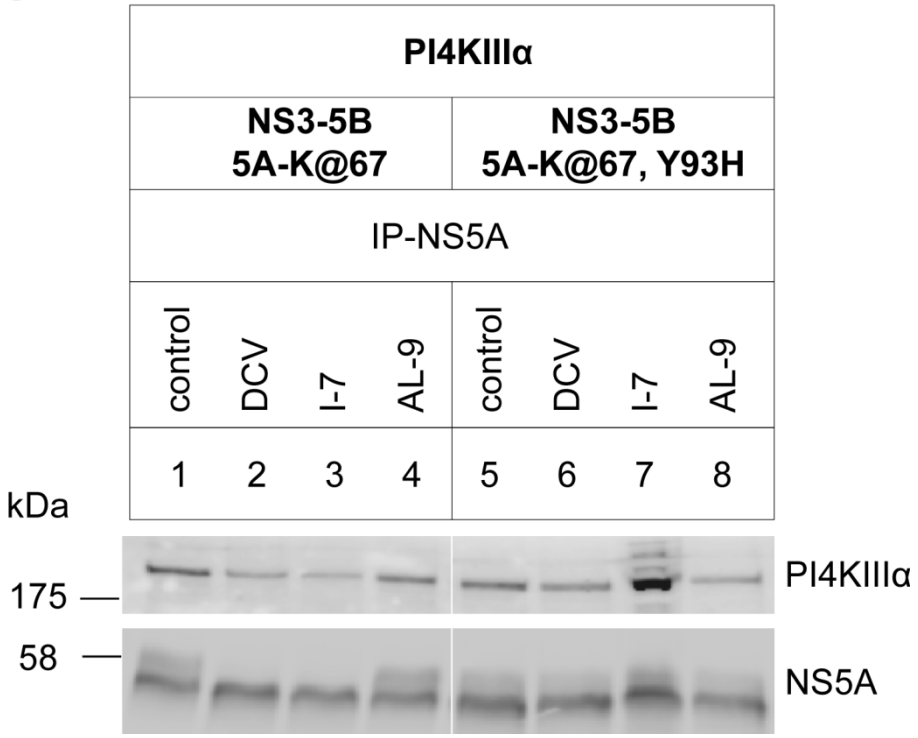
The same type of experiment was repeated with NS5A harboring the resistance mutation Y93H (NS5A-Y93H). In the DMSO-treated cells (control), PI4KIIIα could be less efficiently co-immunoprecipitated when compared to the parental NS5A sequence (66 %, compare lanes 4 and 9). However, PI4KIIIα present in the complex with NS5A is unaffected by DCV at concentrations as high as 1250 pM. This result demonstrates that DCV interferes with the formation of the NS5A-PI4KIIIα complex and that the Y93H resistance mutation renders the interaction between NS5A and PI4KIIIα insensitive to the treatment with DCV. We next asked whether a piperazinyl-N-pheylbenzamide NS5A inhibitor or PI4KIIIα inhibitor AL-9 could also interfere with the NS5A- PI4KIIIα complex formation (Fig. 5C-D). To this aim, the HCV polyproteins were expressed either without inhibitor (DMSO control) or with DCV, I-7 or AL-9. NS5A was then immunoprecipitated as described in Fig. 5A. As already described in Fig. 5A, DCV reduced PI4KIIIα present in a complex with NS5A (55 %) and a similar effect could be observed in the presence of I-7 (46 %, lane 3). Importantly, inhibition of the enzymatic activity of PI4KIIIα by AL-9 had no effect on

complex formation (103 %, lane 4), indicating that the decreased amounts of NS5A- PI4KIIIα complex observed with NS5A inhibitor are not the mere consequence of a low local PI4P concentration. In the presence of the Y93H mutation in NS5A, complex stability remains unaffected by both classes of NS5A inhibitors as well as AL-9 (lanes 6-9). Overall, these data suggest that impairment of the NS5A - PI4KIIIα complex formation is a specific effect of NS5A inhibitors and not just the consequence of a decreased local PI4P concentration, as it has been shown above for NS5A cluster formation.


A
PI4KIIIα
NS3-5B
5A-K@67
NS3-5B
5A-K@67, Y93H
Input
IP-NS5A
Input
control
DCV
10 1250
control
DCV
10 50 250 1250
control
DCV
10 50 250 1250
control
DCV
10 1250
pM
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
kDa
175
58
PI4KIIIα
NS5A


B
PI4KIIIα / NS5A; % of control
150
100
50
0
***
***
***
***
5A-K@67
5A-K@67, Y93H
0
10
50
250
1250
0
10
50
250
1250
DCV (pM)

C


PI4KIIIα
NS3-5B 5A-K@67
NS3-5B 5A-K@67, Y93H
IP-NS5A
control
DCV
I-7
AL-9
control
DCV
I-7
AL-9
1 2 3 4 5 6 7 8
kDa
175
58
PI4KIIIα
NS5A

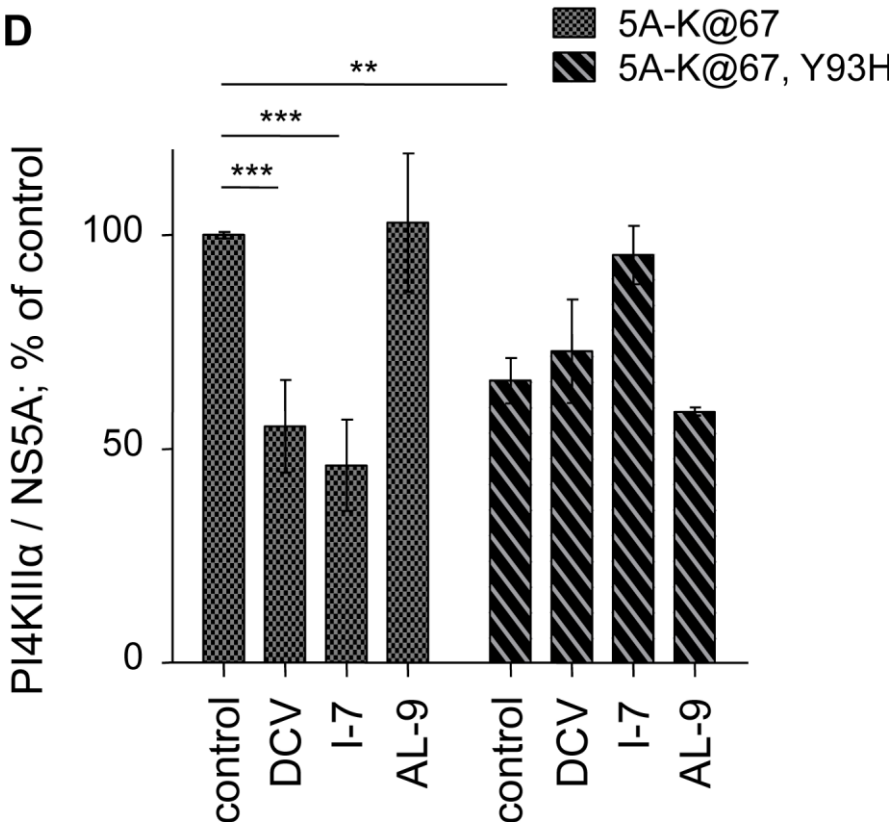


Fig. 5 NS5A inhibitors interfere with the NS5A-PI4KIIIα protein complex formation.

