Characterization of functional determinants in the C-terminal part of hepatitis C virus E1 glycoprotein ectodomain

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Caractérisation de déterminants fonctionnels dans la partie C-terminale de l'ectodomaine de la glycoprotéine E1 du virus de l'hépatite C

Characterization of functional determinants in the C-terminal part of hepatitis C virus E1 glycoprotein ectodomain

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“When one door closes another door opens; but we so often look so long and so regretfully upon the closed door, that we do not see the ones which open for us.”

— Alexander Graham Bell

“It ain’t about how hard you hit. It’s about how hard you can get hit and keep moving forward; how much you can take and keep moving forward. That’s how winning is done!”

-Rocky Balboa

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Abstract

Hepatitis C virus is currently estimated to infect around 71 million people around the world. However, recent advances in drug development led to the generation of pangenotypic direct acting antivirals (DAA), which may make it possible to eliminate HCV by 2030 as planned by the World health organization (WHO). HCV is a small RNA enveloped virus of positive sense. The RNA is encapsidated and surrounded by a lipid bilayer in which the E1 and E2 envelope glycoproteins are anchored on the surface. Thus, E1 and E2 are the first viral proteins to encounter the hepatocytes and mediate the entry step. HCV entry into hepatocytes is a sophisticated process that includes several steps ranging from interaction of glycoproteins with cellular host attachment factors and HCV specific-receptors, which is followed by internalization via clathrin-mediated endocytosis. Finally, viral and endosomal membranes merge at acidic pH leading to the release of viral RNA into the cytoplasm. Among the two glycoproteins, E2 has been the better characterized, as it is responsible for binding to cellular receptors and targeted by neutralizing antibodies. As a member of the Flaviviridae family, it has been suggested by analogy that HCV encodes class II fusion proteins and that E2 is the fusion protein. Nevertheless, the recent crystal structures of E2 revealed that it lacks structural features of class II fusion proteins. Thus, E1 glycoprotein became under the spotlight with the assumption that it is responsible for the fusion step whether alone or with the help of E2. Indeed, the N-terminal part of E1 ectodomain was recently crystallized, and the characterization of conserved residues within this region demonstrated its importance for virus infectivity, E1E2 interaction as well as its involvement in the interplay with HCV receptors. Supporting the potential role of E1 in the fusion process, different segments in the C-terminal of the ectodomain have been reported to be involved in interactions with model membranes. In particular, we investigated two regions of interest. The first one located in the putative fusion peptide (PFP) region between amino acid 270 and 291, containing hydrophobic sequences, supporting its involvement in the fusion step. The second region spanning amino acids 314-342, a membranotropic region located proximal to the transmembrane region of E1 and has been shown by X-ray crystallography and NMR-studies to comprise two α-helices (α2 and α3). We introduced 22 mutations in the C-terminal part of E1 ectodomain in the context of a JFH1 infectious clone. We replaced the most conserved residues with alanine and analyzed the effect of the mutations on the viral life cycle. Twenty out of the 22 mutants were either attenuated or lost their infectivity, indicating their importance for the viral life cycle. We observed different phenotypes; some mutations

modulated the dependence of the virus on CLDN1 and SRBI receptors for cellular entry. Most mutations in the PFP region affected virus secretion and assembly as well as E1E2 heterodimerization. Nevertheless, the majority of mutations in the α2-helix (aa 315-324) led to severe attenuation or complete loss of infectivity without affecting E1E2 folding or viral morphogenesis. Further characterization of some mutants within this region suggested the involvement of the α2-helix in a late step of HCV entry. Finally, our results show the important role of E1 played in E1E2 heterodimerization, virus morphogenesis, interaction with HCV receptors and its potential involvement in the fusion step.

Résumé

Aujourd'hui, le Virus de l'Hépatite C (VHC) infecte plus 70 millions de personnes dans le monde. L'Organisation mondiale de la santé prévoit l'élimination du virus VHC d'ici 2030, grâce aux récentes découvertes dans le milieu du développement médical. Ces derniers ont conduit à la production des antiviraux pangenotypiques à action directe (ADD). Le VHC est un virus enveloppé de l'ARN, avec une polarité positive. Il est constitué de nucléocapside entouré d'une membrane lipidique. La nucléocapside contient l'acide ribonucléique (ARN) et la protéine core. La membrane lipidique quant à elle contient à la surface les glycoprotéines E1 et E2. Ainsi ces protéines, sont les premières à rencontrer les hépatocytes, c'est donc grâce à elles que le virus parvient à entrer dans les cellules. Parmi les deux protéines, l'E2 a été la mieux caractérisée pour ses fonctions de liaisons aux récepteurs spécifiques. De plus les anticorps neutralisants ciblent majoritairement cette protéine. En se basant sur le fait que ce virus est membre de la famille des Flaviviridae, il a été suggéré par analogie, que le VHC contient des protéines de fusion de classe II et que la protéine E2 est la protéine de fusion. Cependant, les structures cristallines récentes d'E2 ont révélé qu'il lui manquait les caractéristiques structurelles des protéines de fusion de classe II. Ainsi, tous les regards se sont tournés sur la glycoprotéine E1, suggérant qu'elle est responsable de l'étape de fusion, seule ou à l'aide d'E2. En effet, la partie N-terminale de l'ectodomaine E1 a été récemment cristallisée. La caractérisation des résidus conservés dans cette région a démontré son importance pour l'infectivité du virus, pour l'interaction entre E1 et E2, ainsi que pour son implication dans l'interaction avec les récepteurs du VHC. En soutenant le rôle potentiel d'E1 dans le processus de fusion, différents segments de l'extrémité C-terminale de l'ectodomaine seraient impliqués dans les interactions avec les membranes modèles. Nous avons étudié en particulier deux régions d'intérêt. La première située dans la zone du peptide de fusion putatif (PFP) entre les acides aminés 270 et 291. Cette région se compose des séquences hydrophobes, soutenant son implication dans l'étape de fusion. La deuxième région englobant les acides aminés 314-342, d'une activité membranotrope située à proximité de la zone transmembranaire d'E1, a été démontrée par la cristallographie aux rayons X et les études de RMN comme comprenant deux hélices α (α2 et α3).

Nous avons introduit 22 mutations dans la partie C-terminale de l'ectodomaine E1 dans le contexte d'un clone infectieux JFH1. Nous avons remplacé les résidus les plus conservés par de l'alanine, puis analysé l'effet des mutations sur le cycle de vie du virus. Vingt des vingt-deux mutants ont été atténué ou ont perdu leur pouvoir infectieux, ce qui indique leur

importance dans le cycle viral. Nous avons observé différents phénotypes; certaines mutations ont modulé la dépendance du virus vis-à-vis des récepteurs CLDN1 et SRBI pour l'entrée cellulaire. Plusieurs mutations dans la région PFP, ont affecté la sécrétion et l'assemblage du virus, ainsi que l'hétérodimérisation E1E2. D'autres mutations, telles que les mutations de l'hélice α2 ont entraîné une atténuation grave ou une perte complète d'infectivité, sans affecter le repliement d'E1 et E2, ni la morphogenèse virale. Une caractérisation plus poussée de certains mutants au sein de la région hélice α2 a suggéré l'implication de cette région dans une étape tardive de l'entrée du VHC. Enfin, nos résultats montrent le rôle important joué par la glycoprotéine E1 dans l'hétérodimérisation de E1E2, la morphogenèse du virus, ainsi que son interaction avec les récepteurs du VHC et son implication potentielle dans l'étape de fusion.

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ABBREVIATIONS

A

ACSL3	Long chain acyl-CoA synthetase 3
Ag	Antigen
ALT	Alanine Aminotransferase
Apo	Apolipoproteins
ARF	ADP ribosylation factor
ARFGAP1 GTPase	GTPase-activating protein for ARF1
ARFP	Alternate reading frame protein
ATP	Adenosine triphosphate

B

BVDV	BovineViral Diarrhea Virus

C

CAS9	CRISPR-associated protein containing 2 nuclease domains that is programmed by small RNAs to cleave DNA
CD4+	Cluster of differentiation 4
CD8+	Cluster of differentiation 8
cDNA	Complementary DNA
CIA	Chemiluminescence immunoassay
CLDN1	Claudin-1
CMV	Cytomegalovirus
CRISPR/CAS9	Clustered Regularly Interspaced Short Palindromic Repeats/ CAS9
CSFV	Classical Swine Fever Virus

CypA	Cyclophilin A

D

DAA	Direct acting antivirals
DC	Dendritic cells
DC-SIGN	Dendritric cell-specific ICAM-grabbing non-integrin
DGAT1	Diacylglycerol acyltransferase-1
DMV	Double membrane vesicles

E

E2c	Core domain of E2
EGFR	Epidermal growth factor tyrosine kinase receptor
EIA	Enzyme immunoassay
eIF2	Eukaryotic Initiation Factor 2
eIF3	Eukaryotic initiation factor 3
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic Reticulum
ESCRT	Endosomal-sorting complex required for transport
ESLD	End-stage liver disease

F

FRET	Fluorescence resonance energy transfer

G

GAGs	Glycosaminoglycans
GBF1	Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1
GP	Glycoprotein
GTP	Guanosine-5'-triphosphate

H

HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCVcc	Cell-cultured HCV
HCVpp	HCV pseudoparticle
HDL	High density lipoproteins
Hek-293T	Human embryonic kidney cells
HEV	Hepatitis E virus
HIV	Human immunodeficiency virus
HNF4a	Hepatocyte nuclear factor 4a
HSPG	Heparan sulfate proteoglycans
HVR	Hyper variable region

I

ICR	Institute of Cancer Research

IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgVR	Intergenotypic variable region
IL10	Interleukin 10
IL28B	Interleukin 28B
IRES	Internal ribosome entry site
IRF-3	Interferon regulatory factor-3
ISGs	IFN-stimulated genes
J	
JAK-STAT	Janus kinase (JAK), Signal Transducer and Activator of Transcription proteins (STATs)
JFH-1	Japanese fulminant hepatitis
L	
LD	Lipid droplets
LDL	Low Density Lipoprotein
LDLr	Low Density Lipoprotein receptor
LEL	Large extracelullar loop
L-SIGN	Liver-specific ICAM-grabbing non-integrin
LVP	Lipoviroparticles
M	
MAPK	Mitogen-activated protein kinases
MAVS	Mitochondrial antiviral-signaling protein
MHC	Major histocompatibility complex
miR-122	MicroRNA-122
MLV	Murine leukemia virus
MTTP	Microsomal triglyceride transfer protein
MVA	Modified vaccinia Ankara
N	
nAbs	Neutralizing antibodies
NANBH	Non-A non-B hepatitis
NFκB	Nuclear factor κB
NK	Natural Killer
NMR	Nuclear magnetic resonance
NPC1L1	Niemann-Pick C1-like1
NPHV	Non-primate *hepacivirus*
NrHV	Norway rats *hepacivirus*
NS	Non-structural
O	
OAS	Oligoadenylate synthetase
OCLN	Occludin

ORF	Open reading frame

P

PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PDB	Protein database
PDI	Protein disulfide isomerase
PEG-IFNa	Pegylated-interferon alpha
PFP	Putative fusion peptide
PI3K/AKT	Phosphatidylinositol 3-kinase/ Protein kinase B(AKT)
PI4KIIIa	Phosphatidylinositol-4 kinase III a
PKR	Protein kinase R
PLA2G4	Group IVA phospholipase A2
polyU/C	Polypyrimidine
PRRs	Pattern recognition receptors

R

RBV	Ribavirin
RdRp	RNA-dependent RNA polymerase
RIG-I	Retinoic acid-inducible gene-I
RLRs	RIG-I like receptors
RNA	Ribonucleic acid

S

SCID	Severe combined immunodeficiency
sE2	Soluble form of E2 glycoprotein
siRNA	Small interfering RNA
SL	Stem-loop
SOC	Standard-of-care
SRBI	Scavenger receptor B-I
ssRNA	Single-stranded RNA
STING	Stimulator of interferon genes
SVR	Sustained virological response

T

TAP1	Transporter associated with Antigen Processing 1
TfR	Transferrin receptor
TGFβ	Transforming growth factor beta
TIP47	Tail-interacting protein 47
TLRs	Toll like receptors
TM	Transmembrane
TMA	Transcription-mediated amplification
TMD	Transmembrane domain
TNFα	Tumor necrosis factor alpha
TRIF	TLR-domain-containing adapter-inducing IFNβ
UTR	Untranslated region

UTR

v

VAPA	Vesicle-associated membrane protein-associated protein A
VLDL	Very-low-density lipoprotein
vRF	Viral replication factories

1 Introduction

1.1 Hepatitis C virus: an overview

1.1.1 Hepatitis

Hepatitis is defined as an inflammation of the liver. It might be self-limiting but it might also become chronic and progress to fibrosis, cirrhosis or hepatocellular carcinoma (HCC). The main cause of hepatitis is the infection by viruses, yet it can also be induced by other factors such as toxic substances (alcohol, drugs) or autoimmune diseases. Whereas a large number of viruses, including Epstein-Barr, Herpes simplex virus and the cytomegalovirus can cause inflammatory disease of the liver, viral hepatitis is the result of infection by the five well described hepatotropic viruses of the type A, B, C, D and E (Tsega, 2000). Hepatitis A and E viruses are enterically transmitted by the faecal-oral route and are mainly associated with acute infections, although some rare cases of chronic hepatitis have been reported upon HEV infection in immunocompromised patients (Bihl and Negro, 2009). Hepatitis B, C and D viruses are parenterally transmitted and can lead to acute or chronic infection that can induce cirrhosis and hepatocellular carcinoma. Despite the fact they cause resembling diseases, hepatitis viruses belong to different virus family.

1.1.2 Discovery of hepatitis C virus

In the early seventies, the only viruses known to be responsible for transfusion-associated hepatitis were hepatitis A virus (HAV) (Bayer et al., 1968) and hepatitis B virus (HBV) (Feinstone et al., 1975) for which serological tests were available. Hepatitis A and hepatitis B diseases differ in their mode of transmission and symptoms. Hepatitis A is transmitted by person to person contact or through consumption of contaminated food or water, characterized by a short incubation time (1-3 weeks) and results in an acute serious sickness but does not lead to chronic hepatitis. While hepatitis B is transmitted through blood or body fluid, has a longer incubation period (1-3 months) and causes a chronic infection (Krugman et al., 1967). However, at that time, it was discovered that an important number of post-transfusion hepatitis was not due to infection by HAV and HBV, suggesting the existence of a yet unknown hepatitis virus (Alter et al., 1975; Feinstone et al., 1975). As a consequence, these hepatitis were called the non-A non-B hepatitis (NANBH). In 1989, the identification

of the hepatitis C virus could be achieved by screening a cDNA library obtained from a highly infectious chimpanzee plasma with antibodies from infected patient sera (Choo et al., 1989). Further experimentation revealed that the infectious agent was a small enveloped virus carrying a single-stranded RNA (ssRNA) genome of ~ 10 kb with a single open reading frame (ORF). The new virus was named hepatitis C virus (HCV) and was classified in the genus *hepacivirus* of the *Flaviviridae* family (Choo et al., 1989). This discovery allowed for the development of HCV diagnosis tests, which led to a great decrease of the risk of infection by the virus.

1.1.3 HCV classification and genetic variability

HCV belongs to the h*epacivirus* genus in the *Flaviviridae* family (Fig.1) which comprises 3 further genera: the *flaviviruses* (e.g. dengue virus), *pestiviruses* (e.g. bovine viral diarrhoea virus) and *pegiviruses* (e.g.GB viruses) (Simmonds et al., 2017). Members of the *Flaviviridae* family share similarities in terms of virion morphology, genome organization and replication strategy. Until recently, there was no evidence for the existence of HCV animal homologs. In 2011, however, the first evidence for a wider hepaciviral host range emerged, with the isolation of a novel *hepacivirus* species in dogs and horses (Burbelo et al., 2012; Kapoor et al., 2011; Pfaender et al., 2015; Ramsay et al., 2015). Since then, HCV homologs were isolated in bats, rodents, primates, bovines and sharks (Hartlage et al., 2016). Thus, the identification of animal *hepaciviruses* contributes to increase our understanding of *hepacivirus* origin and their host range determinants.

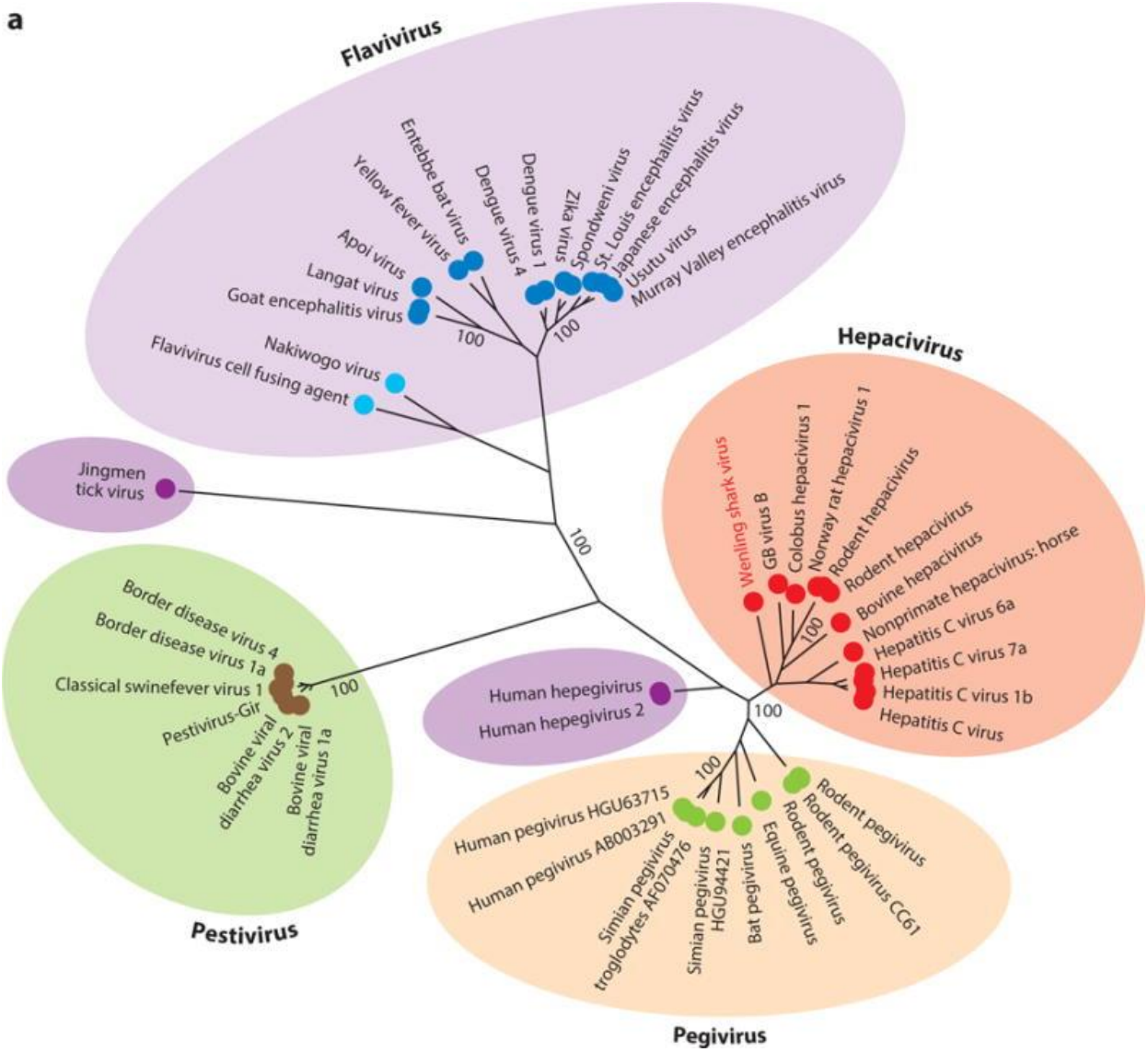

Figure 1 Phylogenetic tree of *Flaviviradea* family. Phylogenetic tree including all four genera of the *Flaviviradea* family in addition to newly identified viruses *(Hartlage et al., 2016).*

HCV shows a high genetic variability. According to phylogenetic analyses of the different HCV strains sequences, HCV was classified into seven major genotypes, labeled 1-7 and each genotype is composed of numerous subtypes, designated by letters (a, b, c). The nucleotide sequences of the genomes from the different genotypes differ by up to 30%, and among each genotype the different subtypes differ by up to 25% over their genome sequence (Simmonds, 2013, 2004). Furthermore, the virus exists as constantly evolving quasispecies within an individual patient (Forns et al., 1999). This genetic heterogeneity is due to the lack of proof reading activity of the RNA-dependent RNA polymerase (RdRp) encoded by HCV. Besides being error prone, the RNA replication is highly efficient with a production of 10^{12}

virions/day (Neumann et al., 1998), which results in continuous introduction of mutations in the virus genome.

HCV genotypes are differently distributed across the world with genotype 1 and 3 being the most prominent genotypes, accounting for 46% and 30% of all infections, respectively. Genotype 1 is widely distributed in Europe, North and South America, Asia and Australia and genotype 3, is mainly present in South Asia. Genotypes 2, 4, and 6 are mainly responsible for the remaining cases of HCV around the world. Genotype 2 is mainly found in west and central Africa, while genotype 4 is the most prevalent in North Africa and the Middle East, particularly Egypt. Genotype 6 is present in South East Asia, genotype 5 in South Africa (Fig.2), in addition to genotype 7 that was recently identified in Canada recovered from patients, presumably infected in Central Africa (Gottwein et al., 2009; Messina et al., 2015; Mohamed et al., 2015; Murphy et al., 2015). Furthermore, the severity of the disease differs among HCV genotypes, as well as the establishment of persistent infection and response to therapy.

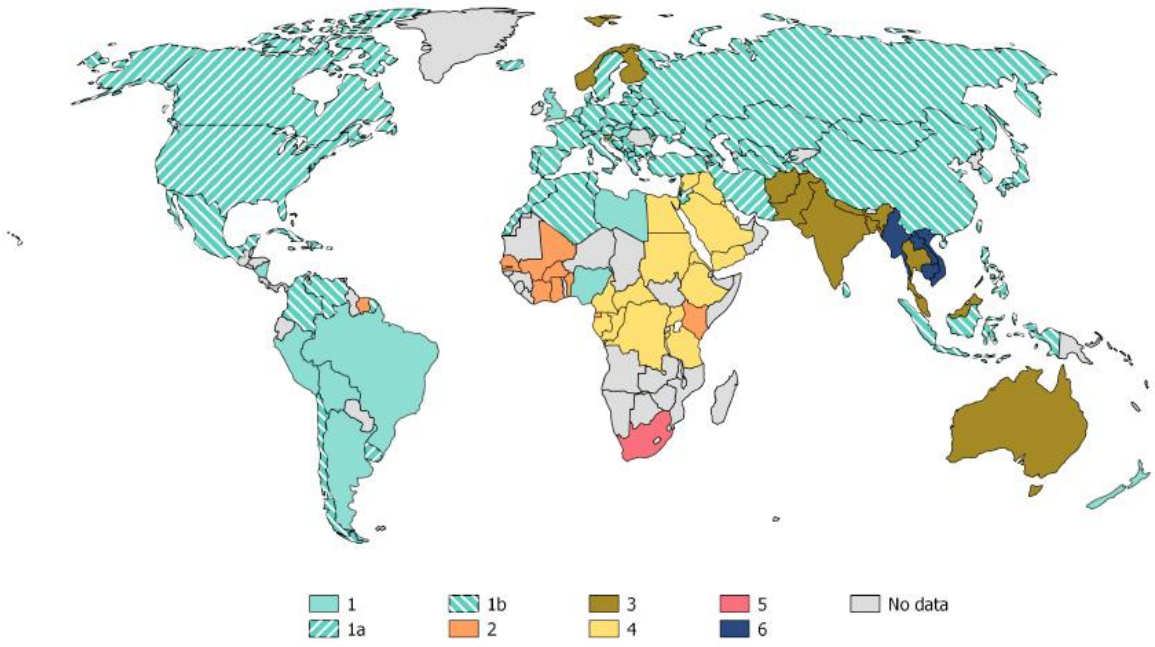

Figure 2 HCV genotypes distribution HCV genotype distribution: global map of HCV genotype predominance in individual countries (Cuypers et al., 2016).

1.1.4 HCV transmission

HCV is mainly transmitted parenterally through exposure to contaminated blood or blood products. This might be due to blood transfusion, medical interventions, haemodialysis, contaminated equipment, or sharing of syringes, especially among injection drug users (Shepard et al., 2005). Sexual transmission and mother to child transmission have also been reported, but are rare (Prasad and Honegger, 2013; Terrault et al., 2013). However, since the implementation of blood screening for HCV in the 1990s, the main source accounting for HCV infection in developed countries is unsafe injection drug use, while blood transfusion associated infection nearly vanished. Nonetheless, risk factors vary between different countries, such as reuse of glass syringes for medical injections remains the key risk factor in a country like Pakistan (Hajarizadeh et al., 2013). Other routes of transmission include receiving a tattoo or piercing in unregulated settings, needle-stick injuries among healthcare workers and patient-to-patient transmission. However, as most infections remain asymptomatic at the time of the contamination, the transmission route of HCV remains unknown in 20% of the cases.

1.1.5 HCV epidemiology

HCV has been reported to infect more than 170 million people around the world, which corresponds to 3% of the global population (Mohd Hanafiah et al., 2013). These figures were based on testing seroprevalance of antibodies to HCV in the population. Nevertheless, the presence of anti-HCV antibodies in the serum constitutes evidence of past or present HCV infection. Eventually, recent figures of HCV global prevalence based on HCV RNA positivity show lower prevalence, with around 71 million HCV infected individuals in 2015 (The Polaris Observatory HCV Collaborators, 2017).

HCV prevalence varies among countries around the world (Fig.3). High prevalence of HCV infection can be attributed in some countries to the use of contaminated medical devices and equipment. One example is Egypt, which has a high seroprevalence of > 10% and is considered to have the highest HCV prevalence worldwilde. This could be traced back to anti-schistosomiasis (flat worms) campaigns between the 1960s and 1980s (Strickland, 2006; Arafa et al., 2005; Frank et al., 2000), during which, contaminated needles and syringes might have been re-used. In addition to Egypt, other developing countries like Cameroon, Gabon, Georgia, Pakistan, Mongolia and Uzbekistan (Baatarkhuu et al., 2017; Karchava et al., 2015; Nerrienet et al., 2005; Njouom et al., 2012; Ruzibakiev et al., 2001) are reported to have >5% of anti-HCV antibody prevalence, where iatrogenic infection (infections

transmitted during medical treatment and care) plays the main role for infection in these countries. Variously, in western countries as North America and Western Europe only a low percentage of HCV global prevalence (< 2%) exists and the main risk factor for HCV infection is the unsafe injection drug use (Gower et al., 2014).

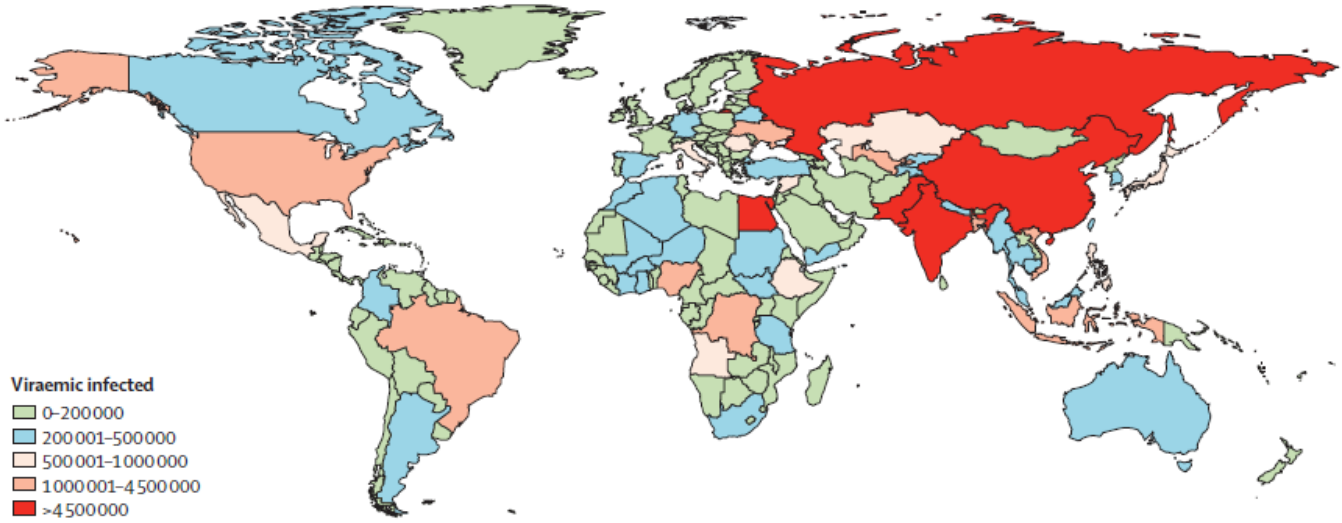

Figure 3 HCV global prevalence: number of HCV infected people in countries all over the world at the end of 2015 (The Polaris Observatory HCV Collaborators, 2017).

1.1.6 HCV Pathogenesis

Hepatitis C is a long-lasting disease that evolves slowly. The first phase of hepatitis C consists in an acute infection that is in most cases asymptomatic. In 80% of the cases, patients develop a chronic infection that can lead in 20% of the cases, to cirrhosis and ultimately to hepatocellular carcinoma over a 20 years period (Fig.4) (Freeman et al., 2001; Stanaway et al., 2016). Importantly, hepatitis C is the major cause for liver transplantation in many parts of the world (Gower et al., 2014).

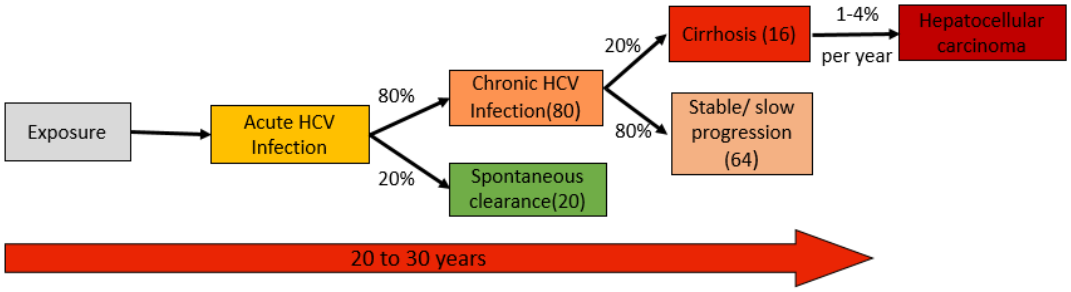

Figure 4 Hepatitis C virus natural history (adapted from (Lauer and Walker, 2001)).

1.1.6.1 Acute hepatitis

In most cases acute HCV infection is asymptomatic and eventually undiagnosed, which limits studies on the early phase of HCV infection. Data on this stage come from experimentally infected chimpanzees and prospective studies on human at high risk of HCV exposure. Only 30% of the patients develop symptoms. Some unspecific symptoms may include fatigue, loss of appetite, anorexia, fever and jaundice, while fulminant hepatitis (characterized by severe necrosis of liver cells) is less common (Hoofnagle, 1997; Thimme et al., 2001). In most cases, these symptoms are transitory. Following HCV infection, viral RNA can be detected 7-21 days after exposure (Farci et al., 1991), while seroconversion appears after 2-8 weeks of infection (Tremolada et al., 1991). An increase in liver transaminase enzymes can be observed 10-14 weeks after exposure (Bowen and Walker, 2005; Farci et al., 1991). About 15-20 % patients are likely to recover from acute hepatitis and clear the virus. In this case, a decline in viral RNA is observed until it becomes undetectable within approximately 4 months of infection. However, in the majority of the cases, symptoms of acute hepatitis resolve but HCV persists and the infection progresses to chronicity which is associated with an increase in viremia (Racanelli and Rehermann, 2003; Thomson et al., 2011). Of note, some factors have been associated with spontaneous viral clearance in some patients such as presence of jaundice (indicating sever liver injury), raised Alanine Aminotransferase (ALT) levels, decline in HCV-RNA and IL28B polymorphisms (Beinhardt et al., 2013).

1.1.6.2 Chronic hepatitis

HCV infection that persists for more than 6 months is considered as a chronic infection. Approximately 80% of patients are unable to clear the infection and develop chronic hepatitis. At this stage patients are mostly symptomless but might experience intermittent and vague symptoms resembling those observed during the acute phase. Transaminase levels do not always correlate with disease progression as well as viral RNA, which is relatively stable at 10^{12} virions produced per day (Neumann et al., 1998). Over time chronic hepatitis develops into hepatic fibrosis that subsequently progress within a period of 20 years in about 20-30% of HCV chronic patients to cirrhosis (Poynard et al., 1997). Disease progression is affected by several factors, such as viral genotype, age, gender, co-infection with HIV (human immunodeficiency virus) or HBV, obesity and alcohol consumption (Thomas and Seeff, 2005). About 25% of cirrhotic patients might progress to end-stage liver disease (ESLD) or hepatocellular carcinoma (HCC). ESLD is associated with several complications, e.g. hepatic encephalopathy, esophageal varices leading to gastrointestinal bleeding, ascites and eventually the need for liver transplantation. The main reason for liver transplantation was considered to be end-stage liver failure due to HCV infection in the United States, but recurrent infection of the graft is frequently observed (Brown, 2005). The risk of death from these complications is 4% per year and the risk of developing HCC lies between 1 and 5% per year (Scott and Gretch, 2007; Thomas and Seeff, 2005). The mechanism by which HCV establishes liver cirrhosis and HCC is not completely understood. Indeed, while the characterization of the molecular virology of HCV infection has greatly progressed, the molecular mechanisms underlining disease progression to fibrosis, cirrhosis and HCC remain unclear. Experimental studies indicate that HCV induces hepatocarcinogenesis directly via its proteins or transcripts or indirectly through induction of liver inflammation. Indeed, HCV has been shown to transmit signal and modulate hepatocytes gene expression. Moreover, viral proteins have been involved in disrupting signal transduction pathways affecting cell survival, proliferation and leading to transformation (Bandiera et al., 2016; Mesri et al., 2014). Thus, at least 3 HCV proteins, the core, NS3 and NS5A, are involved in hepatic carcinogenesis (Kasprzak and Adamek, 2008). Therefore, virus-host interactions and signaling during infection might contribute to cellular transformation and development of HCC.

1.1.7 Immunology

Immune response plays an essential role in controlling HCV infection (Dustin and Rice, 2007). Innate immunity acts as the first line of unspecific defense against exposure to any pathogen. It mediates the protection against a great number of potential infections by the activation of pattern recognition receptors (PRRs). Subsequently, the specific adaptive immune response sets in, which targets the pathogen more specifically and involves immunological memory. For spontaneous clearance of HCV a broad, strong and persistent HCV-specific adaptive immune response is required. Nevertheless, HCV has developed immune evasion mechanisms to overcome anti-viral immune responses (Klenerman and Thimme, 2012; Lechner et al., 2000).

1.1.7.1 Innate Immunity

Innate immunity is the first line of defense against any viral invasion, including HCV infection. Upon infection, the microorganisms are recognized by PRRs as non-self through identification of conserved motifs, found within the nucleic acids and/or proteins, known as pathogen-associated molecular patterns (PAMPs). The main classes of PRRs used by the innate immune system to detect HCV are toll like receptors (TLRs) and retinoic acid-inducible gene-I (RIG-I) like receptors (RLRs) (Saito et al., 2008). These receptors are highly expressed in Kupffer cells (liver based macrophages), hepatic dendritic cells, as well as hepatocytes (Protzer et al., 2012).

HCV is sensed by RIG-I at an early stage of the infection. RIG-I detects the 3' untranslated region of the viral genome (Saito et al., 2008). HCV can also be recognized by TLR receptors. Indeed, the virus double strand RNA can be recognized by TLR3 that localizes in the endosomes (Li et al., 2012).

Recognition of HCV components by PRR triggers the activation of key transcriptional factors, nuclear factor κB (NFκB), the interferon regulatory factor-3 (IRF-3). These transcriptional factors induce the production and secretion of type I IFN (IFN-α/β) (Saito et al., 2008). IFN-β leads to an antiviral state in infected and neighboring hepatocytes by inducing the IFN-stimulated genes (ISGs) expression via the JAK-STAT signaling cascade. Many ISGs control viral infection by directly targeting pathways and functions required during pathogen life cycles (Sadler and Williams, 2008). Although several ISGs involved in the anti-HCV response could be identified, the mechanisms of action of a small number of

them only could be elucidated. Thus, the activation of OAS (2', 5'- oligoadenylate synthetase) causes the degradation of viral and host RNA by activating latent endoribonuclease (Schneider et al., 2014). Moreover, the viperin, which localizes in the endoplasmic reticulum and the lipid droplets interacts with HCV Core and NS5A and interferes with the viral replication (Metz et al. 2013).

However, HCV has evolved some mechanisms to evade innate immunity to establish persistent infection. Thus, several viral non-structural proteins participate to the resistance against the host antiviral response. Indeed, the NS3-NS4A can induce cleavage of members of TLR signaling pathway, especially the TLR-domain-containing adapter-inducing IFNβ (TRIF) leading to the inhibition of TLR3 signalling pathway. Additionally, NS3/4A cleaves MAVS (Mitochondrial antiviral-signaling protein) leading to the inactivation of the RIG-I signaling pathway (Chen et al., 2007; X.-D. Li et al., 2005). Furthermore, NS5A was reported to interfere with TLR signal transduction in immune cells (Abe et al., 2007). Besides, HCV E2 and NS5A proteins have been shown to bind to PKR catalytic domain thus inhibiting the blockade of protein translation (Tan and Katze, 2001; Taylor et al., 1999). Furthermore, NS5A binds in a similar manner to the N-terminal part of the OAS protein inhibiting its anti-viral infectivity (Taguchi et al., 2004). HCV NS4B was also reported to block RIG-I mediated IFN production (Ding et al., 2013; Nitta et al., 2013). Altogether, these interferences contribute to the alteration of innate immune response, which becomes insufficient to eliminate the virus (Feld and Hoofnagle, 2005; Horner and Gale, 2013).

Natural Killer (NK) cells are an important component of innate immunity and participate in the elimination of HCV (Jost and Altfeld, 2013). Indeed, in individuals who spontaneously clear HCV infection, viral control occurs before the onset of the adaptive immune response and thus involves the innate immune effector cells NK and NKT cells. NK and NKT cells release IFN-γ which inhibits HCV replication (Guidotti and Chisari, 2006; Jost and Altfeld, 2013). It is thought that IFN-mediated clearance of the virus is more important than direct cytolysis of the virus by NK cells. Apart from their role during the innate immune response, NK cells play a critical role during the development of adaptive immunity (Altfeld et al., 2011; Vivier et al., 2008). Indeed, NK cells have been shown to have important roles in editing the function of dendritic cells (DC), thereby affecting the ability of DCs to prime antiviral effector T cells.

Interestingly, HCV has also evolved several mechanisms to evade NK cells response.

Immobilization of E2 or HCV virions on a support can inhibit NK cells effector functions and IFN-γ production by crosslinking CD81 on the NK cells surface (Crotta et al., 2002; Tseng and Klimpel, 2002). However, HCV particles in suspension had no effect on NK cells functions. Moreover, direct contact between NK cells and infected hepatoma cells impacts NK cells functions through down regulation of NK cells activating receptors. This effect has been associated with NS3-4A protease activity in the infected cells (Yoon et al., 2016).

Additionally, HCV NS5A protein has been shown to stimulate monocytes through TLR4, which induces the secretion of IL10 and TGF-β and leads to the downregulation of the expression of the activating receptor NKG2D on NK cells. This results in a functional impairment of NK cells (Sène et al., 2010). Moreover, core protein of HCV induces the p53-dependent TAP1 (Transporter associated with Antigen Processing 1) gene expression in liver cells, which is followed by the subsequent up-regulation of major histocompatibility complex (MHC) class I. The increased level of MHC class I results in an inhibition of NK cells cytotoxicity against HCV core transfected liver cells (Herzer et al., 2003).

Thus, HCV modulates NK cell functions by different mechanisms. Altogether these functional interferences lead to an overall NK cell level decrease, an altered subset distribution and a change in NK receptor expression in the setting of chronic HCV (Golden-Mason and Rosen, 2013).

1.1.7.2 Adaptive Immunity

Adaptive immunity to HCV appears 6-8 weeks after infection and constitutes the last barrier against HCV infection. The innate immune response allows the activation of antigen presenting cells and cytokines secretion that will induce the adaptive humoral and cellular immune responses. While the humoral immunity involves antibody producing B-cells, the cellular immunity involves CD4+ helper and CD8+ cytotoxic T cells. In a minority of HCV infected patients, the early production of neutralizing antibodies and a sustained anti HCV response of CD4+ and CD8+ T cells can lead to the clearance of the virus (Guidotti and Chisari, 2006). However, in the majority of the cases, HCV has developed strategies to bypass the immune response and to persist in the host.

1.1.7.2.1 Humoral immune response

Humoral immune response following HCV infection involves activation of B cells upon interaction between the viral antigens and the B-lymphocytes receptors. Acute humoral response to HCV has been difficult to study since it is asymptomatic in most cases. Thus, most of the studies are retrospective. IgM is the first immunoglobulin isotype produced in response to infection (Lau et al., 1994; Tokushige et al., 2000). However, HCV-specific IgM are also detected in chronically infected patients and are thus not good marker of acute infection. Interestingly, the delay between IgG and IgM production is very weak in acute infections (Chen et al., 1992; Nikolaeva et al., 2002). Antibodies directed against structural and non-structural HCV proteins can be detected in HCV infected patients (Logvinoff et al., 2004). Nonstructural protein specific antibodies are thought to be produced in response to debris of damaged cells (Dustin et al., 2014). Only a weak proportion of HCV-specific antibodies correspond to neutralizing antibodies (nAbs) that can inhibit the virus entry into the cells. All known nAbs target epitopes within the envelope glycoproteins E1 and E2 that are present at the surface of the viral particle.