(A) 10A-IFN cells were co-transfected with the construct pEF1A-PIK4CA (PI4KIIIα) and pCD-BlaRep-K@67 (NS3-5B 5A-K@67) or pCD-BlaRep-K@67-Y93H (NS3-5B 5A-K@67, Y93H), respectively and HCV polyprotein was expressed together with PI4KIIIα using the Vaccinia T7-infection/transfection system exactly as described in Fig. 2. Cells were incubated for 8 hours with 0.2% of DMSO as control or 10, 50, 250 or 1250 pM of DCV. Total cell lysates were prepared (Input: lanes 1-3 and 14-16) and NS5A was immunoprecipitated as described in Materials and Methods (IP NS5A: lanes 4-13). Proteins were separated by SDS-PAGE and NS5A and PI4KIIIα were detected by Western blot analysis using specific antibodies. The molecular weight markers are indicated on the left, the positions of NS5A and PI4KIIIα are indicated on the right. (B) Quantification of the image shown in FIG 5A. 5A-K@67: NS3-5B 5A-K@67; 5A- K@67-Y93H: NS3-5B 5A-K@67-Y93H. For each sample, PI4KIIIα and NS5A were quantified by densitometric analysis with the MultiGauge software and the amount of co-immunoprecipitated PI4KIIIα together with NS5A was normalized for the quantity of NS5A. Shown are relative levels of PI4KIIIα with respect to the DMSO control of 5A-K@67. The data are representative for at least three independent experiments and present the average ± SD. ***, $p < 0.001$. (C)

The experiment was performed as described in (A). 10A-IFN cells were treated with the following compounds: control: 0.2 % DMSO, DCV: 250 pM, I-7: 4 µM or AL-9: 8 µM; Shown are proteins after immunoprecipitation of NS5A with NS5A-specific antibody (IP NS5A). The molecular weight markers are indicated on the left, the positions of NS5A and PI4KIIIα are indicated on the right. (D) The quantification of the image shown in Fig. 5C as described in (B). The data are representative for at least three independent experiments and shown as average ± SD. **, $p < 0.01$; ***, $p < 0.001$.

5 Both PI4KIIIα- and NS5A inhibitors reduce cholesterol concentration in the HCV-induced membranous web

HCV RNA replication takes place on specialized membrane compartments that are highly enriched for PI4P and contain cholesterol-rich lipid rafts [43-45]. High cholesterol content is required for efficient replication and depletion of cholesterol causes inhibition of viral replication [44, 46]. We have shown in Fig. 4 that treatment with NS5A inhibitors causes a reduction of PI4P concentration in the MW. In the next experiment, we investigated whether NS5A inhibitors also influence free cholesterol levels in the MW. Intracellular PI4P and intracellular free cholesterol were revealed by fluorescence microscopy in Huh7.5 cells containing HCV replicons of gt 1b (Huh7.5-10A) or gt 2a (JFH), or in Huh7.5 cells as control (Fig. 6A). For cholesterol staining, we used Filipin, an autofluorescent molecule that inserts into the membrane and binds free cholesterol. In parallel, PI4P present in internal cellular membranes is visualized as described before [29]. The results are described in Fig. 6A. In HCV(-) Huh7.5 cells, cholesterol is evenly distributed throughout the entire cell without any

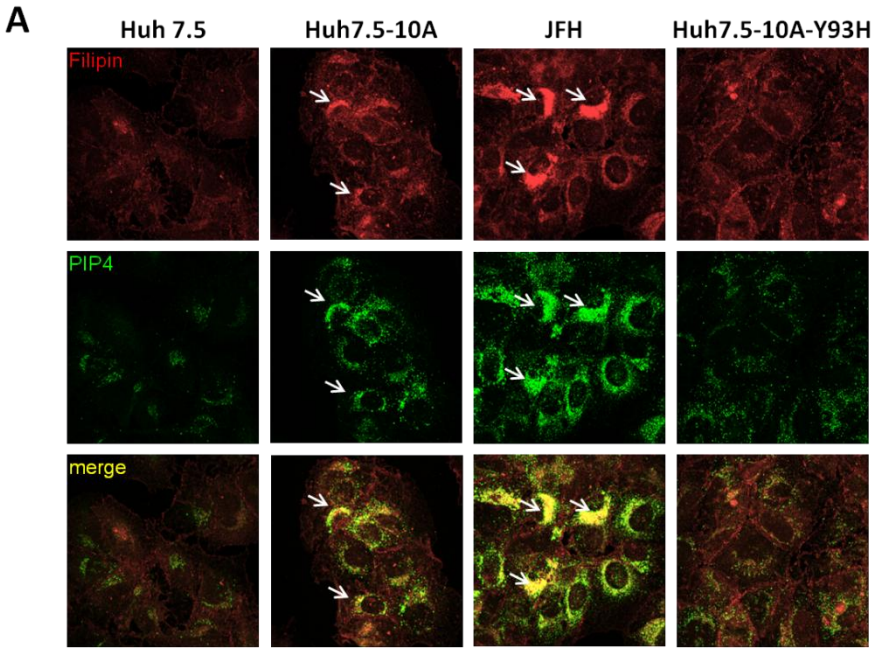
marked accumulation. The pattern is different in cells containing HCV replicons, where cholesterol accumulates in cholesterol-rich bodies and this accumulation depends on replication efficiency of HCV. As a consequence of this, cholesterol accumulation is much more evident in Huh7.5 cells expressing genotype 2a replicons compared to cells expressing genotype 1b. A similar result is obtained with the PI4P-specific staining showing an increased accumulation of PI4P the HCV-induced MW. Interestingly, cholesterol and PI4P show a high degree of colocalization (shown by the yellow colour of the merged images).