The role of the humoral immune response in the control of HCV infection is not well understood. However, the early production of broadly crossreactive nAbs during acute infection has been associated with the spontaneous clearance of the virus in several studies (Dowd et al., 2009; Osburn et al., 2010; Pestka et al., 2007; Raghuraman et al., 2012; von Hahn et al., 2007). Inversely, virus persistence correlates with a delayed production of nAbs (Pestka et al., 2007). Moreover, humans and chimpanzees, who spontaneously cleared infection, are less prone to reinfection (Bassett et al., 2001). Passive immunizations with HCV nAbs have been shown to mediate protection in chimpanzees (Farci et al., 1996; Morin et al., 2012). During the chronic phase of the infection, nAbs are thought to contribute to the control of the viral load in the patient serum (Ball et al., 2014).

Thus, neutralizing antibodies that are produced during infection can contribute in some patients to the spontaneous clearance of the virus. However, in the majority of cases, HCV evades the humoral immune response and progress to chronicity. Several mechanisms contribute to HCV evasion from the humoral immune response. The first is mediated by the high genetic variability of the virus that circulates as quasispecies in the patients. Thus, HCV error prone replication permits the rapid emergence of nAbs resistant variants (von Hahn et al., 2007). Another mechanism of evasion consists in masking the epitopes of the nAbs by E1

and E2 associated glycans (Helle et al., 2007; Lavie and Dubuisson, 2017). Furthermore, the association of HCV with lipoproteins greatly decreases its sensitivity to nAbs (Grove et al., 2008). Interestingly, the apolipoprotein E that is present on the viral particle is also involved in viral escape from antibody neutralization (Bankwitz et al., 2017). Finally, the ability of the virus to spread by cell-to-cell transmission allows bypassing extracellular fluids, thereby impeding nAbs access to viral particles (Brimacombe et al., 2011; Timpe et al., 2008).

1.1.7.2.2 Cell mediated immune response

Cell mediated immunity consists of two main arms; CD4+ and CD8+ T cells. The cytokines produced during the innate immune response as well as the antigen presenting cells activate T lymphocytes. Some data suggest that dendritic cells could be partially permissive to HCV infection, which could interfere with their antigen presenting cells function and impact the quality of the activation of T cells (Pachiadakis et al., 2005). During acute HCV infection, vigorous CD4+ and CD8+ T cell responses targeting different regions of HCV proteins and associated with the production of IFNγ have been observed (Bowen and Walker, 2005; Lechner et al., 2000; Shin et al., 2006; Thimme et al., 2001). Moreover, several studies have reported the existence of a correlation between the T cell response and the control of the infection (Lechner et al., 2000; Takaki et al., 2000; Thimme et al., 2002, 2001). The protective function of CD4+ T cells relies on their capacity to recognize the viral antigens and to activate B and cytolytic CD8+ T cells. Importantly HCV clearance has been shown to correlate with a rapid and strong proliferation of specific CD4+ T cells and the production of IL2 and IFNγ (Diepolder et al., 1995; Kapadia et al., 2007; Missale et al., 1996; Urbani et al., 2006). Conversely delayed and weak or inexistent HCV specific CD4+ T cells responses have been observed in chronic HCV infection (Urbani et al., 2006). Thus, CD4+ T cells help during acute infection is important for recovery, and its maintaining during time allows the development of a protective response (Smyk-Pearson et al., 2008; Urbani et al., 2006). CD8+ T lymphocytes exert their antiviral activities by secreting the proinflammatory cytokines IFNγ and TFNα and through their cytolytic activity (Tsai et al., 1997). Several studies suggest that HCV infection affects CD8+ T cells cytotoxicity, proliferation as well as their capacity to secrete cytokines (Neumann-Haefelin et al., 2005). The impact of the CD8+ T cells response on the infection outcome during acute infection is not clear. Whereas some studies suggest that CD8+ T cells response has no major effect on the outcome of infection (Francavilla et al., 2004; Kaplan et al., 2007; Urbani et al., 2006), others suggest that it

participates to HCV clearance (Grüner et al., 2000; Thimme et al., 2001) . During the chronic phase, the CD8+ T cell response participates to the regulation of the viral replication rate. Finally, spontaneous recovery from acute HCV infection is associated with effective T cells responses. Progression to chronic persistent infection is favored by immune-evading viral mutations as well as the capacity of the virus to inhibit CD8+ T cell response.

1.1.8 Diagnosis of HCV

Since in most HCV infected patients the acute phase of infection is asymptomatic the development of efficient screening tools is of great importance to limit the virus spread.
HCV diagnosis methods comprise direct or indirect tests. The indirect tests correspond to the detection of the antibodies induced by the virus infection, IgM for a very recent infection and IgG for both a recent or past infection. The direct tests comprise the detection and quantification of viral components such as the viral genome or viral antigens. However, these tests do not evaluate the severity nor the outcome of the disease. HCV RNA can be detected in an early phase of infection; approximately 2 weeks post infection, while seroconversion might take place up to 6 weeks later (Fig.5) and might be delayed or missing in immunocompromised patients such as HIV positive individuals (Klenerman and Kim, 2007).

1.1.8.1 Serologic Assays

Several Immunoassays are used for the detection of anti-HCV antibodies in plasma or serum. Two enzyme immunoassays (EIA) have been approved for clinical use, HCV EIA 2.0 (Abbott) and Ortho HCV Version 3.0 (Ortho-Clinical Diagnostics), in addition to an enhanced chemiluminescence immunoassay (CIA) VITROS anti-HCV assay, (Ortho-Clinical Diagnostics) (Pondé, 2013). Current EIA's are quite sensitive with a high specificity towards anti-HCV and relatively inexpensive (Colin et al., 2001). However, the serological assays do not allow differentiating active infections from past infections and can lead to the generation of false positive diagnosis.

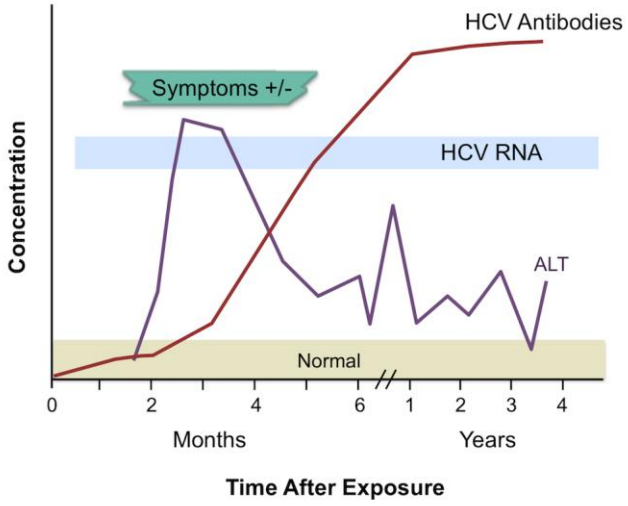

Figure 5. Appearance of laboratory markers over time during HCV infection. Viral RNA became first detectable, which is followed by a raise in ALT levels and delayed appearance of antibodies ("Hepatitis C Online," 2018).

1.1.8.2 Molecular Assays

HCV RNA detection is the main diagnostic tool to determine active infection (replicating virus). It is a useful method to identify patients that are potential candidates for therapy and to evaluate response to antivirals as well as detecting resistance to DAA treatment (European Association for Study of Liver, 2015). Detection and quantification of RNA are based on real time polymerase chain reaction (PCR) or transcription-mediated amplification (TMA) assays. Both methods are highly specific and sensitive and results are expressed in IU/ml rather than viral copies (Pawlotsky, 2002; Scott and Gretch, 2007).

A recent quantitative core antigen assay (ARCHITECT, Abbott, Diagnostics) composed of 5 distinct antibodies directed towards the core protein of HCV was developed. This assay showed high specificity and sensitivity for identification of persistent HCV infection, besides being adequately efficient against all HCV genotypes. Some studies showed a correlation between HCV core antigen and HCV RNA quantification, suggesting that nucleic acid testing could be replaced by core Ag quantification for monitoring antiviral responses during the course of treatment. This could be a less expensive alternative, yet less sensitive (Lamoury et al., 2017; Mederacke et al., 2009; Vermehren et al., 2012).

Detection of HCV specific antibodies and HCV RNA indicates an active infection, while the presence HCV-specific Abs in the absence of viral RNA are signs of a past infection. It's

therefore essential to perform HCV nucleic acid testing following positive serologic tests. It is also recommended to screen HCV RNA in immunocompromised patients, such as HIV infected patients, as humoral immune responses might be deficient.

1.1.8.3 HCV genotyping

HCV genotyping was considered as a prerequisite before starting antiviral therapy at the era of interferon based therapy, as response to therapy differed according to the genotype (Bowden and Berzsenyi, 2006). Besides, the duration of the treatment to reach sustained viral response is also affected by the genotype (González et al., 2013). Additionally, genotype and subtype determination increases our comprehension of the mechanisms of appearance of resistance-associated variants either occurring before treatment or emerging during the treatment course (Cuypers et al., 2016).

Two methods allow the genotyping of the virus. The serologic method is based on the detection of HCV-specific antibodies using competitive Enzyme Immunoassay technique (Pawlotsky et al. 1997). Currently, the available assay allows the identification of the 6 HCV genotypes but not the subtypes (Montenegro et al. 2013). The molecular methods correspond to the sequencing of the viral genome. Early assays analyzed only the 5'UTR region, which was associated with a high percentage of false classification on the subtype level. Meanwhile, specificity has been improved by analyzing the coding regions, especially NS5B and core protein encoding genes, which presents more variability between the different genotypes and subtypes (Avó et al., 2013; Mauss et al., 2018).

1.1.9 Treatment of HCV infection

1.1.9.1 Current Treatment

Most patients exposed to HCV infection are likely to develop chronic hepatitis, while about 15%- 25% might clear the virus spontaneously. The primary goal of therapy is to eradicate HCV infection by achieving a sustained virological response (SVR), which is defined as undetectable HCV RNA in blood 12 weeks (SVR12) or 24 weeks (SVR24) after end of treatment (EASL, 2018).

Previously and for more than a decade the standard-of-care (SOC) treatment of HCV consisted in the administration of pegylated-interferon alpha (PEG-IFNα) plus the nucleoside analogue ribavirin (RBV) (Fried et al., 2002). IFNα acts via inducing host antiviral responses and the PEG moiety increases its stability and half-life in patients, while RBV enhances the antiviral activity of interferon (Feld and Hoofnagle, 2005; Pawlotsky et al., 2004). Nevertheless, this treatment showed modest SVR rates, especially in patients infected with genotype 1 and 4 achieving a SVR in less than 50% of cases. Patients infected with genotype 2 or 3 HCV were the best responders with 80% of them developing a SVR upon treatment (Fig.6) (Zeuzem 2008). Moreover, this treatment was associated with various and frequent adverse effects, e.g. haemolytic anemia, psychiatric disturbances and flu-like symptoms (Kish et al., 2017; Pawlotsky et al., 2015).

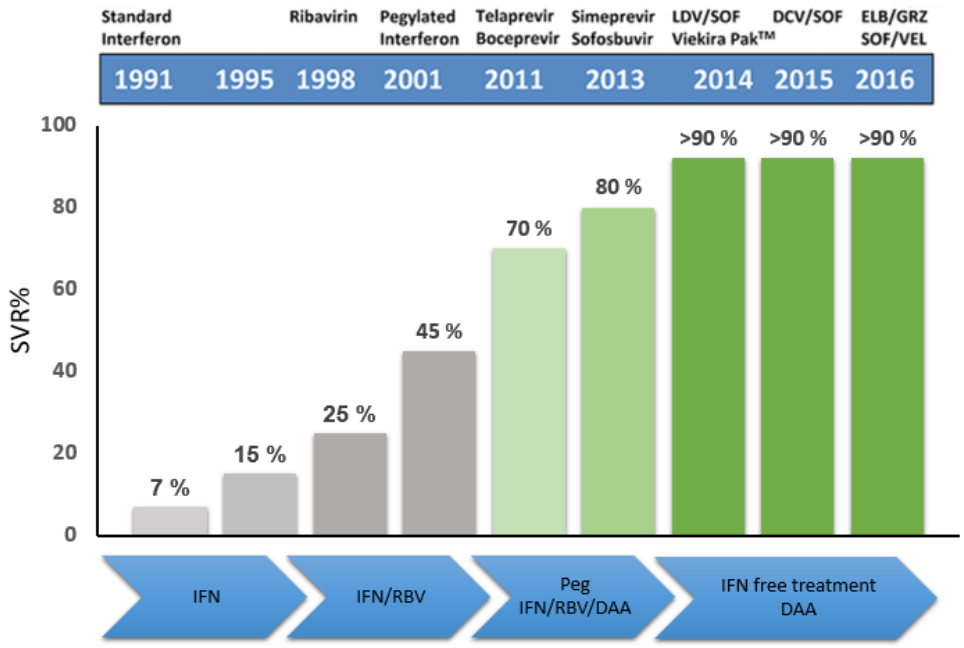

Figure 6 Sustained virological response rates and their improvement with evolution of treatment overtime, adapted from (Carter et al., 2017; Webster et al., 2015), DCV:daclatasvir, SOF:sofosbuvir, VEL: velpatasvir. LDV: ledipasvir, ELB:elbasvir, GRZ: grazoprevir.

Following the isolation of HCV, several tools of cell culture could be developed to characterize the viral cycle. The breakthroughs in the understanding of HCV molecular virology, especially revealing the 3D structures of HCV key enzymes, led to the identification of several targets for drug discovery (Pawlotsky et al., 2015). By combining compounds screening and drug design, small molecules with high potency against different HCV proteins were identified. A milestone in HCV treatment has been achieved by the introduction of the first two DAA (direct acting antivirals), telaprevir and boceprevir. They

represented the first-generation of NS3-4A protease inhibitors (Fig.7). They exerted their effect through binding to the catalytic site of the enzyme and blocking post-translational processing of the viral polyprotein. Both drugs were used in combination with PEG IFN-α and RBV for the treatment of chronic HCV patients infected with genotype 1 in 2011 reaching a SVR in nearly 70% of cases. However, these medications were associated with undesirable adverse effects and low barriers to resistance (Ghany et al., 2011; Pawlotsky et al., 2015). In 2013, simeprevir another NS3-4A protease inhibitor was approved; it showed potent activity against genotypes 1, 2 and 4 but not towards genotype 3. It was better tolerated, but it still had a low barrier to resistance. Second-generation NS3-4A protease inhibitors were developed to have pangenotypic activity with an enhanced barrier to resistance. They also showed antiviral potency against genotype 3, however with lower efficacy against other genotypes (Lahser et al., 2016). A third-generation of NS3-4A protease inhibitors are under clinical development and are expected to have equal antiviral effectiveness against all HCV genotypes and a high barrier to resistance (Pawlotsky, 2014).

Protease inhibitors constitute powerful antivirals and have become key components of all treatments for HCV genotype 1 infection. Nevertheless, the low conservancy of the active site of NS3 among different HCV genotypes, has urged to broaden the treatment spectrum (C. Clark et al., 2013). Thus, more focus has been put in developing inhibitors to other NS proteins, especially NS5A.

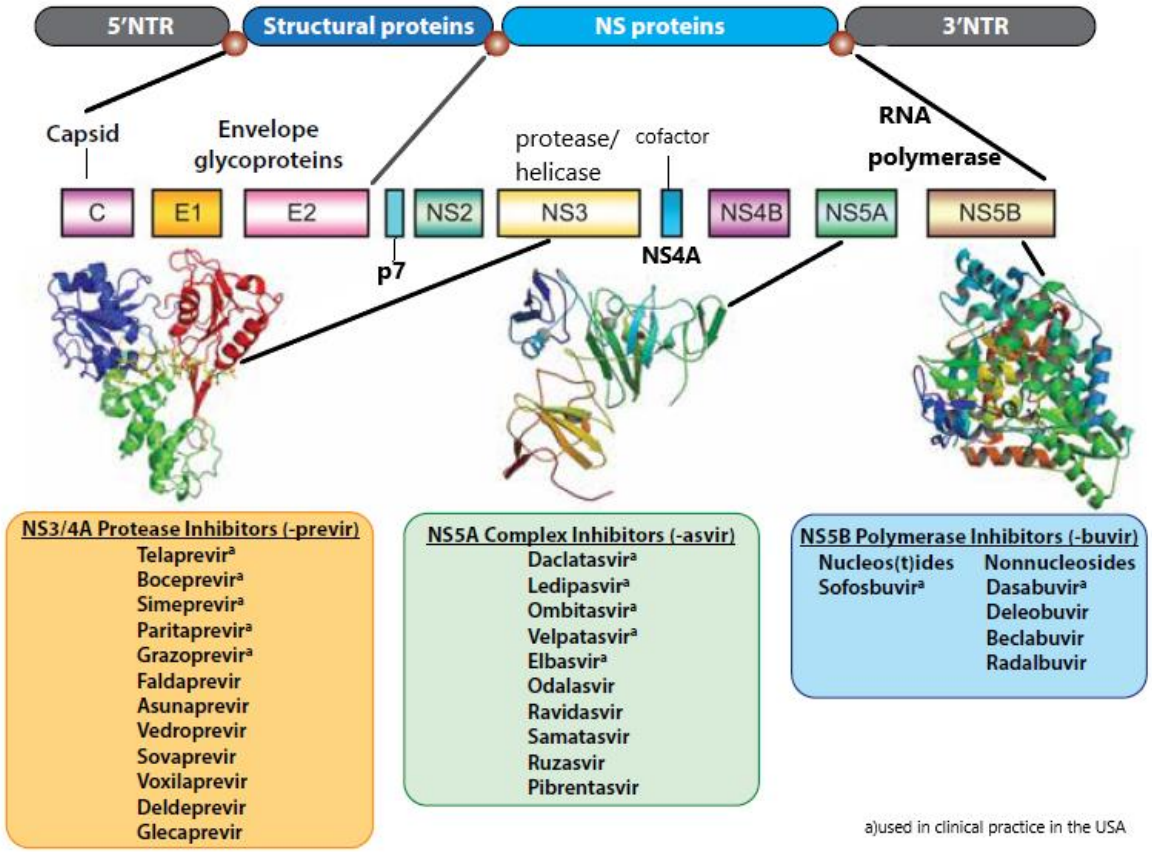

Figure 7 Different classes of direct acting antivirals and their associated viral protein target adapted from (Horsley-Silva and Vargas, 2017).

NS5A has been shown to be an essential component of the replication complex of the virus responsible for regulating viral replication and assembly (Fig.7). Daclatasvir was the first introduced NS5A inhibitor, which was followed by the development of ombitasvir, ledipasvir and samatasvir (Pawlotsky, 2014). These drugs have shown a highly potent antiviral activity against all HCV genotypes in vitro, nevertheless a lower sensitivity of genotype 2 and/or 3 than genotype 1 and 4 has been reported (Gao et al., 2010; Scheel et al., 2011). Although these drugs target the NS5A domain I, their precise mode of action is not clearly known, it is suggested that their effect might go beyond just inhibiting viral replication (Berger et al., 2014; Bukh, 2016). Nowadays, NS5A inhibitors have become an essential component of DAA combined therapy, nevertheless they have a relatively low barrier of resistance (Pawlotsky, 2016). Second-generation NS5A inhibitors e.g. Elbasvir and Velpatasvir are also highly active against all HCV genotypes with a higher barrier to resistance compared with first-generation NS5A inhibitors, but some of them are less active against genotypes 2 and 3 than other genotypes (Lahser et al., 2016).

The RNA-dependent RNA polymerase (RdRp) NS5B has always been considered as an important target for antiviral drugs. DAA targeting NS5B are divided in two categories: the nucleoside and the non-nucleoside inhibitors (Fig.7). Revealing the crystal structure of the NS5B polymerase (Lesburg et al., 1999; Love et al., 2003) paved the way for the development of nucleoside analogs that act as false substrates for the HCV RdRp and subsequently block RNA chain elongation. Due to the high conservation of NS5B active site, nucleoside analogues have pan-genotypic activity against HCV and a high barrier to resistance (Pawlotsky, 2016). Sofosbuvir is the first approved nucleoside analogue that is active against all HCV genotypes (Sofia et al., 2010). It is a pro-drug that becomes active upon metabolization by the liver. Moreover, combined therapy of Sofosbuvir with Velpatasvir (NS5A inhibitor) has been shown in clinical trials to be effective against HCV genotypes 1-6 (Feld et al., 2015; Foster et al., 2015). Sofosbuvir is included in most of the current HCV treatments. Non-nucleoside inhibitors of NS5B act by binding to allosteric sites of the polymerase, thus inducing conformational changes that block its catalytic function leading indirectly to inhibition of RNA replication (Haudecoeur et al., 2013). Non-nucleoside inhibitors such as the Beclabuvir and Dasabuvir, show a limited efficacy mainly against genotype 1 and present a low barrier to resistance (Pawlotsky, 2016).

With the approval of an increasing number of DAA, HCV treatments free of IFN became available for all genotypes. Current approved treatments include combinations of two to three DAA (Table 1). They are mostly well tolerated and can cure HCV infection in more than 90% of the treated patients (Fig.6).

Table 1 Available Hepatitis C direct acting antivirals against different genotypes (Carter et al., 2017).

Drug Class	Generic Name	Associations	Trade Name	HCV Genotype with approved indication
NS3A/4A protease inhibitors	Simeprevir		Olysio 45	1, 4
	Paritaprevir	Ritonavir, ombitasvir, and copackaged with dasabuvir	Viekira Pak 40	1
	Paritaprevir	Ombitasvir, ritonavir	Technivie 44	4
	Grazoprevir	Elbasvir	Zepatier™41	1, 4
NS5B polymerase inhibitors/ nucleotide	Sofosbuvir		Sovaldi 37	1, 2, 3, 4
NS5B polymerase inhibitors/ nonnucleoside	Dasabuvir	copackaged with combination product Ombitasvir, paritaprevir, ritonavir	Viekira Pak 40	1
NS5A inhibitors	Ledipasvir	Sofosbuvir	Harvoni 39	1, 4, 5, 6
	Ombitasvir	Paritaprevir, ritonavir, and copackaged with dasabuvir	Viekira Pak 40	1
	Ombitasvir	Paritaprevir, ritonavir	Technivie 29	4
	Daclatasvir		Daklinza™42	1, 3
	Elbasvir	Grazoprevir	Zepatier™41	1, 4
	Velpatasvir	Sofosbuvir	Epclusa 43	1, 2, 3, 4, 5, 6

1.1.9.2 HCV vaccine

The development of a vaccine would be the best way to definitely eradicate HCV. However, several difficulties have to be overcome to reach that goal. Indeed, the main goal of vaccination is providing sterilizing immunity against any reencounter with the pathogen. Unfortunately, HCV can escape innate and adaptive immune response and natural infection does not provide protective immunity against reinfection. Moreover, HCV's great genetic diversity, the high rate of mutation appearance during replication and the lack of small animal immunocompetent models make the development of a prophylactic vaccine against HCV a great challenge (Smith et al., 2014).

However, there is evidence of some level of protective immunity against HCV. Some *in vivo* studies in chimpanzees suggest that immunity resulting from a resolved infection would prevent persistence following challenge with viruses of the same or different genotype (Grakoui et al., 2003; Major et al., 2002; Nascimbeni et al., 2003; Shoukry et al., 2003; Weiner et al., 2001).

There are two main strategies for developing a prophylactic vaccine. The first approach targets HCV particles structural elements such as the E1E2 envelope glycoproteins to induce the humoral immune response. The second approach uses the conserved non-structural proteins to induce the T cell response (Table 2).

Table 2 Current hepatitis C virus vaccine strategies (Shoukry, 2018).

Main Target	Stage	Immunogen	Vaccine regimen	Induced immune response	Potential improvements
T cells	Phase 2	NS3–NS5	Chimpanzee adenovirus 3 priming + modified vaccinia Ankara boost	Polyfunctional CD4 and CD8 T cells • No antibodies (Abs)	•More potent vectors (e.g., CMV) • Invariant chain combination (enhanced Ag presentation) • Combination with recombinant proteins • Combination with immune check point blockade (for direct-acting antiviral-treated subjects)
Antibodies	Phase 1	gpE1/gpE2	Recombinant gpE1/ gpE2 + adjuvant (MF59C.1)	• Some CD4 T cells • Broadly neutralizing antibodies	• Better adjuvants • Better CD8 T cell response inducers • Combination with nonstructural proteins

Prophylactic B-cell vaccines

These vaccines are mainly based on the use of E1/ E2 envelope glycoproteins as antigens. Indeed, E1 and E2 constitute the main targets of the humoral immune response during HCV infection, inducing the production of a wide range of neutralizing antibodies (Ball et al., 2014; Sautto et al., 2012). These vaccines are the first to have been tested in chimpanzees. Recombinant proteins are produced into yeast, bacteria or mammalian cells and purified to be used in the vaccine. While some recombinant proteins are potent enough to induce immune responses by themselves, others need adjuvants. Advantages of this approach are that neither the pathogen nor its genetic material are included in the vaccine and there is no need for organism culture.

Recently, a vaccine composed of recombinant E1 and E2 proteins from the genotype 1a reached the clinical phase I. This vaccine developed by Chiron (now Novartis) can induce the production of broadly neutralizing antibodies and a robust anti-HCV CD4+ T cell response (Law et al., 2013; Wong et al., 2014). However, despite these results the capacity of the

vaccine to efficiently protect from a persistent infection has still to be evaluated (Houghton, 2011; Walker and Grakoui, 2015).

Prophylactic T-cell vaccines

Another vaccine approach is directed towards priming broadly potent CD4+ and CD8+ T cells by HCV nonstructural proteins. Thus, a vaccine corresponding to a recombinant viral vector encoding the non-structural proteins NS3 to NS5B of the 1b genotype (developed by Okairos now GlaxoSmithKline) is currently in phase 2 clinical trials as a prophylactic vaccine in high-risk injection drug users. Due to a mutation in the NS5B gene, these vectors were replication deficient. The heterologous prime-boost vaccination strategy based on the use of a replication defective adenoviral vector and modified vaccinia Ankara (MVA) vector encoding the NS3 to NS5B proteins has been previously tested in healthy volunteers. This protocol led to the generation of high level of CD4+ and CD8+ HCV-specific T cells targeting multiple HCV antigens. Moreover, sustained memory and effector T-cell populations were generated and the quality of memory T cells improved over time after the MVA boost (Swadling et al., 2014; Walker and Grakoui, 2015).

New generations of HCV vaccines will most probably use a combination of antibody and T cell-based vaccines to boost potency. Thus, a recent study demonstrated that combining adenovirus vector expressing NS proteins with E1E2 protein antigens could induce strong antibody and T cell response that surpass immune response obtained by either vaccine alone (Chmielewska et al., 2014). Furthermore, the follow up of HCV immune response in a patient who spontaneously cleared HCV revealed the importance of both antibody and T-cells responses in spontaneous resolution of HCV (Raghuraman et al., 2012). Based on their mode of action, it is believed that most probably nAbs limit acute infection, allowing T cells to resolve the infection.

1.2 HCV viral structure and function

1.2.1 HCV viral particle

HCV is a small-enveloped virus with a positive single stranded RNA genome. HCV particles present heterogeneous size and density ranging from 30 to 100 nm and from 1.03 to 1.20 g/cm3, respectively (André et al., 2002; De Vos et al., 2002; Falcón et al., 2017, 2003). This

heterogeneity is due to the association of the viral particles with lipoproteins that modulate the virus infectivity. Thus different apolipoproteins (ApoA1, ApoB, ApoC1 and ApoE) are present at the surface of these hybrid particles named lipoviroparticles (LVP). The recent lipidomic characterization of HCVcc demonstrated that viral particles shared similar lipid composition to very low density lipoprotein (VLDL) and low density lipoprotein (LDL) with cholesteryl esters accounting for almost half of the total HCV lipids (Merz et al., 2011; Andre et al. 2002; Gastaminza et al. 2010; Nielsen et al. 2006).

The viral envelope is composed of a lipid bilayer in which the E1 and E2 glycoproteins are embedded (Bartenschlager et al., 2011). Under the lipid membrane, the nucleocapsid formed by the Core protein contains the genomic single strand RNA (Fig.8). E1 and E2 envelope proteins play an essential role in virus entry. They are type I transmembrane proteins forming non covalent E1E2 heterodimers inside infected cells while they associate in large covalent complexes on HCV virions (Vieyres et al., 2014).

Due to limitations in purification, HCV particles have been difficult to characterize by electron microscopy. Recently an approach of HCV particles immunocapture allowed the imaging of intact particles by direct transmission electron microscopy (Piver et al., 2017). The visualized particles present a central disc corresponding to the capsid, surrounded by an irregular ring and an external electron-light crescent corresponding to the lipids associated to the virus.

The association of HCV with lipoproteins is thought to facilitate HCV entry into hepatocytes and protect the viral particles from neutralizing Abs (André et al., 2002).

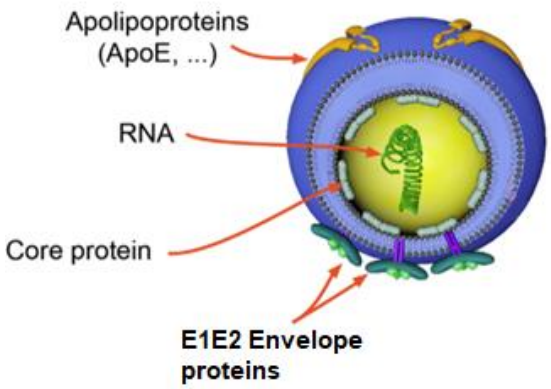

Figure 8 Structure of hepatitis C viral particle. HCV virions are associated with lipoproteins forming a hybrid particle termed lypoviroparticle (Lavie and Dubuisson, 2017).

1.2.2 HCV genome organization and function

The genome of the hepatitis C virus comprises a single stranded RNA which is composed of 9.6 kb. It is a positive sense RNA and hence it is directly translated after delivery into the cells. The genome contains one open reading frame (ORF) that is flanked by two untranslated regions (UTR) and encodes a polyprotein of approximately 3000 amino acids. The 5' end serves as an internal ribosome entry site (IRES) while the 3' end is crucial for replication. Consequently, this polyprotein is co- and posttranslationally cleaved by viral and cellular peptidases into mature proteins (Fig.9), the structural proteins (core, E1 and E2), the nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) and the p7 protein (Bartenschlager et al., 2013a; Gottwein and Bukh, 2008; Moradpour et al., 2007).

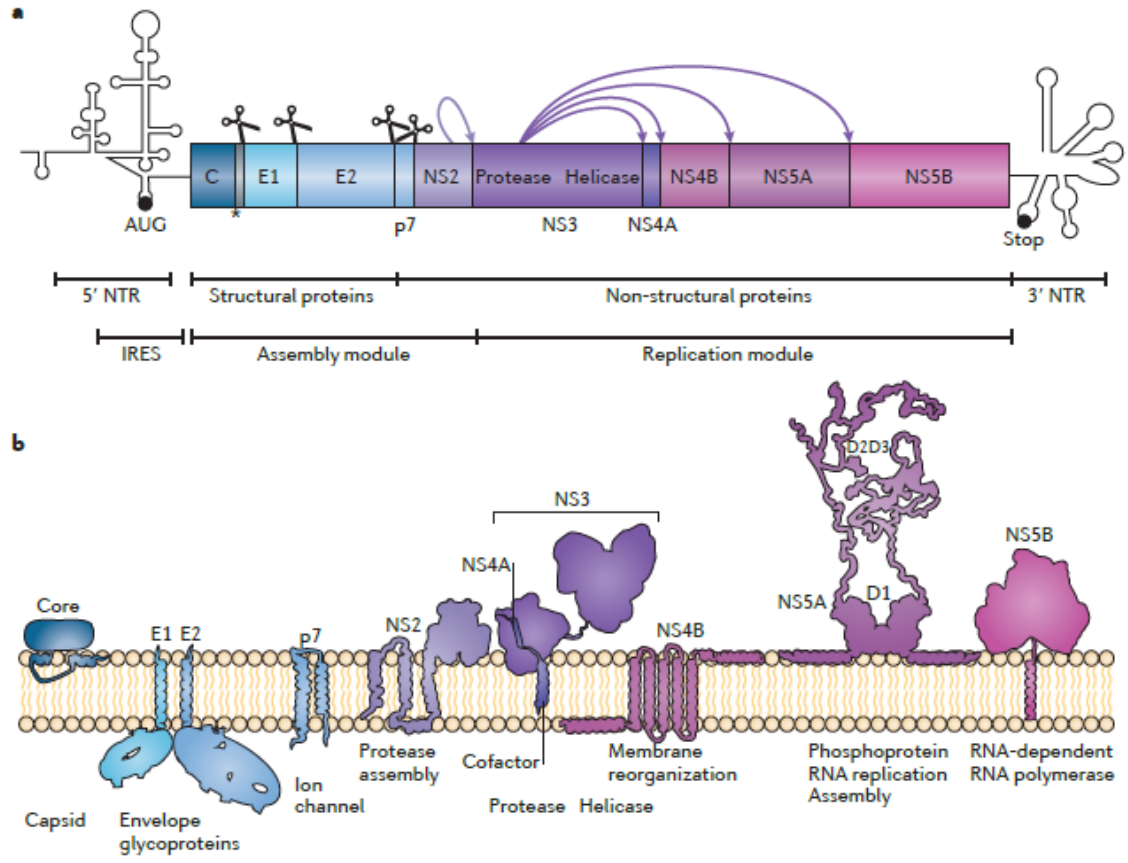

Figure 9 Genome organization of hepatitis C virus and membrane arrangements of its viral proteins.a) HCV viral genome consists of a single open reading frame encoding the polyprotein that is flanked by two non-translated regions at its 3'- and 5'ends. An internal ribosome entry site (IRES) is found in the 5'NTR region. Cellular signal peptidases that cleave the polyprotein are designated by scissors, while arrows indicate cleavage sites by viral proteases. b) Viral proteins' membrane topology and their different functions. Viral proteins are anchored to the ER by transmembrane domains or by an α-helix as for core and NS5A proteins. NS3 is tethered to the ER via its cofactor NS4A and an α-helix (Bartenschlager et al., 2013b).

1.2.2.1 The 5'UTR

The 5' UTR consists of 341 nucleotides and is the most conserved region among HCV genotypes. It is composed of four structured domains (I to IV) (Fricke et al., 2015). The domains I and II are crucial for HCV replication (Friebe et al., 2001; Kim et al., 2002), as they comprise 2 binding sites for the microRNA-122 (miR-122) which stabilize the viral genome and regulate its replication (Henke et al., 2008; Jopling et al., 2008; Sedano and Sarnow, 2014). The domains II, III and IV in addition to the first 42 nucleotides of the core coding region form the IRES that binds directly to the 40S ribosomal subunit initiating cap-independent translation of the polyprotein (Ji et al., 2004; Otto and Puglisi, 2004). Moreover, the domain III interacts with eIF3 (eukaryotic initiation factor 3), which induces the translation of the viral RNA (Kieft et al., 2002).

1.2.2.2 The 3'UTR

The 3'UTR region contains approximately 225 nt and comprises 3 regions. The first one is a genetically variable region of 30-70 nucleotides, which starts directly after the termination codon. It is followed by a polypyrimidine (polyU/C) sequence, which differs in length and composition among genotypes. The last segment corresponds to 98 highly conserved nucleotides that form the 3' terminal domain (3'X), which consists of 3 stem-loops (SL1, SL2 and SL3) (Kolykhalov et al., 1996; Tanaka et al., 1996; Yi and Lemon, 2003). The 3'X and a minimal poly(U/C) tract are indispensable for replication, while the variable region modulates the efficacy of the replication (Murayama et al., 2010; Niepmann et al., 2018; Yi and Lemon, 2003).

1.2.2.3 Core

The core protein, which is presumed to form the nucleocapsid, is required for genome packaging. It represents the first 191 residues of the viral polyprotein. It is first released from the polyprotein by a host signal peptidase cleavage at the C-terminus (McLauchlan et al., 2002), after being addressed to the endoplasmic reticulum by a signal peptide located in its C terminal part. There, the mature form of the protein is produced after processing by a host signal peptide peptidase (Santolini et al., 1994). Only the mature form of the protein leads to the production of infectious viral particles (Targett-Adams et al., 2008). These proteins

oligomerize and associate with the viral genome to form the virus nucleocapsid (Kao et al., 2016; Moradpour and Penin, 2013a). The mature core protein is a dimeric alpha-helical protein, which behaves as a membrane protein (Boulant et al., 2005). This protein comprises two main domains and several disulfide bonds that stabilize its structure. The N terminal hydrophilic domain (D1) is composed of 120 residues and contains several positively charged amino acids (Klein et al. 2005). It shares characteristics with capsids proteins of related pestiviruses and flaviviruses (Boulant et al., 2005; McLauchlan, 2000). D1 is mainly involved in the binding with RNA and core-core interactions that might lead to capsid assembly (Cristofari et al., 2004; Klein et al., 2005). Furthermore this domain interacts with several viral and cellular factors (Roohvand et al., 2009).

The second hydrophobic domain (D2) of about 50 residues is necessary for proper folding of D1 and is responsible for the membrane association of core (McLauchlan, 2009; McLauchlan et al., 2002; Rouillé et al., 2006; Schwer et al., 2004). When expressed in the context of heterologous expression systems or HCV replicon, core is found attached to the Endoplasmic reticulum (ER) and at the surface of lipid droplets. Moreover core was also found to localize in the mitochondria in full-length HCV replicon expressing Huh7 cells (Schwer et al., 2004). Nevertheless, in infected cells, core was only found in association with lipid droplets (Rouillé et al., 2006). Since the export of the protein from the ER to the lipid droplets has been shown to depend on the cleavage of the C terminal part of the protein by signal peptide peptidase, it is likely that this step is quickly achieved in the course of an infection. Localization of core at the surface of lipid droplets is essential for the production of viral particles (Boulant et al., 2007; Lyn et al., 2013).

Besides its role in nucleocapsid formation, core protein has been reported to interact with several cellular proteins and signaling pathways (McLauchlan, 2000). HCV core modulates apoptosis in a pro- and anti-apoptotic way (Chou et al., 2005; Kountouras et al., 2003). It also induces Huh-7 cell proliferation (Fukutomi et al., 2005) and is involved in liver injury and fibrogenesis (Núñez et al., 2004) as well as steatosis and HCC (Lerat et al., 2002; Moriya et al., 1998). There has also been evidences that core interacts with HCV E1 glycoprotein (Baumert et al., 1998; Nakai et al., 2006), p7 and NS2 (Murray et al., 2007).

Frame shift protein

An unusual ribosomal frameshift during translation of the core encoding region results in the generation of an alternate reading frame protein (ARFP). ARFP is composed of 160 residues

with a molecular weight of 17 kDa (Boulant et al., 2003). Supporting the existence of this protein, ARFP-specific antibodies and T-cell immune response have been detected in HCV infected patients (Karamitros et al., 2012; Walewski et al., 2001). However, some studies showed that ARFP is unnecessary for HCV replication in vivo or in cell culture but pointed out the presence of a functionally important RNA element in the ARFP coding region (McMullan et al., 2007). Interestingly, recent studies suggested a role of ARFP in virus associated pathogenesis and development of HCC and that ARFP-specific antibodies levels might constitute biological markers for disease progression (Kassela et al., 2017; Moustafa et al., 2018).

1.2.2.4 HCV envelope glycoproteins E1E2

HCV E1E2 envelope glycoproteins will be illustrated in details in Chapter 1.5 of the Introduction.

1.2.2.5 p7 protein

P7 is a small polypeptide composed of 63 amino acids, positioned at the junction between the structural and nonstructural proteins. It has been shown to consist of two transmembrane domains (TM1 and TM2) that are connected via a short cytoplasmic loop (Fig.10), with the N and C- terminus ends directed toward the ER lumen (Carrère-Kremer et al., 2002). P7 protein belongs to the viroporin family that can form an ion channel, facilitating the virus production (Nieva et al., 2012; Premkumar et al., 2004). Interestingly, this ion channel activity could be blocked by amantadine, an antiviral drug blocking the M2 ion channels of influenza virus (Griffin et al., 2003; Steinmann et al., 2007b). P7 is not essential for HCV RNA replication but for the assembly and release of infectious viral particles in chimpanzees and *in vitro* (Sakai et al., 2003; Steinmann et al., 2007a). Thus, the absence of p7 leads to the accumulation of capsid intermediates that have partially incorporated viral RNA (Gentzsch et al., 2013). Several functions have been proposed for p7 during infection. First, by forming an ion channel, p7 might regulate the pH of some intracellular compartments which is important for the protection of infectious virions during their secretion, as low pH might induce misfolding and inactivation of the HCV glycoprotein (Wozniak et al., 2010). Second, during the virus morphogenesis, p7 modulates NS2 complexes formation with E2, NS3 and NS5A, thus regulating early assembly events (Popescu et al., 2011a; Shanmugam and Yi, 2013;

Stapleford and Lindenbach, 2011). Third, p7 in association with NS2 regulates the core protein relocalization from lipid droplets to ER assembly sites (Boson et al., 2011).

1.2.2.6 NS2 protein

NS2 is an integral membrane protein of 23 kDa localized in the ER. Before cleavage from the polyprotein, NS2 participates together with the N terminal part of NS3 in a protease activity responsible for the cleavage at the NS2/NS3 junction (Grakoui et al., 1993a; Hijikata et al., 1993). Indeed, NS2/NS3 is a cysteine protease whose activity is zinc-dependent and therefore classified as a metalloprotease (Gouttenoire et al., 2006; Lorenz et al., 2006; Pallaoro et al., 2001; Tedbury and Harris, 2007). NS2 by itself is not required for RNA replication, but the cleavage at the NS2/NS3 junction is required to release the NS3 protein which is crucial for HCV replication (Welbourn et al., 2005). Moreover, NS2 has been shown to be necessary for the production of infectious HCVcc (Jones et al., 2007). Recent studies demonstrated that beside its protease activity, NS2 plays a pivotal role during virus assembly by interacting with structural and nonstructural proteins such as E1, E2, p7, NS3 and NS5A (Boson et al., 2011; Jirasko et al., 2010; Popescu et al., 2011a; Stapleford and Lindenbach, 2011). It has been reported that the C-terminus of NS2 is responsible for its catalytic activity while the N-terminus encodes a membrane domain that is composed of 3 presumed transmembrane segments (Fig.10) (Jirasko et al., 2010; Yamaga and Ou, 2002).

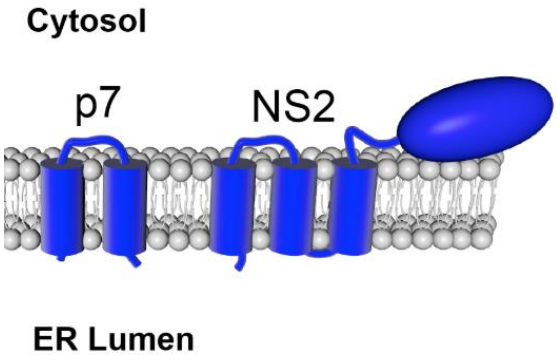

Figure 10 Schematic diagram of the topology of p7 and NS2 proteins (Popescu et al., 2011a).

1.2.2.7 NS3 and NS4A

NS3 is multifunctional protein of 67 kDa with a N-terminal serine protease domain and a C-terminal nucleoside triphosphatase (NTPase)/RNA helicase domain (Fig.11) (Gallinari et al.,

1998). The 8 kDA NS4A protein acts as a cofactor for NS3 protease, forming the NS3-4A complex. NS4A is anchored in the ER membrane via its transmembrane N-terminal domain thus allowing the association of NS3 with the ER membrane (Fig.12). The NS3-NS4A protease is indispensable for HCV replication. It catalyzes the cleavage of the polyprotein at the junctions between NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B. This activity is thus essential for the setting up of the viral RNA replication complex. The structure of the NS3/NS4A protease complex has been resolved (Yao et al., 1999) and the catalytic triad of the protease has been shown to consist of the residues His 57, Asp 81 and Ser 139 (Bartenschlager et al., 1993; Grakoui et al., 1993b).

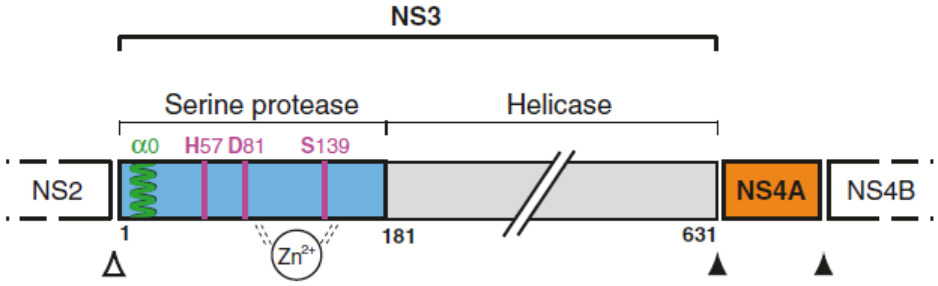

Figure 11 Schematic diagram of the NS3/4A region of the HCV polyprotein. The serine protease is indicated in blue, while the helicase in grey. NS4A is presented in orange, white arrow indicate cleavage by NS2 protease while black arrows stand for cleavages by NS3/4A protease (Moradpour and Penin, 2013b).

In addition to its role in the processing of the polyprotein, NS3-NS4A protease can cleave and inactivate several cellular factors (Sumpter et al. 2005). In fact, NS3/4A cleaves the key proteins of the innate immune response, MAVS and TRIF. This leads to the inactivation of the innate immune response induced by the RIG-I and TLR3 sensors of double stranded RNA signaling pathways (Li et al. 2005; Chen et al. 2007).

The NS3 helicase-NTPase function is required for viral replication. It allows unwinding RNA regions of dense secondary structures before RNA replication and separating nascent RNA from template strands during the replication. Moreover it may displace RNA bound proteins that could interfere with RNA synthesis. The helicase function of NS3 is enhanced by NS4A, and the NTPase activity seems to provide the energy required for the helicase function (Kuang et al., 2004; Lindenbach et al., 2007; Pang et al., 2002).

These data demonstrate the importance of the NS3/NS4A protease in HCV replication as well as in pathogenesis and persistence. Therefore, NS3/NS4A has been the first target for the development of anti-HCV DAA (K. Li et al., 2005).

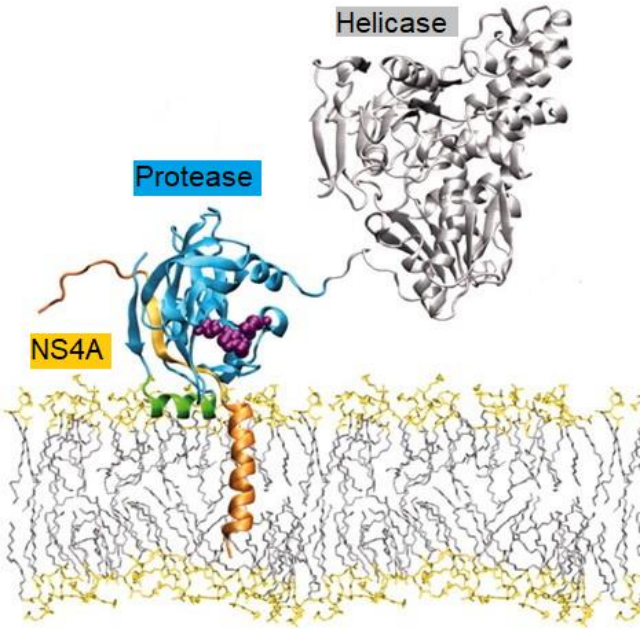

Figure 12 3D structure of the NS3 protease/helicase complexed with its cofactor NS4A (Bartenschlager et al., 2013b). The NS3 C-terminal helicase domain is shown in grey and the N-terminal protease domain in blue. The NS4A cofactor that anchors NS3 to intracellular membranes is depicted in orange.

1.2.2.8 NS4B

NS4B is an integral membrane protein of 27 kDa localized in the ER membrane (Hugle et al., 2001; Lundin et al., 2003). It is predicted to contain 4 transmembrane domains (TMD) encoded by its central region and cytosolic N-terminal and C-terminal regions (Elazar et al., 2004; Hugle et al., 2001; Lundin et al., 2003). Its N-terminal part contains two amphipathic α-helices. The second of them can cross the membrane bilayer likely upon oligomerization of NS4B. The C terminal part of NS4B harbors a highly conserved α -helix, a membrane associated amphipathic α-helix and two palmitoylation sites (Fig.13) (Gouttenoire et al., 2009; Yu et al., 2006). Likewise other HCV nonstructural proteins, NS4B can form oligomers. Thus, some studies demonstrated that NS4B can form at least trimers and that the palmitoylation sites at its C-terminal part are involved in this process (Yu et al., 2006).

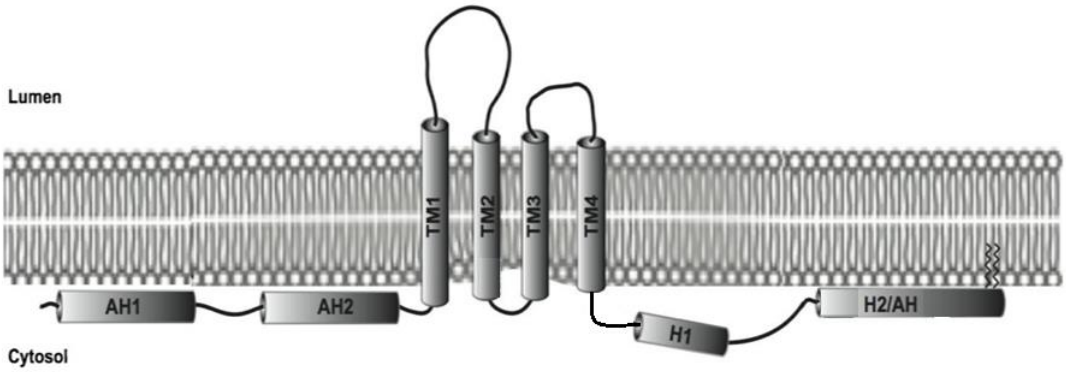

Figure 13 Schematic presentation of NS4B. NS4B is presumed to have 4 transmembrane domains (TM1-TM4) that traverse the ER, having both C-and N-terminal parts directed towards the cytosol. Two amphipathic helices are present at the N-terminus that are essential for HCV replication. The C- terminus contains a palmitoylation site and 2 helices (adapted from (Lemon et al., 2010)).

NS4B has been shown to induce the formation of the membranous web, arising from an alteration of the ER membrane and constituting the site of anchoring of the HCV replication complex (Egger et al., 2002; Gosert et al., 2003). However, a more recent study indicates that NS5A, rather than NS4B, induces alteration of intracellular membranes that resemble more the double-membrane vesicles observed during HCV replication (Romero-Brey et al., 2012). NS4B interacts with other viral non-structural protein and plays an essential role in HCV replication. Moreover, NS4B can bind and hydrolyze ATP and GTP and its role in the replication depends on its capacity to bind GTP (Thompson et al., 2009).

As observed for NS3/NS4A, NS4B can interfere with the innate immune response. Indeed, NS4B can inhibit the RIG-I–like receptors-mediated interferon signaling by targeting STING, an adaptor protein facilitating the phosphorylation of IRF3 (Ding et al., 2013; Nitta et al., 2013; Yi et al., 2016). More recently, NS4B has also been shown to antagonize the TLR3-mediated interferon signaling by inducing the degradation of TRIF (Liang et al., 2018).

1.2.2.9 NS5A

NS5A is a membrane-associated phosphoprotein of 447 aa containing a unique amphipathic α-helix that serves as membrane anchor (Tellinghuisen et al., 2004). It is organized in three structural domains (Fig.14) separated by low complexity sequences. Domain I (aa36-213) and Domain II (aa250-342) are implicated in viral replication, while domain III (aa 356-447) is crucial for assembly and secretion (Appel et al., 2008; Kim et al., 2011; Tellinghuisen et al., 2008). The domain I has been crystallized as a dimer and can bind RNA (Moradpour et

al., 2005). Moreover domain I mediates the association of the protein with lipid droplets and is important for the release of infectious virus (Miyanari et al., 2007).

Domain III is dispensable for genome replication, while the C terminal 38 residues of this domain contain the major determinant for viral assembly. This region contains 3 serine residues that are required for the interaction of NS5A with core protein (Masaki et al. 2008).

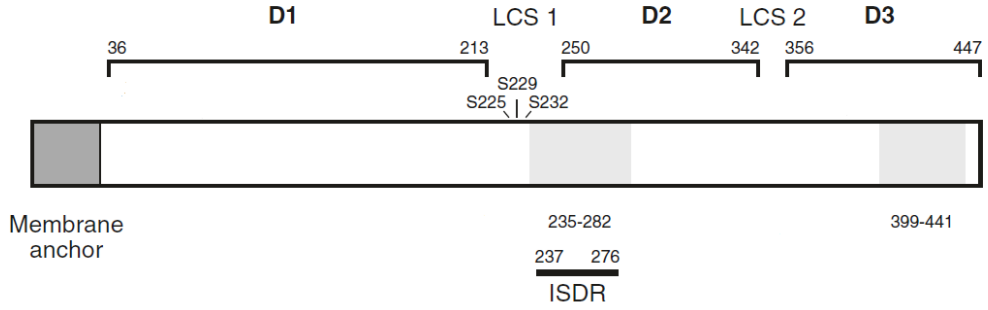

Figure 14 Schematic illustration of NS5A. Amino acid positions refer to Con1 strain of genotype 1b. The three domains of NS5A (D1–D3) are separated by LCS 1 and 2 (low complexity sequences). An amphipathic α-helix at the N-terminus serves as a membrane anchor for NS5A referred to by a dark grey box. Serine residues impacting NS5A hyperphosphorylation are also indicated (Moradpour and Penin, 2013b).

NS5A can be extensively phosphorylated. Two main forms of different molecular weights can be observed that are referred to as the basal (56 kDa) and hyperphosphorylated (58 kDa) form. A great number of phosphorylation sites have been identified that are the target of different kinases. Several studies showed a negative effect of hyperphosphorylation on viral replication in genotype 1 (Appel et al., 2005; Evans et al., 2004). However these observations were not confirmed in genotype 2a JFH1 virus. This suggests that the impact of phosphorylation of NS5A may vary among different genotypes. Several phosphorylated residues have been identified in the domain III that are important for virus assembly (Masaki et al., 2008). Nevertheless, recent studies showed that a certain ratio of the basally and hyperphosphorylated forms of NS5A (p56: p58) is required for optimal RNA replication (Y. Huang et al., 2007; Liu et al., 2012; Qiu et al., 2011; Tellinghuisen et al., 2008). Thus, the phosphorylation of NS5A is presumed to be involved in regulating a switch from genome replication to assembly, based on the fact that a decrease in NS5A phosphorylation or hyperphosphorylation, enhance RNA replication, but reduce viral particle production (Appel et al., 2005; Masaki et al., 2008; Ross-Thriepland et al., 2015; Ross-Thriepland and Harris, 2014). Interestingly, it has likewise been reported for closely related *flaviviruses* such as the West Nile virus and Dengue virus that phosphorylation might similarly regulate switching

between virus replication and assembly (Chu and Yang, 2007; Hirsch et al., 2005; Tellinghuisen et al., 2008). Thus, NS5A phosphorylation is clearly important for its function, but the molecular mechanisms that are behind the modulation of the protein function have still to be elucidated.

NS5A interacts with a great number of viral and cellular protein partners. Indeed, domains II and III present disordered structure that can adopt wide range conformations allowing the interaction of the protein with an important number of partners (Feuerstein et al., 2012). Notably, NS5A interacts with NS5B and modulates its polymerase activity (Shirota et al., 2002). Moreover interactions with cellular factors involved in the regulation of transcription, apoptosis or the cell cycle control have been reported (Lan et al., 2002).

Finally, accumulated data led to a model in which NS5A recruits nascent genome from the ER-associated replication complex to lipid droplets where they interact with core to be encapsidated in viral particles.

1.2.2.10 NS5B

NS5B, RNA-dependent RNA polymerase (RdRp) is the key enzyme of HCV replication complex, which is composed of NS3 and the downstream nonstructural proteins (Paul et al., 2014). NS5B is classified as a tail-anchored protein. Indeed, its C-terminal region (21 aa) that contains an α-helical transmembrane domain is responsible for its association with the ER membrane (Ivashkina et al., 2002; Moradpour et al., 2004a; Schmidt-Mende et al., 2001). Some studies have shown that this membrane anchor is not required for polymerase function in vitro, but is essential for viral replication in cells (Moradpour et al., 2004a). The N-terminal 530 amino acids form the RNA polymerase. The crystal structure of the NS5B catalytic domain revealed the classic "right hand structure" of polymerases (Fig. 15) that includes thumb, fingers and palm subdomains (Ago et al., 1999; Bressanelli et al., 1999). Fingers and thumb domains form a tunnel to which the single strand RNA template binds, leading it to the catalytic site, which is located in the palm domain (Bressanelli et al., 2002). Nucleotides reach the active site via a second positively charged tunnel.

The RdRp initiates de novo RNA synthesis from an RNA template without the need of a primer (Luo et al., 2000; Zhong et al., 2000). Replication starts by using the genome as a template to generate intermediate complementary minus stranded RNA that serves in turn as

a template for consecutive production of genomic plus stranded RNA. The generated RNA is error prone due to the lack of proofreading activity of the RdRp. The mutation rate is estimated at one error/replicated genome (Choi, 2012). This high error rate is the reason behind HCV's high genetic variability and emergence of resistance to antiviral therapies.

The polymerase activity of NS5B is modulated by interaction with NS3 and NS5A viral proteins (Bartenschlager et al., 2004). Furthermore, NS5B interacts with several host cell factors such as cyclophilin B that affect the efficacy of the replication (Ishii et al., 2006). Due to its crucial role in RNA replication, NS5B is considered as the major target for antiviral drug development.

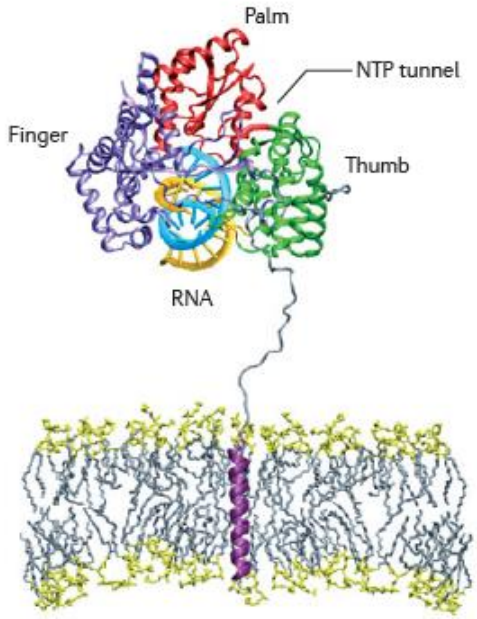

Figure 15 Structure of HCV NS5B protein. NS5B full-length protein associated with ER through its C-terminal transmembrane tail (PDB accession 1GX6). Right hand structure shown with finger, thumb and palm subdomains interacting with RNA strand (Bartenschlager et al., 2013b).

1.3 Models for the study of HCV

1.3.1 The HCV replicon system

From its identification in 1989, the characterization of HCV life cycle remained for a long time limited by the lack of an efficient cell culture system. Nonetheless, in 1997 the complete sequence of the viral genome obtained *in vitro* could be validated since intrahepatic injection of the viral RNA in chimpanzee led to the development of the disease and the production of infectious HCV (Kolykhalov et al., 1997). However neither replication nor viral production

was detectable after transfection of hepatoma cells with different HCV genomic RNAs. To overcome this difficulty, the strategy used was to focus on the replicative part of the genome by eliminating the structural protein sequences to generate a minimal autonomous replicative unit. Thus, selectable HCV replicons were developed that contained the 5' and 3' UTR, NS3 to NS5B coding sequences from the genotype 1b Con1 strain (Lohmann et al., 1999). This sequence was introduced in a bicistronic construct containing the neomycin resistance gene (Lohmann et al., 1999). Subgenomic replicons are generally bicistronic RNAs having two IRES. Starting from the 5' end, HCV IRES initiates the translation of an antibiotic resistant gene which is pursued by EMCV (encephalomyocarditis virus) IRES driving the translation of the nonstructural proteins (NS3 - NS5B). The replicative capacity of this replicon was further improved by the selection of adaptive mutations and the identification of cellular clones presenting increased permissiveness to viral replication (Fig.16). Replicons derived from strains of different genotypes were subsequently developed (Kato et al., 2003; Saeed et al., 2012; Yu et al., 2014). Following the development of replicons, attempts to reconstitute the full HCV genome sequence by reintroduction of the structural protein sequences were unsuccessful for the production of virus *in vitro* as well as *in vivo* (Bukh et al., 2002; Pietschmann et al., 2002). This was due to the fact that the non-structural proteins are involved in the assembly of the virus and that adaptive mutations providing an improved replication had a deleterious effect on the assembly function of NS proteins (Murray et al., 2008; Pietschmann et al., 2009).

The difficulties to develop a cell culture system for HCV are also partly due to the induction of the innate immune response. Moreover, required pro-viral factors might not be expressed in the Huh7 cells currently used to study HCV. Accordingly, a recent study has shown that Huh7 hepatoma cells do not express the SEC14L2 factor and that the rescuing of its expression allows the replication of all HCV genotypes in several hepatoma cell lines (Saeed et al., 2015).

The subgenomic replicons have been shown to be a valuable tool for testing and identifying antiviral compounds affecting HCV replication as well as characterizing virus–host interactions and intracellular localization of viral proteins (Lohmann, 2013). Indeed, these systems had a major role in identifying and developing DAA's (Bartenschlager et al., 2013b).

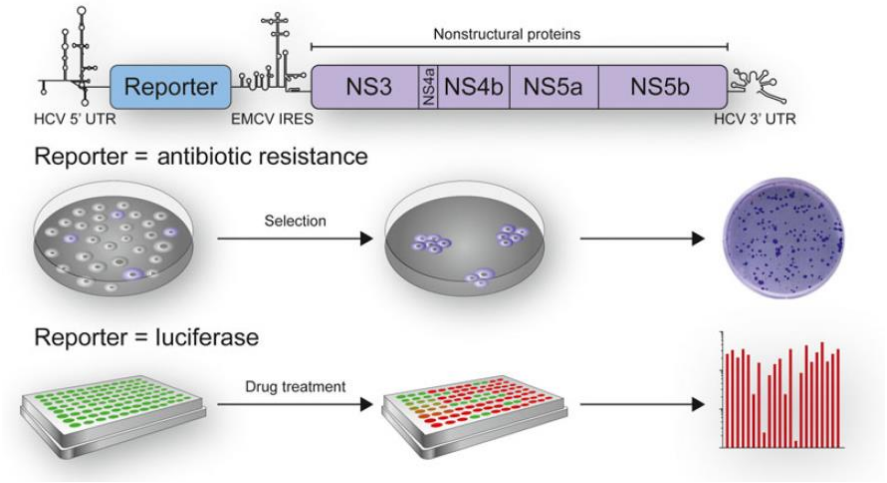

Figure 16 Replicon system of HCV. Schematic illustration of the replicon system. Reporter genes are introduced in the replicon sequence. An antibiotic resistance gene allows for the selection of positive clones and fluorescent proteins encoding genes allow measuring the efficiency of replication (Ortega-Prieto and Dorner, 2016).

1.3.2 HCV pseudoparticles

The HCV pseudoparticle (HCVpp) model has been shown to be a valid tool for the study of viral entry. HCVpp are retroviral particles harboring unmodified HCV envelope glycoproteins (Bartosch et al., 2003a; Drummer et al., 2003; Hsu et al., 2003). They are produced in human embryonic kidney cells (Hek-293T) by co-transfection of three expression vectors: (a) an expression vector encoding HCV E1 and E2 glycoproteins, (b) a plasmid encoding the retroviral gag-pol precursor of either human immunodeficiency virus (HIV) or murine leukemia virus (MLV) that will provide the virus core and (c) a transfer vector containing a packaging-competent retroviral minigenome encoding a reporter gene and including retroviral sequences necessary for reverse transcription and integration of the reporter gene in the infected cell genome. This results in the production of pseudotyped particles containing HIV or MLV nucleocapsids and carrying HCV glycoproteins at their surface. Supernatants containing secreted HCVpp are then used in turn to transduce Huh7 hepatoma cells. Entry of HCVpp into target cells results in the release of the retroviral capsid into the cytoplasm, which is followed subsequently by reverse transcription and integration of the viral genome into the host genome. The infectivity can then be measured by quantification of the reporter gene activity (Fig.17). HCVpp are replication deficient and therefore support a single-round infection. Therefore the reporter gene expression correlates with HCVpp entry. This system allowed demonstrating that HCV entry step is mediated by E1 and E2 envelope proteins. Thus, it has been shown that HCVpp infectivity could be

neutralized by anti-E1 anti-E2 antibodies as well as HCV infected patient sera (Bartosch et al., 2003a). Furthermore, the HCVpp system has been utilized to evaluate the efficiency of neutralizing Abs targeting HCV E1 and E2 (Hsu et al., 2003; Meunier et al., 2005; Tarr et al., 2007; Wasilewski et al., 2016; Yu et al., 2004). Various HCVpp panels have been developed by incorporation of the glycoprotein sequences of major genotypes and have been used to study the potency of cross neutralizing antibodies (Meunier et al., 2005; Owsianka et al., 2005).

The HCVpp model is an interesting tool to analyze only the viral cell entry process apart from other steps of HCV life cycle. This system made a great contribution in identifying new HCV receptors such as Claudin-1 (Evans et al., 2007) and Occludin (Ploss et al., 2009) in addition to verifying the crucial role of other receptors or co-factors in viral entry as CD81 and SRBI (Cormier et al., 2004b; Dreux et al., 2009; Lavillette et al., 2005; Zhang et al., 2004). HCVpp could also be used to study the role of certain domains or residues of the glycoproteins in viral entry through reverse genetics (Bartosch et al., 2005; Owsianka et al., 2006; Prentoe et al., 2014; Russell et al., 2009). Nevertheless, there are some restrictions regarding the HCVpp model. Indeed, HCVpp are produced in non-liver cells that do not secrete lipoproteins. Thus, contrarily to authentic HCV particles, HCVpp do not associate with lipoproteins (Bartosch et al., 2003a; Keck et al., 2007; Popescu and Dubuisson, 2010). Additionally, they assemble in a post Golgi-compartment similar to retroviruses. This led to some differences of phenotypes observed between HCVpp and HCVcc (Keck et al., 2005; Wasilewski et al., 2016). Indeed, lipoprotein association of HCV can impact antibody neutralization and the role in entry played by lipid receptors LDLr, SRBI, and NPC1L1 (Lavie and Dubuisson, 2017).

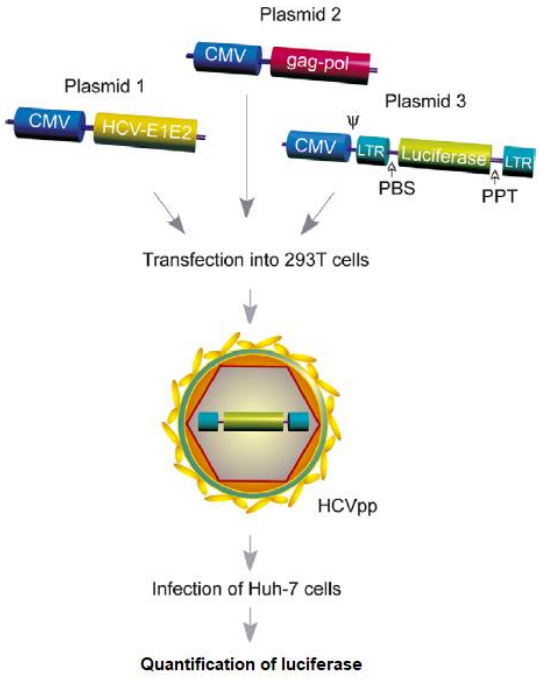

Figure 17 Production of HCV pseudoparticles. For generation of HCVpp, Hek-293T are co-transfected with three expression vectors: (a) an expression vector encoding HCV E1 and E2 glycoproteins, (b) a plasmid encoding the retroviral gag-pol precursor providing the virus core and (c) a transfer vector encoding a reporter gene (luciferase) (Voisset and Dubuisson, 2004).

1.3.3 HCVcc culture system

1.3.3.1 HCVcc

The establishment of replicon and HCVpp systems has enabled the characterization of the replication and entry steps of HCV life cycle, while later steps, such as assembly and release of nascent virions remained elusive. The difficulty to produce infectious HCV virions could be overcome in 2005. Indeed, three teams published the development of the based cell-cultured HCV (HCVcc) system (Fig.18) that supported the whole viral life cycle (Lindenbach et al. 2005; Wakita et al., 2005; Zhong et al. 2005). Thus, Wakita et al. could isolate a clone of a full-length HCV genome of genotype 2a from a Japanese patient suffering from fulminant hepatitis that was capable to replicate without adaptive mutations and to propagate spontaneously in cell culture. (Kato et al. 2001; Kato et al., 2003; Wakita et al., 2005). However, the reason behind that specific feature remains elusive. Thus, for the first time since HCV discovery, it was possible to produce infectious virus in cell culture upon transfection of hepatoma cells with genomic HCV RNA transcribed *in vitro*.

The infectivity of produced HCVcc could be demonstrated *in vivo* in chimpanzees and human liver chimeric mice (Bukh and Purcell, 2006; Kato et al., 2008; Lindenbach et al., 2006). This system allowed confirming the results obtained with HCVpp or replicons systems in the characterization of HCV entry and replication, respectively. Thus the role in entry of CD81, SRBI, CLDN1 and OCLN cellular receptor could be confirmed (Evans et al., 2007; Grove et al., 2007; Lindenbach et al., 2005; Ploss et al., 2009). Moreover, the involvement of cellular lipids in HCV replication could also be observed with the HCVcc system (Y. Huang et al., 2007; Kapadia and Chisari, 2005).

Importantly, HCVcc allowed for the first time to study HCV assembly and release (Diaz et al., 2006; Gastaminza et al., 2006; Lindenbach et al., 2006). Furthermore the association of HCVcc with lipoproteins enabled to characterize their influence on infectivity.

Additionally, electron microscopy studies showed that cell cultured derived HCV particles are spherical in shape and density gradient analysis showed that they had similar densities to serum-derived viruses (Lindenbach et al., 2005; Piver et al., 2017; Wakita et al., 2005; Zhong et al., 2005).

However, one limitation of HCVcc system was that it relied on a single viral strain and used a single cell line. To better study the life cycle of other HCV genotypes, recombinant viruses between the sequences of the structural proteins of these genotypes and the non-structural proteins of JFH1 have been developed (Gottwein et al., 2011, 2009, 2007; Scheel et al., 2008). Generally, these genomic replacements reduce the replicative fitness, which can be overcome by adaptive mutations that enhance the replication of chimeras to a similar level as the wild-type virus. These recombinant viruses allowed the identification of new antiviral molecules and broadly neutralizing antibodies (Giang et al., 2012; Gottwein et al., 2013, 2011; Keck et al., 2013).

Recently, full-length infectious cell culture systems have been developed that allow the production of viruses of genotype 1a, 2a, 2b, 3a (Kim et al., 2014; Li et al., 2015; Ramirez et al., 2016, 2014) and more recently of genotype 6a (Pham et al., 2018). The development of these systems has been laborious since it required the identification of adaptive mutations conferring culture propagation to the patient consensus clones that are inherently non-viable in vitro (Ramirez and Bukh, 2018). Developing robust HCV cell culture systems for different genotype isolates plays an important role in drug and vaccine development for different

genotypes. Thus, it is important to establish other full length culture systems for other subtypes and for genotypes 4, 5 and 7 (reviewed in Ramirez and Bukh, 2018).

One limitation of these HCVcc systems is their dependency on a single hepatoma cell line, which genetically differs from hepatocytes. Consequently, cell culture systems have been developed to better mimic hepatocytes, such as primary human hepatocytes as well as hepatocyte-like cells originating from pluripotent stem cells (Helle et al., 2013; Ploss et al., 2010; Roelandt et al., 2012).

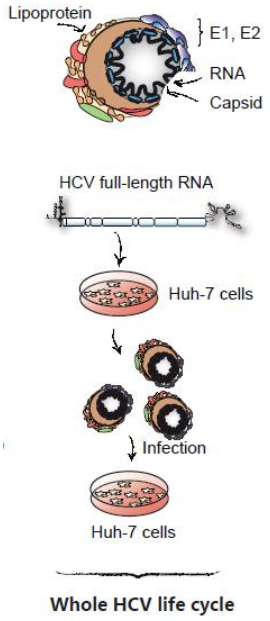

Figure 18 Cell culture derived HCV (HCVcc) production. Hepatoma cells are transfected with in vitro-transcribed JFH-1 genomic RNA. This results in the translation and replication of the RNA leading to the production of infectious viral particles, which in turn infect naïve target cells. This model representing the whole viral life cycle of HCV enables studying all the steps of the viral life cycle (Gerold and Pietschmann, 2014).

1.3.3.2 HCV Permissive cell lines

Due to its hepatotropic nature, HCV has been found to replicate in human hepatocytes as well as hepatoma cell lines. The Huh-7 hepatoma cell line, originating from a 57-year-old Japanese hepatocellular carcinoma patient, was the first to be used for the study of HCV infection. Other Huh-7 derivatives such as Huh-7.5 have been shown to be highly permissive to HCV RNA replication with or without the aid of adaptive mutations (Blight et al., 2002). The increased permissiveness of Huh-7.5 cells was acquired by curing Huh-7 cells containing

replicons with IFN-α. This led to a defect in the IFN pathway due to a mutation in retinoic acid-inducible gene I (RIG-I) (Sumpter et al., 2005) and an increased expression level of CD81 on the cell surface (Koutsoudakis et al., 2007). Huh-7.5.1 were obtained by curing GFP-HCV replicon containing Huh-7.5 cell line with IFN-γ, permitting increased HCV production efficiency (Moradpour et al., 2004b). The fact that Huh-7 cell line and its derivatives are not polarized and are consequently not reflecting the polarized nature of hepatocytes in the liver, might hamper the study of some aspects of HCV entry, assembly and cell to cell transmission (Steinmann and Pietschmann, 2013). Nevertheless, HepG2 hepatoma cells, that overexpress human CD81 and miR-122, are capable to polarize in cell culture and have been shown to be permissive to HCV infection (Narbus et al., 2011). Similarly, Belouzard et al. isolated Huh-7 clones able to polarize and showed that productive HCV infection occurred from the basolateral domain of the cells (Belouzard et al., 2017). Primary human hepatocytes (PHH) that constitute a more physiologically relevant cell type to study HCV infection have been used in HCVcc infection assays (Helle et al., 2013, 2010; Podevin et al., 2010). However, PHH rapidly lose their phenotype when isolated from the liver, thus resulting in poor permissivity to HCV infection *in vitro (*Lowey et al. 2018). Thus, the use of this system meets several limitations which are their limited availability, metabolic instability, high donor diversity and their dedifferentiation in culture (Steinmann and Pietschmann, 2013).

1.3.4 HCV Animal models

Human being is the only natural host for HCV. However, chimpanzees as well as tupaia (tree shrews) can be infected experimentally and are the only alternative animal model (Kolykhalov et al., 1997; Weiner et al., 1990; Xie et al., 1998). However, the use of chimpanzees in medical research is ethically not accepted anymore, even though previous studies in chimpanzees contributed to greatly increase our knowledge of HCV biology.

1.3.4.1 Chimpanzee

Chimpanzees have been involved in many studies and played an important role in the discovery of HCV. Indeed, HCV viral genome could be cloned from a chimpanzee that was experimentally infected with non-A non-B hepatitis (Weiner et al., 1990). Moreover this model allowed to validate HCV viral genome by intrahepatic injection of the nucleic acids

and the monitoring of the virus propagation (Kolykhalov et al., 1997) (reviewed in (Catanese and Dorner, 2015). Since acute infections in human are asymptomatic, they are difficult to study. Thus, experimental infection of chimpanzees provided essential data on this stage of the disease. This model also allowed the completion of immunologic studies that led to the development and evaluation of several candidate vaccines (Bukh et al., 2001b; Folgori et al., 2006). It has also been used in several antiviral efficacy studies (Carroll et al., 2009; Chen et al., 2007; Morin et al., 2012; Olsen et al., 2011). However, there are some differences between humans and chimpanzees in response to HCV infection. Whereas around 70-80% of infected humans develop chronic hepatitis, only 30-50% of infected chimpanzees evolve to chronicity (Lanford et al., 2001). Additionally, no fibrosis has been reported in chimpanzees, while only one case of hepatocellular carcinoma has been observed in this model (Vercauteren et al., 2015). Furthermore, contrarily to humans, chimpanzees do not respond to IFNα treatment (Lanford et al., 2007)).

Although chimpanzee model fulfills many requirements of a good animal model, the limited availability of the animals, the expensiveness of these experiments and ethical issues constitute major drawbacks to the use of this model. Moreover, the fact that chimpanzee yet count among endangered animal led in 2013 to the banning of their use in invasive experimental research.

Besides primates, other species have been assessed for HCV infection susceptibility, yet only the tree shrew (*Tupaia belangeri)* showed susceptibility. They are non-rodent small squirrel-like mammals. Upon HCV infection, they present low levels of viremia that are associated with HCV-related liver disorders (Amako et al., 2010; Xie et al., 1998). Nonetheless, the lack of robust HCV replication, the limited tools available for studying host responses and difficulties in breeding tree shrews limit the use of this model for pathogenesis studies (Catanese and Dorner, 2015).

The narrow host tropism of HCV led to the development of small practical HCV animal models. Due to the fact that rodents are resistant to HCV infection, several strategies have been developed to permit the study of HCV in mice (reviewed in (Mailly et al., 2013).

1.3.4.2 Genetically humanized mouse models

Mice are naturally not susceptible to HCV infection, mostly due to species difference. It has been shown that viral entry and replication are impaired in murine cells and that human

CD81 and OCLN are responsible for this species tropism (Ploss et al., 2009). In order to overcome the entry blockade in murine hepatocytes, an approach consisted in the transient expression of the minimal human factors CD81, OCLN, CLDN1 and SRBI by adenoviral delivery (Fig.19). This allowed entry of HCVcc in mouse hepatocyte *in vitro* and *in vivo* (Dorner et al., 2013, 2011). This model was found suitable for evaluating vaccine candidates as well as entry inhibitors (Giang et al., 2012). Nevertheless, HCV replication is not supported by murine cells (Dorner et al., 2011). The blockage of replication was reported to be caused by induction of innate immune responses especially the PKR- and IRF3 mediated pathways (Chang et al., 2006; Lin et al., 2010). Thus, C57BL/6 mice expressing the 4 human receptors with deficiencies in several innate immune signaling pathways (STAT1-/-) supported entry and a low level of replication. The infection induced antiviral cellular and humoral responses but was not associated with liver disease (Dorner et al., 2013; Vogt et al., 2013).

Chen et al. developed an immunocompetent animal model by generating transgenic mice expressing human OCLN and CD81 on an outbred ICR background. In this model, HCV infection leads to the development of liver disease without reaching HCC stage (Chen et al. 2014). Surprisingly, similar approach in C57BL/6 mice did not allow reaching sustained HCV replication. Thus, it seems that the genetic background of the mice greatly affects HCV replication rate. Notably, the level of apoE expression as well as the miR-122 pool are crucial parameters that affect HCV production.

1.3.4.3 Human liver-chimeric mice

Another approach to render mice susceptible to HCV infection and overcoming the species barrier is humanizing mice liver through transplantation of human hepatocytes (Fig.19). This approach renders the mice permissive to HCV and any other human hepatotropic pathogen. Nevertheless, to avoid xenograft rejection by mice immune system after transplantation, immunocompromised SCID mice are used for severe combined immunodeficiency (Bumgardner et al., 1998). Additionally, recipient mice suffer from a constitutive or inducible liver injury, which provides a competitive growth advantage to the human donor hepatocytes over the resident mouse hepatocytes (von Schaewen et al., 2014). This approach takes advantage of the capacity of hepatocytes to proliferate and regenerate an injured liver

(Overturf et al., 1996; Sandgren et al., 1991). Two main mice recipient lines have been used to produce human-liver chimeric mice: the uPA-SCID mouse and the FRG mouse (Vercauteren et al., 2014). Others such as MUP-uPA (Tesfaye et al., 2013) and the TK-NOG mice (Kosaka et al., 2013) have also been shown to permit persistent and reproductive HCV infection of both cell-culture produced HCV and natural virus isolates (Bissig et al., 2010; Bukh et al., 2010; Lindenbach et al., 2006; Meuleman et al., 2011). These human chimeric mice constitute an important tool for studying basic aspects of the HCV viral life cycle and evaluating new antiviral therapies (Vercauteren et al., 2015). They have been used for the study of further hepatotropic human pathogens such as HBV, HDV or *Plasmodium falciparum* (Vercauteren et al., 2014). Moreover, due to the fact that human hepatocytes residing in the mouse liver retain most of their properties, the use of these humanized mice have been extended to the study of human metabolism and potential toxicity of medicinal compounds (reviewed in (Vercauteren et al., 2014)).

One of the major drawbacks of human liver xenograft mouse models is their lack of functional immune system that precludes study of HCV-specific immune response, HCV vaccines as well as immunopathogenesis. Moreover, contrarily to what is observed in chronically HCV infected patients, HCV infection has not been shown to induce fibrosis, cirrhosis or HCC in this model. This supports the hypothesis that inflammatory response contributes to disease progression. In order to overcome this hurdle, immunocompetent xenograft models have been developed. In these models, immunodeficient mice are engrafted with both human hepatocytes and human immune cells. This has been achieved by injecting human CD34+ hematopoietic stem cells into mice that have been engrafted with adult human hepatocytes from different donors (Gutti et al., 2014; Wilson et al., 2014). Recently, in addition to this allogeneic system, a syngeneic model has been developed by injecting both human hepatocyte progenitor cells and human CD34+ hematopoietic stem cells from the same fetal donor (Washburn et al., 2011). Upon HCV infection, challenged mice developed hepatic fibrosis as well as human HCV specific T-cell responses (Bility et al., 2016; Washburn et al., 2011). However, HCV RNA has been detected in liver extracts but not in plasma, most likely due to the low level of human liver engraftment in these mice (Vercauteren et al., 2014; Washburn et al., 2011). This model allows studying HCV specific T cell responses as well as HCV pathogenesis, while the lack of functional B-cells hinders antibody response studies and vaccine research. In spite of these challenges, these models are considered as the best model for HCV study in its natural host settings.

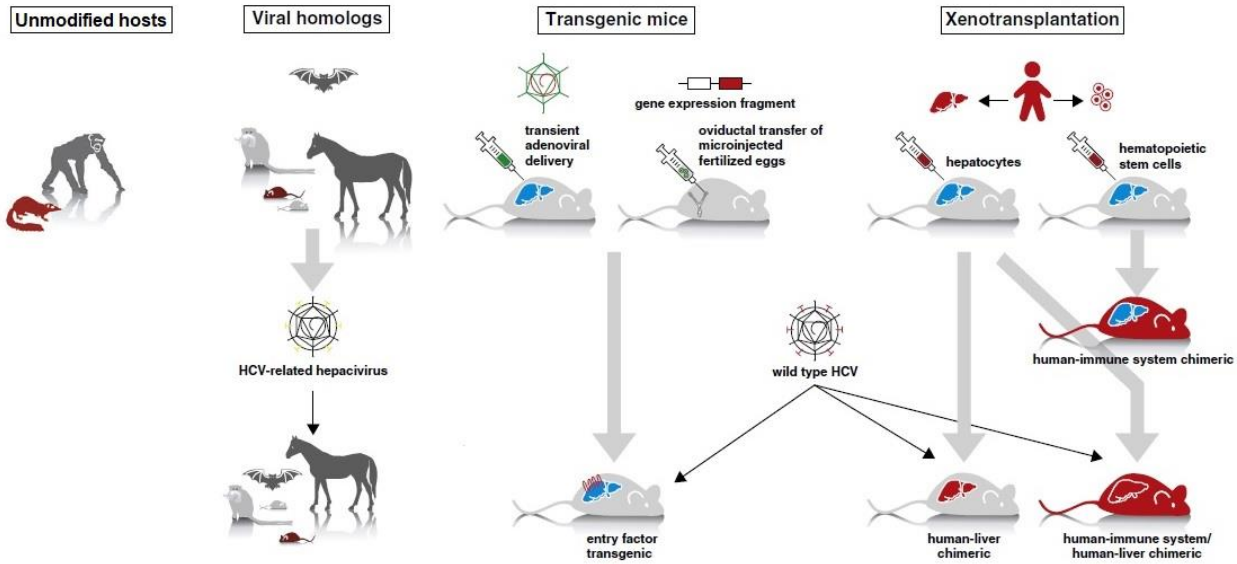

Figure 19 Animal models for HCV research. Animal models for HCV research. Several models have been proposed for the study of HCV in animals. The first group involves animals that are naturally susceptible to HCV infection (chimpanzees & tree shrews). The second model consists of *hepaciviruses* that infect dogs, horses, bats and rodents and cause an infection similar to HCV in their hosts. The third model corresponds to the expression of human factors in mice to render them susceptible to HCV infection. In the fourth approach mice are rendered susceptible to HCV upon xenotransplantation of human hepatocytes and/or human immune cells (adapted from (Burm et al., 2018)).