In order to follow cholesterol- and PI4P accumulation upon treatment with different HCV inhibitors, we performed the following experiment with the JFH-1 replicon containing cells. These cells were chosen because of the more intense staining pattern.

JFH-1 replicon-containg cells were plated 24 hours before the experiment and incubated with DMSO as negative control or with DCV, AL-9, TLV or HCV-796 for 8 hours. After treatment, cells were labeled for cholesterol or PI4P as described above. The results are shown in the upper portion of Fig. 6B. Importantly, as also shown before [29], RNA replication efficiency was not significantly affected after 8 hours of treatment (data not shown). DMSO treatment showed no effect on cholesterol- and PI4P accumulation. Incubation with the PI4KIIIα inhibitor AL-9, as expected, drastically reduced PI4P concentration (20 % residual, Fig. 6C) and concomitant with PI4P reduction we also observe a sharp reduction in cholesterol staining (12 %), strengthening the recently published finding that cholesterol trafficking to HCV membranous structures requires active PI4KIIIα [19]. Importantly, we

observed a similar decrease of PI4P and cholesterol upon incubation with the NS5A inhibitor DCV. PI4P was reduced to 15 % and cholesterol to 10 % (Fig. 6B-C). This effect was specific for agents that affect the NS5A/PI4KIIIα complex function (*i.e.*, maintenance of high PI4P levels in the replication compartment membranes), as the NS5B inhibitor HCV-796 and the NS3 inhibitor TLV did not change PI4P or cholesterol concentration.

To demonstrate that the observed effect on cholesterol staining is specifically mediated by the interaction of the NS5A inhibitors with the NS5A protein, we performed an experiment in order to investigate whether DCV-resistant replicons maintain baseline MW PI4P- and cholesterol levels upon incubation with the drug (Fig. 6B, bottom part). To select for a DCV-resistant population, cells containing a *Neo*-selectable JFH-1 replicon were grown for 4 weeks in the presence of appropriate amounts of G418 and 500 pM DCV. The resulting JFH-1 replicon-containing cells (termed here JFH-DCV) were 4000 fold resistant to DCV when compared to the parental JFH-1 replicon (data not shown). As already shown for cells harboring DCV-resistant genotype 1b replicon (Fig. 4A), also cells containing genotype 2a DCV-resistant replicons (JFH-DCV) presented a reduction of baseline PI4P and cholesterol (50% and 65%, respectively, Fig. 6D). However, treatment with NS5A-, NS3- or NS5B-inhibitors did not result in further decrease of PI4P or cholesterol levels. In contrast, treatment with AL-9 continued to inhibit PI4P as well as cholesterol concentration in the presence of the DCV-mutation.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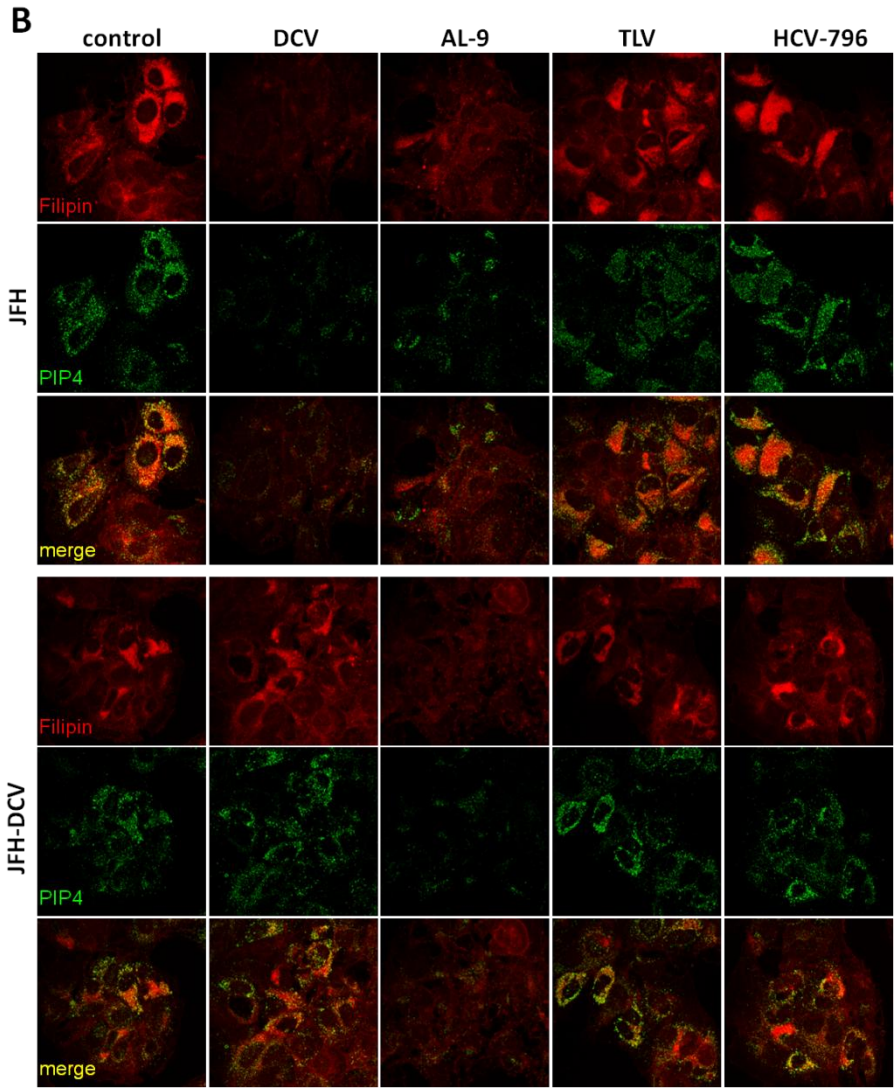
Huh 7.5
Huh7.5-10A
JFH
Huh7.5-10A-Y93H
Filipin
PIP4
merge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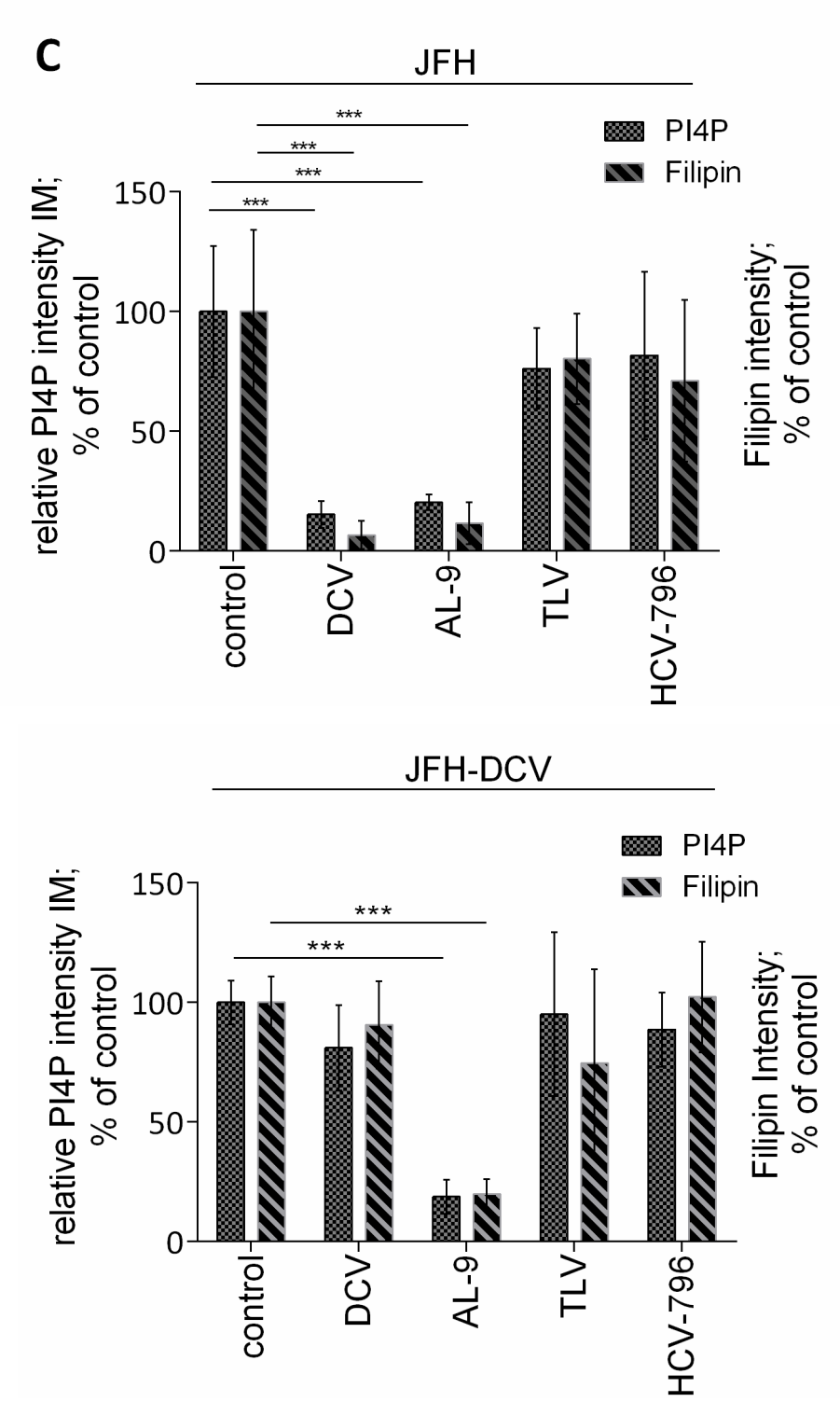
C
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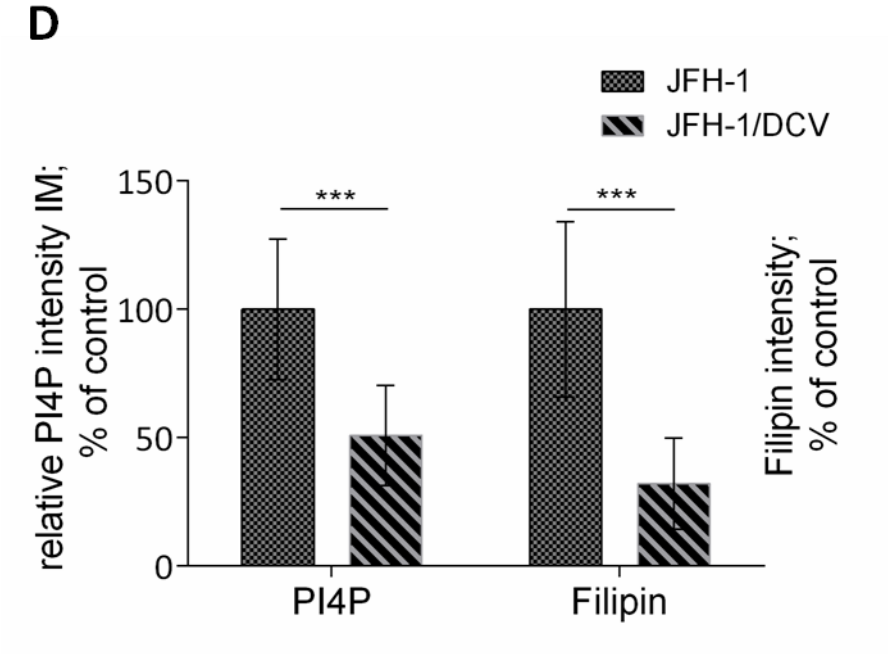

Fig. 6 Both PI4KIIIα- and NS5A inhibitors reduce cholesterol concentration in the HCV-induced membranous web.

(A) Huh7.5, Huh7.5-10A, Huh7.5-10A-Y93H and JFH cells were plated 24 hours prior to analysis by immunofluorescence microscopy. Detection of PI4P (green) and Filipin (red) was performed as detailed in Materials and Methods. The last row shows the merge of the two images. Arrows indicate regions of PI4P-Filipin colocalization. (B) JFH cells (upper panels) or JFH-DCV cells (lower panels) were incubated with the indicated compounds for 8 hours. Control: 0.2 % DMSO, DCV: 250 pM, AL-9: 8 µM, TLV: 5 µM, HCV-796: 0.2 µM. PI4P (green) and Filipin (red) were detected as described in Materials and Methods. For each condition are shown the merged images. (C) Quantification of PI4P in internal membranes (IM) and Filipin present in membranes from images shown in (B) is performed by measuring PI4P and Filipin fluorescence intensity as described in Materials in Methods. Changes in mean fluorescence intensity relative to the control are shown. Data are presented as average ± SD. ***, $p < 0.001$. (D) Quantification of PI4P in internal membranes (IM) and Filipin as described in C relative to the JFH cells. Data are presented as average ± SD. ***, $p < 0.001$.