1.3.4.4 HCV homologs

Another promising approach is based on using HCV homologs. Several animal *hepaciviruses* have been recently identified that infect dogs, horses, bats and rodents. They could constitute interesting surrogate models for studying HCV host immune responses and pathogenesis (Fig.19) (Hartlage et al., 2016). The GB virus B (GBV-B) has been the only known HCV homolog until the year 2011. It was named after a surgeon (G.B. as initials) suffering from acute hepatitis, whose serum was experimentally used to infect tamarins, which consequently developed acute hepatitis (Deinhardt et al., 1967; Stapleton et al., 2011). GBV-B infection in tamarins served as a surrogate model for functional in vivo studies (Bright et al., 2004; Bukh et al., 2001a). Nevertheless, GBV-V infections rarely developed to chronicity and its natural host remains to be identified (Martin et al., 2003; Scheel et al., 2015; Takikawa et al., 2010). In fact, besides hepatotropism, an HCV homolog should establish persistent infection and should be associated with similar immune responses and pathogenesis. Thus, the non-primate *hepacivirus* (NPHV) has been reported to share common characteristics with HCV. It causes similar infection in horses as HCV in humans, since it is hepatotropic and capable to establish persistent infection. Moreover induced host immune responses also resemble those of HCV

in humans, especially liver pathogenesis and delayed seroconversion (Scheel et al., 2015). This makes of NPHV a potential surrogate model for HCV. However, the animal size and the accompanied costs of animal care makes it unpractical for scientific research (Scheel et al., 2015). Conversely, rodents have always been considered as the most convenient animal models because of their small size, being easy to handle and genetically modifiable. For these reasons, recently isolated rodent *hepaciviruses* have been of great interest (Drexler et al., 2013; Firth et al., 2014; Kapoor et al., 2011). Indeed, a rodent *hepacivirus*, NrHV has been isolated in Norway rats from New York city (Firth et al., 2014). NrHV presents HCV genomic features including similar polyprotein cleavage profile and secondary structures in 5' and 3' UTR. It has been reported to establish high-titer infections in laboratory mice with immunological responses close to that observed in human infections (Billerbeck et al., 2017). In immune-compromised mice, the virus establishes persistent infections while it is cleared in immune-competent mice within several weeks. Transient depletion of CD4+ T cells before infection allowed establishing chronic infection in immune-competent mice. More recently, Trivedi and collaborators further investigated NrHV in rats in order to develop an immunocompetent model that could establish persistent infection (Trivedi et al., 2018). Interestingly, NrHV infection in rat resembles HCV infection through its hepatotropism, its propensity to persist and its ability to induce gradual liver damages. Thus, NrHV infection in rat constitutes an immunocompetent surrogate model to study the mechanisms of HCV persistence, immunity and pathogenesis.

1.4 HCV life cycle

HCV life cycle is a multistep process that can be divided into 4 steps: 1. Virus entry, 2. RNA translation and protein maturation, 3. Genome replication and finally 4. Assembly and release of viral particles from host cells. HCV interacts with several host cell factors and takes advantage of the host cell machinery at different stage of its life cycle.

1.4.1 HCV entry

HCV entry into the host cell is a complex process that involves several cell surface molecules. It can be subdivided into three steps: the attachment of the virus to the cell surface via non-specific interactions, the interaction of the virus with specific receptor that leads to the internalization of the particle and the fusion between the viral envelope and the endosomal membrane that allows to release of the viral genome into the cell cytoplasm.

1.4.1.1 Attachment factor

1.4.1.1.1 Glycosaminoglycans

Glycosaminoglycans (GAGs) are large polysaccharides present on the surface of most mammalian cells functioning as primary attachment molecules for HCV as well as many other viruses (Lin et al., 2013). There are several types of highly sulfated GAGs. However interactions have been only reported between HCV and the heparan sulfate proteoglycans (HSPG). Similarly, HSPG has been shown to constitute an attachment factor prior to binding to specific receptors for other *Flaviviridae* viruses such as Dengue virus and classical swine fever virus (CSFV) (Hulst et al., 2001). Indeed, it has been reported that both heparin, a heparan sulfate homolog, and heparinase treatment (an enzyme that degrades heparan sulfate at the cell surface), could inhibit HCV binding to the cell surface (Barth et al., 2003; Koutsoudakis et al., 2006). Whereas it has been initially proposed that HCV envelope glycoproteins directly interacted with HSPG, more recent results have shown that the viral particle interacted with these molecules via apoE apolipropotein that is present at the surface of HCVcc (Jiang et al., 2012; Xu et al., 2015).

1.4.1.1.2 Lectins: DC-SIGN/L-SIGN

DC-SIGN (dendritric cell-specific ICAM-grabbing non-integrin) and L-SIGN (liver-specific ICAM-grabbing non-integrin) that belong to the C-type lectin family serve as adhesion receptors for many viruses such as HIV type I (Geijtenbeek et al., 2000). They are type II transmembrane proteins that interact with glycans. Both lectins are not expressed in hepatocytes, L-SIGN is present in liver sinusoidal endothelial cells, while DC-SIGN is found on Kuppfer cells, dendritic cells and lymphocytes. Both lectins interact with carbohydrate structures on pathogens (Koppel et al., 2005) and have been reported to bind E2 glycoprotein (Gardner et al., 2003; Pöhlmann et al., 2003). Moreover, it has been reported that DC-SIGN and L-SIGN expressed on HeLa or Radji B cells were able to bind HCVpp and transmit it to Huh7 cells in a coculture model (Cormier et al., 2004a; Lozach et al., 2004). Therefore, the capture and transmission of circulating HCV particles by hepatic sinusoidal endothelial cells might promote HCV infection of adjacent cells that are not directly in contact with circulating blood (Cormier et al., 2004a; Gardner et al., 2003).

1.4.1.1.3 Low Density Lipoprotein Receptors

Low density lipoprotein receptor (LDLr) is expressed in a wide range of tissues. Its main function in the liver is to mediate the clathrin-dependent endocytosis of the cholesterol rich LDL. The fact that HCV was found to associate with LDL and VLDL in sera of HCV infected patients (Agnello et al., 1999; André et al., 2002; Thomssen et al., 1992), led to the presumption that LDLr may be involved in HCV cell attachment. In agreement with this hypothesis, it has been shown that serum derived HCV could interact with LDLr via virion-associated lipoproteins (Agnello et al., 1999). Correlating with the fact that HCVpp do not associate with lipoproteins, it has been shown that LDLr are not involved in HCVpp entry into Huh7 cells (Bartosch et al., 2003a). The development of the HCVcc system allowed to further study the role of LDLr during HCV infection. Thus, the down regulation of LDLr expression with siRNA inhibited HCVcc infection (Owen et al., 2009). Moreover, antibodies targeting VLDL or apoE interfered with HCVcc infection whereas apoB-specific antibodies have no effect (Chang et al., 2007; Owen et al., 2009). Different kinetics of internalization were observed for infectious particles and lipoproteins, suggesting that they follow distinct uptake pathway (Albecka et al., 2012). Pretreatment of the virus with lipoprotein lipase reduces HCV infectivity while increasing its internalization, which suggests that LDLr-mediated internalization leads to non-productive viral entry (Albecka et al., 2012).

Thus, the precise role of LDLr during infection remains controversial. Studies suggest that LDLr mediates virus attachment to the cell surface through apoE interaction (Hishiki et al., 2010; Owen et al., 2009). But some data suggest that it participates to other steps such as replication (Albecka et al., 2012).

Importantly, apoE can interact with other receptors at the cell surface and notably SRBI. Recently, the lipoprotein receptors LDLr, SRBI and VLDLr have been shown to be redundant for HCV entry (Yamamoto et al., 2016).

1.4.1.2 HCV-specific receptors

1.4.1.2.1 CD81 tetraspanin

CD81 is a member of the tetraspanin family, which broadly expressed proteins involved in regulation of several cell functions such as morphology, signaling, invasion, motility and fusion (Hemler, 2005). CD81 contains 4 transmembrane domains, two extracellular loops and short intracellular domains. The extracellular loops are termed the large extracelullar loop (LEL) and small extracellular loop (Fig.20). CD81 is the best and first characterized HCV entry factor. Due to the lack of infectious HCV cell culture system, the first approaches to identify a potential HCV receptor relied on the use of a soluble form of E2 glycoprotein (sE2). This way, CD81 was shown to interact with sE2 (Pileri et al., 1998). This interaction is species specific since sE2 was not interacting with rodent CD81 (Flint et al., 2006). Residues in CD81 that are responsible for the binding of sE2 have been identified in CD81 LEL (Bertaux and Dragic, 2006; Drummer et al., 2005). The role of CD81 in HCV entry could be further confirmed with HCVpp and HCVcc systems. Thus, soluble CD81 LEL and CD81-specific antibodies have been demonstrated to inhibit entry of HCVcc and HCVpp into Huh7 cells and human hepatocytes (Cormier et al., 2004b; McKeating et al., 2004; Zhang et al., 2004). Additionally, silencing of CD81 expression by siRNA in Huh7 cells inhibited HCVcc and HCVpp entry. Reciprocally, the rescuing of CD81 expression in hepatoma cells HepG2 and HH29 that do not express CD81 and are resistant to HCV infection rendered them sensitive to infection (Bartosch et al., 2003c; Cormier et al., 2004b; McKeating et al., 2004; Zhang et al., 2004). In a similar way, CD81 expression level correlated with HCV infectivity in Huh7 cells (Akazawa et al., 2007; Koutsoudakis et al., 2007). Interestingly, anti-CD81 antibodies were able to block HCV entry at a post-binding step, which suggests that CD81 serves as a co-receptor for HCV entry after virus attachment to the cell surface (Cormier et al., 2004b; Koutsoudakis et al., 2006). HCV binding to CD81 triggers multiple signaling pathways. Thus, this interaction has been shown to activate the MAPK and PI3K/AKT signaling pathways and Rho GTPases family members. This leads to an actin-dependent relocalization of the virus/CD81 complexes at the apical membranes of hepatocytes for the internalization step (Baktash et al., 2018; Farquhar et al., 2012; Harris et al., 2010, 2008).

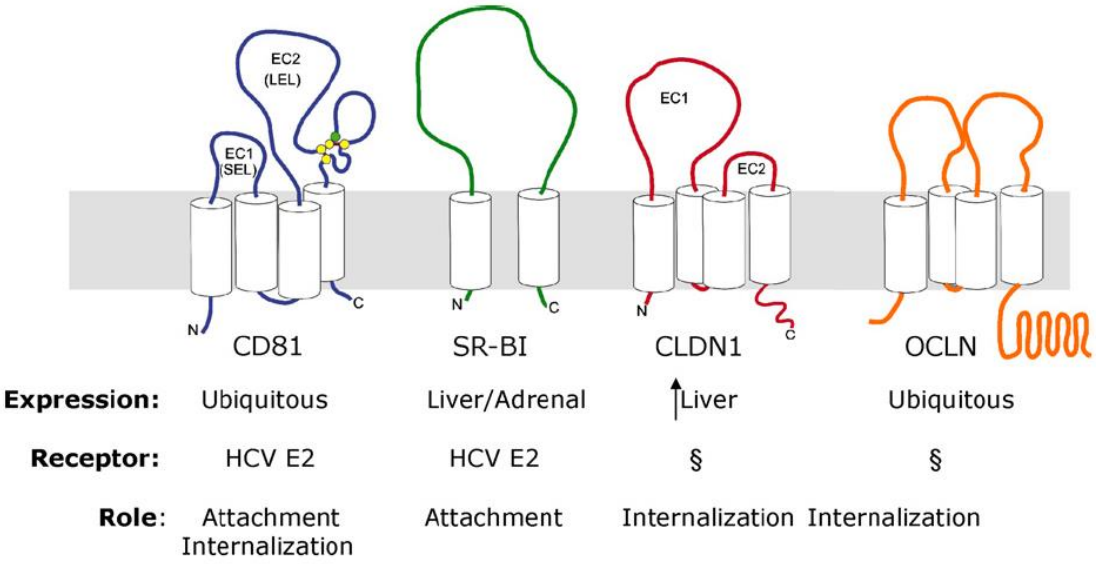

Figure 20 Illustration of different cell surface molecules implicated in HCV entry. Tissue tropism and function of HCV entry factors CD81, SRBI and tight junction proteins CLDN1 and OCLN are depicted. Large extracellular loop (LEL) and small extracellular loop (SEL) or extracellular loops 1 and 2 (EC1 and EC2) are illustrated (Lemon et al., 2010).

1.4.1.2.2 SRBI

The scavenger receptor B-I (SRBI) is expressed on various cell types, yet it is highly expressed on hepatocytes and in steroidogenic tissues. SRBI is composed of N- and C-terminal cytoplasmic domains and an extracellular domain formed by a large loop that is responsible for its receptor function (Fig.20) (Thi et al., 2011). SRBI plays a role in lipid metabolism. It is a multiligand receptor that can bind high density lipoproteins (HDL), LDL and VLDL as well as apolipoproteins (Eck et al., 2008). SRBI mediates the selective cholesterol ester uptake from HDL and the bidirectional transfer of free cholesterol (Acton et al., 1996). SRBI was identified as an HCV co-receptor for its capability to bind HCV sE2 glycoprotein on HepG2 cells, that are characterized by the absence of CD81 (Scarselli et al., 2002). Moreover, hypervariable region 1 (HVR1) of E2 has been shown to interact with SRBI, and SRBI-specific residues involved in this interaction have been identified (Bartosch et al., 2003c; Scarselli et al., 2002). Furthermore, SRB1 overexpression increased HCVcc infection (Grove et al., 2007), and its downregulation inhibited HCVcc infection (Zeisel et al., 2007). Interestingly, it was shown that the physiological ligands of SRBI, HDL and oxidized LDL, could modulate HCV infection. Indeed, HDL has been shown to enhance HCVpp entry, while oxidized LDL inhibit it (Bartosch et al., 2005; Voisset et al., 2005; von Hahn et al., 2006).

SRBI seems to participate in multiple steps during viral entry. First, SRBI interacts with virus-associated lipoproteins, which would facilitate the cell surface attachment of the virions. Second, by its lipid transfer activity, SRBI mediates a post-binding event that could modify the lipoprotein profile of viral particles by dissociating viral particles from associated lipoproteins (Dao Thi et al., 2012a; Zahid et al., 2013). Third, the interaction of SRBI with E2 glycoprotein HVR1 leads to enhanced cell entry (Dao Thi et al., 2012a; Scarselli et al., 2002). Importantly, the interaction with SRBI has been proposed to be required for the subsequent interaction of the virus with CD81, since this interaction might lead to the exposure of the CD81 binding region on E2 (Farquhar et al., 2012; Harris et al., 2010).

As previously mentioned, LDLr and SRBI have been shown to have redundant functions during HCV entry (Yamamoto et al., 2016). Indeed, LDLr/SRBI double knockout Huh7 cells generated with the CRISPR/CAS9 genome editing technique could be rescued for HCV infection by the exogenous expression of either LDLr or SRBI. The ability of the receptors to complement cells for infection relied on their lipid binding/uptake activities.

1.4.1.2.3 CLDN1

The tight junction protein, Claudin-1 (CLDN1) was identified as a crucial HCV entry factor. It is composed of two extracellular domains, EL1 and EL2 (for extracellular loop 1 and 2) anchored in the cell membrane through four transmembrane domains (Fig.20). The identification of CLDN1 as a HCV co-receptor has been made through the screening of a hepatocyte cDNA library expressed in HEK293 cells and infected by HCVpp (Evans et al., 2007). Indeed, the non-permissive HEK293 cells could be infected by HCVpp upon complementation with CLDN1 expressing cDNA. The role of CLDN1 in HCV entry could be further confirmed by the fact that its down regulation in Huh7.5 cells drastically inhibited HCV infection (Evans et al., 2007). Further studies revealed that EL1 is required for viral entry. Moreover, among the 24 members of the claudin family, only CLDN1, CLDN6 and CLDN9 can mediate HCV entry but CLDN6 and CLDN9 are weakly expressed in liver cells (Meertens et al., 2008; Zheng et al., 2007). However no direct interaction between HCV and CLDN1 could be demonstrated. Interestingly, CLDN1 overexpression in HepG2 cells that lack CD81 did not allow HCV infection, while overexpression of both CLDN1 and CD81 in these cells increased their permissiveness to HCV when compared to HepG2 cells in which

only CD81 was overexpressed. These data indicate that CLDN1 cannot substitute the CD81 entry pathway (Evans et al., 2007). HCV entry neutralization kinetics suggests that CLDN1 is involved in a step subsequent to SRB1 and CD81 binding (Evans et al., 2007). HCV binding to CD81 is thought to induce the recruitment of CLDN1 that forms a CD81-CLDN1 complex involved in HCV internalization (Farquhar et al., 2012). In agreement with this model, anti-CLDN1 antibodies that prevent CD81-CLDN1 interaction neutralize infection, and HCV induces the internalization of CD81-CLDN1 complexes (Farquhar et al., 2012; Krieger et al., 2010). The importance of the localization of CLDN1 to tight junctions for HCV infection is controversial. Indeed, whereas some studies suggest that there is a correlation between HCV infection and the localization of CLDN1 to tight junctions, other reports have shown that CD81-CLDN1 complexes were mainly found at the basolateral membrane where infection takes place (Belouzard et al., 2017; Coller et al., 2009; Harris et al., 2008; Liu et al., 2009; Yang et al., 2008).

1.4.1.2.4 OCLN

Occludin (OCLN) is another tight junction protein that was identified as a pivotal host factor for HCV entry. Similarly to CD81 and CLDN1, OCLN contains two extracellular loops, EL1 and EL2 (Fig.20). It has been shown that EL2 was required for HCV entry (Liu et al., 2009; Sourisseau et al., 2013). An interaction between OCLN and E2 in the lysates of infected cells could be observed through co-immunoprecipitation assays (Benedicto et al., 2008; Liu et al., 2010, 2009). However no interaction between HCV particle components and OCLN expressed at the cell surface has been reported. Contrarily to CLDN1, OCLN constitutes a crucial determinant of the human tropism of HCV together with CD81 (Ploss et al., 2009), thus mice harboring hepatocytes that express human OCLN and CD81 have been shown to be susceptible to HCV infection (Dorner et al. 2011; 2013). Moreover, synchronized infection assays revealed that OCLN acted subsequently to CD81 and CLDN1 at a late entry step (Sourisseau et al., 2013). Nevertheless, the precise role played by OCLN during infection is unknown.

The four entry factors CD81, SRBI, CLDN1 and OCLN are essential for HCV entry into cells and their expression in the hepatocytes of transgenic mice allowed to confer HCV susceptibility (Dorner et al., 2011; Vercauteren et al., 2015). In addition to these four receptors, several other entry factors have been identified such as the epidermal growth factor

receptor tyrosine kinase (EGFR), the Niemann-Pick C1-like1 cholesterol absorption receptor (NPC1L1), as well as the transferrin receptor (TfR1) (Lupberger et al., 2011; Martin and Uprichard, 2013; Sainz et al., 2012). The interaction of the viral particles with cellular receptors and factors leads to molecular rearrangements at the plasma membrane that result in the internalization of virus-receptor complexes through clathrin-dependent endocytosis. This step is followed by the fusion between the viral envelope and the endosomal membrane that allows the release of the viral genome into the cytosol.

1.4.1.3 Fusion of viral and host membranes

The fusion mechanism includes several steps. First the fusion protein undergoes conformational changes that lead to the exposure of the fusion peptide, then the latter inserts into the host membrane, this is followed by lipid mixing of outer membranes leaflets (hemifusion) and finally the complete fusion of the viral and cellular membranes results in a single unified membrane (Fig. 21). Behind that general mechanism lies more diversity among the fusion processes used by the different viruses. The activity of fusion proteins is highly regulated to control the place and the moment of fusion reaction. Thus, during the virus morphogenesis and maturation, the fusion protein adopts an inactive prefusion conformation. Moreover, the conformational change required to induce fusion is triggered by different signals depending on the virus. Thus, flaviviruses fuse with the host cell upon exposure to acidic pH of the endosomes, whereas for other viruses, such as HIV-1, the fusion occurs at the plasma membrane and neutral pH upon interaction with cellular receptors (Kielian et al. 2008).

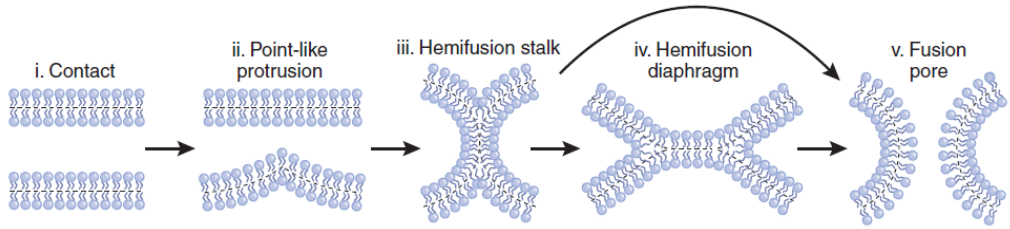

Figure 21 Fusion via hemifusion mechanism of lipid bilayers. i) pre-fusion state: initial contact ii) point like membrane protrusion getting the two membrane bilayers (contacting leaflets) in close contact iii) a hemifusion stalk: merging of only the outer (contacting) leaflets of membranes without affecting the inner membranes of the bilayers iv) expansion of the hemifusion stalk leads to a hemifusion diaphragm v) finally a fusion pore forms in the diaphragm or directly from the fusion stalk (Chernomordik and Kozlov, 2008).

Classes of Fusion proteins

Fusion proteins are classified into three classes according to their structures (Fig.22). Class I fusion proteins consist mainly of α-helical structures and contain a fusion peptide at their N-termini. They form trimers on the surface of viral particles in pre- and post-fusion states (Kielian and Rey, 2006). They are constituted of a single-chain precursor that requires a proteolytic cleavage by host cell proteases to become fusogenic. This step generates two disulphide-bonded subunits with no effect on the overall structure of the fusion protein. One of the two subunits is responsible for the receptor binding while the other mediates fusion. The N-terminal fusion peptide is buried at the trimer interface in the metastable fusion subunit. External triggers destabilize the trimer contacts, which leads to the exposure of the fusion peptide. One of the best-characterized class I fusion protein is the influenza-virus haemagglutinin (Skehel and Wiley, 2000).

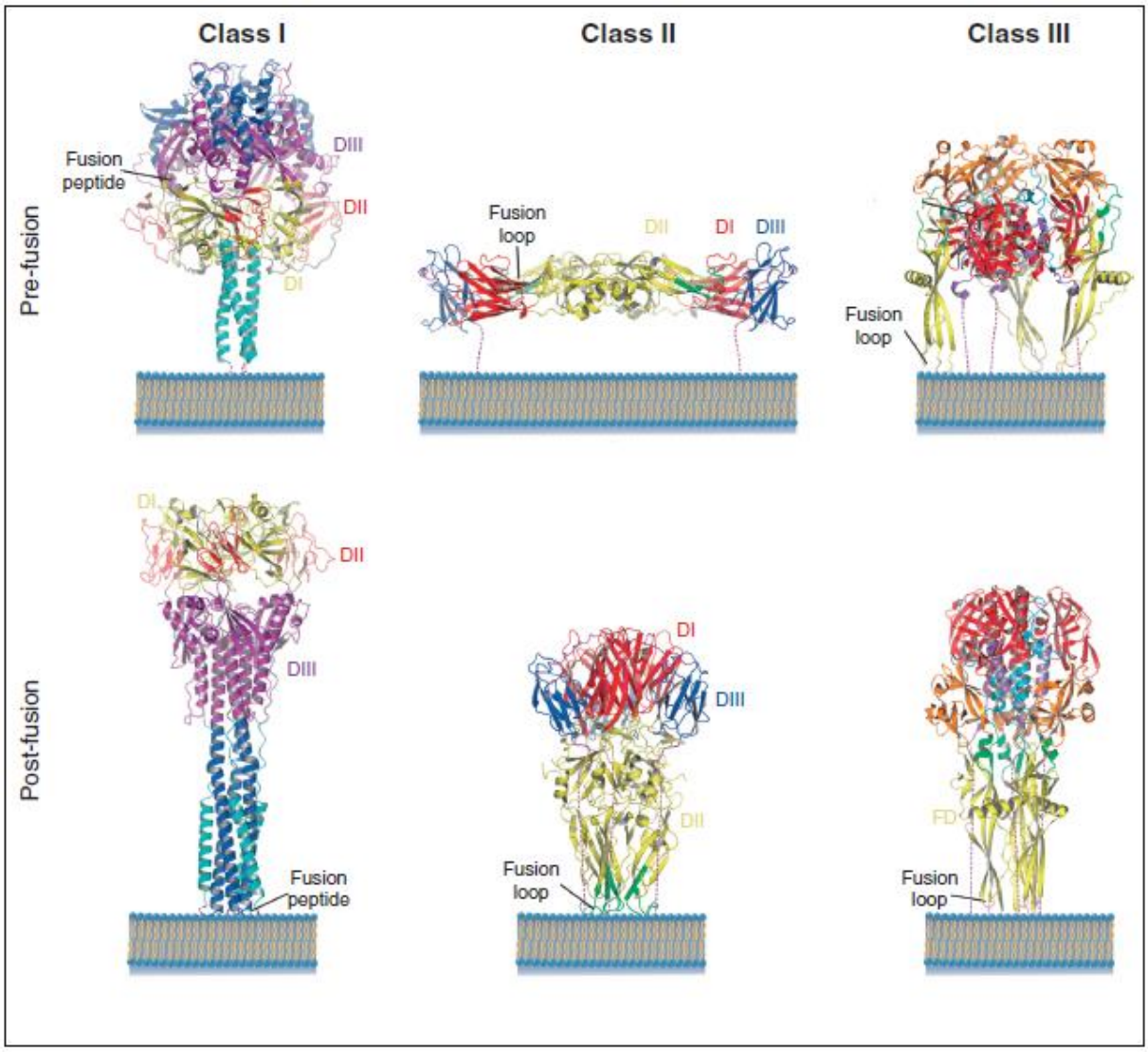

Figure 22 Structure representation of the 3 classes of fusion proteins of enveloped viruses. Pre-fusion states are presented on the top and the post-fusion states below showing conformational changes associated with the

fusion process. Functional domains are identified by color. In all fusion classes, a fusion domain (colored in yellow) is concealed in the prefusion state and gets uncovered upon exposure to environmental factors such as receptor binding or low pH. The fusion motif penetrates the cell membrane and the protein folds back on itself bringing the fusion motif and TMD (not shown) close to each other. The proteins have a trimeric conformation in the post fusion state (Baquero et al., 2013).

Class II fusion proteins have been found in viruses of the *Flaviviridae, Togaviridae* and *Bunyaviridae* families (Modis, 2013). They present a three globular domains structure, essentially constituted by β-sheets. They are anchored in the viral membrane through their C-terminus part. The N-terminal domain I is a β-barrel, the domain II corresponds to an elongated β-stranded region bearing the fusion loop, the domain III presents an immunoglobulin superfamily fold. Class II fusion proteins are co-translated with a partner protein that chaperones the fusion protein during its folding and transport. The cleavage of the chaperone protein makes the fusion protein ready for triggering. Unlike class I fusion proteins, class II fusion proteins associate in metastable homo or hetero-dimers that parallel the viral envelope. The rearrangement undergone during fusion leads to formation of more stable homotrimers in which the fusion peptide loops is exposed.

Class III fusion proteins are found in herpesviruses, rhabdoviruses and baculoviruses. They are organized in 5 domains that contain α-helices and β-sheets (Backovic and Jardetzky, 2011). The proteins are associated in trimers in their pre- and post-fusion conformations. They share structural features with both class I and class II proteins. Indeed, in their post-fusion conformation, these proteins form α-helices trimers similar to class I fusion proteins. Moreover, as found in class II proteins, they possess a central β-stranded fusion domain. No priming event is required to render the protein sensitive to external trigger. Furthermore, for the best characterized class III fusion protein, the G protein from rhabdoviruses, the conformational change induced by low pH is reversible (Baquero et al., 2013).

HCV membrane fusion

Due to the relatedness of HCV with *Flaviviridae* virus family, and in the absence of crystal structures of E1 and E2 glycoproteins, E2 was postulated to be a class II fusion protein (Garry and Dash, 2003; Krey et al., 2010). However, this hypothesis has recently been disputed by the resolution of E2 core domain crystal structure in two different studies (A. G. Khan et al., 2014; Kong et al., 2013a). These studies showed that E2 glycoprotein does not bear the features of class II fusion proteins. Indeed, it does not have the expected three-domain structure shared by class II viral fusion proteins. Instead a globular structure has been

reported that includes many regions with no regular secondary structure. The potential fusion peptide previously proposed is located in secondary structure elements within the hydrophobic core of the protein. Moreover, contrarily to what is expected for a fusion protein, E2 does not undergo structural rearrangement at low pH. This led to the presumption that E2 might not have a direct role in the fusion step and that rather E1 alone or in association with E2 might be responsible for this step. In agreement with this hypothesis, several regions in the E1 glycoprotein have been suggested to take part in the fusion process, which will be discussed later in more details.

Table 3 Comparison of the characteristic features of the three classes of viral fusion proteins (Falanga et al., 2018; Kielian and Rey, 2006; White et al., 2008).

Characteristics	Class I	Class II	Class III
Pre-fusion structure	trimeric	dimeric and parallel to viral envelope	Trimeric
Predominant secondary structure	α- helix	β-sheet	α- helix and β-sheet
Post-fusion structure	trimer of hairpins with central α- helical coiled coil	trimer of hairpins composed of β structures	trimer of hairpins with central α- helical coiled coil
Fusion peptide location	N-terminal peptide buried in trimer interface	internal loops buried in dimer interface	fusion loops positioned toward the viral envelope and domains composed of β sheets
Fusion trigger	low pH/ receptor binding/ low pH + receptor binding	low pH	low pH/ receptor binding
proteolytic processing required for fusion	yes	yes	No
viruses	Influenza virus/ HIV/ Ebola virus	dengue virus/ tick-borne encephalitis virus/ Semliki forest virus	vesicular stomatitis virus/ herpes simplex virus 1

Of note, pestivirus bovine viral diarrhea virus (BVDV), that was also initially postulated to harbor a class II fusion protein, was recently shown to present a novel class of fusion proteins. The crystal structure of the BVDV E2 protein did not show the three domain structure shared by alpha and flavivirus class II fusion proteins, instead it is composed of linearly organized domains thus, differing from any known fusion proteins (El Omari et al., 2013; Yue Li et al., 2013). Additionally, HCV and pestiviruses share some similarities; both contain a small E1- and a large E2 glycoprotein that binds to host cell receptors. Also, both viruses undergo post-attachment priming steps, which allow the conformational change induced by low pH. On the opposite, flavivirus fusion proteins do not need this step (reviewed in (Douam et al., 2015; Lindenbach and Rice, 2013; Ogden and Tang, 2015)).

Hence, the fact that E2 proteins of BVDV and HCV lack features of class II fusion proteins led to the hypothesis that E1 might be responsible for the fusion step in both viruses and that they might be using new mechanisms of fusion.

1.4.1.4 Mechanism of HCV entry

Right after infection, HCV is transported via the blood stream and crosses the fenestrated endothelium of the liver sinusoids to reach the hepatocytes. Once it gets in contact with the basolateral membranes of hepatocytes, the entry process is initiated via capture of HCV virions by attachment molecules and specific receptors in a time and space controlled manner (Fig.23). First, HCV attaches to hepatocytes through HSPG syndecans (Lefèvre et al., 2014; Shi et al., 2013) or SRBI (Dao Thi et al., 2012a) depending on particle density. It was first believed that binding of HCV virions to HSPG or SRBI was mediated by HCV glycoproteins (Barth et al., 2003; Scarselli et al., 2002). However, it was recently suggested that HCV associated ApoE and not the glycoproteins could be responsible for the first contact (Dao Thi et al., 2012a; Jiang et al., 2012). Due to the interaction between lipoproteins and HCV particles, the LDL receptor has also been proposed to be involved in attachment of HCV particles (Agnello et al., 1999). Nonetheless, HCV-LDLr interaction may lead to a nonproductive entry pathway that might lead to degradation of viral particles (Albecka et al., 2012). Attachment of viral particles to the cell surface is not sufficient to induce cell entry process. Thus, for the virus to enter the cell, capture of viral particles is followed by several molecular mechanisms that involve different host factors. Those include the four essential entry factors: SRBI, CD81, CLDN1 and OCLN. However HCV entry mechanisms are far from being fully understood. SRBI has been shown to play an important role in the transition phase between viral capture and entry of the virus. Indeed, through its interaction with HCV-associated lipoproteins it participates to virus capture. Additionally, its lipid transfer activity is involved in a post binding process that is required for viral entry. This step could modify the lipoprotein profile of viral particles as well as the lipid content of cellular membranes (Dao Thi et al., 2012a; Zahid et al., 2013). These rearrangements may affect the movement and localization of cell surface molecules used as receptors. Moreover, these viral particle morphological changes have been proposed to induce the exposure of the CD81 binding site on E2 glycoprotein, thus allowing the subsequent binding of HCV to this receptor (Dao Thi et al., 2012a; Scarselli et al., 2002). The uncovering of this epitope could be due to the

modification of the viral particle composition and lipoprotein rearrangements, or resulting from E1E2 conformational changes. The interaction sites of HCV and CD81 have been mapped to CD81 LEL (Drummer et al., 2002) and a conformational region of E2 (A. G. Khan et al., 2014; Kong et al., 2013a). HCV binding to CD81 triggers several signaling pathways and is thought to induce the migration of virus-receptor complexes to the site of internalization (Brazzoli et al., 2008; Farquhar et al., 2012; Harris et al., 2010, 2008). The lateral migration of HCV-CD81 complex allows its interaction with the tight-junction protein CLDN1. While CD81-CLDN1 interaction could be demonstrated, no direct interaction between CLDN1 and HCV glycoproteins was reported (Evans et al., 2007; Harris et al., 2010). HCV-CD81 trafficking is induced through different signaling pathways including EGFR (epidermal growth factor), RAS GTPase and RHO GTPase signaling (Brazzoli et al., 2005; Diao et al., 2012; Lupberger et al., 2011). Following the formation of HCV-CD81-CLDN1 complex, the PI3K/AKT signaling pathway is transiently activated, which facilitates virus entry (Liu et al., 2012). FRET (fluorescence resonance energy transfer) and stoichiometric imaging have shown that virus-CD81-CLDN1 complexes were internalized via clathrin-dependent endocytosis (Farquhar et al., 2012; Harris et al., 2010, 2008). The Tight-junction receptor OCLN is also an essential factor for HCV entry, however, its exact role is not fully understood. It has been proposed to be involved in a late entry step, subsequently to CD81 and CLDN1 (Ploss et al., 2009; Sourisseau et al., 2013).

Since CLDN1 and OCLN are tight-junction proteins, it had been proposed that upon binding to CD81 HCV would migrate to tight junctions for internalization. However several data obtained in polarized hepatocytes contradict this hypothesis, being more in favor of an entry of the virus via the basolateral pole of the cell with no specific involvement of tight junctions (Belouzard et al., 2017; Harris et al., 2010, 2008; Mee et al., 2009, 2008). Nevertheless, the importance of tight junctions for HCV infection might depend on the cell culture system used. Indeed, the recent imaging of HCV entry in a three-dimensional polarized hepatoma system revealed an initial colocalization of HCV with basolateral entry factors SRBI, CD81 and EGFR. This step was followed by an actin-mediated accumulation of the virus at the tight junctions and its association with OCLN and CLDN. This led to the internalization of the virus via clathrin-mediated endocytosis. This 3D polarized hepatoma system is closer than 2D systems to the *in vivo* situation since it reconstitutes the complex polarization profile of hepatocytes (Baktash et al., 2018).

During the endocytosis process, the plasma membrane buds inwards and forms a clathrin pit that internalizes the HCV-receptor complex. Subsequently, the virus-receptor complexes are located in Rab5A-containing early endosomal compartments (Coller et al., 2009; Farquhar et al., 2012). Acidification of endosomal compartments induces initiation of the fusion of viral and endosomal membranes (Blanchard et al., 2006). Knowing that secreted HCV particles are pH resistant, it is believed that the interaction of CD81 with E2 might be responsible for priming HCV glycoproteins to respond to the low pH and induce fusion of viral and endosomal membranes (Sharma et al., 2011). Moreover, it was suggested that within endosomes, SRBI lipid transfer activities might further modify the viral particles and the associated lipoproteins (Dao Thi et al., 2012a). The optimal pH for fusion *in vitro* is 5.5 in the HCVpp model and 5 in the HCVcc model (Haid et al., 2009; Lavillette et al., 2006). Recently, several chemical compound families have been shown to specifically inhibit HCV fusion step (Perin et al., 2016; Vausselin et al., 2016). Finally, following the fusion step, the viral RNA is released into the cytosol ready to be translated into viral proteins and to start HCV replication (Niepmann, 2013).

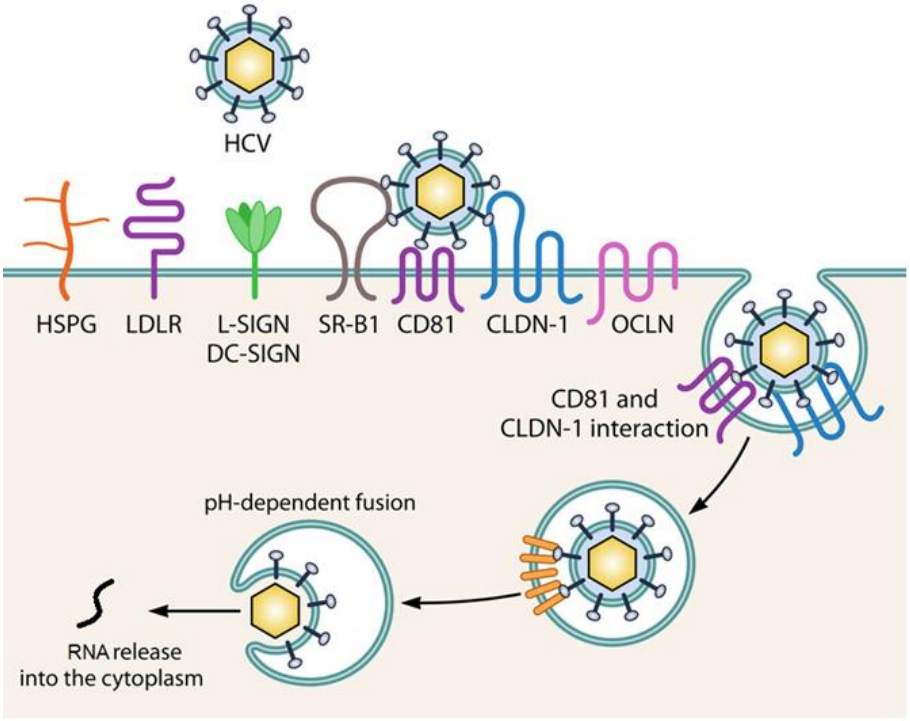

Figure 23 Cell entry of hepatitis C virus. Entry of HCV into hepatocytes is initiated via capture by attachment factors as HSPG, L-SIGN/DC-SIGN or LDLr. This step is followed by specific interactions with the four main HCV receptors SR-B1, CD81, CLDN1 and OCLN in a temporally and spatially coordinated manner. The virus is then internalized in a clathrin-dependent manner and the fusion between the viral and the endosomal envelope leads to the release of the capsid into the cell cytosol and finally the RNA (adapted from (van Dongen et al., 2016).

1.4.1.5 Cell-to-cell transmission

Hepatocytes can be infected by HCV via two different mechanisms either cell-free entry or via cell-to-cell transmission. The cell free entry corresponds to the classical route, in which extracellular virus reaches hepatocytes and interacts with several receptors in order to enter the cell. This happens typically upon primary infection of the host with HCV. However, HCV can also propagate from infected cells to neighboring cells independently of the classical entry pathway (Timpe et al., 2008). Cell-to-cell transmission was first demonstrated by co-culturing HCV-infected hepatoma cells with naïve cells in the presence of neutralizing Abs, which neutralized cell free infection. While reducing more than 95% of cell-free virus infectivity, neutralizing antibodies had minimal effects on the frequency of infected cells in the culture (Timpe et al., 2008). It is thought that cell-to-cell transfer of virus infection is an important route of virus propagation in liver tissue. Moreover this entry pathway might be a virus strategy to escape from the host neutralizing response (Brimacombe et al., 2011; Catanese et al., 2013).

Cell-to-cell transmission involves SRBI, CLDN1 and OCLN. However, the role of CD81 is controversial. Indeed, while two studies reported that CD81 was not required in that process (Timpe et al., 2008; Witteveldt et al., 2009), three more recent works concluded that CD81 was necessary for cell-to-cell transfer (Brimacombe et al., 2011; Catanese et al., 2013; Fan et al., 2017).

Additionally, it has been shown that DAA resistant HCV variants utilize mostly cell-to-cell route for virus transmission. Blocking this route has been shown to decrease the spreading of resistant viruses in vitro, resulting in virus elimination (Xiao et al., 2014). Thus, both cell-to-cell and cell-free transmission should be targeted by inhibitors for efficient virus clearance.

1.4.2 HCV translation

Following fusion, HCV capsid releases viral genome in the cytoplasm, where it is translated and replicated. Translation is initiated by the IRES present in the 5’ UTR while downstream elements like the *cis*-replication element in the coding region and the 3’ UTR participate in the translation regulation (Niepmann et al., 2018). The IRES directly recruits the 40S ribosomal subunit to the viral RNA in the absence of host translation initiation factors

(reviewed in (Niepmann, 2013)). Following this step, the eIF2-GTP-Met-tRNAi ternary complex and eIF3 join the 40S-IRES complex to form the 48S complex (Fraser et al., 2007; Hellen, 2009). After the binding of the ternary complex and eIF3, GTP hydrolysis is mediated by eIF5, which leads to the release of eIF2-GDP (Hellen, 2009; Locker et al., 2007). Following the release of eIF3, the 60S ribosomal subunit is joined to constitute the translationally competent 80S ribosome that proceeds to elongation and termination. Translation of the E1 signal peptide directs the nascent polyprotein to the ER. Thus, translation takes place in association with ER membranes that contain the enzymes required for the processing of the structural proteins. After co- and post-translational cleavage of the polyprotein by cellular and viral proteases, 10 viral proteins are produced. They include 3 structural proteins Core E1, E2; the p7 ion channel and 6 nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Fig. 24).

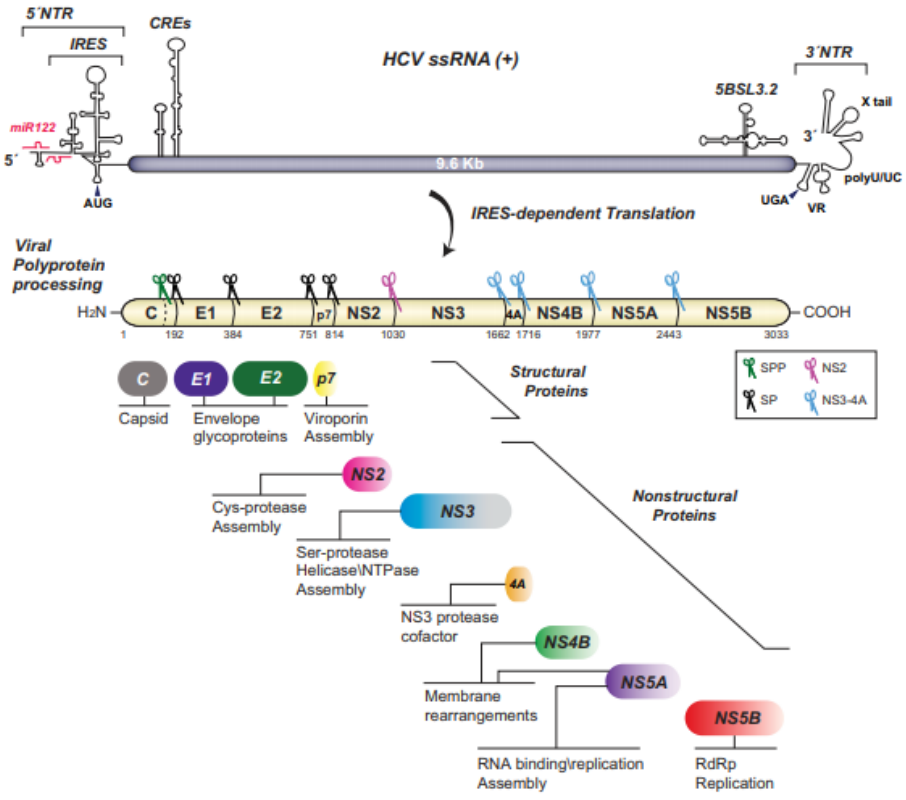

Figure 24 HCV translation and polyprotein processing. HCV positive single strand RNA is translated into a single polyprotein which is further processed by signal peptide peptidase, signal peptidases and viral proteases into its structural and nonstructural proteins. Function of each protein is illustrated below (Paul et al., 2014).

1.4.3 HCV replication

Following polyprotein cleavage, the viral replicase complex is constituted. It is composed of NS3 to NS5B and the genomic viral RNA. Whereas NS2 in itself is not required, it indirectly affects replication through the cleavage of NS2/NS3 junction, which has to be completed for the replication to start (Madan et al., 2014). The NS3 bifunctional protein is responsible for the processing of NS proteins through its serine-protease domain that interacts with NS4A. NS3 C-terminal domain has a crucial role of helicase and could unwind RNA secondary structure and dissociate double–stranded RNA intermediates (Appleby et al., 2011; Dumont et al., 2006; Gu and Rice, 2010). NS4A that anchors NS3 to the ER membrane regulates the replication by stimulating protease and helicase activities of NS3 (Lindenbach et al., 2007). As observed for positive-strand RNA viruses, HCV replication occurs in specialized compartments resulting from massive membrane rearrangements in the ER, termed the membranous web or double membrane vesicles (DMV). Moreover, in the context of HCV replication, lipid droplets converge with the membranous web and are involved in replication (Targett-Adams et al., 2008). These specific organelle-like membranous structures are also designed as viral replication factories (vRF). Formation of DMVs can be triggered by the expression of viral proteins NS3-NS5B in absence of replication. NS4B has been proposed to be the primary inducer of DMV (Egger et al., 2002; Gouttenoire et al., 2010; Paul et al., 2011). However recent data suggest that formation of DMV requires the concerted action of HCV replicase proteins NS3-5B. NS5A is a RNA-binding phosphoprotein. It has been shown to activate NS5B RNA-dependent RNA polymerase (RdRp) independently of its phosphorylation status. This activation might rely on the RNA-binding ability of NS5A or on its interaction with NS5B (Quezada and Kane, 2013; Shirota et al., 2002). No enzymatic activity could be ascribed to NS5A. Nevertheless, its functions might depend on its interactions with several cellular factors such as VAPA (vesicle-associated membrane protein-associated protein A), CypA (cyclophilin A), PI4KIIIα phosphatidylinositol-4 kinase III or ApoE. The RdRp NS5B is the key enzyme catalyzing viral RNA replication. Initiation of RNA synthesis depends on highly structured elements in the 3' UTR. It is thought that synthesis of negative-strand RNA starts at the 3' end of viral RNA. Moreover, this step seems to be rate limiting. The negative strand is then used for the synthesis of positive strand RNAs that are stabilized by miR-122. Indeed miR-122 binds to the 5'UTR and protects the genomic RNA against 5'-3' exonucleases degradation (You Li et al., 2013; Machlin et al., 2011; Sedano and Sarnow, 2014; Shimakami et al., 2012). Neosynthesized RNA genomes are used for translation, replication or participate to the assembly of new viral particles.

Several cellular factors have been shown to participate in HCV replication (Germain et al., 2014; Shulla and Randall, 2012). Thus, CypA that interacts with NS5A contributes to the formation of HCV viral replication factories. HCV replication has also been shown to strongly depend on PI4KIIIα and its product PI4P (phosphatidylinositol 4 phosphate). Thus HCV infection leads to an alteration of PI4KIIIα localization and a concomitant increase in PI4P intracellular levels (Bianco et al., 2012; Reiss et al., 2011). In absence of PI4KIIIα activity, HCV replication and DMVs morphology are impaired (Reiss et al., 2011). Moreover, elevated levels of PI4P during HCV infection are also due to the hijacking of the ARFGAP1 GTPase (GTPase-activating protein for ARF1) by NS5A (Li et al., 2014). Thus, PI4P phosphatase Sac1 is removed from the replication site by NS5A-activated ARFGAP1, which contributes to maintain high levels of PI4P. The role of PI4P in infected cells appears to consist in recruiting host factors at replication complexes. Thus, two PI4P-interacting lipid transfer protein OSBP (oxysterol-binding protein) and FAPP2 (Golgi-associated four-phosphate adaptor protein 2) are recruited to replication complexes in a PI4P –dependent way and are necessary for HCV replication (I. Khan et al., 2014; Wang et al., 2014). This way, HCV subverts the nonvesicular cholesterol transport mediated by OSBP and the glucosylceramide transport mediated by FAPP2 for vRF biogenesis.

The generation of new membranes during HCV replication requires the synthesis of phospholipids. Accordingly, HCV modulates fatty acids and phospholipids metabolism in infected cells (Diamond et al., 2010; Popescu et al., 2014).

Interestingly, the guanine nucleotide exchange factor for G-proteins of the ARF (ADP ribosylation factor) family, GBF1 (Golgi-specific Brefeldin A-resistance guanine nucleotide exchange factor 1) that regulates membrane dynamics in the early secretory pathway is critical for HCV replication (Goueslain et al., 2010). Moreover, GBF1 role during HCV replication relies on the activation of ARF4 and ARF5 (Farhat et al., 2016).

Lipid droplets (LD)s accumulate at vRF (viral replication factories) sites and are involved in replication (Targett-Adams et al., 2008). Thus, LD-binding protein TIP47 (tail-interacting protein 47) regulates HCV RNA replication through its interaction with NS5A (Ploen et al., 2013; Vogt et al., 2013). Importantly, LDs play a key role in the coordination of viral RNA synthesis and particles morphogenesis (Miyanari et al., 2007).

1.4.4 HCV assembly

Subsequent to RNA replication, HCV particles assembly requires the gathering of nascent viral genomes and structural proteins core, E1 and E2. The assembly process can be divided in three steps: first the nucleocapsid formation through interaction of the viral RNA with the core protein, then the acquisition of an envelope with anchored E1E2 proteins through budding in the ER lumen and finally particles maturation and transport through the secretory pathways, that overlaps with VLDL secretion pathway (Gastaminza et al., 2008). A characteristic shared by HCV with other members of the *Flaviviridae* family is the involvement of non-structural proteins in the virus assembly process (Murray et al., 2008). All the viral factors involved in assembly localize in the vicinity of LD that are assumed to be the site of HCV assembly (Fig.25).

As the main component of the viral particle, core protein plays an essential role in viral assembly. After its synthesis and cleavage by signal peptide peptidases at ER membranes, core undergoes homodimerization (Boulant et al., 2005) and is subsequently trafficked to cytosolic LDs (Barba et al., 1997; Moradpour et al., 1996), which are intracellular stores for cholesterol esters and triglycerides (Martin and Parton, 2006). Core-LD interaction is thought to be crucial for recruiting other viral molecules involved in the assembly process (Miyanari et al., 2007), as any interference that prevents this interaction has been shown to inhibit virus assembly (Boulant et al., 2007; Miyanari et al., 2007; Shavinskaya et al., 2007). As mentioned previously, HCV infection induces a delocalization of the LDs from the cytoplasm to perinuclear region at vRF sites (Olofsson et al., 2008; Popescu et al., 2011b). NS5A is presumed to be released from the viral replicase complex to reach the LD surface where it interacts with core. Core–NS5A interaction mediates the recruitment of viral RNA to LD for nucleocapsid assembly (Lindenbach, 2013; Masaki et al., 2008). The interaction between core and the LDs is modulated by several cellular proteins such as DGAT1 (diacylglycerol acyltransferase-1) that is involved in LD morphogenesis and PLA2G4 (group IVA phospholipase A2). These two cellular factors have been shown to be required for infectious virus production (Herker et al., 2010; Menzel et al., 2012). NS5A has been shown to play a pivotal role in the assembly process, especially in the transition between replication and assembly (Lindenbach, 2013). Indeed, the balance between the hyperphosphorylated and hypophosphorylated forms of NS5A seems to regulate the transition between replication and

assembly. Thus hyperphosphorylated form of NS5A is associated with assembly by decreasing interaction between NS5A and the viral RNA (Masaki et al., 2014). This form of NS5A can interact with core as well as with P7-NS2 complex (Jirasko et al., 2010; Ma et al., 2011; Popescu et al., 2011a; Scheel et al., 2012).

Another major component of the viral particle is the HCV E1E2 heterodimer that is retained in the ER (Dubuisson et al., 1994) and requires to be transferred to LDs, where the viral particle assembles (Miyanari et al., 2007). This is thought to be mediated by NS2 and p7, which interact with E1 and E2 triggering the transport of the heterodimer to the LDs (Jirasko et al., 2010; Ma et al., 2011; Popescu et al., 2011a; Stapleford and Lindenbach, 2011). It has been shown that E1E2 heterodimer, p7 and NS2 form a functional unit that moves close to the LDs (Popescu et al., 2011a). In addition to its role in transferring HCV glycoproteins to LDs, p7 also has a role in the late steps of capsid envelopment and assembly (Gentzsch et al., 2013). Oligomerized p7, through its ions channel properties, is thought to balance the pH in the particles secretion compartment to protect envelope proteins from low pH-induced conformational changes (Wozniak et al., 2010). During the assembly process, NS2 has also been shown to interact with the NS3-4A complex that is involved in viral RNA encapsidation (Ma et al., 2008; Yi et al., 2007), since mutations in the helicase domain of NS3 and in the C-terminus of NS4A lead to assembly defects (Phan et al., 2011; Pietschmann et al., 2009). Moreover, NS4B and NS5B are also involved in assembly, but their precise contribution is unknown (Gouklani et al., 2012; Jones et al., 2009; Paul et al., 2011).

As suggested by the association of lipoproteins with HCV particles, HCV morphogenesis is tightly linked to VLDL assembly pathway. Indeed, RNA interference and/or inhibitors targeting cellular factors involved in VLDL biogenesis such as MTTP (microsomal triglyceride transfer protein), ACSL3 (long chain acyl-CoA synthetase 3) or HNF4a (hepatocyte nuclear factor 4a) affect the production of viral particles (Gastaminza et al., 2008; H. Huang et al., 2007; Li et al., 2014). ApoA and apoC have been shown to redundantly participate to HCV particle formation (Fukuhara et al., 2014).

Assembled HCV virions are thought to follow the conventional secretory pathway to the Golgi where E1 and E2 envelope proteins undergo complex modifications (Vieyres et al., 2014). Microtubular transport machinery and the endocytic recycling compartment have been shown to participate in HCV egress (Coller et al., 2012). Moreover components of the ESCRT (endosomal-sorting complex required for transport) machinery are required for HCV

particle release, but its precise contribution is unknown (Corless et al., 2010; Tamai et al., 2012).

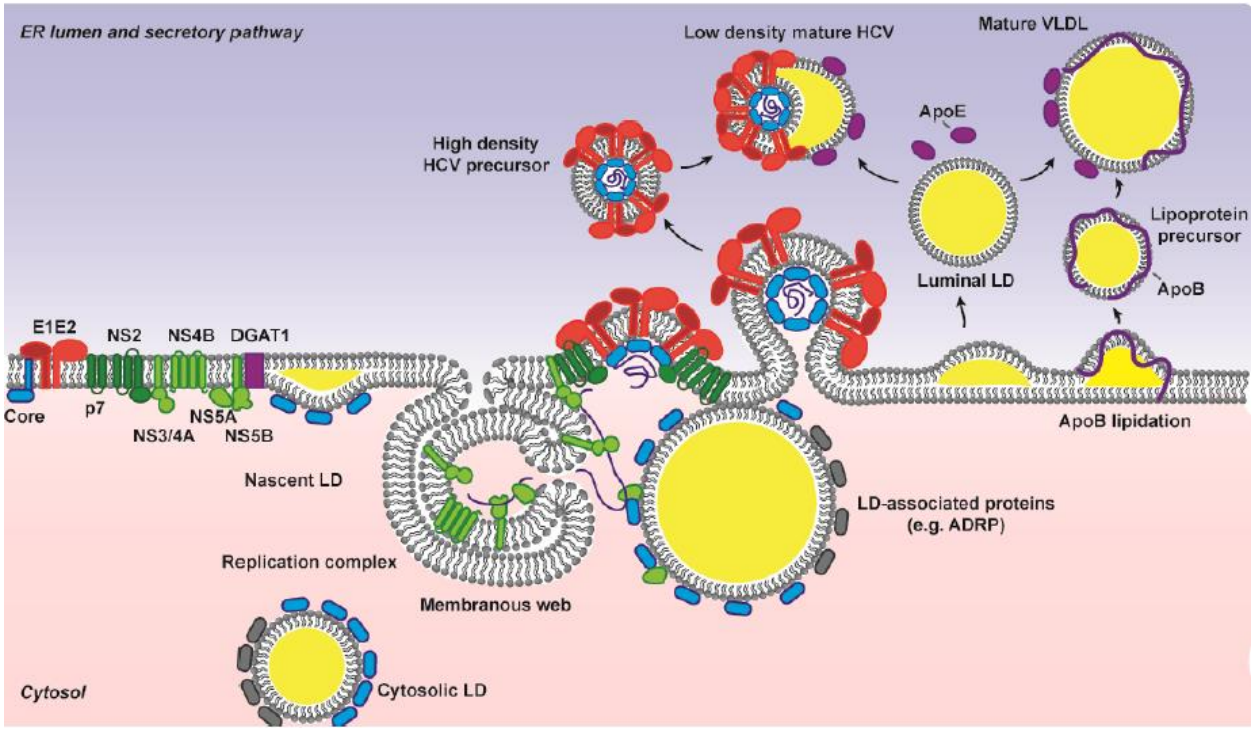

Figure 25 HCV assembly model. After its synthesis and maturation, core is trafficked to cytosolic LDs, which triggers recruitment of other viral molecules involved in the assembly process. LDs translocate upon HCV infection from cytoplasm to perinuclear vicinity. Nascent HCV RNA is trafficked from viral replication site to LD surface, which is mediated by NS5A. There it interacts with core for nucleocapsid assembly. NS2 and p7 interact with E1E2 heterodimer mediating their translocation from the ER to LDs. Due to virus association with lipoproteins, it is presumed that the process of budding into the ER, maturation and release of HCV virions is closely linked to VLDL assembly pathway. Following the assembly and budding of virions in the ER, HCV particles exit the cells through the secretory pathway, where virions acquire their low buoyant density and envelope proteins associated glycans undergo complex modifications (Vieyres et al., 2014).

1.5 HCV glycoproteins E1 and E2

HCV E1 and E2 glycoproteins are type I transmembrane proteins that associate in noncovalent heterodimers within infected cells, while they form large covalent complexes stabilized by disulfide bonds on the virions (Vieyres et al., 2010). Their N-terminal ectodomains are composed of 160 aa and 360 aa for E1 and E2, respectively, while their C-terminal TMDs include 30 aa. E1 and E2 play a major role in virus entry by mediating the binding of the virus to receptors as well as the fusion between viral and host target membranes. They also constitute a key element for HCV assembly.

1.5.1 E1 and E2 biogenesis

1.5.1.1 E1E2 heterodimer formation

Directly after RNA translation, the polyprotein precursor is processed by HCV proteases NS2-3 and NS3-4A to release the non-structural proteins, while the structural part is cleaved by host signal peptidases located in the ER at cleavage sites (C/E1, E1/E2, E2/p7 and p7/NS2) reviewed in (Reed and Rice, 2000)). Cleavages at C/E1 and E1/E2 are directly completed after translation (Dubuisson et al., 2000, 1994), whereas cleavage at E2/p7 and p7/NS2 are delayed thus resulting in the presence of E2-p7-NS2 precursor molecules in infected cells. While most of NS2 is cleaved from the E2-p7-NS2 precursor, the cleavage between E2 and p7 remains incomplete which leads to the existence of uncleaved E2-p7 products (reviewed in (Reed and Rice, 2000).

Translocation of E1 and E2 to the ER is induced by signal sequences present in the C terminus region of the upstream protein, respectively. Thus, E1 signal peptide is part of core sequence and E2 signal peptide consists of E1 C-terminus (Santolini et al., 1994), while E1 and E2 TMDs are responsible for their retention in the ER (Cocquerel et al., 2000). During their synthesis, E1 and E2 ectodomains are translocated into the ER lumen, while the transmembrane domains are anchored in the ER (Cocquerel et al., 2002). E1 and E2 associate in non-covalent heterodimers. The TMDs contain two hydrophobic amino acids stretches linked by hydrophilic polar conserved residues that play an important role in membrane anchoring, ER retention and E1E2 heterodimerization (reviewed in (Vieyres et al., 2014). In addition to the TMs, several other regions located in E1 and E2 ectodomains have been shown to play a role in the interplay between E1 and E2 (Albecka et al., 2011; Douam et al., 2014; Drummer and Poumbourios, 2004). E1E2 heterodimers formation is a slow process. Moreover, E1 and E2 folding are dependent on each other and on cellular ER chaperones such as calnexin and protein disulfide isomerase (PDI) (Lavie et al., 2007). Nevertheless, E2 ectodomain can be expressed alone and fold properly (Heile et al., 2000).

Although E1E2 heterodimers are retained in the ER of Huh7 cells, it has been shown that their expression in polarized Caco2 and HepG2 cells that are competent for lipoprotein assembly leads to the secretion of a fraction of E1E2 with triglyceride rich lipoproteins (Icard et al., 2009). This result might rely on the interaction between HCV glycoproteins and apolipoproteins (Boyer et al., 2014; Lee et al., 2014).

Intergenotypic incompatibilities exist between E1 and E2 from different genotypes. Thus, for some genotypes combination, HCVcc harboring E1 and E2 from two different genotypes are not infectious (Albecka et al., 2011; Carlsen et al., 2013; Douam et al., 2014; Maurin et al., 2011).

1.5.1.2 Folding, glycosylation and disulphide bonds formation

Following their synthesis, HCV E1 and E2 mature in the ER where they undergo the formation of intramolecular disulfide bonds by the PDI and are glycosylated by the N-glycosylation machinery (Dubuisson and Rice, 1996; Goffard and Dubuisson, 2003). HCV E1 and E2 are highly glycosylated proteins, since N-linked glycans contribute to one third of the mass of the E1E2 heterodimer. N-glycans are linked to aspargine (Asn) within the Asn–X–Thr/Ser motif where X corresponds to any residue except proline. E1 harbors four and E2 up to eleven conserved N-glycosylation sites (Fig.26 & 27). Although HCV is highly heterogeneous, most N-glycosylation sites are conserved among the various genotypes indicating that occupation of these sites by glycans is essential for HCV life cycle (Deleersnyder et al., 1997; Goffard et al., 2005). In agreement with that hypothesis, mutagenesis studies have shown that some glycans were crucial for virus assembly and infectivity. Indeed, they modulate E1E2 heterodimerization, folding and their interaction with HCV receptors and neutralizing antibodies (Helle et al., 2010; Lavie et al., 2018). In the ER, processes of glycan modification are limited and result in the generation of high mannose glycans. A more important structural diversity is introduced in the Golgi apparatus, which leads to the formation of complex glycans. Thus, the type of glycan decorating the protein gives some information on their intracellular trafficking during biogenesis. Due to their transit in the Golgi, HCVcc associated E1E2 harbor high-mannose and complex type N-glycans. In the HCVpp context, E1E2 display a majority of complex glycans. This result reflects the existence of differences between the assembly processes of HCVpp and HCVcc (Vieyres et al., 2010).

The crystal structure (4MWF) presented by Kong and coworkers included most of the N-glycosylation sites of E2. It showed that 7 of the 11 N-glycans form a glycan shield that covers the E2 neutralizing epitopes (Kong et al., 2013a), supporting that glycans mediate immune evasion.

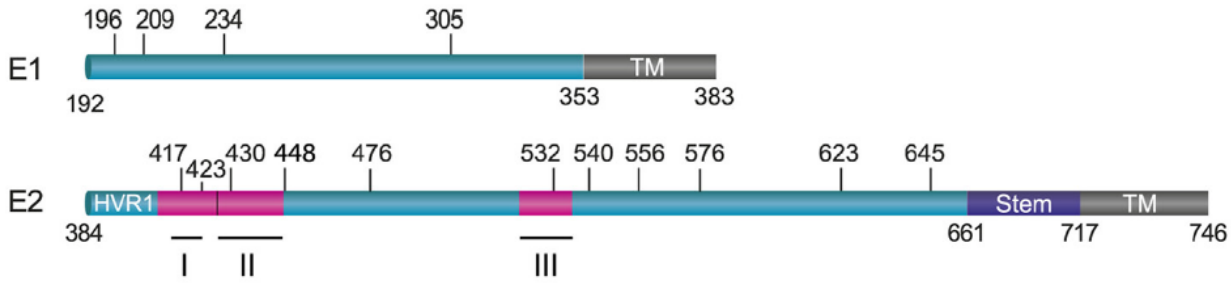

Figure 26 Schematic presentation of E1 and E2 glycoproteins. TMDs are represented as grey boxes. Glycosylation sites are shown by vertical rods. The 3 main immunogenic epitopes of E2 glycoprotein are depicted by pink boxes (Lavie et al., 2018).

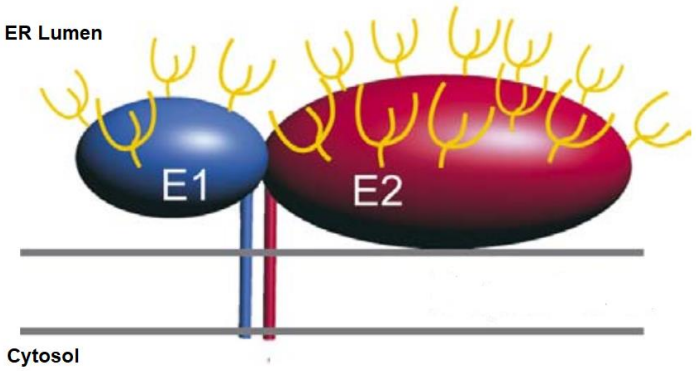

Figure 27 Schematic diagram of HCV envelope glycoproteins (Voisset and Dubuisson, 2004).

The ectodomains of HCV E1 and E2 of genotype 2a (JFH-1) include 8 and 18 conserved cysteine residues, respectively. Intracellular HCV glycoproteins associate in non-covalent heterodimers and the cysteines are engaged in intramolecular disulfide bonds. In most heterologous expression systems, a great part of E1E2 proteins follows a non-productive folding process and forms misfolded aggregates stabilized by intermolecular disulfide bridges. As mentioned above, the folding process leading to functional E1E2 heterodimers is slow and involves chaperone assistance. In that context, E1 and E2 assemble in non-covalent heterodimers and cysteines are involved in intramolecular disulfide bridges (Lavie et al., 2007). These functional forms are recognized by conformational antibodies (Cocquerel et al., 2003; Deleersnyder et al., 1997; Dubuisson and Rice, 1996). In the HCVpp system, non-covalent E1E2 heterodimers are incorporated in the retroviral particle envelope (Flint et al., 2004; Op De Beeck et al., 2004). In contrast, HCVcc associated E1 and E2 proteins form large natively folded complexes that are linked through disulfide bridges (Vieyres et al., 2010). Disulfide bridges may contribute to the resistance of the particles to low pH. This implies that a rearrangement of disulfide bonds is necessary to induce fusion at low pH

(Tscherne et al., 2006). Surprisingly, mutation of individual cysteine residues in E1 leads to attenuated virus infectivity, whereas mutation of any cysteine of E2 abolishes virus infectivity (McCaffrey et al., 2011; Wahid et al., 2013).

1.5.2 E2 glycoprotein

1.5.2.1 E2 structural organization

Among the E1E2 heterodimer, E2 has been the best characterized and studied HCV glycoprotein during the past years. As a matter of fact, E2 is considered as the receptor binding protein, due to its interaction with CD81 tetraspanin (Pileri et al., 1998) and scavenger receptor B1 (SRB1) (Scarselli et al., 2002). Moreover, it is the major target of neutralizing Abs. E2 harbors several highly variable regions (HVR) (Fig.28A). The 27 N-terminal residues correspond to the hypervariable region 1 (HVR1; 384-410aa). This region is targeted by neutralizing antibodies and is thus under high immune pressure. Hypervariable region 2 (HVR2) is located at position 461-481aa downstream of HVR1 and is surrounded by conserved cysteine residues forming a disulfide linked loop (Kong et al., 2013a; McCaffrey et al., 2007). A third region has also been reported to be a hypervariable region and to be targeted by some neutralizing antibodies, the hypervariable region 3 (HVR3, aa 434-450) (Troesch et al., 2006). At last, an intergenotypic variable region (IgVR) aa 570–580 has also been identified. This region is highly heterogenous between genotypes but conserved within a genotype (McCaffrey et al., 2007). HVR2 and IgVR are required for E1 and E2 heterodimerization and for the virus infectivity (McCaffrey et al., 2011). However, HVR1, HVR2 and IgVR can be deleted in a soluble form of E2 without affecting the global folding of E2 (McCaffrey et al., 2007). Nevertheless, deletion of these three regions leads to a loss of infectivity (Bankwitz et al., 2010; Forns et al., 2000; McCaffrey et al., 2011).

HVR1 has been shown to be an important determinant of particle binding to SRBI (Bankwitz et al., 2014, 2010). Thus, deletion of HVR1 and antibodies targeting this domain inhibit HCV cell entry (Bankwitz et al., 2014; Bartosch et al., 2003b; Catanese et al., 2007; Dao Thi et al., 2012b; Scarselli et al., 2002). Moreover, deletion of HVR1 renders HCV resistant to anti-SRBI antibody neutralization and abrogates SRBI binding (Bankwitz et al., 2014). Interestingly, HVR1 seems to constitute an important shielding domain since its deletion greatly increases the accessibility of E2 CD81-binding domain to E2-specific antibodies

(Bankwitz et al., 2014). In agreement with this result, deletion of HVR1 increases sE2 binding to CD81 (Roccasecca et al., 2003; Scarselli et al., 2002). This finding suggests that binding of HCV with SRBI and CD81 are interdependent events during entry.

The region of E2 that contains the receptor-binding site (aa 384-661) is connected to the TMD (aa716-746) by a highly conserved segment comprising heptad repeats (aa675-699). These repeats have been shown to be important for E1E2 heterodimerization and could be involved in E1E2 complex rearrangement during the fusion process (Albecka et al., 2011; Drummer and Poumbourios, 2004; Pérez-Berná et al., 2006).

Resolving the tertiary structures of HCV E1 and E2 glycoproteins has been a setback for a long time due to the difficulties of obtaining proteins in their native forms. This was due to the fact that both proteins are highly glycosylated, harbor many disulfide bridges and their folding is interdependent (Lavie et al., 2007). The lack of crystal structures of HCV envelope glycoproteins and the close relation and similarities of HCV with flaviviruses, led to the hypothesis that HCV harbored a class II fusion protein (Garry and Dash, 2003) and that E2 was the fusion protein (Krey et al., 2010). Nonetheless, this assumption has been challenged after revealing the tertiary structure of the core domain of E2 (E2c) in 2 separate crystallographic studies (A. G. Khan et al., 2014; Kong et al., 2013b). Of note, E2 proteins in both studies were truncated, folded in the absence of E1, and expressed in a soluble form. Both studies showed similar structures for E2c pointing out that E2 does not share any similarity with other viral fusion proteins contrary to what was expected (Fig.28B). As a matter of fact, E2c does not have a three-domain structure as expected for class II fusion proteins, instead it presents a globular structure with many regions with no regular secondary structure. E2c possesses a central immunoglobulin-fold β domain shared by other fusion proteins. This β sandwich is flanked by front and back layers containing loops, short helices and β sheets (A. G. Khan et al., 2014; Kong et al., 2013b). Kong's study allowed localizing CD81-binding site in the front layer of the protein. The structural data obtained by Khan and Kong show that E2 does not harbor the structural hallmarks of fusion proteins. Moreover, structural analyses at low pH reveal that E2 does not undergo structural rearrangement, thus indicating that it is not prone to play a direct role in fusion. Furthermore, it has been shown that E2 putative fusion peptide suggested by Krey and coworkers is rather involved in modulating virus binding than in fusion (Lavie et al., 2014a). This led to the presumption that E2 might not have a direct role in the fusion step and that rather E1 alone or in association with E2 might be responsible for this step.

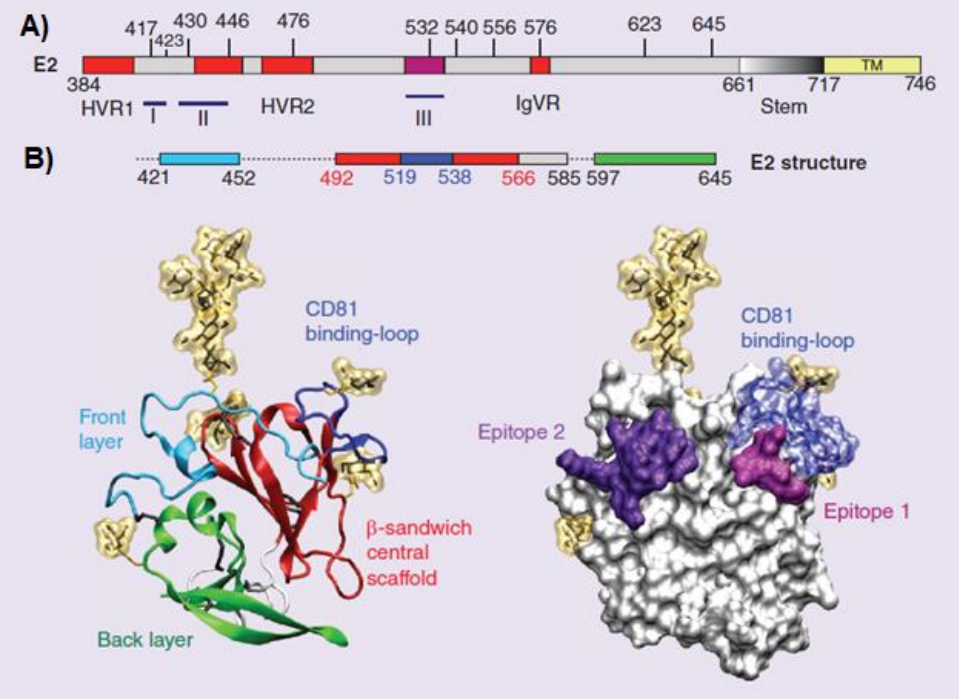

Figure 28 HCV E2 glycoprotein. A) E2 represented as linear diagram with its TM represented as a yellow box. The glycosylation sites are depicted as vertical bars. The hypervariable regions (HVR1, HVR2, HVR3, IgVR) are represented as red boxes and immunogenic epitopes (I, II, III) are underlined (HVRIII not depicted due to overlap with epitope II). B) Linear illustration of HCV E2 core region that was crystallized. Crystal structure of E2 shown below on the left as a ribbon representation (PDB accession code 4MWF)(Kong et al., 2013b), with its structural components colored similar to the above linear presentation. Right figure showing surface representation of E2 core with similar orientation as left diagram. Neutralizing epitopes 1 and 2 are shown in magenta and purple, respectively. CD81 binding site is represented in blue. (Lavie et al., 2015).

1.5.2.2 E2 Neutralization

Neutralizing Abs (nAbs) inhibit viral infection either directly via binding to HCV particles preventing further interaction with receptors or by inhibiting post-entry steps by preventing conformational changes of the envelope glycoproteins required for the fusion step. Neutralizing antibodies mainly target E2 glycoprotein. This has been shown by characterizing the reactivity of antibodies isolated from HCV-infected patients and immunized animals (Ball et al., 2014). Several regions in E2 glycoprotein have been shown to be targeted by neutralizing antibodies. Notably, HVR1 consisting of the first 27 aa of E2 (aa 384–410) was considered as a major immunodominant neutralization region. As mentioned before, this region is involved in interaction with SRBI, virion assembly and release, and exposure of ApoE epitope on the virions (Bankwitz et al., 2014, 2010; Prentoe et al., 2011). Due to the high variability of HVR1, antibodies directed against HVR1 have poor cross-neutralization efficacy against different HCV genotypes (Wang et al., 2011). Recently, it has been shown that viruses missing HVR1 are more sensitive to neutralization (Bankwitz et al., 2010;

Prentoe et al., 2011), presuming that HVR1 masks CD81 receptor binding site on E2, which is considered as the main target of nAbs. Indeed, antibodies that have broad neutralizing activities target conformational epitopes within E2 and are likely to interfere with the interaction between E2 and CD81 (Ball et al., 2014). NAbs directed against CD81 binding site are classified to Abs that recognize linear epitopes within E2 located at aa 412-423, conformational epitopes with contact residues in the region 523-535aa or epitopes spanning both binding regions. The first group of antibodies recognizing linear epitopes within the region aa 412-423 referred to as epitope I include Abs AP33 and 3/11 (Zhang et al., 2007). This region is directly found adjacent to HVR1 and is highly conserved. Recently, several Abs targeting this region have been described, such as HCV1, which has been shown to bind predominantly at position L413 and W320 within E2 (Kong et al., 2012). Interestingly, residue W420, which is essential for CD81 binding, was common to all Abs targeting the region 412-423 as an essential contact residue (Owsianka et al., 2006). However, antibodies directed to this region have shown a very low seroprevalence, suggesting that it is not naturally highly immunogenic, which is likely due to its poor accessibility (Tarr et al., 2007). The segment in E2 situated between aa 523-535 (Owsianka et al., 2006) present several conserved residues that are targeted by broadly nAbs (Ball et al., 2014). Moreover, this sequence seems to be immunogenic in natural occurring infections, as human mAbs directed against several overlapping epitopes in this region could be isolated from sera of infected patients (Ball et al., 2014). A third class of antibodies targets epitopes spanning 412-423 and 523-535 regions, which suggests that these regions are close to each other on the surface of virus associated-E2 (Perotti et al., 2008). Finally, another antigenic region in E2 called epitope II that has been previously reported by Zhang spanning aa 427 to 446 (Zhang et al., 2007), has also been shown to play a role in CD81 binding (Drummer et al., 2006). Interestingly, antibodies directed against this epitope might be neutralizing, while others might not be. Moreover, non-neutralizing antibodies targeting epitope II have been shown to interfere with neutralizing antibodies directed toward epitope I (Sautto et al., 2012; Zhang et al., 2009). The crystal structures of epitope II in complex with a neutralizing and non-neutralizing antibody were obtained (Deng et al., 2014, 2013). They revealed different spatial organizations of the epitope depending on the associated antibody. Thus, the ability of epitope II to induce the production of neutralizing or non-neutralizing antibodies could depend on the different conformational changes undergone by this region during the viral life cycle.

CD81 binding site is highly conserved and resistant to the appearance of adaptive mutations (Keck et al., 2011; Owsianka et al., 2006). Thus, it might be a good target for developing therapeutic Abs.

Antigenic regions 4 and 5 could be identified outside CD81 binding region by screening patients' antibodies libraries (Clementi et al., 2012; Giang et al., 2012). Among the monoclonal antibodies targeting this region, AR4A that recognizes a discontinuous epitope on E1 and E2, presents a broad neutralization spectrum and can inhibit infection *in vivo*.

1.5.2.3 Role of E2 in attachment and binding

HCV entry into hepatocytes is a complex process which involves multiple host cell factors and cell surface molecules. HCV E2 glycoprotein has been shown to be responsible for receptor binding. In particular, the two HCV receptors, SRBI and CD81, have been shown to interact directly with E2. Indeed, CD81 was the first identified entry factor for HCV (Pileri et al., 1998). Multiple regions have been identified in E2 that interact with CD81, depending on the proper folding of E2. Additionally, most E2 nAbs are directed toward CD81 binding region. Therefore, the characterization of nAbs binding sites via site directed mutagenesis led to the identification of important domains for CD81 binding within E2 (reviewed in (Fénéant et al., 2014)). Three regions at least have been identified to be involved in direct interaction with CD81, whereas further regions might modulate this interaction. The first region is located between (aa 474–494) spanning HVR2, second region lies at aa 522–551 and the last one extends from residue 612 to 620 (Fénéant et al., 2014). The mutagenesis study of Kong and colleagues showed interactions of CD81 with certain residues within the front layer of E2 (aa 427–430 and 442–444) and in the CD81 binding loop including aa 525 in the 522–551aa region (Kong et al., 2013a). The deletion of a part of the 474-492aa region in E2c does not affect its interaction with CD81. In a similar manner, the 612-619aa segment is not localized on the same face than CD81 binding region and forms an α-helix that could be crucial for E2 structure (Kong et al., 2013b). Thus, this region could be involved in an indirect manner in E2/CD81 interaction. On the whole, it is difficult to precisely identify residues directly involved in E2/CD81 interaction, since residues might indirectly influence CD81 binding. Notably, E2 conformation is influenced by its heterodimerization with E1. Accordingly, some E1 mutations have been shown to affect E2 interaction with CD81 (Haddad et al., 2017; Wahid et al., 2013).

Several regions of E2 modulate E2 and CD81 interaction. Indeed, HVR1 deletion and some point mutations (G451A, V514A) have been reported to increase the interaction with CD81 while reducing the dependence of the virus on SRBI for entry (Grove et al., 2008; Lavie et al., 2014b).

HVR1 of E2 has been shown to be involved in HCV binding to SRBI. Deletion of HVR1 as well as antibodies directed toward this region have been reported to inhibit viral entry into the cells (Bankwitz et al., 2010; Bartosch et al., 2003c; Scarselli et al., 2002). Moreover, the deletion of HVR1 led to loss of inhibitory activity of anti –SRBI Abs and virus binding to SRBI (Bankwitz et al., 2010). Additionally, HVR1 deletion enhances CD81 binding site exposure making it more accessible to anti-CD81 Abs (Bankwitz et al., 2010). Consequently, the deletion of HVR1 has been reported to increase binding of soluble E2 to CD81 (Roccasecca et al., 2003; Scarselli et al., 2002). To that end, SRBI and CD81 binding are suggested to be two tightly linked steps in HCV entry.

1.5.3 E1 glycoprotein

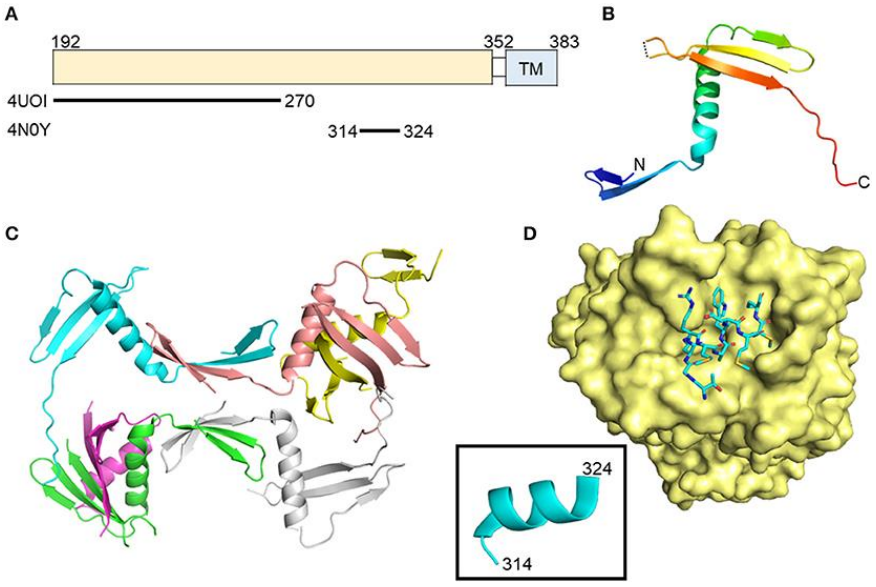

Figure 29 Crystal structure of the N-terminal domain of E1 and an E1 peptide-mAb complex. A) E1 represented as a linear diagram with the crystallized regions depicted below. B) Monomeric crystal structure of E1 N-terminus (PDB ID: 4 UOI) C) Association of 6 monomers of E1 N-terminal domain in an asymmetric structure. D) E1 peptide (aa314-324) crystal structure in complex with Ab IGH 526. Ab is colored in yellow, while peptide is represented by blue bars. Moreover, the peptide is represented as a blue helical structure in a box (Yost et al., 2018).