D. Discussion

NS5A inhibitors currently include several of the most potent antiviral agents that target HCV. Although the elucidation of the MOA of these HCV inhibitors has been the focus of intensive research, the precise MOA is still enigmatic. The high efficacy of this class of compounds may be partly explained by the prediction to block at least two distinct stages of the viral life cycle, namely viral RNA replication [9] and virion assembly/secretion [47]. The aim of the work presented in this paper is that of elucidating the MOA by which NS5A inhibitors interfere with HCV RNA replication.

Currently, there are two phenotypic markers that have been associated with the MOA of some NS5A inhibitors. These include downregulation of NS5A hyperphosphorylation [21, 24] and alterations in NS5A localization at the onset of inhibition [25, 41]. Different chemotypes have been classified as NS5A inhibitors due to their profile of resistance mutations (for review see [48]). However, limited information has been obtained with regards to how these agents act at the molecular basis.

The piperazinyl-N-phenylbenzamides class of "monomeric" HCV inhibitors was tentatively reported as "NS5A inhibitors" solely based on the appearance of mutations in NS5A conferring resistance to this class of compounds [20]. These mutations included Y93H, a primary drug-induced mutation by DCV. We demonstrate here that the action of piperazinyl-N-phenylbenzamides, in this study represented by I-7 and I-19, is also associated with inhibition of NS5A

hyperphosphorylation and with altered NS5A subcellular distribution. As shown in Figure 2, compounds I-7 and I-19 efficiently inhibit NS5A hyperphosphorylation and induce NS5A relocalization into large clusters (Fig. 3). Even though alteration of NS5A subcellular localization of is a hallmark of NS5A inhibitors [25, 41], the precise distribution is variable and might depend on the replication system and the chemotype of NS5A inhibitors used . In some cases, NS5A-clusters colocalize with lipid droplets [25], a phenotype we and others did not observe ([41] and McGivern, DR et al., submitted).

In order to demonstrate that the formation of NS5A cluster is not just a consequence of replication inhibition, we used NS5B polymerase or NS3 protease inhibitors, agents which block HCV replication by completely different mechanisms. NS5B polymerase inhibitors inhibit synthesis of viral RNA by pre-formed replication complexes, while protease inhibitors prevent the formation of new replication complexes, but do not affect synthesis of viral RNA from pre-existing ones. The latter mechanism has also been proposed for NS5A inhibitors [25]. Among these HCV- replication inhibitors, only NS5A inhibitors change NS5A localization (Fig. 3) indicating that NS5A-clustering is a specific marker of targeting NS5A inhibitors. Interestingly, similar NS5A cluster formation has also been observed upon inhibition of PI4KIIIα [17, 29]. Ultrastructural analysis of HCV-replicating cells revealed that viral RNA replication takes place on heterogenous double-membrane vesicles (DMV) and multi-membrane vesicles (MMV) [39, 45, 49, 50]. Silencing or pharmacologic inhibition of PI4KIIIα induces the formation of smaller and homogenous DMV that is

believed to reflect the NS5A-positive clusters observed by immunofluorescence [17, 19, 29]. The similar phenotypes of the subcellular alterations induced by NS5A or PI4KIIIα inhibition led us to formulate the hypothesis that these two inhibitor classes interfere with the same pathway.

NS5A associates with PI4KIIIα and stimulates its enzymatic activity in order to generate PI4P at the site of replication [16-18]. Inhibition of PI4KIIIα abolishes PI4P induction and abrogated HCV replication. Similarly, NS5A with mutations in the PI4KIIIα binding site shows impaired capability to associate with PI4KIIIα, which correlates with reduced PI4P induction and reduced HCV replication. At the same time, NS5A cluster formation correlates well with impaired PI4P induction [37]. Taken all this information together, NS5A clusters observed upon treatment with NS5A inhibitors might be an indicator of shortage of PI4P in the HCV replicative membranes. We performed experiments in order to follow how treatment with NS5A inhibitors affected PI4P levels in intracellular membranes of cells harboring the HCV replicon. The results showed that NS5A inhibitors cause a reduction of PI4P comparable to the effect of the PI4KIIIα inhibitor AL-9 (Fig. 4). Thus, decreased PI4P concentration in intracellular membranes and NS5A cluster formation appear to be correlated and we propose that both effects can be considered as a consequence of inhibition of the NS5A / PI4KIIIα complex function.

These results are consistent with a mechanism by which NS5A inhibitors interfere with the protein complex normally formed between NS5A and PI4KIIIα. We demonstrate here for the first time

that treatment of NS5A inhibitors lower the amount of NS5A- PI4KIIIα complex detectable by co-immuneprecipitation (Fig. 5). Inhibition of complex formation is dose-dependent and is specific for NS5A inhibitors. It is not just a consequence of a lowering in the local PI4P concentration, as no significant effect on the complex is observed upon treatment with the PI4KIIIα inhibitor AL-9.

Binding of NS5A inhibitors to domain I of NS5A [51] could affect binding to PI4KIIIα either directly or indirectly, possibly by inducing a conformational change of NS5A that prevents productive association of PI4KIIIα. Initial binding studies with fragmented small monomeric inhibitors stated that binding appears to be necessary but not sufficient for inhibition. A second function was postulated to transmit the inhibitory potential of the compounds. Symmetrical or non-symmetrical structures of the most potent NS5A inhibitors might be required to bind across the NS5A dimer interface [51] and induce a conformational change of the NS5A dimer that finally results in failure to interact with PI4KIIIα. Dissociation of the NS5A dimer as causative reason for dissociation of PI4KIIIα from the complex is less plausible because the effect of NS5A inhibitors on NS5A dimers, if any, was suggested to promote and not prevent dimer formation [51]. In light of this latter consideration, an alternative explanation could be that it binding to PI4KIIIα requires in fact the NS5A monomer and not the dimeric form. Further work is required to address the exact stoichiometry of the NS5A- PI4KIIIα complex.

It is worth pointing out that the Y93H mutation renders NS5A resistant to NS5A inhibitors at the cost of binding efficiency of PI4KIIIα.

We observe a 40 % reduction of PI4KIIIα present in a complex with NS5A-Y93H when compared to the parental NS5A sequence (Fig. 5). This result favors the hypothesis of a conformational change in NS5A induced by the Y93H mutation that affects PI4KIIIα binding but at the same time makes NS5A refractory to the action of the inhibitor(s). Interestingly, the reduced binding capability of PI4KIIIα correlates well with reduced PI4P concentration in the MW of the DCV-resistant replicon 10A-Y93H (reduction of 42 %, Fig. 3B) and reduced replication efficiency [35] .

Many (+) strand RNA viruses stimulate PI4-phosphate kinases to generate PI4P at sites of replication. [46, 52] However, the exact role of PI4P remains unclear. PI4P-enriched membranes are targeted by lipid-transfer proteins [53, 54]. Of special interest is oxysterol-binding protein (OSBP), which has been suggested to play a functional role for HCV replication and viral release [55, 56]. OSBP localizes to PI4P-containing membranes via its pleckstrin-homology (PH) domain and regulates the membrane lipid composition by sensing and exchanging sterols and phosphoinositol phosphate with the C-terminal lipid transport domain. The relative concentrations of phosphoinositides and cholesterol within a specific membrane compartment is decisive for fluidity and curvature of the membranes [57]. The right homeostasis between these lipids might be important to provide the correct platform for the assembly of the replication complex.

High cholesterol content in the HCV replicative membranes is required for efficient replication and depletion of cholesterol causes inhibition of replication [44, 46]. It has recently been proposed that OSBP

is recruited to the HCV MW in a PI4KIIIα-dependent manner and that both factors are required for cholesterol trafficking to the MW [19]. A similar, PI4KIIIβ-dependent mechanism has been proposed very recently for poliovirus [58]. In this study we further underline the observed correlation between PI4P- and cholesterol-concentration within the MW. Both, AL-9 and DCV cause a decrease of PI4P concentration in the HCV replication-associated membranes, concomitantly with a decrease in cholesterol levels and inhibition of replication (Fig. 5). PI4P and cholesterol levels are not affected upon inhibition of HCV replication with NS3 or NS5B inhibitors, arguing that the decrease of the HCV-induced lipids is a consequence specific for the inhibition of the function NS5A / PI4KIIIα complex.

In conclusion, we confirm that downregulation of NS5A hyperphosphorylation is a phenotypic marker associated with the action of NS5A inhibitors belonging to different chemotypes. Importantly, NS5A inhibitors drastically reduce PIP4 within the HCV replication compartment membranes, apparently a consequence of the inhibition of the NS5A- PI4KIIIα complex formation. Together with the PI4P decrease, we observe a concomitant decrease of membrane cholesterol. Based on our findings we propose that interference with the NS5A - PI4KIIIα complex formation can be considered an important component of the MOA of this class of HCV inhibitors and that the decrease of PI4P together with cholesterol and the formation of NS5A-containing clusters are consequences of the inhibition of NS5A-PI4KIIIα complex function.

E. Materials and Methods

1 Reagents and Plasmids

Plasmids were assembled by standard recombinant DNA technology and the correct sequence was confirmed by automated nucleotide sequencing. The plasmid pHCVNeo17B used for the generation of the Huh7.5-10A cell line was described elsewhere [26]. pHCVNeo17B-Y93H is identical to pHCVNeo17B, but contains a mutation Y93H in NS5A.

The plasmid pEF1A-PIK4CA untagged is a kind gift from G. Randall (Department of Microbiology, University of Chicago). The plasmid pcD-BLARep-Wt contains the entire Con1 HCV sequence cloned into the pcDNA3 expression vector (Invitrogen) [27]. The plasmid pCD-BlaRep-K@67 is identical to pcD-BLARep-Wt but contains the insertion of lysine after V67 in NS5A. Plasmid pCDBlaRep-K@67-Y93H contains the insertion of lysine after V67 and Y93H in NS5A.

The HCV RNA polymerase inhibitor HCV-796 was gift from Arrow Pharmaceuticals and the HCV NS5A inhibitor Daclatasvir was a gift from Okairos. The NS3 inhibitor Telaprevir was purchased from Aurogene S.R.L. The synthesis of the CKIα inhibitor H-479 [28], AL-9 [29] and the compounds I-7 and I-19 [20] were described previously.

2 Cell lines and culture conditions

The human hepatoma-derived cell lines Huh7.5 and 10A-IFN [26], both highly permissive for HCV replication, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml Penicillin, 100 mg/ml Streptomycin and 2 mM L-glutamine. Stable cell lines harboring the subgenomic replicons of the JFH-1 isolate [30] or the adapted Con 1 10A isolate [26] were generated by electroporation of *in vitro*-transcribed HCV RNA into Huh7.5 as described [29] and following selection with G418 (0.8 mg/ml, Sigma-Aldrich) for three weeks. Replicon cell lines were maintained in the presence of 0.8 mg/ml G418. The Con1 10A subgenomic replicon contains the adaptive mutations E176G in NS3 and an insertion of lysine after amino acid V67 in NS5A. The replicon Con1 10A-Y93H replicon is identical to Con1 10A but contains an additional mutation Y93H in NS5A. The stable cell line containing the JFH-1 replicon resistant to DCV (JFH-DCV) was generated as follows: Cells expressing the replicon JFH-1 (JFH) were plated in 100 mm tissue culture dishes and maintained in medium supplemented with 0.5 mg/ml G418 and 500 pM Daclatasvir for 5 weeks. Cells were maintained as subconfluent monolayers and medium was changed twice weekly. The resistance phenotype of the cell pool was validated at the end of selection.

3 Replication assays

Calculation of EC_{50} (50% effective concentration) values of HCV inhibitors was performed as follows: Huh7.5-10A or Huh7.5-10A-Y93H

were plated at the density of 45000 or 70000 cells/well, respectively, in 24-well dishes. After 24 h, cells were treated with HCV inhibitors in a final concentration of 0.2% DMSO. After three days of treatment, RNA was extracted using the RNeasy Mini Kit (Qiagen) and HCV RNA was quantified by real time PCR using the protocol described previously [31].

4 Protein expression with the Vaccinia Virus T7 infection/transfection system, immunoprecipitation and Western Blotting

HCV nonstructural proteins were expressed using the Vaccinia T7-infection/transfection system as described previously [32]. Briefly, 10A-IFN cells were seeded at a density of 9 x 10^5 / 60-mm-diameter dish. After 24 h, cells were infected with Vaccinia Virus vTF7-3 and successively transfected either with 7 µg of NS5A-expressing plasmids (pcD-BLARep-Wt, pCD-BlaRep-K@67 or pCDBlaRep-K@67-Y93H, Fig. 2) or in combination with 5 µg of PI4KIIIα-expressing plasmid (pEF1A-PIK4CA, Fig. 5) using the transfection reagent Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. Compounds were added as indicated in the figure legends for 8 hours and cells were harvested with TEN buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA, 150 mM NaCl). Cells were lysed for 30 min in ice with NP_{40}-lysis buffer containing 20 mM Tris/HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% NP_{40}, 2 mM dithiothreitol, 10 mM sodium fluoride and protease inhibitors (Complete, Roche Applied Science)