See Annex: **Function of the hepatitis C virus E1 envelope glycoprotein in viral entry and assembly**

2 Aims of the study:

HCV E1 and E2 heterodimer comprise the major viral determinant for viral entry and is a crucial component of assembly. The E2 glycoprotein has been the better characterized glycoprotein, since it is the receptor binding protein and is targeted by neutralizing antibodies. By analogy with other members of the *Flaviviridae* family, it has been suggested that HCV possesses a class II fusion protein and that E2 is the fusion protein. Nevertheless, the lately obtained crystal structures of E2 revealed that it lacks structural hallmarks of class II fusion proteins. Therefore more focus has been put on E1 glycoprotein assuming it to be responsible for the fusion step either alone or in association with E2. Recently, the N-terminal part of E1 ectodomain was crystallized and the characterization of the conserved residues of this region revealed that it is important for virus infectivity, E1E2 interaction and in the interplay of HCV with CLDN1.

In agreement with the putative involvement of E1 in viral fusion, several regions of the protein, including C terminal regions aa265–296 and aa309–340, have been reported to be involved in interactions with model membranes. Moreover several adaptive mutations conferring resistance to inhibitors of late entry steps emerged in these regions. In that context, we sought to investigate the functional role of the C-terminal part of E1 ectodomain in the viral life cycle. In particular two regions of interest were investigated. The first one located in the putative fusion peptide (PFP) region between amino acid 270 and 291, containing hydrophobic sequences, which suggested its involvement in the fusion step. The second region spanning amino acids 314-342, which is a membranotropic region located proximal to the transmembrane region of E1. It has been shown by X-ray crystallography and NMR-studies to comprise two α-helices (α2 and α3). This peptide has also been shown to interact with membranes. We introduced 22 mutations in the C-terminal part of the E1 ectodomain in the context of a JFH1 infectious clone. We replaced the most conserved residues with alanine and analyzed the effect of the mutations on the viral life cycle. The obtained results of the functional study of the C-terminal part of HCV E1 glycoprotein were published in the article:

Moustafa, R.I., Haddad, J.G., Linna, L., Hanoulle, X., Descamps, V., Mesalam, A.A., Baumert,T.F., Duverlie, G., Meuleman, P., Dubuisson, J., Lavie, M., 2018. Functional study of the C-terminal part of hepatitis C virus E1 ectodomain. J. Virol. JVI.00939-18. https://doi.org/10.1128/JVI.00939-18

3 Results:

Amino acid conservation in the second half of the E1 ectodomain and mutated residues. Amino acid conservation in E1 region from aa 270 to 350 among HCV genotypes is represented in Fig. 30. This region contains the putative fusion peptide aa270-291 (PFP) (Drummer et al., 2007) and two α helices, α2 (aa315-324) and α3 (aa331-338), as revealed by NMR and X-ray crystallography studies performed on the aa314-342 and aa314-324 E1 peptides (Spadaccini et al., 2010). The less-variable residues of this region among HCV genotypes were individually replaced by alanine in the context of the JFH1 infectious clone. This led to the generation of 22 mutants. Among them, 5 belong to the PFP region, whereas 8 are located in the α2-helix region. Unexpectedly, the residues of the α3 helix were more variable, which suggested a less important role for this region in the HCV life cycle. Mutations were introduced in a modified version of the plasmid encoding the fulllength JFH1 genome in which the N-terminal E1 sequence has been modified to reconstitute the A4 epitope, which is present in E1 of genotype 1a (Goueslain et al., 2010), and therefore allows for the identification of this modified E1 of genotype 2a for which there is no antibody readily available. It is worth noting that introduction of the A4 epitope does not affect HCV infectivity and thus does not interfere with the characterization of the phenotypes of E1 mutants.

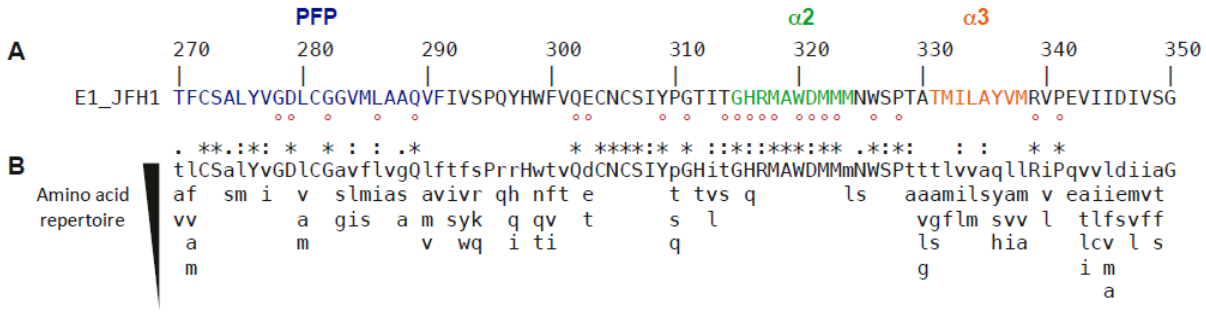

Figure 30 E1 C-terminal region sequence analyses. (A) The E1 aa270-350 sequence from the HCV JFH1 strain (AB047639; genotype 2a) is indicated with respect to the polyprotein numbering. Amino acids mutated in this study are indicated by a red dot. (B) Amino acid repertoires of the C-terminal region of E1. The amino acid (aa) repertoire was deduced from the ClustalW multiple alignment of the 28 representative E1 sequences from confirmed genotypes and subtypes in the European HCV database (https://euhcvdb.ibcp.fr/euHCVdb/jsp/nomen_tab1.jsp). Amino acids observed at a given position in fewer than two distinct sequences were not included. Amino acids observed at a given position in more than 25 distinct sequences are shown in capital letters. The degree of amino acid conservation at each position can be inferred from the extent of variability (with the observed amino acids listed in decreasing order of frequency from top to bottom), together with the similarity index according to ClustalW convention (asterisk [*], invariant; colon [:], highly similar; dot [.], similar).

Effect of E1 mutations on HCV replication. In a first step, we assessed the ability of the produced mutants to replicate. For this purpose, the expression of several HCV proteins (E1, E2, and NS5A) was examined at 48 h postelectroporation of Huh-7 hepatoma cells with wt and mutant HCV RNA. For all mutants, similar levels of protein expression could be observed; hence, any effect of the mutations on viral replication could be excluded (Fig. 31). We included in our analysis the GND nonreplicative HCV mutant and the ΔE1E2 assembly-deficient mutant that carries an in-frame deletion in E1E2 coding region. Interestingly, D279 and Q289 mutations in the PFP and Q302 in the region between PFP and α2 led to the detection of an additional band of lower molecular weight, which likely corresponds to an alternative glycoform of E1 as previously observed when E1 is expressed as a recombinant protein (Duvet et al., 2002).

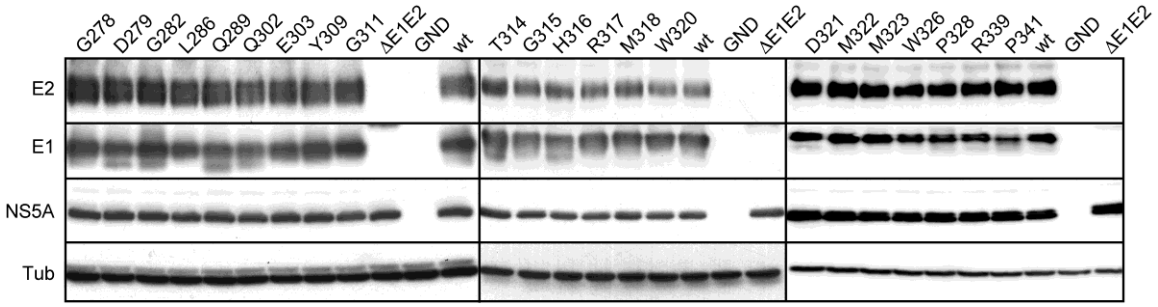

Figure 31. Effect of E1 mutations on the expression of viral proteins. Viral RNA transcribed from JFH1-derived mutants was electroporated into Huh-7 cells that were lysed 48 h later. Viral proteins were separated by SDS-PAGE and revealed by Western blotting with MAbs A4 (anti-E1), 3/11 (anti-E2), and anti-NS5A, as well as anti-beta-tubulin antibody, to verify loading of equal amounts of cell lysates. The protein detected by MAb A4 in cells expressing ΔE1E2 mutant corresponds to a fusion protein between the N terminus of E1 and the C terminus of E2.

Effect of E1 mutations on HCV infectivity. Since the introduced mutations did not affect viral replication, we assessed their impact on the production of infectious virus. To do so, we determined the intracellular and extracellular infectivity after electroporation of Huh-7 cells with viral RNAs. We observed different phenotypes of virus infectivity: (i) complete loss of infectivity for mutants G278A, D279A, G282A, Q302A, Y309A, M318A, W320A, D321A, and M322A; (ii) severe attenuation of infectivity for mutants G311A, T314A, G315A, H316A, R339A, and P341A; (iii) slight attenuation of infectivity for mutants L286A, E303A, M323A, W326A, and P328A; and (iv) no effect on infectivity for mutants Q289A and R317A. In most cases, intra- and extracellular infectivity profiles were similar, suggesting that the mutations did not affect infectious virus release. However, the P341A mutant showed a 3-log decrease in its extracellular infectivity level compared to wild-type infectivity, while its intracellular infectivity was reduced by only 1 log at 96 h postelectroporation. This result

is in favor of an effect of this mutation on the secretion of infectious virus. These initial data show that most mutations in the putative fusion peptide and α2-helix regions result in a loss of infectivity or a severe attenuation, suggesting that these regions are important for the HCV life cycle (Fig. 32).

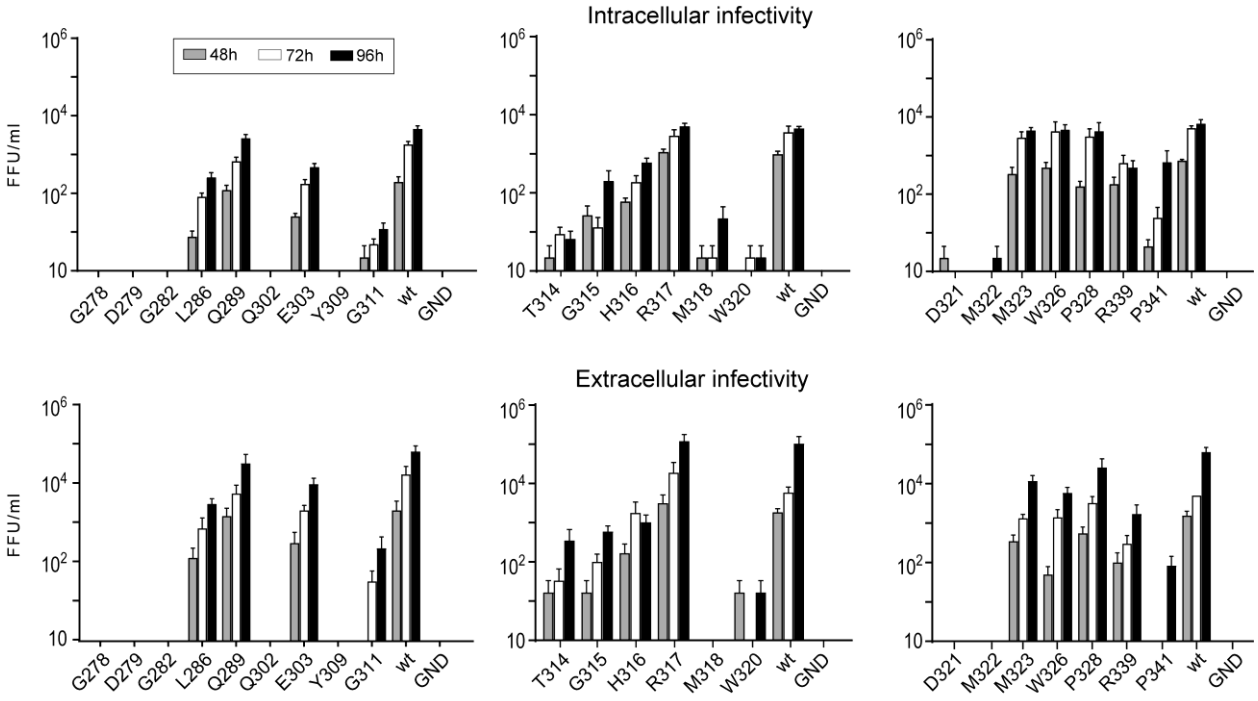

Figure 32 Effect of mutations on extracellular and intracellular infectivities. Viral RNA transcribed from JFH1-derived mutants was electroporated into Huh-7 cells. The infectivities of the supernatants and intracellular viruses were determined at 48, 72, and 96 h postelectroporation by titration. Error bars indicate standard errors of the means from at least three independent experiments. Values were compared to the wild-type virus. Differences were considered statistically significant for the extracellular infectivity of mutants G278A, D279A, G282A, L286A, Q302A, E303A Y309A, G311A, T314A, G315A, H316A, M318A, W320A, D321A, M322A, W326A, R339A, and P341A ($P < 0.05$) and for the intracellular infectivity of mutants G278A, D279A, G282A, L286A, Q302A, E303A Y309A, G311A, T314A, G315A, H316A, M318A, W320A, D321A, M322A, R339A, and P341A ($P < 0.05$) at 96 h postinfection.

Effect of E1 mutations on virion release. To determine whether the mutations affected the release of viral particles, the intra- and extracellular levels of HCV core protein at 48 h postelectroporation were quantified. For all mutants, the level of intracellular core protein was similar to the wild-type virus, confirming the absence of effect of E1 mutations on viral replication. In contrast, the levels of extracellular core proteins were reduced for most mutants in the potential fusion peptide region that presented impaired infectivity, as well as for the severely attenuated P341A mutant, indicating a defect in the secretion or assembly of viral particles (Fig. 33). Interestingly, most of the mutations in or close to the E1 α2 helix (G311A, T314A, G315A, H316A, M318A, W320A, D321A, and M322A) affecting virus

infectivity had no impact on the secretion of core protein, suggesting that these mutations led to the release of noninfectious viral particles.

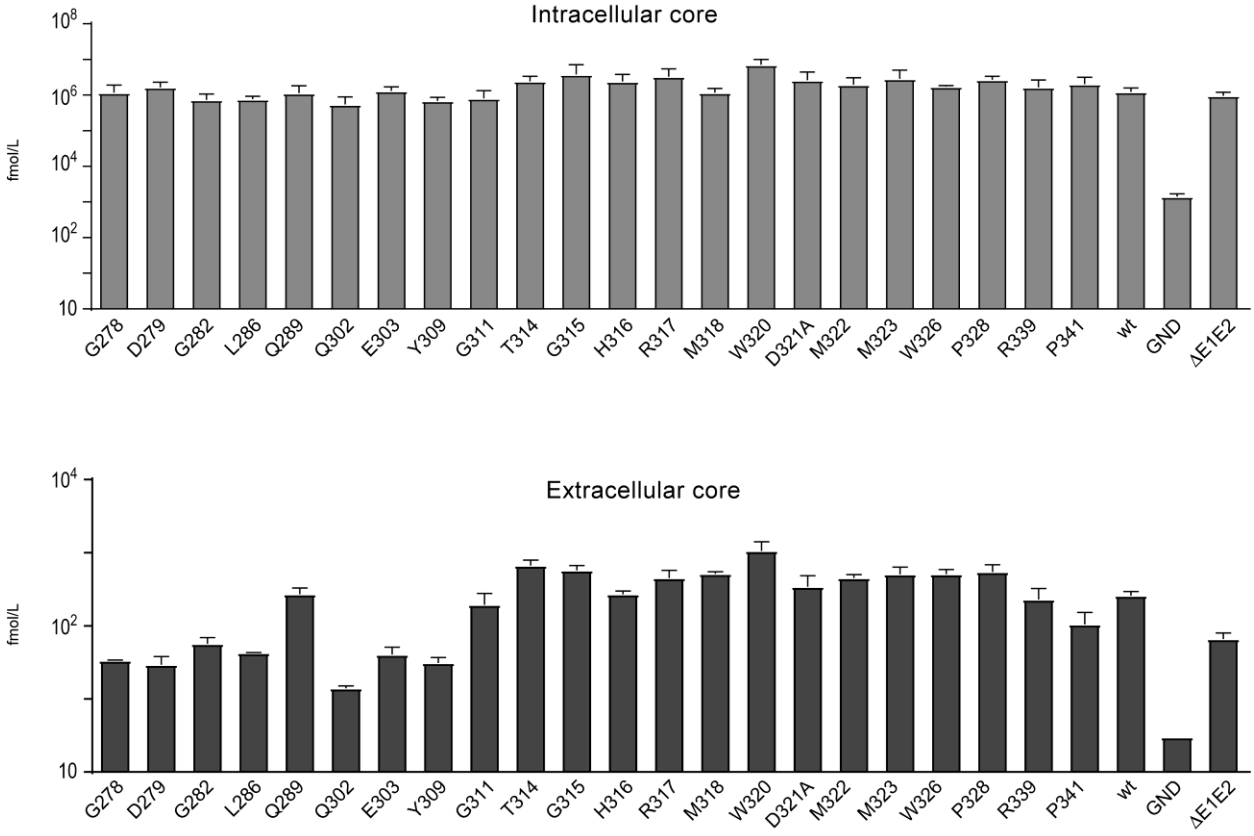

Figure 33 Effects of E1 mutations on HCV core protein secretion. Huh-7 cells were electroporated with wild-type or mutant viral RNAs. The levels of core protein in supernatants and cell lysates were determined at 48 h postelectroporation. Error bars indicate standard error of the means from at least three independent experiments. Values of core protein were compared to the wild-type value. Differences were considered statistically significant for extracellular mutants G278A, D279A, G282A, L286A, Q302A, E303A Y309A, and P341A ($P < 0.05$).

Effect of E1 mutations on HCV glycoprotein folding and E1E2 heterodimerization. Since E1 and E2 cooperate for their respective folding, we analyzed the effect of the mutations on the formation of E1E2 heterodimers (Wahid et al., 2013). For this purpose, we performed pulldown assays using the CD81 large extracellular loop (CD81-LEL), which recognizes correctly folded E2 (Fig. 34A). E2 protein from all mutants could be precipitated by CD81-LEL, indicating that the E1 mutations had no effect on E2-folding. In the PFP segment and the downstream E1 region (aa274-309), nearly all mutations impacting infectivity affected the coprecipitation of E1. Thus, for the attenuated mutants (L286A and E303A), a lower signal on the E1 Western blot was observed after CD81-LEL pulldown. For the noninfectious mutants (G278A, D279A, Q302A, and Y309A), the E1 protein was not

detectable. The impairment in E1 coprecipitation indicates that these mutations affect the interaction between E1 and E2, at least in the context of properly folded E2. These results suggest that the potential fusion peptide and the downstream E1 region (aa274-309) are involved in E1E2 interaction. Thus, the loss of infectivity of the mutants in this region might be due to the associated alteration in protein folding.

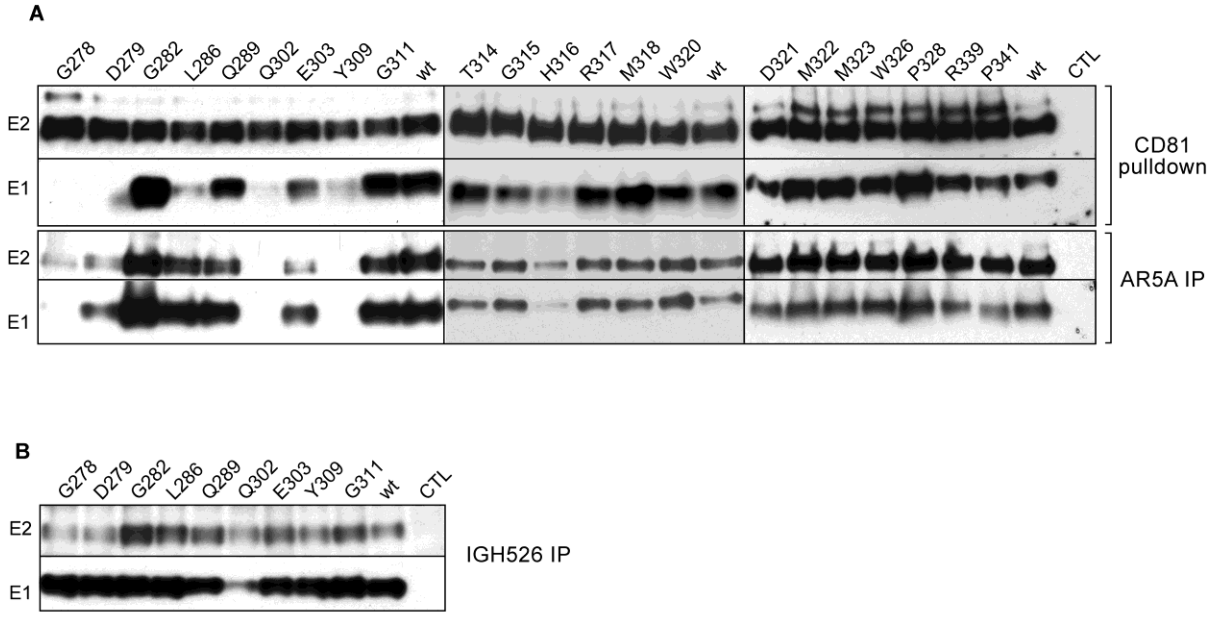

Figure 34 Effect of E1 mutations on E1E2 conformation. (A, upper panel) Interaction of HCV glycoproteins and CD81 (HCV entrybfactor). E1 and E2 from cell lysates were analyzed by GST pulldown at 48 h postelectroporation using a CD81-LEL-GST fusion protein. Pulled-down E1 and E2 were separated by SDS-PAGE and revealed by Western blotting with MAbs anti-E1 (A4) and anti-E2 (3/11). (A, lower panel, and B) Recognition of HCV E1 and E2 glycoproteins by conformation-sensitive anti-E1E2 MAb AR5A and anti-E1 MAb IGH526, as indicated. At 48 h postelectroporation, E1 and E2 proteins from cell lysates were analyzed by immunoprecipitation with MAbs AR5A and IGH526. Immunoprecipitated proteins were revealed by Western blotting using MAbs A4 and 3/11.

The G282A mutation in the PFP and the majority of the mutations in α2 and the downstream region (aa325-341) of E1 had no impact on the formation of the E1E2 heterodimer since E1 could be efficiently precipitated with E2 in CD81-LEL pulldown for all mutants, except for one (H316A) in this region. Thus, mutations G311A, T314A, G315A, M318A, W320A, D321A, M322A, M323A, W326A, P328A, R339A, and P341A, which led to the production of attenuated or noninfectious virus, had no impact on E1E2 interaction. These mutants do not present conformational or heterodimerization defects that could explain their loss of infectivity.

To further characterize the effect of E1 mutations on the folding of the E1E2 heterodimer, we performed immunoprecipitation experiments with conformationsensitive antibodies. In a first step, we used the human monoclonal antibody (MAb) AR5A, which recognizes an

epitope shared by E1 and E2 (Giang et al., 2012)(Fig. 34A). Data obtained in this assay correlated with the results of the CD81 pulldown assay. Indeed, for the noninfectious or attenuated mutants in the potential fusion peptide and downstream region (G278A, D279A, Q302A, E303A, and Y309A), E1E2 glycoproteins were either weakly or not recognized by MAb AR5A. These findings confirm that these mutations affected E1E2 conformation and that the loss of infectivity or attenuation was due to an alteration in protein folding. Unexpectedly, E1 and E2 were well recognized by the AR5A MAb for the mutant L286A, for which CD81 pulldown assay showed a weak signal for E1 coprecipitation, suggesting an effect on the interaction between E1 and E2. This might be due to a partial alteration of the affinity between E1 and E2, which is in agreement with its attenuated infectivity.

In the case of the G282A mutant in the PFP region and most of the mutants in the α2 and downstream region (G311A, T314A, G315A, M318A, W320A, D321A, M322A, M323A, W326A, P328A, R339A, and P341A), which are either attenuated or noninfectious, E1 and E2 were recognized by AR5A MAb, excluding any effect on the heterodimerization or folding for these mutations. The absence of effects on E1E2 heterodimerization and folding is in agreement with the unimpaired virus secretion observed for the mutants in the α2 helix and downstream region. Altogether, these results suggest that these mutations lead to the production of noninfectious viral particles.

For the mutants in the PFP region, E1 folding was further characterized by immunoprecipitation with the E1-specific antibody IGH-526 (Fig. 34B), which recognizes a discontinuous epitope that includes a linear region spanning residues 313 to 327 (Kong et al., 2015). Due to the overlap between α2-helix residues and the IGH-526 epitope, we could not use this antibody to characterize E1 folding for α2-helix mutants. For all tested mutants except for the mutant Q302A, the glycoprotein E1 was recognized by the MAb IGH-526, indicating that these mutations have no drastic effect on the conformation of the E1 glycoprotein. E2 coprecipitated with E1 for most mutants. However, the signal for E2 was lower for G278A, D279A, Q302A, and Y309A mutants compared to the wild type, which is in agreement with the defect in the heterodimerization of the envelope proteins observed in the CD81 pulldown assay and AR5A immunoprecipitation. In addition, this test further confirms a remaining interaction between E1 and E2 of the mutant L286A, which might be responsible for the attenuated infection observed for this mutant.

Whereas the defects in infectivity of most PFP and aa292-309 downstream region mutants can be attributed to impairment in virus assembly and envelope protein folding, the loss of infectivity of most mutants in the α2 helix and downstream region remains unexplained.

Effect of E1 mutations on HCV neutralization and inhibition by CD81. During their incorporation at the surface of viral particles, envelope glycoproteins undergo structural changes (Falson et al., 2015; Wahid et al., 2013). However, due to the low particle production yield of the HCV cell culture system, biochemical analyses of particle-associated envelope proteins are difficult to implement. Alternatively, the effect of the mutations on the folding of virus associated envelope proteins can be determined by the analysis of the sensitivity of the virus to neutralization with the help of conformational neutralizing antibodies or CD81-LEL. However, this approach is only possible for the characterization of attenuated viruses. Thus, neutralization assays were performed with mutants showing a decrease in infectivity of ≤ 1 log10 (L286A, E303A, M323A, W326A, P328A, and R339A) (Fig. 35).

The L286A and E303A mutants did not show any difference in sensitivity to inhibition by CD81-LEL and AR5A. This result contrasts with the effect of the L286A and E303A mutations on the heterodimer formation observed in biochemical interactions assays. Thus, although these mutations affect intracellular envelope protein heterodimerization, they have no major impact on E1E2 folding at the surfaces of the viral particles.

Conversely, α2-region mutations that had no impact on intracellular E1E2 recognition by AR5A or CD81-LEL led to an increase in virus sensitivity to inhibition by AR5A. Thus, the M323A, W326A, P328A, and R339A mutations likely induce a conformational change of virion-associated E1E2, leading to a better access of the AR5A epitope. To further confirm the results obtained with AR5A, we used AR4A MAb, which recognizes a discontinuous epitope on E1 and E2, in neutralization and immunoprecipitation assays (Giang et al., 2012). As shown on Fig. 35D, AR4A MAb could precipitate E1 and E2 from M323A, W326A, P328A, and R339A mutants with the same efficiency as wt E1E2. As found in AR5A-mediated neutralization, AR4A inhibited the infectivity of M323A, P328A, W326A, and R339A mutants with a higher efficiency (Fig. 35C). This result further supports a specific impact of these mutations on the virus-associated E1E2 conformation.

On another hand, the recognition of E1E2 of E303A mutant by AR4A in immunoprecipitation assays was slightly affected, while L286A mutation had no impact on E1E2 recognition (Fig. 35D). This confirms the effect of the E303A mutation on the

conformation of intracellular E1E2 glycoproteins, as shown by the results obtained in AR5A immunoprecipitation. As observed in AR5A neutralization experiments, E303A and L286A do not significantly affect the neutralization efficiency of AR4A (Fig. 35C). These findings support a specific impact of E303A mutation on intracellular forms of E1E2 glycoproteins.

Effect of E1 mutations on the recognition of HCV receptors. We further characterized the phenotypes of attenuated mutants by analyzing their dependence on the main known HCV receptors. For this, we determined their sensitivity to inhibition by anti-receptor MAbs, previously reported to affect HCV entry (Fig. 36).

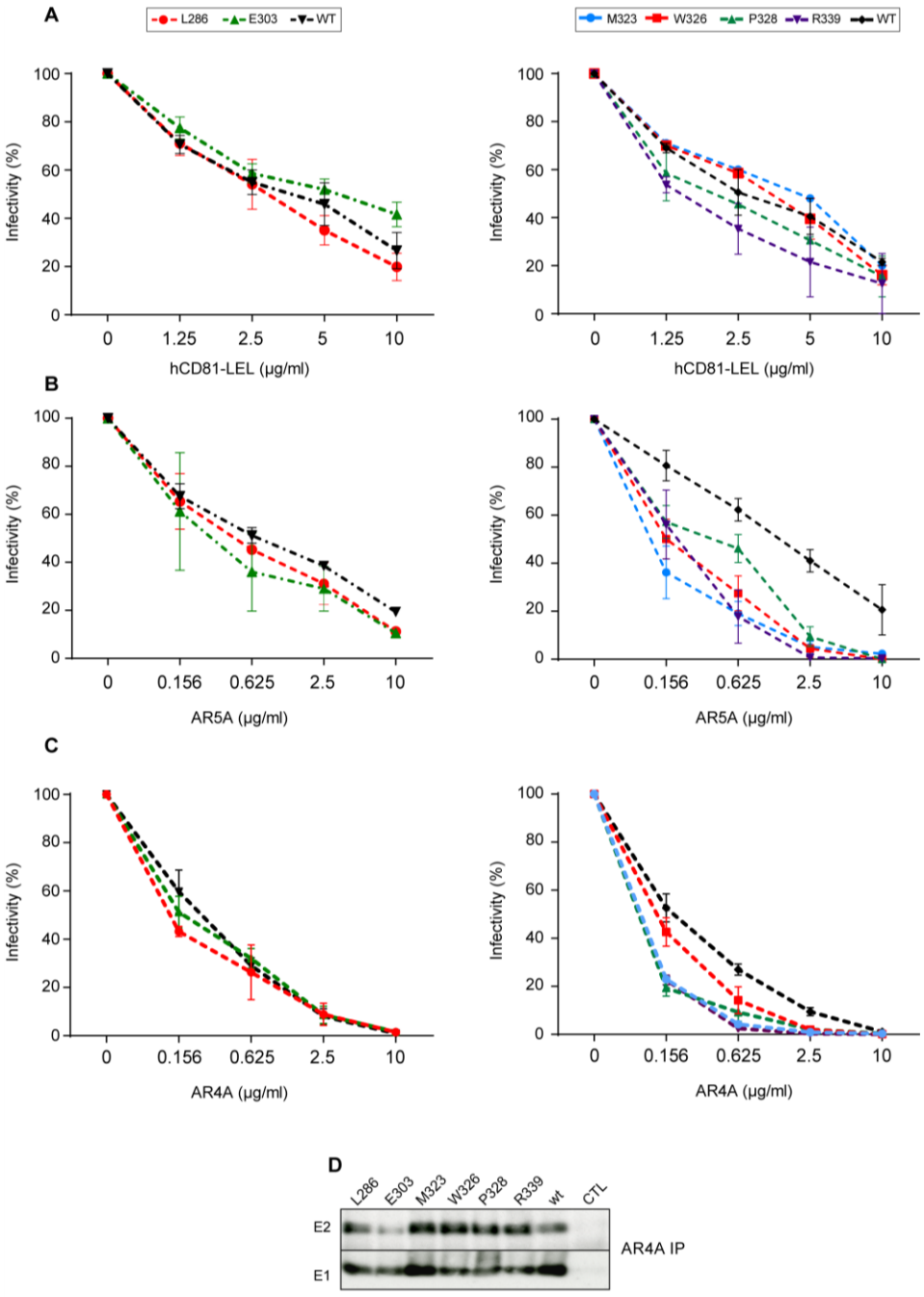

Figure 35 Effect of E1 mutations on E1E2 interaction with HCV neutralizing antibodies and CD81. CD81 inhibition assays (A) and AR5A (B) and AR4A (C) neutralization experiments were carried out by incubating E1 mutants or wild-type virus with increasing concentrations of human CD81-LEL, MAb AR5A, or MAb AR4A at 37°C for 2 h. The mixture was then added to naive Huh-7 cells that were plated 1 day before. At 72 h postinfection, infectivity was determined by immunofluorescence. The values are the combined data from three independent experiments. The error bars represent standard errors of the means. Results were compared to those of the wild type and a P value of < 0.05 was obtained for mutants M323A, W326A, P328A, and R339A in the AR5A and AR4A neutralization experiments. (D) Recognition of HCV E1 and E2 glycoproteins by conformation-sensitive anti-E1E2 MAb AR4A. At 48 h postelectroporation, E1 and E2 proteins from cell lysates were analyzed by immunoprecipitation with MAb AR4A. Immunoprecipitated proteins were revealed by Western blotting with MAbs A4 and 3/11.

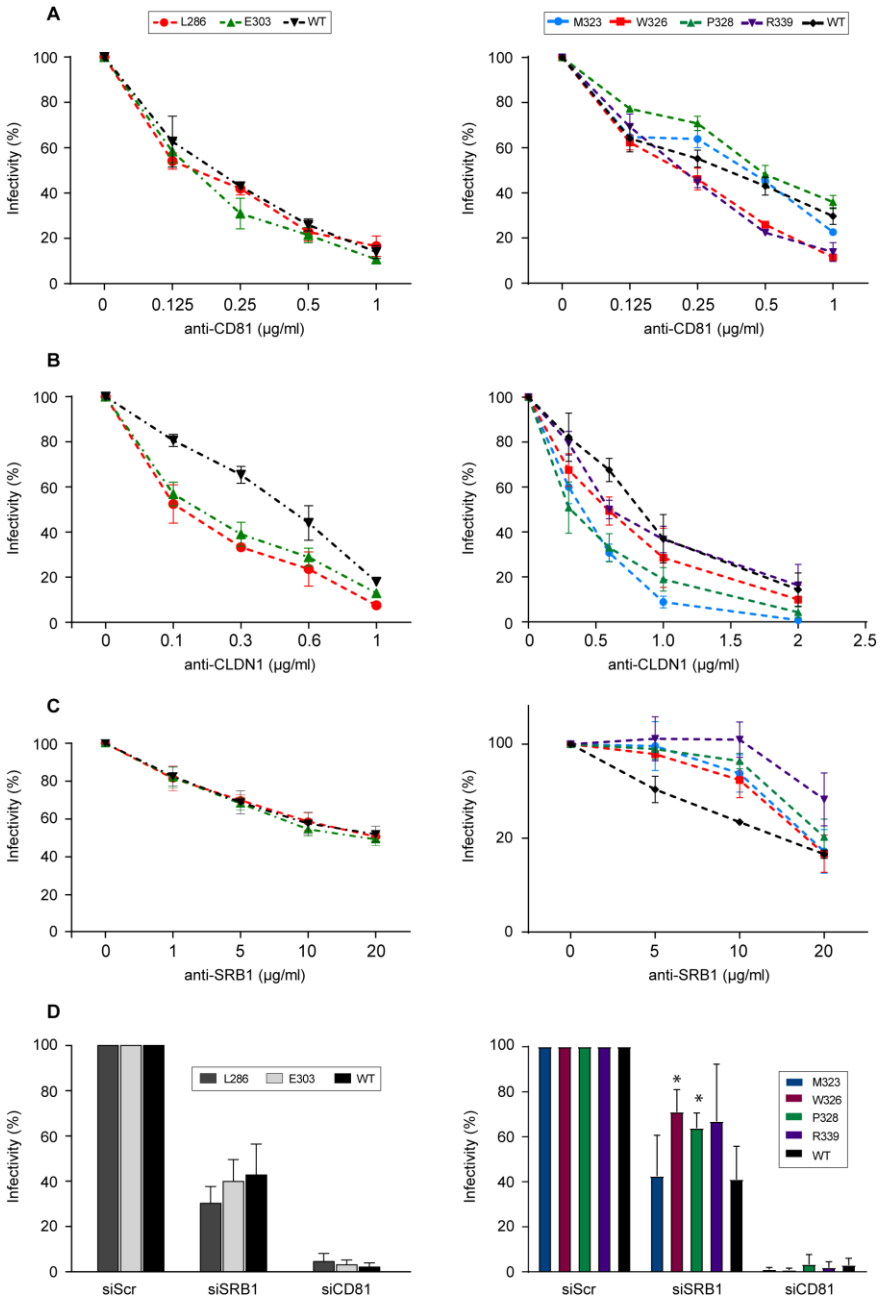

Figure 36 Effect of E1 mutations on the recognition of HCV receptors. Huh-7 cells were preincubated at 37°C for 2 h with increasing concentrations of antibodies targeting HCV receptors: anti-CD81 MAb JS81 (A), anti-CLDN1 MAb OM8A9-A3 (B), and anti-SR-BI MAb Cla-I (C). E1 mutants or wild-type virus were then inoculated onto the cells. At 72 h postinfection, the residual infectivity was determined by immunofluorescence. The values are the combined data from three independent experiments. The error bars represent standard errors of the means. Results were compared to those of the wild type. A P value of < 0.05 was determined for mutants L286A, E303A, M323A, and P328A in the presence of anti-CLDN1 MAbs and for mutants M323A, W326A, P328A, and R339A in the presence of anti-SR-BI MAbs. (D) SRB1 or CD81 expression was downregulated by siRNA targeting SRB1 or CD81 mRNA. Infectivity is expressed as the percentage of infection performed in the presence of the control siRNA. Mean values and standard deviations from three independent experiments are shown. The unpaired t test was used to compare the infectivities of the wild-type and mutant viruses. Differences were considered statistically significant if the P value was < 0.05.

No significant difference in sensitivity to inhibition by the anti-CD81 antibody was observed for the mutants. The absence of effect of the mutations on the dependence on CD81 for entry was confirmed by silencing the expression of CD81 with small interfering RNA (siRNA).

Interestingly, mutants in the α2 helix or downstream region (M323A, W326A, P328A, and R339A) were less sensitive to inhibition by the SR-BI-specific antibody, whereas the sensitivity to the SR-BI antibody was not affected for L286A and E303A mutants. Moreover, for the W326A and P328A mutants, infection was significantly less inhibited by the downregulation of SR-BI expression with siRNA. This suggests that residues M323, W326, P328, and R339 modulate HCV dependence on the SR-BI receptor. Similar phenotypes were obtained regarding CLDN1 dependence for mutants in the PFP and α2-helix regions. Indeed, L286A (PFP), E303A (PFP downstream region), M323A (α2 helix), and P328A (α2-helix downstream region) were more sensitive to inhibition by the anti-CLDN1 MAb than the wild type, suggesting that they are more dependent on CLDN1 for entry. However, the W326A and R339A mutants showed the same sensitivity as the wild-type virus to anti-CLDN1 inhibition, suggesting that only specific residues in the α2 helix and downstream region modulate the dependence of the virus on CLDN1.

Due to the absence of an anti-OCLN MAb capable of neutralizing HCV infection, the dependence on the OCLN receptor was tested using a knockout cell line (OKH4) (Shirasago et al., 2016). As found for the wild-type virus, all of the mutants failed to infect the cells, indicating that the mutations have no effect on the dependence on the OCLN receptor.

Characterization of SR-BI independent mutants. HCV associates with lipoproteins to form lipo-viro particles (Bartenschlager et al., 2011). Moreover, HCV-associated lipoproteins modulate HCV infectivity and play a role in virus interaction with SR-BI (Andréo Ursula et al., 2007; Chang et al., 2007; Maillard et al., 2006). Accordingly, E2 mutations that modulate HCV dependence on SR-BI have been associated with a shift in virion density (Bankwitz et al., 2010; Grove et al., 2008; Prentoe et al., 2011). In this context, we sought to determine whether E1 mutations that led to a decrease in SR-BI dependence were also associated with a change in viral particle density. For this, we analyzed the density of infectious viral particles obtained for the W326A and P328A mutants. After ultracentrifugation, the distribution of infectious particles in density gradients was determined by quantification of infectivity in the different fractions. As shown in Fig.37, no difference was observed between the distribution of infectious wild-type virus and mutant W326A and P328A viruses, which were mainly

concentrated in the 1.05 density fraction. Thus, W326A and P328A mutations that affect SR-BI dependence of the virus do not appear to affect virus association with lipoproteins.

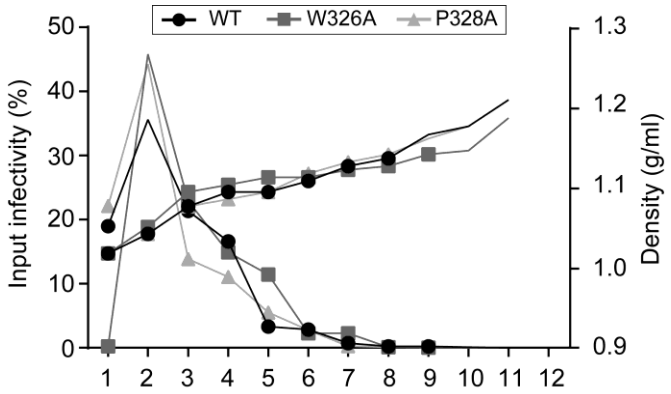

Figure 37 Density gradient analyses of SRBI independent mutants. Concentrated supernatants of cells electroporated with HCV RNA were separated by sedimentation through a 10 to 50% iodixanol gradient. Fractions were collected from the top and analyzed for their infectivity by titration and for their density.