and protein extract was cleared by centrifugation. Co-immunoprecipitation experiments were performed as follows: For each sample, 3.5 µg of mouse anti-NS5A antibody (Austral Biologicals, Cat. No HCM-131-5) were incubated with 30 µl packed volume of protein A-Sepharose (PAS) for 1 h at 4°C in 300 µl of immunoprecipitation buffer (IPB_{150}: 20 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP40). After antibody binding, the resin was washed twice with IPB_{150}, and incubated with 400 µg of protein extract for 2 h at 4°C in final volume of 300 µl of IPB_{150} supplemented with 2 mM dithiothreitol, 10 mM sodium fluoride and protease inhibitors. Immune complexes were washed three times with IPB_{150} and twice with PBS and proteins were detached from the PAS-resin by boiling in SDS sample buffer. For Western Blot analysis, 20 µg of total extract or immune complexes were separated by 7.5% polyacrylamide-SDS gel electrophoresis and proteins were transferred to nitrocellulose membrane (Protran, Whatman). NS5A was detected with affinity-purified rabbit anti-NS5A antibody (1:10000, [33]) and PI4KIIIα was detected with PI4KIIIα-specific antibody (1:250, Cell Signaling, Cat No. 4902) overnight at 4°C. Nitrocellulose membranes were incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature and NS5A was detected using the ECL Prime Western blotting Detection Reagent (GE Healthcare) while PI4KIIIα was revealed using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

Proteins were quantified by densitometry analysis using the Multigauge software (Fuji Phosphoimager). The quantity of PI4KIIIα present in a complex with NS5A was normalized for the amount of immunoprecipitated NS5A present in each sample.

5 Indirect immunofluorescence

Cells were plated in 24-well plates (50000 cells/well for Huh7.5 and Huh7.5-10A , 80000 cells/well for Huh7.5--10A-Y93H and JFH cells and 100000 cells/well for JFH-DCV cells) 24 hours prior to the experiment and were treated for 8 h as indicated in the figure legends. PI4P staining of the internal membranes and NS5A staining was performed exactly as previously described [29]. Primary antibodies used were: anti-PI4P IgM (Echelon, Cat.No. Z-P004, 1:300) or affinity-purified rabbit anti-NS5A antibody ([33], 1:2000). Secondary antibodies: goat anti-mouse IgM or goat anti-rabbit IgG conjugated to Alexa Fluor 568 (Invitrogen, Cat. No. A-21042) or Alexa Fluor 488 (Invitrogen, Cat.-No. A-11011) (1:600). Nuclei were stained with Hoechst dye 33342 (Sigma-Aldrich, 1:4000). For staining of free cholesterol, cells were incubated with Filipin III (Sigma-Aldrich, Cat. No. F4767) at a concentration of 0.2 mg/ml in TBS for 45 min. at room temperature during the final washing steps. In this case, nuclei were stained with RedDot™2 (Biotium, Cat. No. 40061). Slides were then mounted with 5 µl Dako Fluorescence Mounting Medium (Cat.-NoS3023, Dako North America, Inc.) and analyzed by using an inverted Leica TCS SP5 scanning laser confocal microscope. Digital images were taken using LAS AF software (Leica) and analyzed using Volocity software (Perkin Elmer). Quantification of

fluorescence intensity was determined using at least six randomly picked fields for each condition. For the quantification of relative PI4P levels in internal membranes, PI4P fluorescence intensity was normalized to the cell number. For the quantification of the relative cholesterol levels localized to cell membranes, the fluorescence intensity of Filipin was normalized to the cell number after setting the threshold of the signal intensity such that predominantly HCV-induced cholesterol accumulations were measured. Quantitative immunofluorescence data are presented as means ± the standard deviation (SD). For the calculation of statistical significance, a two-tailed, unpaired T-test was performed.

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CHAPTER IV

SUMMARY, CONCLUSIONS AND

TRANSLATIONAL IMPACT OF THE PROJECTS

A. Discovery of HCV inhibitors targeting host factors

The new gold standard therapy for chronic genotype 1 HCV infection is an NS3/4A protease inhibitor (boceprevir or telaprevir) (Jacobson et al., 2011; Poordad et al., 2011) in combination with pegylated alpha interferon and ribavirin (PegIFN/RBV). This new standard of care still has limited efficacy, particularly among treatment-experienced patients who previously failed to respond to PegIFN/RBV therapy, and is associated with several adverse reactions. Research into more effective antiviral therapies has, in part, focused on host factors targets that may lead to broadly active drugs with pangenotype activity. These may also provide greater barriers to drug resistance. The availability of multiple new therapeutic options could lead to regimens that are free of PegIFN and/or RBV.

An increasing understanding of the molecular interactions between the virus and its host cell has allowed the identification of promising host targets for anti-HCV therapy and host-targeting agents (HTAs) are currently under development (Baugh et al., 2013). The most advanced compound is an inhibitor of cyclophilin A, a host factor known to be critical for viral RNA replication and possible virion assembly or release. This compound, alisporivir, has demonstrated *in vivo* efficacy and is now in a phase 3 trial. Several other HTAs with very different host targets are further upstream in the development pipeline.

Several groups have performed screens to identify cellular cofactors involved in HCV replication and infection. A common target identified

in the various screens is phosphatidylinositol-4-kinase IIIα (PI4KIIIα) (Berger et al.,2009; Borawski et al.,2009; Tai et al., 2009; Trotard et al., 2009; Reiss et al.,2011). PI4KIIIα belongs to the family of type III phosphatidylinositol 4-kinases, enzymes that catalyze the conversion of phosphatidylinositol to phosphatidylinositol 4-phosphate (PI4P). PI4P is the most abundant monophosphorylated inositol phospholipid in mammalian cells and the importance of this phospholipid is just started to be unraveled (D'Angelo et al., 2008). In addition to playing important roles in intracellular signaling and membrane trafficking, phosphatidylinositol lipids and their metabolizing enzymes are also exploited by many different viruses in order to transform cellular membranes in structures supporting their replication (Miller et al., 2008; Heaton et al., 2008). PI4KIIIβ was shown to be a host factor required for enterovirus replication (Hsu et a., 2009), whereas several reports have demonstrated that PI4KIIIα is crucial for HCV replication (Berger et al., 2009; Borawski et al., 2009; Reiss et al., 2011; Vaillancourt et al., 2011). Owing to the importance of this pathway, the need for specific inhibitors of PI4III kinases is increasing. Only recently, some enviroxime-like compounds with antiviral activity against enterovirus have been demonstrated to target PI4KIIIβ. One of these agents is a very specific inhibitor of the β-isoform of the type III PI4-kinases (Arita et al.,2012). Until 2012, no such compound exists for the PI4KIIIα isoform.