Characterization of noninfectious, assembly-competent E1 mutants. In this study, we identified several mutants that either lost their infectivity (M318A, W320A, D321A, and M322A) or were severely attenuated (G311A, T314A, and G315A) but showed a level of core release similar to the wild-type virus. Thus, these mutations did not affect viral assembly and led to the secretion of noninfectious particles. Moreover, they had no impact on E1 folding or on E1E2 heterodimerization. Since specific mutations in E1 can induce the release of viral particles devoid of genomic RNA (Haddad et al., 2017), we sought to determine whether the impaired infectivity of these mutants was due to similar defects. We chose to quantify the RNA content of the particles released for the two severely attenuated mutants, G315A in the α2 helix and G311A in the upstream region, and the noninfectious mutant W320A in the α2 helix. After electroporation of viral RNA in Huh-7 cells, viral particles released in the supernatant were precipitated with polyethylene glycol, which allowed the removal of the free RNA present in the medium after electroporation. Following this step, viral RNA was extracted and quantified by quantitative reverse transcription-PCR. In parallel, intracellular viral RNA content was determined. As expected, lower levels of extracellular viral RNA were obtained for the nonreplicative GND mutant and the assembly-deficient ΔE1E2 mutant. On the opposite, viral RNA of G311A, G315A, and W320A mutants accumulated at similar levels as the wild type, both intra- and extracellularly (Fig. 38). These findings indicate that the loss of infectivity of these mutants was not due to a defect in RNA uptake in the viral particle.

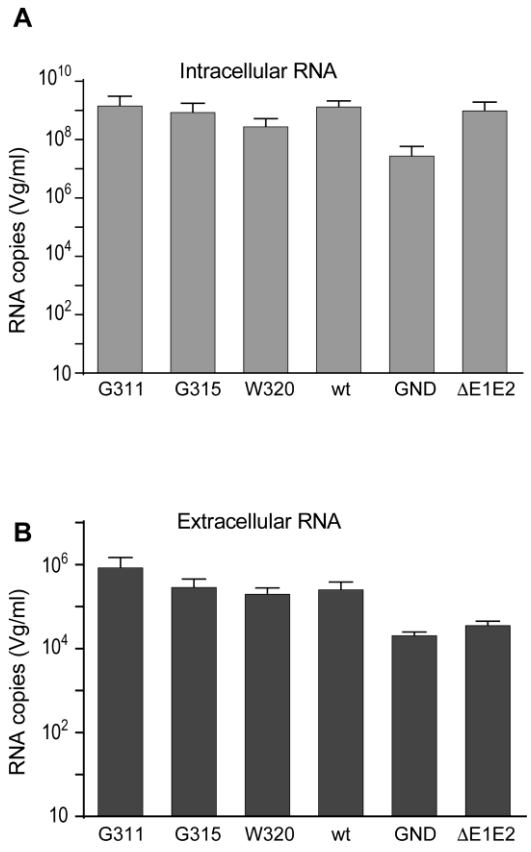

Figure 38 Effect of E1 mutations on viral RNA incorporation. Huh-7 cells were electroporated with mutants and wt RNA. (A) At 48 h postelectroporation, intracellular viral RNA was extracted and quantified by quantitative RT-PCR. In parallel, the viral particles were precipitated from the supernatant with polyethylene glycol and concentrated by ultracentrifugation. (B) Extracellular viral RNA contained in the concentrated virus was extracted and quantified by quantitative RT-PCR.

During morphogenesis, HCV glycoprotein E1 assembles to form noncovalent trimers which is essential for infectivity (Falson et al., 2015). Therefore, we determined the effect of the G311A, G315A, and W320A mutations on the capacity of E1 to form trimers in infected cells. Thus, after electroporation of viral RNA into Huh7 cells, envelope proteins from the lysate were concentrated by pulldown with *Galanthus nivalis* lectin and analyzed by Western blotting without thermal denaturation as previously described (Falson et al., 2015). Similar amounts of E1 trimers could be observed for the G311A, G315A, and W320A mutants than for the wt virus, indicating that the mutations do not alter E1 trimerization (Fig. 39).

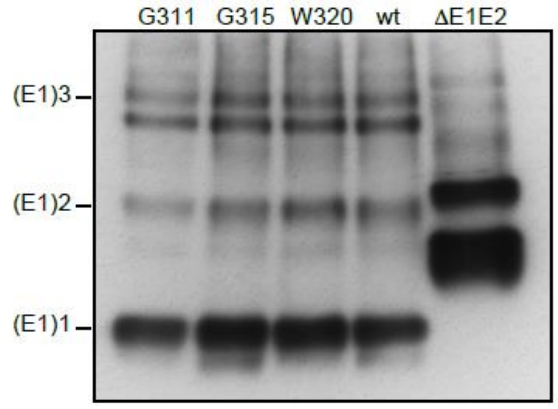

Figure 39 Effect of E1 mutations on E1 trimerization (not shown in the article). Huh-7 cells were electroporated with mutant and wt RNA and 48hpe cells were lysed in PBS 1% Triton X-100. HCV envelope glycoproteins were pulled down with GNA, treated with Laemmli sample buffer, heated for 5 min at 37°C, separated by SDS-PAGE and detected Western blot analyses with anti-E1 MAb A4. The oligomeric forms of E1 are indicated on the left.

Since they did not affect envelope protein folding, core secretion, or RNA encapsidation, the G311A, G315A, and W320A mutations lead to the release of noninfectious viral particles that may be deficient in viral entry. To confirm this hypothesis, we produced retroviral particles pseudotyped with HCV envelope proteins bearing the corresponding mutations in E1. As observed in Fig. 40, infectivity of HCVpp carrying E1 mutations was reduced by 2 log compared to HCVpp wild-type infectivity. This result could be due either to a defect in particle production or to the production of noninfectious particles. We thus assessed the efficiency of the incorporation of envelope proteins in HCVpp particles. To do so, extracellular particles were concentrated on a sucrose cushion by ultracentrifugation and analyzed by Western blotting. Although similar levels of retroviral capsid proteins were observed for all viruses, smaller amounts of E1 and E2 were detected for the G311A and G315A mutants, indicating that these mutations affect E1 and E2 incorporation in HCVpp viral particles. In contrast, the W320A mutation was associated with an increased incorporation of E1 and E2 in the particles, as shown by the detection of larger amounts of E1 and E2 in the concentrated particles samples. Thus, the loss of infectivity of G311A and G315A might be due to an impaired incorporation of the envelope proteins in the viral particles, while the absence of infectivity of the W320A mutant indicates an effect of this mutation on virus entry.

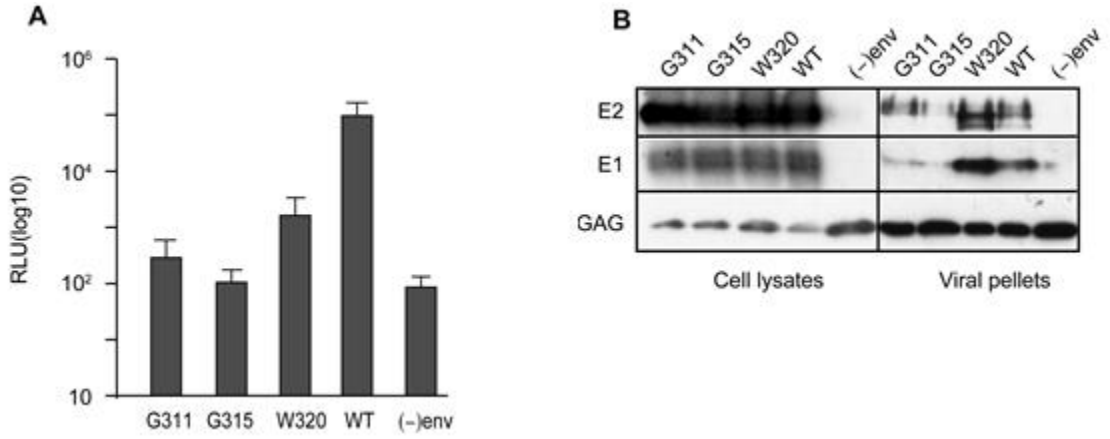

Figure 40 Effect of E1 mutations on HCVpp infectivity. Infectivity of HCVpp harboring E1 with G311A, G315A, or W320A mutation. (A) HCVpp infectivity was determined by measuring the activity of the luciferase reporter gene in infected Huh7 cells. Pseudotyped particles produced in the absence of envelope proteins were used as negative controls. The results are reported as means ± the standard deviations (error bars) of three independent experiments. A P value of <0.05 was obtained for mutants G311A, G315A, and W320A. (B) Effect of E1 mutations on the incorporation of envelope proteins in HCVpp particles. Cells producing HCVpp were lysed and analyzed by Western blotting. HCVpps contained in the supernatants of transfected 293T cells were concentrated on a 20% sucrose cushion by ultracentrifugation and analyzed by Western blotting. E1, E2, and capsid were detected using MAbs A6, 3/11, and CRL1912, respectively.

Table 4 Summary of the phenotypes of E1 mutants

E1 region	Mutant	Infectivity[a]	Core secretion[b]	E1E2 heterodimerization and folding[c]				Infection inhibition assays [d]					
				CD81 PD		IP AR5A	IP AR4A	hCD81 LEL	Anti-E1E2		Anti-CD81	Anti-CLDN1	Anti-SRBI
				E1	E2				AR5A	AR4A			
	Wild type	+++	++	++	++	++		++	++	++	++	++	++
PFP	G278	-	-	-	++	-	ND	ND	ND	ND	ND	ND	ND
PFP	D279	-	-	-	++	+	ND	ND	ND	ND	ND	ND	ND
PFP	G282	-	-	++	++	++	ND	ND	ND	ND	ND	ND	ND
PFP	L286	++	-	+	++	++		++	++		++	+++	++
PFP	Q289	+++	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
	Q302	-	-	-	++	-	ND	ND	ND	ND	ND	ND	ND
	E303	++	-	+	++	+		++	++		++	+++	++
	Y309	-	-	-	++	-	ND	ND	ND	ND	ND	ND	ND
	G311	+	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
	T314	+	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
α2	G315	+	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
α2	H316	+	++	+	++	+	ND	ND	ND	ND	ND	ND	ND
α2	R317	+++	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
α2	M318	-	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
α2	W320	-	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
α2	D321	-	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
α2	M322	-	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
α2	M323	++	++	++	++	++		++	+++		++	+++	-
	W326	++	++	++	++	++		++	+++		++	++	-
	P328	+++	++	++	++	++		++	+++		++	+++	-
	R339	++	++	++	++	++		+	+++		++	++	-
	P341	-	+	++	++	++	ND	ND	ND	ND	ND	ND	ND

[a]The infectivity of HCVcc harbouring the different E1E2 glycoproteins in the supernatant of electroporated Huh-7 cells was quantified 96h post electroporation (Fig. 3). +++: infectious titers higher than 10^4 ffu/mL; ++: infectious titers higher than 10^3 ffu/mL; +: infectious titers higher than 10^2 ffu/mL; -: titer between 0 and 50 ffu/mL.

[b]Secretion of core Ag in the supernatant quantified at 48 h post electroporation of Huh-7 cells (Fig. 4). ++, concentration greater than or equal to the wild type; +, concentration reduced by less than one log.; -, concentration reduced by one log. or more.

[c] The recognition of E1E2 proteins by AR4A and AR5A conformational antibodies and their interaction with hCD81 LEL were determined by precipitation experiments (Fig. 5). ++, similar amount of E1E2 precipitated to that of the wild type; +, lower amount of E1E2 precipitated; -, no E1E2 precipitated. ND, not determined.

[d] The sensitivity of the mutants to inhibition of infectivity by different antibodies or the hCD81 LEL was assessed (Fig. 6 and 7). ++, wild-type sensitivity to neutralization; +++, higher sensitivity to inhibition than the wild type; +, lower sensitivity to inhibition than the wild type; ND, not determined.

Colour code; white: wild type phenotype, green: defective in viral particle assembly/secretion, blue: slightly attenuated mutants, red: non-infectious/ assembly competent mutants

4 Discussion:

HCV E1 and E2 envelope proteins interact forming a heterodimer that constitutes a crucial functional subunit for virus entry and assembly (Bartosch et al., 2003a; Helle et al., 2007). For a long time E2 has been the better characterized and studied envelope glycoprotein, as it is the receptor binding protein and the major target of neutralizing antibodies. Until 2013, the difficulties to produce E1 and E2 in their native forms have hampered their structural characterization. Indeed, since E1 and E2 are highly glycosylated, contain many disulfide bonds and present interdependent folding, their overexpression in eukaryotic cells often led to the formation of large proportions of misfolded aggregates (Lavie et al., 2007). Also, conservation of genomic organization among members of the *Flaviviridae* family, led to the hypothesis that viruses of the *hepacivirus* and *pestivirus* genera resemble *flavivirus* genus in encoding type II fusion proteins (Garry and Dash, 2003). Moreover, HCV E2 was proposed to be the fusion protein (Krey et al., 2010). Nonetheless, this assumption has been disputed by the recent resolution of the tertiary structure of HCV E2 glycoprotein (A. G. Khan et al., 2014; Kong et al., 2013b) as well as E2 of *pestivirus* (El Omari et al., 2013; Yue Li et al., 2013). For both viruses, E2 tertiary structure did not share common features of class II fusion proteins, conversely to what was expected. Moreover, HCV E2 region that had been postulated to be responsible for fusion by (Krey et al., 2010) was found to be located in secondary structure elements within the core region of E2 which makes it unlikely to mediate fusion (A. G. Khan et al., 2014; Kong et al., 2013b). Additionally, E2 has not been shown to go through any oligomeric or conformational changes in response to low pH. These findings suggest that E2 might not play a direct role in the fusion step and that E1 alone or in association with E2 might be responsible for the fusion process. In alignment with this presumption, E1 has been shown to form trimers on the surface of viral particles (Falson et al., 2015), which is a common feature of fusion proteins, since class I, II and III envelope fusion proteins have been reported to have a trimeric post-fusion structure (Baquero et al., 2013). Furthermore, several regions in the E1 protein present fusion peptide peculiarities.

In this study, we investigated the functional role of two regions that might play an important role in the fusion process. The first region, spanning residues 272-291, corresponds to a highly conserved hydrophobic sequence that has been shown to interact with membranes and modify their biophysical properties. These findings would be in agreement with a role of this region during the fusion step (Pérez-Berná et al., 2009). As a consequence this segment was

proposed to constitute a putative fusion peptide (PFP) (Drummer et al., 2007; Garry and Dash, 2003; Pérez-Berná et al., 2009). The second region, spanning amino acids 314-342 is a membranotropic region located in the C terminal part of E1 ectodomain upstream the transmembrane domain. X-ray crystallography and NMR-studies revealed that this region contains two α-helices (α2 and α3) (Kong et al., 2015; Spadaccini et al., 2010).

The role of these two regions in the HCV life cycle has been characterized by point mutagenesis in the context of JFH1 infectious clone. Thus, the most conserved residues of these regions were replaced by alanine. Our data showed that 20 out of 22 mutations within these regions affected viral infectivity, demonstrating the importance of these residues in the viral life cycle. In brief, we showed that most residues in the PFP and its downstream region are involved in E1E2 heterodimerization, assembly and secretion of HCV infectious virions. Moreover, several residues in the PFP and α2 regions were found to modulate virus binding to CLDN1 and/ or SRBI receptors. Importantly, most mutants in the α2 helix caused an impairment of viral infectivity without affecting E1E2 folding and interaction or virion assembly, suggesting that these mutations resulted in the production of noninfectious or attenuated virions, most probably deficient in viral entry.

The majority of the mutations introduced in the PFP and its downstream region (G278A/ D279A/ L286A/ Q302A/ E303A and Y309A) affected the interaction between the glycoproteins E1 and E2. These findings were in alignment with the computational prediction of E1E2 heterodimer structure by Freedman et al (Freedman et al., 2017) confirming the involvement of the E1 region aa 290-360 in E1E2 interaction. These data are also in accordance with several studies showing that regions of E1 and E2 ectodomains participates in the interaction, in addition to their TMD's, which have been shown to be essential for the heterodimerization. In fact, residues 201-206 located in the E1 N-terminus have been found to be crucial for the epitope structure of the conformational antibodies AR4A and AR5A, which span both glycoproteins (Giang et al., 2012). Additionally, residues W239, I262, and D263 also located in E1 N-terminus, have been shown to be involved in E1E2 interaction (Haddad et al., 2017). Moreover, a recent flow-cytometry study probed the binding of alanine mutants covering the full sequence of E1E2 of H77 strain of genotype 1a to multiple antibodies (Gopal et al., 2017). The data obtained were in agreement with our findings showing a reduction in the recognition of E1 mutants G278A, D279A, Q302A, and D303A by AR5A, while mutants G282A, L286A, Q289A, and G311A retained their binding capacity. Also, a study characterizing the function of chimeric heterodimers of different

phenotypes reported the importance of residues 308, 330, and 345 for E1E2 functional interaction (Douam et al., 2014). Nevertheless, G282A was the only mutation among mutations in the PFP region that impaired infectivity without affecting the interaction between E1 and E2. However, further analyses of this mutant showed a deficiency in virion assembly, which could be the reason for the loss of infectivity. Of note, this residue was described in two other studies once in the context of an HCVpp model and in a transcomplementation system of HCV lacking E1 coding sequence (Lavillette et al., 2007; Tong et al., 2017). In that regard, G282A mutation only led to a decrease in infectivity and not a complete loss of infectivity as observed in the HCVcc system. Moreover, in the transcomplementation system this mutation resulted in the secretion of lower levels of extracellular RNA supporting the hypothesis that loss of infectivity was due to a defect in virion assembly.

In addition to the involvement of the PFP region in E1E2 heterodimerization, our data revealed the importance of this region for viral assembly. However, this does not rule out the involvement of the PFP region in the fusion process. As a matter of fact, it has been shown for different viruses that mutations in the fusion peptide might impact different steps of the viral life cycle in addition to the entry step. This has been shown for the Semliki Forest virus, as its fusion peptide affects the interaction between its envelope proteins as well as virion assembly (Duffus et al., 1995; Gibbons et al., 2004). Likewise, mutating a glycine residue in the fusion peptide of the influenza virus resulted in different phenotypes according to the substituted residue (Qiao et al., 1999). Taking into consideration that mutations in the PFP region impact several steps of the viral life cycle, makes the identification of its role in the entry process difficult.

Interestingly, we also showed in this study that some mutants changed their affinity to certain HCV specific receptors. Attenuated mutants L286A and E303A in the PFP and its downstream region as well as M323A and P328A mutants in the α2 helix presented higher sensitivity to inhibition of infectivity by CLDN1-specific Abs than the wild type indicating that they are more dependent on CLDN1 for entry. These findings are in line with previous studies suggesting a potential role for E1 in the interaction between HCV and CLDN1 (Haddad et al., 2017; Hopcraft and Evans, 2015). Although, the so far reported mutations (T213A, I262A and H316A) exhibited an opposite effect on CLDN1/HCV interplay by decreasing the dependence on CLDN1 and increasing the dependence on CLDN6 for cell entry. Consequently, different regions of E1 might have opposite effects on the CLDN1/HCV

interaction. Also these data reinforce the hypothesis that E1 is involved in interactions with CLDNs. Although no direct interaction has been shown until now between E1 and CLDN1, E1 might modulate E2 binding to CLDN1 in a similar way as it does for E2-CD81 interaction (Wahid et al., 2013).

Surprisingly, certain mutations in α2 helix and downstream region (M323A, W326A, P328A, and R339A) exhibited partial resistance to inhibition of infectivity by SRBI specific antibody or siRNA downregulation of SR-BI expression. It thus appears, that residues M323, W326, P328 and R339 play a role in modulating HCV dependence on SRBI receptor. It's noteworthy, that certain mutations in E2 such as G451R, V514A as well as the HVR1 deletion have been reported to reduce the viral dependence on SRBI (Bankwitz et al., 2010; Grove et al., 2008; Lavie et al., 2014b). Interestingly, these mutants also showed increased affinity for CD81 at the same time. While M323A, W326A and P328A mutations did not significantly affect the virus dependence on CD81, some mutants (M323A and P328A) showed increased affinity for CLDN1. Thus, it seems that a change in the affinity of the glycoproteins for one receptor impacts their interaction with other receptors indicating a balanced interplay between HCV envelope proteins and viral receptors. These data are in line with an interdependent involvement of SRBI, CD81, CLDN1 and OCLN in HCV entry process (reviewed in (Douam et al., 2014)).

In agreement with the functional interaction of SRBI with HCV associated lipoproteins, different E2 mutations that caused a decrease in SRBI dependence, were associated with an increase in viral particle density (Grove et al., 2008). The observed shift in particle density is likely due to a change in the lipid composition of HCV particles, which may explain the reason behind the lower dependence on SRBI receptors for entry. In a similar way, cysteine mutants of E1 (C207A and C272) had an impact on the density of viral particles, suggesting that the E1E2 heterodimer affected the interplay between the virus and lipoproteins (Wahid et al., 2013). Furthermore, E1 and E2 have been reported to interact with ApoE, whose association with the viral particle is essential for infectivity (Boyer et al., 2014; Lee et al., 2014; Mazumdar et al., 2011). However, contrary to what was expected, our mutations W326A and P328A did not alter the density of viral particles, suggesting that other factors are involved in virus interaction with SRBI or E1 mutations might have an impact on E2 interaction with SRBI as previously shown for E2-CD81 interplay (Wahid et al., 2013).

Interestingly α2-helix region attenuated mutants M323A, W326A, and P328A modulated the dependence of the virus on CLDN1 and SRBI receptors, but had no effect on E1E2 heterodimer formation nor its conformation, which was in line with a recent study characterizing the binding capacity of HCV E1- and E2 -specific antibodies to an E1E2 mutants library (Gopal et al., 2017). These findings indicate that the α2-helix region of E1 plays an important role in the interplay of HCV with CLDN1 and SRBI during viral entry.

Interestingly, a functional study characterizing IGH526 epitope (Kong et al., 2015) reported a drastic effect on genotype 1a HCVpp infectivity of the mutations G315A, M318A, D321A, and M322A as observed in the HCVcc system. Nevertheless, introducing W320A mutation into genotype 1a only resulted in a 30% decrease of infectivity (Kong et al. 2015), while in the present study it had a more severe effect on the infectivity of HCVcc and HCVpp of genotype 2a. This discrepancy might be due to differences in glycoproteins sequences between both genotypes impacting their functionality.

In our study, the most intriguing phenotypes were observed for most mutants of the α2-helix region of E1 that were either severely attenuated or non-infectious but did not exhibit any deficiency in E1E2 heterodimerization nor viral particle assembly. These findings imply that these specific mutations result in the generation of noninfectious HCV particles. Further characterization of G311A, G315A and W320A mutants for their capacity to incorporate viral genomic RNA into particles or to form trimers of E1, revealed no defects. The fact, that these specific mutations severely affected infectivity, with no effect on E1E2 folding, E1 trimerization, virus assembly nor RNA encapsidation, strongly suggested that HCV entry step was impaired.

Being hampered by the difficulty of producing noninfectious HCVcc particles in large amounts, we introduced our mutations in the HCVpp system, which confirmed the impairment of infectivity for those mutants. Interestingly, for mutants G311A and G315A, we observed a deficiency of E1E2 incorporation into viral particles that might explain the loss of infectivity of these mutants. On the contrary, mutation W320A was not associated with defect in envelope proteins incorporation into HCVpp. In fact, this mutation resulted in loss of infectivity in both HCVcc and HCVpp systems while no deficiency in viral particle assembly, heterodimerization, RNA encapsidation or E1 trimerization could be observed. These data indicate an effect of this mutation on the entry step of the virus. Unfortunately,

further attempts to identify the affected step in the entry process were hampered by the low production yield of viral particles.

Furthermore, based on the fact that the α2-helix region is located close to the E1 transmembrane domain, our findings are in agreement with accumulated data suggesting a role of the region at the junction between fusion protein ectodomain and TM anchor, termed (pre-TM), in the fusion step (Peisajovich and Shai, 2003). Moreover, the hydrophobicity of the α2-helix region supports the hypothesis of its involvement in membrane destabilization (Pérez-Berná et al., 2008). Indeed, many fusion proteins present additional hydrophobic segments in membrane proximal regions. Pre-TMs are often characterized by an unusual clustering of aromatic residues, which is in line with their involvement in the fusion process. Their localization at membrane interfaces make them likely to cooperate with the fusion peptide and TM domain during membrane apposition to mediate membrane distortion required for fusion. Thus, in addition to the fusion peptide, which is responsible for initiating the fusion process, other membranotropic segments in the fusion protein are essential for driving and completing the process (Sáez-Cirión et al., 2003; Suárez et al., 2000). There have been accumulated evidence that pre-TM regions of several fusion proteins play an essential role in membrane fusion reviewed in (Apellániz et al., 2014; Falanga et al., 2018). This has been reported for pre-TM of HSV-1 glycoprotein H that strongly interacts with membranes (Galdiero et al., 2007); pre-TM of foamy virus Gp47 that presents fusogenic activity (Wang et al., 2016) and the pre-TM of Ebola GP2 that perturbs membranes when in a helical structure (Regula et al., 2013).

Eventually, our findings support the hypothesis that α2-helix region has either a direct or indirect role in the fusion step during viral entry as previously suggested due to its membranotropic nature (Spadaccini et al., 2010).

To conclude, our study allowed further characterizing the role of E1 in HCV entry and assembly. Thus, our data showed the involvement of the PFP region in E1E2 heterodimerization and viral particle assembly. Although an involvement of this region in fusion is not excluded, its multifunctionality hampered further characterization of the role of this region in the entry process. Our work could further confirm a role for E1 in modulating HCV interaction with its coreceptors. Furthermore, our data suggest that α2-helix region of E1 is involved in a late step of HCV entry, potentially fusion. Altogether, all these results are in accordance with the fact that fusion of membranes is a complex process that includes the

contribution of several membranotropic peptides. While the fusion peptide initiates the fusion step, other membranotropic segments in the fusion protein interact either directly or indirectly with membranes contributing to membrane merging of viral and host cell membranes (Apellániz et al., 2014; Peisajovich and Shai, 2003).

Finally, our work together with recent functional characterization studies reveal that E1 is involved in E1E2 assembly, virus morphogenesis, interplay with HCV receptors and potentially fusion step and therefore plays a more important role in HCV life cycle than previously thought.

Perspectives:

The HCV entry and assembly steps are two sophisticated processes in the HCV life cycle. The envelope glycoproteins E1 and E2 play crucial roles in these two steps through their involvement in the interaction with lipoproteins as well as with multiple host cell receptors and cofactors. Although partial 3D structures have started to shed some light on these envelope glycoproteins in recent years, we are still far from understanding the dynamics of the fusion and assembly processes in HCV. Indeed, the current structures available indicate that the fusion mechanism of HCV remains elusive while differing from that of flaviviruses. Indeed, HCV envelope glycoproteins seem to belong to another class of fusion proteins. Accumulated data led to rule out a central role of E2 in the fusion process, being more in favor of a crucial involvement of E1. Hence, the development of a robust membrane fusion assay is urgently required for a better characterization of this process. Moreover, HCV fusion process might rely on a strong interplay between E1 and E2. Indeed, E1 and E2 interdependency for their folding and the completion of entry and assembly steps, suggests that they work as a functional complex rather than two independent subunits with specific functions, as generally observed for other fusion proteins. Thus, resolving the structure of HCV E1E2 complex is strongly required for a better understanding of E1 and E2 cross-talks, which will pave the way for unraveling the elusive mechanisms of the HCV entry and assembly.

5 Materials and Methods:

In this study we examined the role of the C-terminal part of the E1 ectodomain in the HCV viral life cycle, especially the putative fusion peptide region as well as the pre-transmembrane proximal region containing the α2- and α3 helices. We performed an alanine scanning by replacing the conserved residues in these regions by alanine. All characterization methods and experiments are described in the article. However, a more detailed description is illustrated hereunder for some experiments that are only briefly outlined in the article.

5.1 Cloning of viral mutants

The virus used in the present study is a modified version of the JFH1 isolate (genotype 2a; GenBank accession number AB237837) (Wakita et al., 2005), kindly provided by T. Wakita (National Institute of Infectious Diseases, Tokyo, Japan). It was engineered to reconstitute the A4 epitope in E1 (pJFH1-CS-A4) (Goueslain et al., 2010) and titer-enhancing mutations have been introduced (Delgrange et al., 2007). Site-directed mutagenesis was used for generating all the JFH1 mutants by replacing selected conserved residues by alanine. To that end, the plasmid pJFH1-CS-A4 was utilized as a template to perform polymerase chain reactions (PCR). The inserts containing the mutations were generated by the fusion PCR method that relies on three polymerase chain reactions (shown in figure 41). The first PCR was done using the outer sense primers containing the alanine mutation and the antisense primers ending by the restriction site BsiWI within the sequence of the E1 gene. This PCR generated segment serves as the DNA segment downstream the mutation. The second PCR was realized by using sense primers starting with the restriction site EcoRI and antisense primers containing the mutation for the production of the DNA fragment upstream the mutation. Importantly, the antisense and sense primers carrying the mutations contain complementary sequences. Finally, the third PCR was performed to fuse the two PCR generated DNA fragments using the sense primers containing the EcoRI and the antisense primers containing the BswI restriction sites. The obtained fusion EcoRI/ BsiWI PCR fragment was digested by the restriction enzymes EcoRI and BsiWI and was purified by migration on agarose gel. Subsequently, the mutated DNA fragment was ligated with the vector pJFH1-CS-A4, which had been previously digested by the restriction enzymes EcoRI/ BsiWI. After ligation, the constructs were transformed into competent *E.coli* bacteria to amplify the plasmid DNA.

Colonies were screened by PCR on colonies. The plasmids from positive screened colonies were extracted and sequenced to verify that the insert contained only the desired mutation.

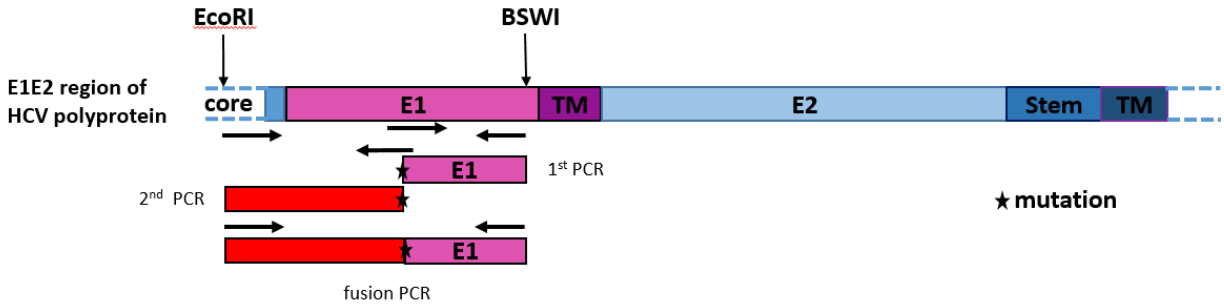

Figure 41 generation of mutated DNA fragment

5.2 RNA interference experiments

For siRNA transfection, 3 µl of lipofectamine RNAi MAX (Life Technologies) were added to 0.5 ml of 1X PBS and incubated for 3 min. In a 6-well plate, 2.5 µl of siRNA at 20 µM either targeting CD81 or SRBI gene was spotted in the center of the wells. Then, the diluted transfection reagent was added to the siRNA, and the mixture was incubated for 30 min at room temperature. Subsequently, $2.5x10^5$ freshly trypsinized Huh7 cells in a volume of 2 ml of DMEM 10% FBS were added to the transfection mix and plates were incubated at 37°C. Cells were trypsinized 48 h later and plated in a 96-well plate, and infected the following day. Infected cells grown in 96-well plates were fixed 30 hours post-infection with ice-cold methanol and then analyzed by immunofluorescence using the E1 specific A4 mAb.

5.3 Viral RNA quantification and virus precipitation by polyethylene glycol (PEG)

Around 4 x 10^6 of Huh7 cells were electroporated with 10 µg RNA of wt or mutants. After 6h of electroporation, supernatants were aspirated and cells were washed twice with sterile 1X PBS and then incubated by DMEM 10%FBS (an essential step to eliminate all the RNA that was not incorporated into the cells during electroporation). At 48h post-electroporation, the efficiency of electroporation was evaluated by immunofluorescence and intracellular RNA was extracted using RNA extraction kit (NucleoSpin RNA, Ref. 740955.250, Macherey-

Nagel). In parallel, supernatants were collected and extracellular virus was precipitated by the addition of PEG 6000 at a final concentration of 8%. The mixture was incubated on ice for one hour with shaking followed by overnight incubation at 4°C. Subsequently, the solution was centrifuged at 8000 rpm for 25 min and the pellet was resuspended in 10 ml of the supernatant and recentrifuged at 8000 rpm for 20 min. Finally, the supernatant was discarded and the pellet resuspended in 11 ml of cold 1X PBS. The virus was then pelleted by ultracentrifugation at 27000 rpm at 4°C for 4 h (SW41 rotor). The supernatant was removed carefully and the pellet was resuspended in 140 µl of complete medium. The viral RNA was isolated using the kit QIAamp Viral RNA mini Kit (Cat. No.52906, QIAGEN) and intra- and extracellular RNA were quantified via RTqPCR as previously described.

Annex:

Functional study of the C-terminal part of hepatitis C virus E1 ectodomain

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Keywords: hepatitis C virus; glycoprotein; envelope proteins; viral entry; viral assembly

Running title: HCV glycoprotein E1 in virus entry

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Performed the experiments: RIM, JGH, LL, XH, VD, AAM, ML

Provided reagents: AAM, TFB, PM

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Wrote the paper: RIM, JGH, JD, ML

Abstract

In the HCV envelope glycoproteins E1 and E2, which form a heterodimer, E2 is the receptor binding protein and the major target of neutralizing antibodies, whereas the function of E1 remains less characterized. To investigate E1 functions, we generated a series of mutants in the conserved residues of the C-terminal region of the E1 ectodomain in the context of an infectious clone. We focused our analyses on two regions of interest. The first region is located in the middle of the E1 glycoprotein (between amino acids (aa) 270 and 291), which contains a conserved hydrophobic sequence and was proposed to constitute a putative fusion peptide. The second series of mutants was generated in the aa314-342 region, which has been shown to contain two alpha helices (α2 and α3) by NMR studies. Twenty out of the twenty-two generated mutants were either attenuated or noninfectious. Several mutations modulated the virus's dependence on claudin-1 and the scavenger receptor BI co-receptors for entry. Most of the mutations in the putative fusion peptide region affected virus assembly. Conversely, mutations in the α-helix 315-324 residues M318, W320, D321, and M322 resulted in a complete loss of infectivity without any impact on E1E2 folding and on viral assembly. Further characterization of the W320A mutant in the HCVpp model indicated that the loss of infectivity was due to a defect in viral entry. Together, these results support a role for E1 in modulating HCV interaction with its co-receptors and in HCV assembly. They also highlight the involvement of α-helix 315-324 in a late step of HCV entry.

Importance:

HCV is a major public health problem worldwide. The virion harbors two envelope proteins, E1 and E2, which are involved at different steps of the viral life cycle. Whereas E2 has been extensively characterized, the function of E1 remains poorly defined. Here we characterized the function of the putative fusion peptide and the region containing alpha helices of the E1 ectodomain, which had been previously suggested to be important for virus entry. We could confirm the importance of these regions for the virus infectivity. Interestingly, we found several residues modulating the virus's dependence on several HCV receptors, thus highlighting the role of E1 in the interaction of the virus with cellular receptors. Whereas mutations in the putative fusion peptide affected HCV infectivity and morphogenesis, several mutations in the α2 helix region led to a loss of infectivity with no effect on assembly, indicating a role of this region in virus entry.

Introduction

With 70 million people infected worldwide, Hepatitis C virus infection is a major health problem (1). With a high propensity for establishing chronic infections, HCV is considered as the major cause of chronic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma. The development of direct-acting antivirals (DAA) against HCV has been a milestone in the treatment of Hepatitis C. This treatment shows high efficacy against all HCV genotypes, reaching high HCV clearance rates. However, the high cost of these antiviral therapies precludes their accessibility to the large majority of HCV infected patients (2). In this context, the development of a preventive HCV vaccine would constitute the most cost-effective means to limit HCV spread. Therefore, a thorough understanding of the contribution of HCV glycoproteins E1 and E2 to viral entry and assembly is still required for the development of therapeutic and preventive vaccines.

HCV is an enveloped virus with a positive stranded RNA genome that belongs to the *Hepacivirus* genus of the *Flaviviridae* family (3). Its genome encodes a single polyprotein that is processed by cellular and viral proteases into 10 polypeptides, which include seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) and three structural proteins (the core and the two envelope glycoproteins E1 and E2) that are the components of the viral particle (4). Thus, the viral particle is composed of a nucleocapsid constituted of the genomic RNA and the core protein, which is surrounded by a lipid membrane in which the heterodimer formed by the two envelope glycoproteins E1 and E2 is anchored. E1 and E2 glycoproteins are type I transmembrane proteins with well conserved C-terminal transmembrane domains and highly glycosylated N-terminal ectodomains (5). As components of the viral particle, E1 and E2 are involved in virion morphogenesis and constitute the major viral determinant of HCV entry (6) ((7). HCV entry into hepatocytes is a complex process that involves several cell surface molecules. Among them, the contributions to HCV entry of the scavenger receptor BI (SR-BI), the tetraspanin CD81, and the tight-junction proteins claudin-1 (CLDN1) and occludin (OCLN) have been the most characterized (8).

For a long time, E2 was the most studied HCV envelope protein. Indeed, E2 is the major target of neutralizing antibodies, it mediates the interaction between the virus and SR-BI and CD81 receptors, and it was postulated to be the fusion protein of the virus (9). However, the structure of E2 does not harbor the features of a fusion protein, which led to the hypothesis that the fusion step would rather rely on E1 (10) (11). Recently, the N-terminal part of E1 (residues 192 to 270) was crystallized. The characterization of the role of the conserved

residues of this region during the HCV life cycle showed that it is important for the virus infectivity and E1E2 heterodimer formation (12). Moreover, this region was shown to contain residues that mediate the dependence of the virus on the claudin-1 receptor for entry. In addition, a cross-talk between HCV glycoprotein E1 and the viral genomic RNA was identified (12).

To further characterize the contribution of E1 to the different steps of the HCV life cycle, we generated a new series of mutants in the conserved residues of the C-terminal region of the E1 ectodomain. We focused our analysis on two regions of interest. The first region is located in the middle of the polypeptide (Putative Fusion Peptide (PFP), between aa270 and 291), which contains conserved hydrophobic sequences that could potentially act as a fusion peptide (13, 14). The second series of mutants was generated in the aa314-342 region, which has been shown to contain two alpha helices (α2 and α3) by NMR and X-ray crystallography studies (15) (16). We thus took advantage of these data to further investigate the functional role of the C-terminal part of the E1 ectodomain by alanine replacement of residues in the context of an infectious clone. Out of the 22 generated mutants, only two exhibited a wild-type phenotype, while nine were no longer infectious. Several mutations modulated the dependence of the virus on claudin-1 and/or SR-BI receptors for entry. Importantly, mutations in the α2 residues M318, W320, D321, and M322 resulted in a complete loss of infectivity without any impact on E1E2 folding or on viral assembly. Further characterization of the W320A mutant in the context of the HCVpp system indicated that the loss of infectivity is due to a defect in viral entry.

Results

Amino acid conservation in the second half of the E1 ectodomain and mutated residues. Amino acid conservation in E1 region from aa 270 to 350 among HCV genotypes is represented in Fig. 1. This region contains the putative fusion peptide aa270-291 (PFP) (13) and two α helices, α2 (aa315-324) and α3 (aa331-338), as revealed by NMR and X-ray crystallography studies performed on the aa314-342 and aa314-324 E1 peptides (15). The less-variable residues of this region among HCV genotypes were individually replaced by alanine in the context of the JFH1 infectious clone. This led to the generation of 22 mutants. Among them, 5 belong to the PFP region, whereas 8 are located in the α2-helix region.

Unexpectedly, the residues of the α3 helix were more variable, which suggested a less important role for this region in the HCV life cycle. Mutations were introduced in a modified version of the plasmid encoding the fulllength JFH1 genome in which the N-terminal E1 sequence has been modified to reconstitute the A4 epitope, which is present in E1 of genotype 1a (17), and therefore allows for the identification of this modified E1 of genotype 2a for which there is no antibody readily available. It is worth noting that introduction of the A4 epitope does not affect HCV infectivity and thus does not interfere with the characterization of the phenotypes of E1 mutants.

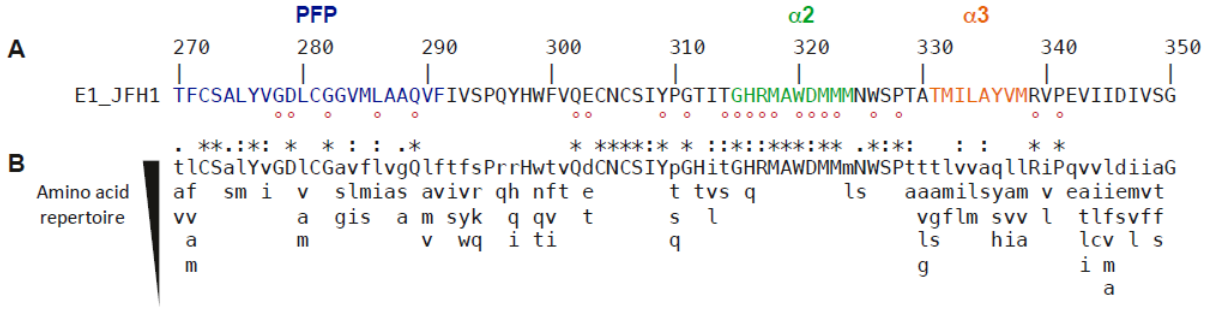

Figure 1 E1 C-terminal region sequence analyses. (A) The E1 aa270-350 sequence from the HCV JFH1 strain (AB047639; genotype 2a) is indicated with respect to the polyprotein numbering. Amino acids mutated in this study are indicated by a red dot. (B) Amino acid repertoires of the C-terminal region of E1. The amino acid (aa) repertoire was deduced from the ClustalW multiple alignment of the 28 representative E1 sequences from confirmed genotypes and subtypes in the European HCV database (https://euhcvdb.ibcp.fr/euHCVdb/jsp/nomen_tab1.jsp). Amino acids observed at a given position in fewer than two distinct sequences were not included. Amino acids observed at a given position in more than 25 distinct sequences are shown in capital letters. The degree of amino acid conservation at each position can be inferred from the extent of variability (with the observed amino acids listed in decreasing order of frequency from top to bottom), together with the similarity index according to ClustalW convention (asterisk [*], invariant; colon [:], highly similar; dot [.], similar).