In CHAPTER II of this thesis we demonstrated that the 4-Amino Quinazoline compound named AL-9, originally proposed to target NS5A, inhibits HCV replication by targeting PI4KIIIα (Bianco et

al.,2012). Compounds targeting host factors may have the general advantage of imposing a higher genetic barrier to the development of resistance. Whether or not PI4KIIIα is a viable target is still a matter of debate. In mice, this enzyme is essential, and even conditional ablation of PI4KIIIα leads to severe pathology (Vaillancourt et al., 2012). However, partial PI4KIIIα inhibition, liver targeting or short treatment duration might still provide a therapeutic window useful for HCV treatment. For this reason, AL-9 is a good lead compound for the development of more potent and specific pharmacological inhibitors of PI4KIIIα to be used both as important research tools as well as leads for initial drug discovery. Indeed, very recently, small inhibitors that display high selectivity for PI4KIIIα and potently inhibit HCV replication *in vitro* were identified from a collection of compounds containing a quinazoline core (Leivers et al., 2013).

B. Elucidation of mode of action of NS5A replication complex inhibitors.

Since the introduction of IFNα- and RBV-based combination therapy, there have been several medically unmet needs in HCV treatment. These include the need for the 'ideal' regimen: an all-oral, IFN-free combination cocktail with pan-genotype coverage, minimal side effects, the reduction of viral resistance, and high virologic cure rates in all patient groups with the improvement of antiviral efficacy in HCV GT 1 and 4. With dozens of compounds already in clinical

development, and many more at preclinical stages, expectations for achieving this goal for HCV are justifiably high.

The discovery of NS5A inhibitors and clinical validation of NS5A as a therapeutic target signaled a new era for HCV drug development. NS5A replication complex inhibitors (Conte et al., 2009; Gao et al., 2010; Fridell et al., 2011), exemplified by Daclatasvir, exhibit a number of preclinical properties that make them excellent candidates for progression into clinical development. A hallmark feature of this class of inhibitor is retention of picomolar activity against all HCV genotypes. Pan-genotype activity is an attractive quality for an HCV DAA due to the worldwide distribution of HCV genotypes and the potential for patients to be co-infected with more than 1 genotype/subtype (Asselah et al., 2003, Giannini et al., 1999, Matsubara et al., 1996). Importantly, this broad spectrum anti-HCV activity does not incur any penalty in terms of cellular toxicity for a range of cell types (Gao et al., 2010 and Lemm et al., 2010). Since NS5A-targeting molecules are identified as a novel class of HCV DAA, they represent the best candidate to achieve the most synergistic antiviral effect when combined with other mechanistically distinct DAAs, by suppressing the emergence of all possible multiple resistant variants. Preclinical data demonstrated that NS5A inhibitors exhibit additive to synergistic antiviral effects when combined with pegIFN and/or RBV, NS3 protease, or NS5B polymerase inhibitors (Fridell et al., 2010, Gao et al., 2010 and Graham et al., 2011). Further lending credence to the suitability of NS5A inhibitors as components of future DAA cocktails are the observations that other HCV DAAs retain their antiviral activity

in Daclatasvir-resistant replicon-containing cell lines (Fridell et al., 2010). Thus, NS5A-associated resistance polymorphisms do not confer cross-resistance to other HCV DAAs; a crucial property for components of combination regimens. Since most of the serious side effects of standard HCV therapy come from the nonspecific actions of IFNα and RBV, the minimization of side effects also could be achieved by an IFNα-free regimen consisting of Daclatasvir and other DAAs. The feasibility of these DCV-containing and IFNα-free DAA combination regimens was already proved by several clinical trials (Chayama et al., 2012; Suzuki et al., 2013). In order to shorten the duration and frequency of current anti-HCV treatment, DCV also would be a valuable combination option, thanks to its extraordinary antiviral potency and desirable pharmacokinetic profile, which make a once-daily dosing possible. Finally, the all-oral delivery of anti-HCV therapeutics could be realized by implementing a DCV-containing, all-DAA regimen in practice, with no need for muscular injections of IFNα. This would provide a great convenience for HCV patients.

Although their target is known, how NS5A inhibitors actually work is still incompletely understood, and this would be useful knowledge for deciding which combinations might be most effective.

Thus, we studied the potential mode of action of the NS5A replication complex inhibitors, represented by Daclatasvir, the first in class of NS5A inhibitors (Gao et al, 2011), and the piperazinyl-*N*-(aryl)benzamides class (Conte et al., 2009).

As previously reported, NS5A inhibitors block NS5A hyperphosrylation (Lemm et al., 2010; Qiu et al., 2011) and specifically

alter its subcellular localization (Targett-Adams et al., 2011; Lee et al., 2011). However these modifications may only be a biomarker for the mechanism of action of NS5A inhibitors.

In the CHAPTER III of this thesis, we demonstrated for the first time that NS5A inhibitors significantly deplete the PI4P and cholesterol of which the HCV virus enriches the membranous web. A high content of both PI4P and cholesterol are required for efficient replication and their depletion causes inhibition of HCV replication (Aizaki et al., 2004; Bianco et al., 2012; Alvisi et al.,2011). The importance of NS5A for HCV replication structures formation has previously been described: NS5A associates with PI4KIIIα (Berger et al., 2009), thereby recruiting the kinase to the membranous web and guiding the production of phosphatidylinositol 4-phosphate, the product of the kinase reaction, to the viral membranous structures. Different groups found that PI4KIIIα directly interacts with the NS5A domain I (Berger et al., 2011; Lim et al., 2011; Tai and Salloum, 2011; Reiss et al., 2011 and 2013) where resistant mutations to NS5A inhibitors map (Fridell et al., 2010). We observed that NS5A inhibitors destabilize the NS5A-PI4KIIIα complex, suggesting that the pertubation of the functional complex between the viral protein NS5A and lipid kinase PI4KIIIα mays represent the real molecular target of this antiviral agents through which the essential supply of PI4P and cholesterol to the membranous web is interrupted.

The identification of the precise mode of action of NS5A inhibitors will not only enhance the possibility of developing more effective combinations of multiple classes to treat patients, but also facilitate

the discovery of new therapeutic targets with improved properties such as a higher barrier to resistance.

The true benefit of NS5A inhibitors will likely be as components of tailored HCV DAA drug cocktails, in which they could play a pivotal role.

C. Chapter IV – References

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