Effect of E1 mutations on HCV replication. In a first step, we assessed the ability of the produced mutants to replicate. For this purpose, the expression of several HCV proteins (E1, E2, and NS5A) was examined at 48 h postelectroporation of Huh-7 hepatoma cells with wt and mutant HCV RNA. For all mutants, similar levels of protein expression could be observ

ed; hence, any effect of the mutations on viral replication could be excluded (Fig. 2). We included in our analysis the GND nonreplicative HCV mutant and the ΔE1E2 assembly-deficient mutant that carries an in-frame deletion in E1E2 coding region. Interestingly, D279 and Q289 mutations in the PFP and Q302 in the region between PFP and α2 led to the detection of an additional band of lower molecular weight, which likely corresponds to an

alternative glycoform of E1 as previously observed when E1 is expressed as a recombinant protein (18).

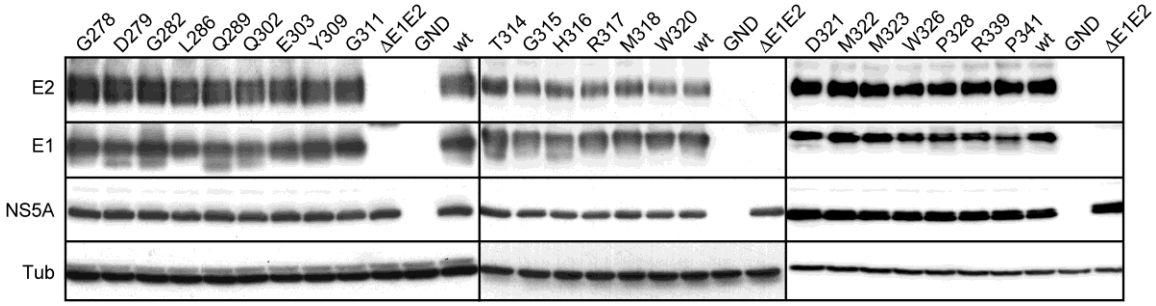

Figure 2. Effect of E1 mutations on the expression of viral proteins. Viral RNA transcribed from JFH1-derived mutants was electroporated into Huh-7 cells that were lysed 48 h later. Viral proteins were separated by SDS-PAGE and revealed by Western blotting with MAbs A4 (anti-E1), 3/11 (anti-E2), and anti-NS5A, as well as anti-beta-tubulin antibody, to verify loading of equal amounts of cell lysates. The protein detected by MAb A4 in cells expressing ΔE1E2 mutant corresponds to a fusion protein between the N terminus of E1 and the C terminus of E2.

Effect of E1 mutations on HCV infectivity. Since the introduced mutations did not affect viral replication, we assessed their impact on the production of infectious virus. To do so, we determined the intracellular and extracellular infectivity after electroporation of Huh-7 cells with viral RNAs. We observed different phenotypes of virus infectivity: (i) complete loss of infectivity for mutants G278A, D279A, G282A, Q302A, Y309A, M318A, W320A, D321A, and M322A; (ii) severe attenuation of infectivity for mutants G311A, T314A, G315A, H316A, R339A, and P341A; (iii) slight attenuation of infectivity for mutants L286A, E303A, M323A, W326A, and P328A; and (iv) no effect on infectivity for mutants Q289A and R317A. In most cases, intra- and extracellular infectivity profiles were similar, suggesting that the mutations did not affect infectious virus release. However, the P341A mutant showed a 3-log decrease in its extracellular infectivity level compared to wild-type infectivity, while its intracellular infectivity was reduced by only 1 log at 96 h postelectroporation. This result is in favor of an effect of this mutation on the secretion of infectious virus. These initial data show that most mutations in the putative fusion peptide and α2-helix regions result in a loss of infectivity or a severe attenuation, suggesting that these regions are important for the HCV life cycle (Fig. 3).

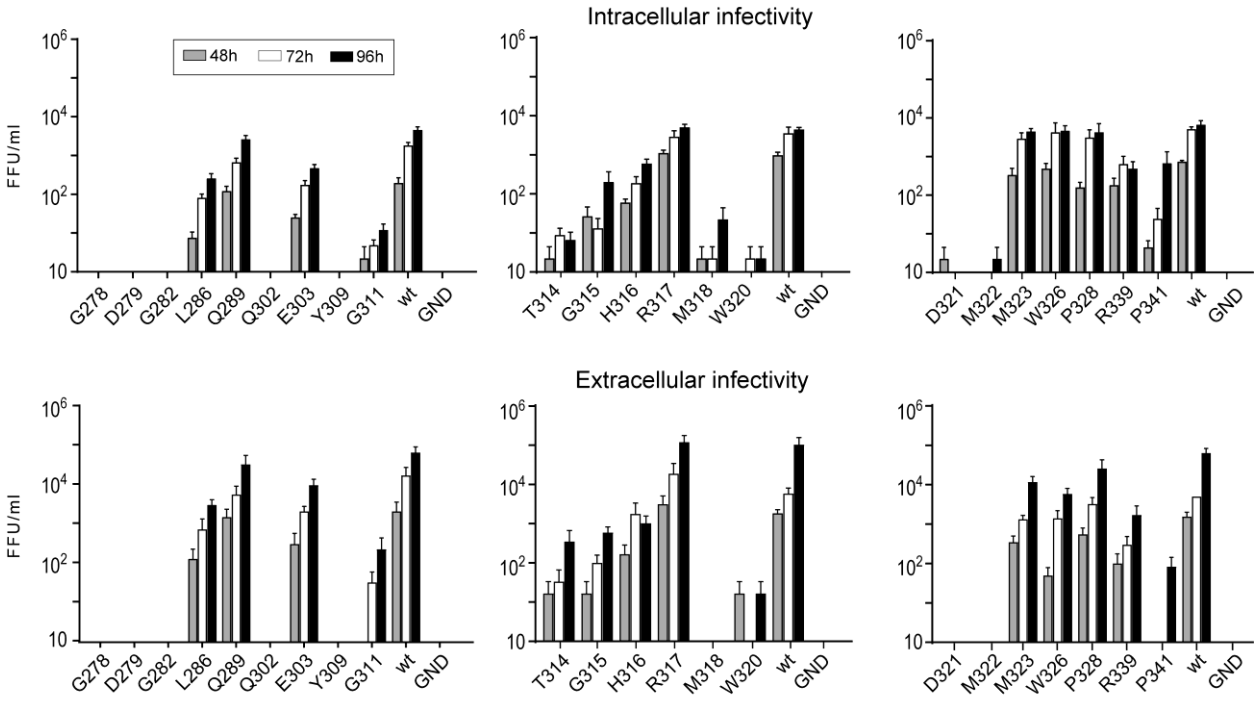

Figure 3 Effect of mutations on extracellular and intracellular infectivities. Viral RNA transcribed from JFH1-derived mutants was electroporated into Huh-7 cells. The infectivities of the supernatants and intracellular viruses were determined at 48, 72, and 96 h postelectroporation by titration. Error bars indicate standard errors of the means from at least three independent experiments. Values were compared to the wild-type virus. Differences were considered statistically significant for the extracellular infectivity of mutants G278A, D279A, G282A, L286A, Q302A, E303A Y309A, G311A, T314A, G315A, H316A, M318A, W320A, D321A, M322A, W326A, R339A, and P341A ($P < 0.05$) and for the intracellular infectivity of mutants G278A, D279A, G282A, L286A, Q302A, E303A Y309A, G311A, T314A, G315A, H316A, M318A, W320A, D321A, M322A, R339A, and P341A ($P < 0.05$) at 96 h postinfection.

Effect of E1 mutations on virion release. To determine whether the mutations affected the release of viral particles, the intra- and extracellular levels of HCV core protein at 48 h postelectroporation were quantified. For all mutants, the level of intracellular core protein was similar to the wild-type virus, confirming the absence of effect of E1 mutations on viral replication. In contrast, the levels of extracellular core proteins were reduced for most mutants in the potential fusion peptide region that presented impaired infectivity, as well as for the severely attenuated P341A mutant, indicating a defect in the secretion or assembly of viral particles (Fig. 4). Interestingly, most of the mutations in or close to the E1 α2 helix (G311A, T314A, G315A, H316A, M318A, W320A, D321A, and M322A) affecting virus infectivity had no impact on the secretion of core protein, suggesting that these mutations led to the release of noninfectious viral particles.

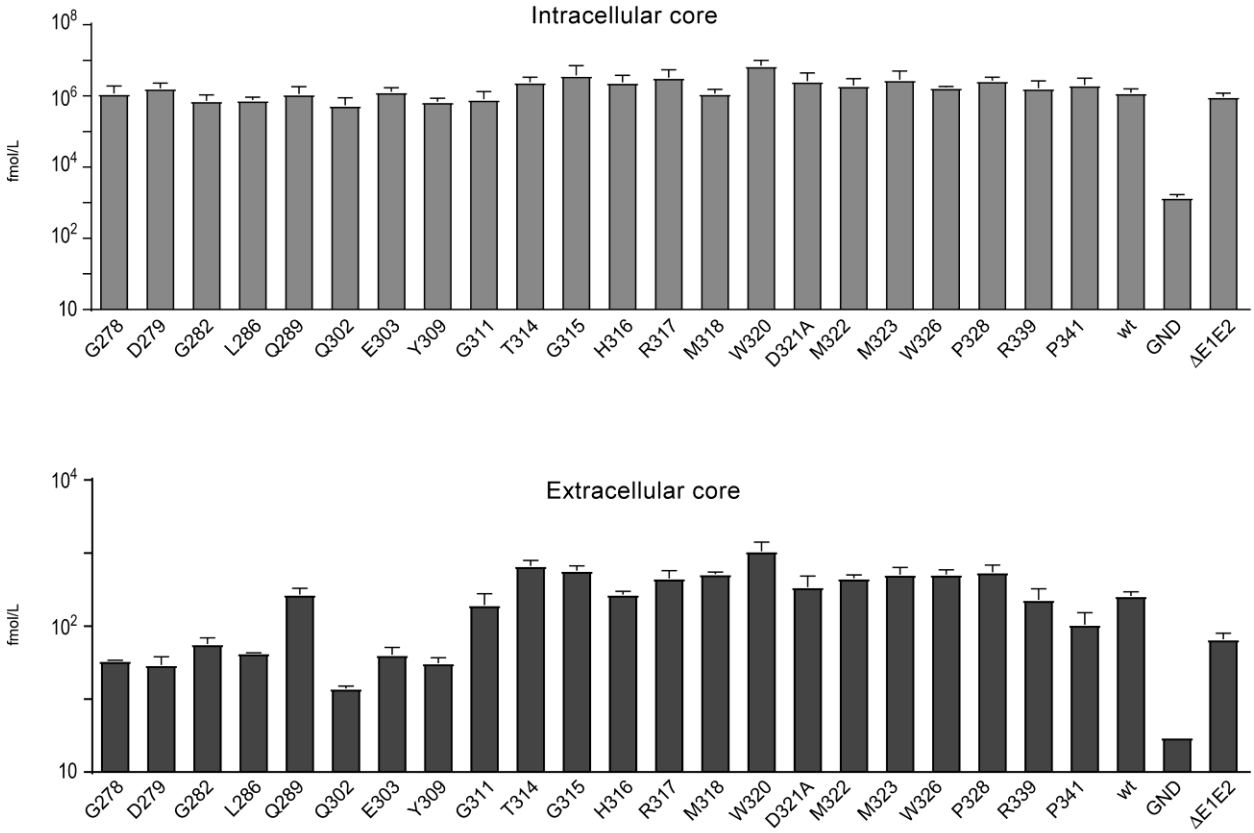

Figure 4 Effects of E1 mutations on HCV core protein secretion. Huh-7 cells were electroporated with wild-type or mutant viral RNAs. The levels of core protein in supernatants and cell lysates were determined at 48 h postelectroporation. Error bars indicate standard error of the means from at least three independent experiments. Values of core protein were compared to the wild-type value. Differences were considered statistically significant for extracellular mutants G278A, D279A, G282A, L286A, Q302A, E303A Y309A, and P341A ($P < 0.05$).

Effect of E1 mutations on HCV glycoprotein folding and E1E2 heterodimerization. Since E1 and E2 cooperate for their respective folding, we analyzed the effect of the mutations on the formation of E1E2 heterodimers (19). For this purpose, we performed pulldown assays using the CD81 large extracellular loop (CD81-LEL), which recognizes correctly folded E2 (Fig. 5A). E2 protein from all mutants could be precipitated by CD81-LEL, indicating that the E1 mutations had no effect on E2-folding. In the PFP segment and the downstream E1 region (aa274-309), nearly all mutations impacting infectivity affected the coprecipitation of E1. Thus, for the attenuated mutants (L286A and E303A), a lower signal on the E1 Western blot was observed after CD81-LEL pulldown. For the noninfectious mutants (G278A, D279A, Q302A, and Y309A), the E1 protein was not detectable. The impairment in E1 coprecipitation indicates that these mutations affect the interaction between E1 and E2, at least in the context of properly folded E2. These results suggest that the potential fusion peptide and the downstream E1 region (aa274-309) are involved in E1E2

interaction. Thus, the loss of infectivity of the mutants in this region might be due to the associated alteration in protein folding.

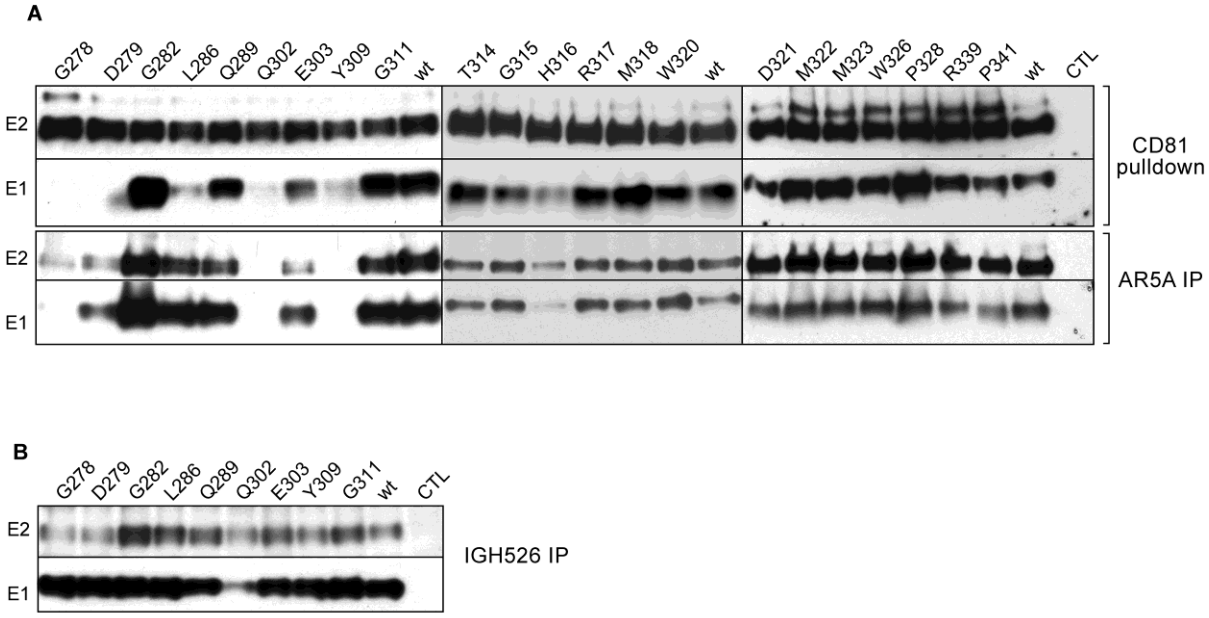

Figure 5 Effect of E1 mutations on E1E2 conformation. (A, upper panel) Interaction of HCV glycoproteins and CD81 (HCV entrybfactor). E1 and E2 from cell lysates were analyzed by GST pulldown at 48 h postelectroporation using a CD81-LEL-GST fusion protein. Pulled-down E1 and E2 were separated by SDS-PAGE and revealed by Western blotting with MAbs anti-E1 (A4) and anti-E2 (3/11). (A, lower panel, and B) Recognition of HCV E1 and E2 glycoproteins by conformation-sensitive anti-E1E2 MAb AR5A and anti-E1 MAb IGH526, as indicated. At 48 h postelectroporation, E1 and E2 proteins from cell lysates were analyzed by immunoprecipitation with MAbs AR5A and IGH526. Immunoprecipitated proteins were revealed by Western blotting using MAbs A4 and 3/11.

The G282A mutation in the PFP and the majority of the mutations in α2 and the downstream region (aa325-341) of E1 had no impact on the formation of the E1E2 heterodimer since E1 could be efficiently precipitated with E2 in CD81-LEL pulldown for all mutants, except for one (H316A) in this region. Thus, mutations G311A, T314A, G315A, M318A, W320A, D321A, M322A, M323A, W326A, P328A, R339A, and P341A, which led to the production of attenuated or noninfectious virus, had no impact on E1E2 interaction. These mutants do not present conformational or heterodimerization defects that could explain their loss of infectivity.

To further characterize the effect of E1 mutations on the folding of the E1E2 heterodimer, we performed immunoprecipitation experiments with conformationsensitive antibodies. In a first step, we used the human monoclonal antibody (MAb) AR5A, which recognizes an epitope shared by E1 and E2 (20)(Fig. 5A). Data obtained in this assay correlated with the results of the CD81 pulldown assay. Indeed, for the noninfectious or attenuated mutants in the potential fusion peptide and downstream region (G278A, D279A, Q302A, E303A, and

Y309A), E1E2 glycoproteins were either weakly or not recognized by MAb AR5A. These findings confirm that these mutations affected E1E2 conformation and that the loss of infectivity or attenuation was due to an alteration in protein folding. Unexpectedly, E1 and E2 were well recognized by the AR5A MAb for the mutant L286A, for which CD81 pulldown assay showed a weak signal for E1 coprecipitation, suggesting an effect on the interaction between E1 and E2. This might be due to a partial alteration of the affinity between E1 and E2, which is in agreement with its attenuated infectivity.

In the case of the G282A mutant in the PFP region and most of the mutants in the α2 and downstream region (G311A, T314A, G315A, M318A, W320A, D321A, M322A, M323A, W326A, P328A, R339A, and P341A), which are either attenuated or noninfectious, E1 and E2 were recognized by AR5A MAb, excluding any effect on the heterodimerization or folding for these mutations. The absence of effects on E1E2 heterodimerization and folding is in agreement with the unimpaired virus secretion observed for the mutants in the α2 helix and downstream region. Altogether, these results suggest that these mutations lead to the production of noninfectious viral particles.

For the mutants in the PFP region, E1 folding was further characterized by immunoprecipitation with the E1-specific antibody IGH-526 (Fig. 5B), which recognizes a discontinuous epitope that includes a linear region spanning residues 313 to 327 (Kong et al., 2015). Due to the overlap between α2-helix residues and the IGH-526 epitope, we could not use this antibody to characterize E1 folding for α2-helix mutants. For all tested mutants except for the mutant Q302A, the glycoprotein E1 was recognized by the MAb IGH-526, indicating that these mutations have no drastic effect on the conformation of the E1 glycoprotein. E2 coprecipitated with E1 for most mutants. However, the signal for E2 was lower for G278A, D279A, Q302A, and Y309A mutants compared to the wild type, which is in agreement with the defect in the heterodimerization of the envelope proteins observed in the CD81 pulldown assay and AR5A immunoprecipitation. In addition, this test further confirms a remaining interaction between E1 and E2 of the mutant L286A, which might be responsible for the attenuated infection observed for this mutant.

Whereas the defects in infectivity of most PFP and aa292-309 downstream region mutants can be attributed to impairment in virus assembly and envelope protein folding, the loss of infectivity of most mutants in the α2 helix and downstream region remains unexplained.

Effect of E1 mutations on HCV neutralization and inhibition by CD81. During their incorporation at the surface of viral particles, envelope glycoproteins undergo structural changes (19, 21). However, due to the low particle production yield of the HCV cell culture system, biochemical analyses of particle-associated envelope proteins are difficult to implement. Alternatively, the effect of the mutations on the folding of virus associated envelope proteins can be determined by the analysis of the sensitivity of the virus to neutralization with the help of conformational neutralizing antibodies or CD81-LEL. However, this approach is only possible for the characterization of attenuated viruses. Thus, neutralization assays were performed with mutants showing a decrease in infectivity of ≤ 1 log10 (L286A, E303A, M323A, W326A, P328A, and R339A) (Fig. 6).

The L286A and E303A mutants did not show any difference in sensitivity to inhibition by CD81-LEL and AR5A. This result contrasts with the effect of the L286A and E303A mutations on the heterodimer formation observed in biochemical interactions assays. Thus, although these mutations affect intracellular envelope protein heterodimerization, they have no major impact on E1E2 folding at the surfaces of the viral particles.

Conversely, α2-region mutations that had no impact on intracellular E1E2 recognition by AR5A or CD81-LEL led to an increase in virus sensitivity to inhibition by AR5A. Thus, the M323A, W326A, P328A, and R339A mutations likely induce a conformational change of virion-associated E1E2, leading to a better access of the AR5A epitope. To further confirm the results obtained with AR5A, we used AR4A MAb, which recognizes a discontinuous epitope on E1 and E2, in neutralization and immunoprecipitation assays (20). As shown on Fig. 6D, AR4A MAb could precipitate E1 and E2 from M323A, W326A, P328A, and R339A mutants with the same efficiency as wt E1E2. As found in AR5A-mediated neutralization, AR4A inhibited the infectivity of M323A, P328A, W326A, and R339A mutants with a higher efficiency (Fig. 6C). This result further supports a specific impact of these mutations on the virus-associated E1E2 conformation.

On another hand, the recognition of E1E2 of E303A mutant by AR4A in immunoprecipitation assays was slightly affected, while L286A mutation had no impact on E1E2 recognition (Fig. 6D). This confirms the effect of the E303A mutation on the conformation of intracellular E1E2 glycoproteins, as shown by the results obtained in AR5A immunoprecipitation. As observed in AR5A neutralization experiments, E303A and L286A

do not significantly affect the neutralization efficiency of AR4A (Fig. 6C). These findings support a specific impact of E303A mutation on intracellular forms of E1E2 glycoproteins.

Effect of E1 mutations on the recognition of HCV receptors. We further characterized the phenotypes of attenuated mutants by analyzing their dependence on the main known HCV receptors. For this, we determined their sensitivity to inhibition by anti-receptor MAbs, previously reported to affect HCV entry (Fig. 7).

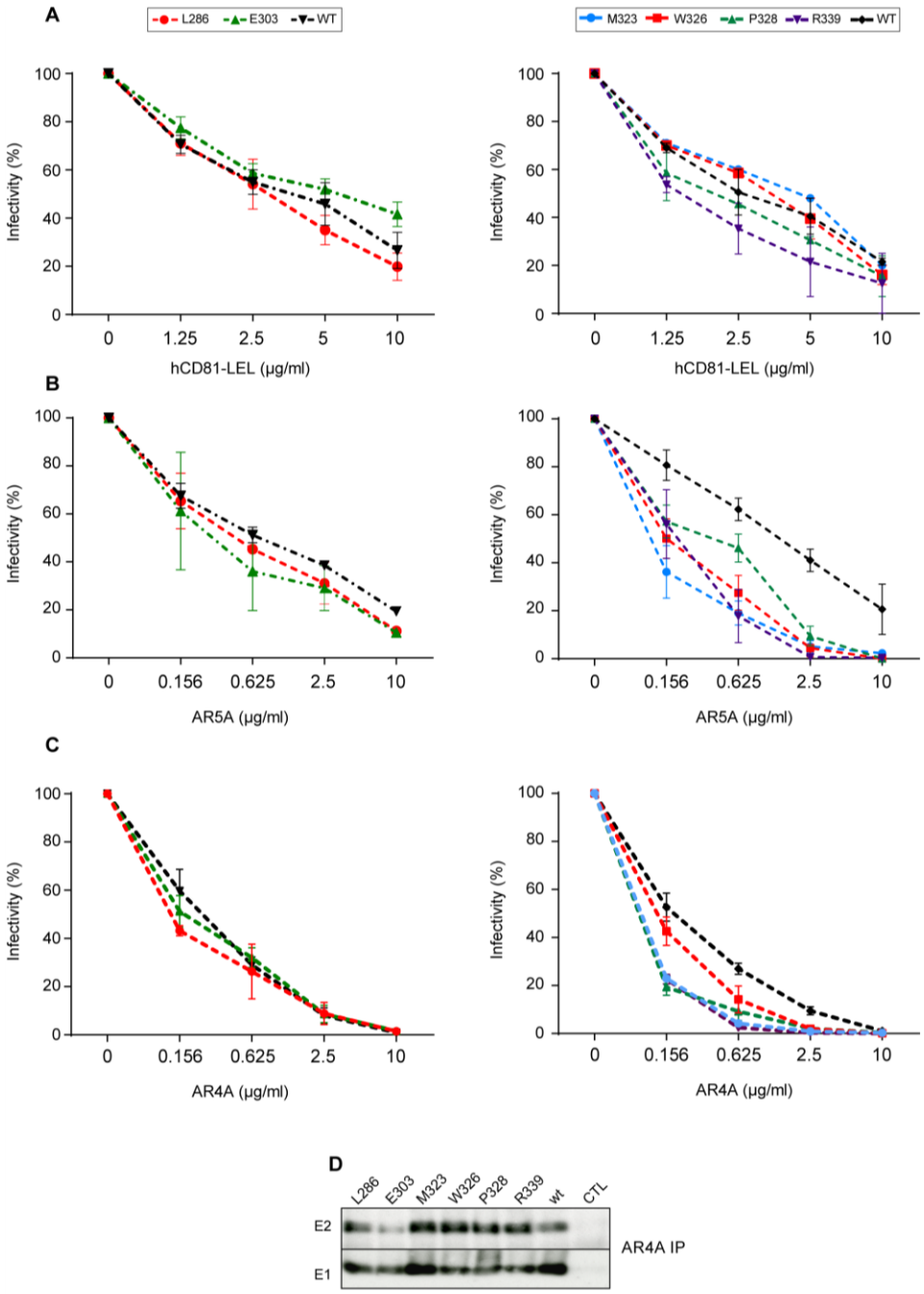

Figure 6 Effect of E1 mutations on E1E2 interaction with HCV neutralizing antibodies and CD81. CD81 inhibition assays (A) and AR5A (B) and AR4A (C) neutralization experiments were carried out by incubating E1 mutants or wild-type virus with increasing concentrations of human CD81-LEL, MAb AR5A, or MAb AR4A at 37°C for 2 h. The mixture was then added to naive Huh-7 cells that were plated 1 day before. At 72 h postinfection, infectivity was determined by immunofluorescence. The values are the combined data from three independent experiments. The error bars represent standard errors of the means. Results were compared to those of the wild type and a P value of < 0.05 was obtained for mutants M323A, W326A, P328A, and R339A in the AR5A and AR4A neutralization experiments. (D) Recognition of HCV E1 and E2 glycoproteins by conformation-sensitive anti-E1E2 MAb AR4A. At 48 h postelectroporation, E1 and E2 proteins from cell lysates were analyzed by immunoprecipitation with MAb AR4A. Immunoprecipitated proteins were revealed by Western blotting with MAbs A4 and 3/11.

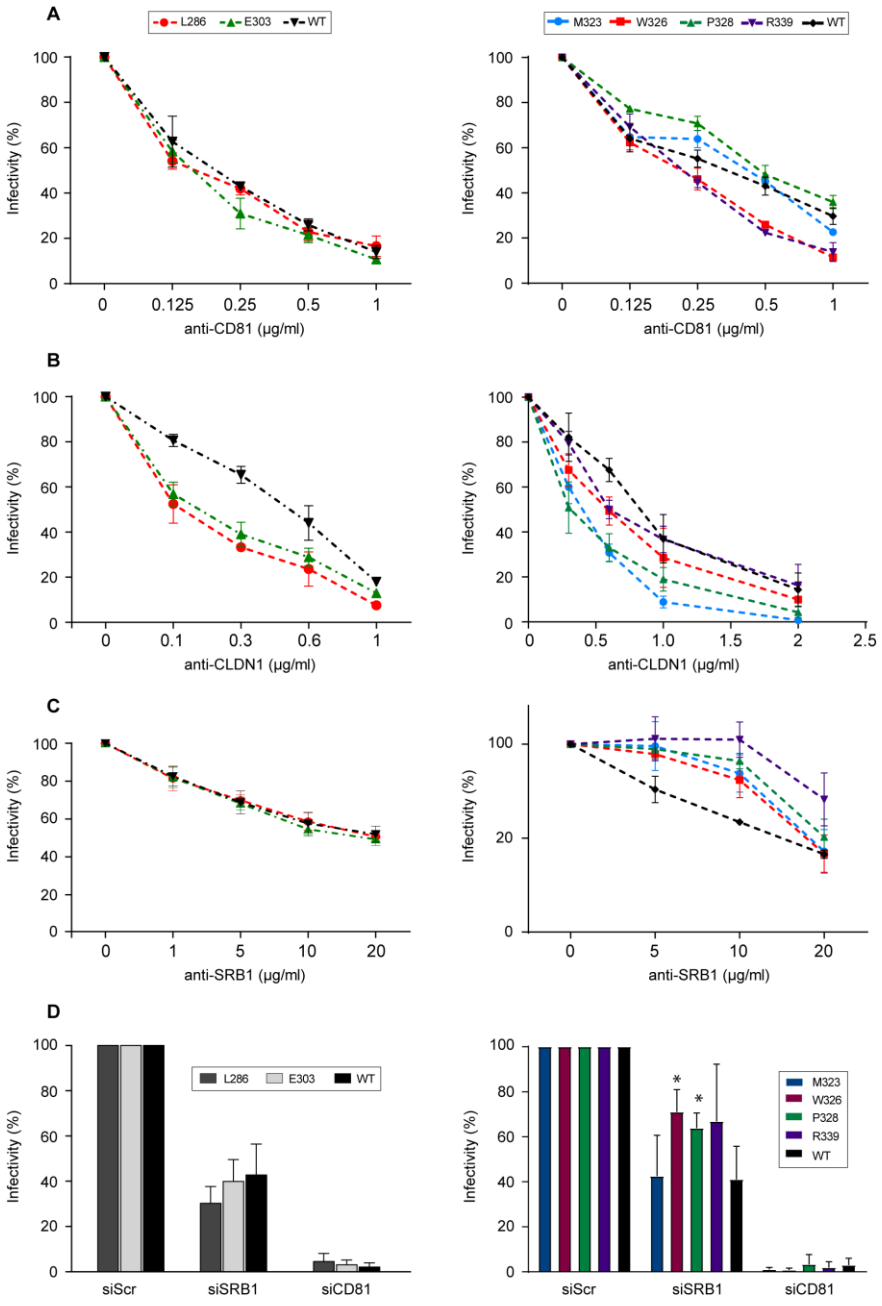

Figure 7 Effect of E1 mutations on the recognition of HCV receptors. Huh-7 cells were preincubated at 37°C for 2 h with increasing concentrations of antibodies targeting HCV receptors: anti-CD81 MAb JS81 (A), anti-CLDN1 MAb OM8A9-A3 (B), and anti-SR-BI MAb Cla-I (C). E1 mutants or wild-type virus were then inoculated onto the cells. At 72 h postinfection, the residual infectivity was determined by immunofluorescence. The values are the combined data from three independent experiments. The error bars represent standard errors of the means. Results were compared to those of the wild type. A P value of < 0.05 was determined for mutants L286A, E303A, M323A, and P328A in the presence of anti-CLDN1 MAbs and for mutants M323A, W326A, P328A, and R339A in the presence of anti-SR-BI MAbs. (D) SRB1 or CD81 expression was downregulated by siRNA targeting SRB1 or CD81 mRNA. Infectivity is expressed as the percentage of infection performed in the presence of the control siRNA. Mean values and standard deviations from three independent experiments are shown. The unpaired t test was used to compare the infectivities of the wild-type and mutant viruses. Differences were considered statistically significant if the P value was < 0.05.

No significant difference in sensitivity to inhibition by the anti-CD81 antibody was observed for the mutants. The absence of effect of the mutations on the dependence on CD81 for entry was confirmed by silencing the expression of CD81 with small interfering RNA (siRNA).

Interestingly, mutants in the α2 helix or downstream region (M323A, W326A, P328A, and R339A) were less sensitive to inhibition by the SR-BI-specific antibody, whereas the sensitivity to the SR-BI antibody was not affected for L286A and E303A mutants. Moreover, for the W326A and P328A mutants, infection was significantly less inhibited by the downregulation of SR-BI expression with siRNA. This suggests that residues M323, W326, P328, and R339 modulate HCV dependence on the SR-BI receptor. Similar phenotypes were obtained regarding CLDN1 dependence for mutants in the PFP and α2-helix regions. Indeed, L286A (PFP), E303A (PFP downstream region), M323A (α2 helix), and P328A (α2-helix downstream region) were more sensitive to inhibition by the anti-CLDN1 MAb than the wild type, suggesting that they are more dependent on CLDN1 for entry. However, the W326A and R339A mutants showed the same sensitivity as the wild-type virus to anti-CLDN1 inhibition, suggesting that only specific residues in the α2 helix and downstream region modulate the dependence of the virus on CLDN1.

Due to the absence of an anti-OCLN MAb capable of neutralizing HCV infection, the dependence on the OCLN receptor was tested using a knockout cell line (OKH4) (22). As found for the wild-type virus, all of the mutants failed to infect the cells, indicating that the mutations have no effect on the dependence on the OCLN receptor.

Characterization of SR-BI independent mutants. HCV associates with lipoproteins to form lipo-viro particles (23). Moreover, HCV-associated lipoproteins modulate HCV infectivity and play a role in virus interaction with SR-BI (24–26). Accordingly, E2 mutations that modulate HCV dependence on SR-BI have been associated with a shift in virion density (27–29). In this context, we sought to determine whether E1 mutations that led to a decrease in SR-BI dependence were also associated with a change in viral particle density. For this, we analyzed the density of infectious viral particles obtained for the W326A and P328A mutants. After ultracentrifugation, the distribution of infectious particles in density gradients was determined by quantification of infectivity in the different fractions. As shown in Fig.8, no difference was observed between the distribution of infectious wild-type virus and mutant W326A and P328A viruses, which were mainly concentrated in the 1.05

density fraction. Thus, W326A and P328A mutations that affect SR-BI dependence of the virus do not appear to affect virus association with lipoproteins.

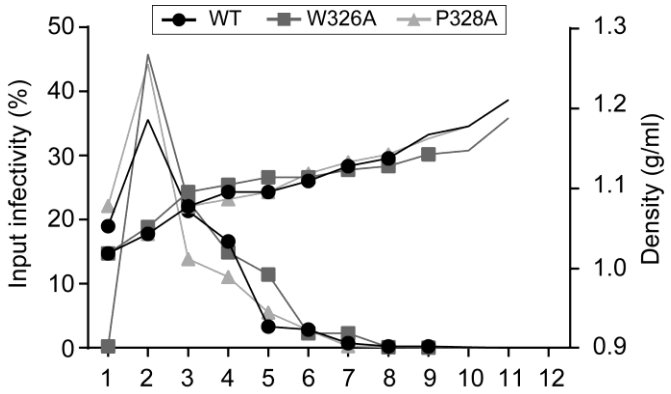

Figure 8 Density gradient analyses of SRBI independent mutants. Concentrated supernatants of cells electroporated with HCV RNA were separated by sedimentation through a 10 to 50% iodixanol gradient. Fractions were collected from the top and analyzed for their infectivity by titration and for their density.

Characterization of noninfectious, assembly-competent E1 mutants. In this study, we identified several mutants that either lost their infectivity (M318A, W320A, D321A, and M322A) or were severely attenuated (G311A, T314A, and G315A) but showed a level of core release similar to the wild-type virus. Thus, these mutations did not affect viral assembly and led to the secretion of noninfectious particles. Moreover, they had no impact on E1 folding or on E1E2 heterodimerization. Since specific mutations in E1 can induce the release of viral particles devoid of genomic RNA (Haddad et al., 2017), we sought to determine whether the impaired infectivity of these mutants was due to similar defects. We chose to quantify the RNA content of the particles released for the two severely attenuated mutants, G315A in the α2 helix and G311A in the upstream region, and the noninfectious mutant W320A in the α2 helix. After electroporation of viral RNA in Huh-7 cells, viral particles released in the supernatant were precipitated with polyethylene glycol, which allowed the removal of the free RNA present in the medium after electroporation. Following this step, viral RNA was extracted and quantified by quantitative reverse transcription-PCR. In parallel, intracellular viral RNA content was determined. As expected, lower levels of extracellular viral RNA were obtained for the nonreplicative GND mutant and the assembly-deficient ΔE1E2 mutant. On the opposite, viral RNA of G311A, G315A, and W320A mutants accumulated at similar levels as the wild type, both intra- and extracellularly (Fig. 9). These findings indicate that the loss of infectivity of these mutants was not due to a defect in RNA uptake in the viral particle.

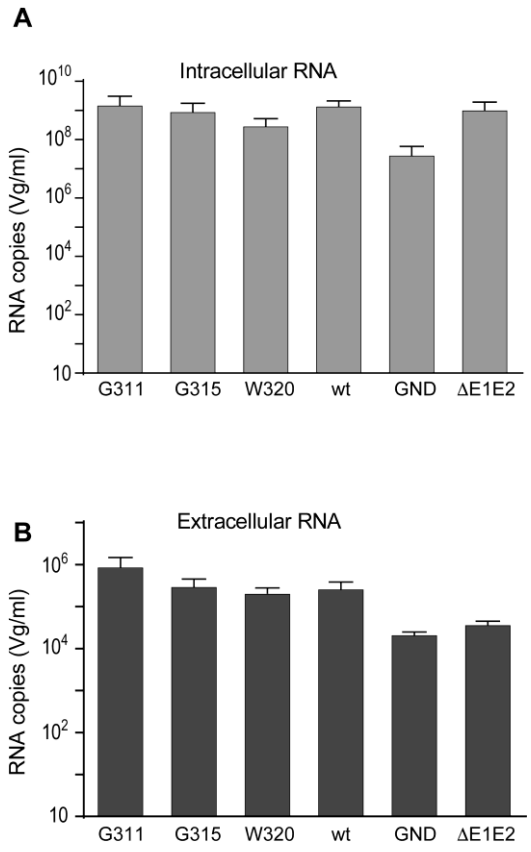

Figure 9 Effect of E1 mutations on viral RNA incorporation. Huh-7 cells were electroporated with mutants and wt RNA. (A) At 48 h postelectroporation, intracellular viral RNA was extracted and quantified by quantitative RT-PCR. In parallel, the viral particles were precipitated from the supernatant with polyethylene glycol and concentrated by ultracentrifugation. (B) Extracellular viral RNA contained in the concentrated virus was extracted and quantified by quantitative RT-PCR.

During morphogenesis, HCV glycoprotein E1 assembles to form noncovalent trimers which is essential for infectivity (21). Therefore, we determined the effect of the G311A, G315A, and W320A mutations on the capacity of E1 to form trimers in infected cells. Thus, after electroporation of viral RNA into Huh7 cells, envelope proteins from the lysate were concentrated by pulldown with *Galanthus nivalis* lectin and analyzed by Western blotting without thermal denaturation as previously described (21). Similar amounts of E1 trimers could be observed for the G311A, G315A, and W320A mutants than for the wt virus, indicating that the mutations do not alter E1 trimerization.

Since they did not affect envelope protein folding, core secretion, or RNA encapsidation, the G311A, G315A, and W320A mutations lead to the release of noninfectious viral particles that may be deficient in viral entry. To confirm this hypothesis, we produced retroviral particles pseudotyped with HCV envelope proteins bearing the corresponding mutations in

E1. As observed in Fig. 10, infectivity of HCVpp carrying E1 mutations was reduced by 2 log compared to HCVpp wild-type infectivity. This result could be due either to a defect in particle production or to the production of noninfectious particles. We thus assessed the efficiency of the incorporation of envelope proteins in HCVpp particles. To do so, extracellular particles were concentrated on a sucrose cushion by ultracentrifugation and analyzed by Western blotting. Although similar levels of retroviral capsid proteins were observed for all viruses, smaller amounts of E1 and E2 were detected for the G311A and G315A mutants, indicating that these mutations affect E1 and E2 incorporation in HCVpp viral particles. In contrast, the W320A mutation was associated with an increased incorporation of E1 and E2 in the particles, as shown by the detection of larger amounts of E1 and E2 in the concentrated particles samples. Thus, the loss of infectivity of G311A and G315A might be due to an impaired incorporation of the envelope proteins in the viral particles, while the absence of infectivity of the W320A mutant indicates an effect of this mutation on virus entry.

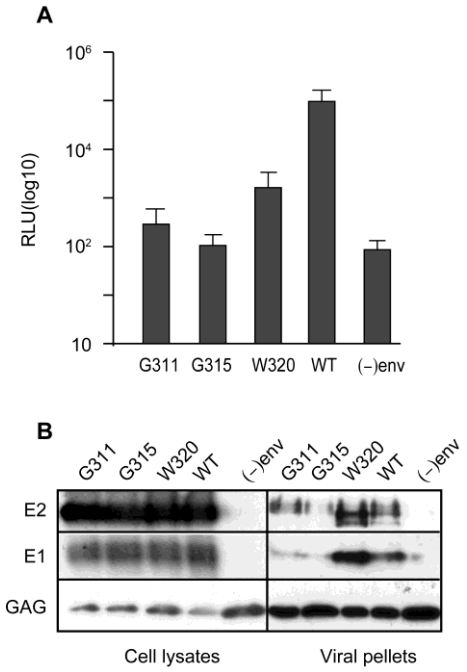

Figure 10 Effect of E1 mutations on HCVpp infectivity. Infectivity of HCVpp harboring E1 with G311A, G315A, or W320A mutation. (A) HCVpp infectivity was determined by measuring the activity of the luciferase reporter gene in infected Huh7 cells. Pseudotyped particles produced in the absence of envelope proteins were used as negative controls. The results are reported as means ± the standard deviations (error bars) of three independent experiments. A P value of <0.05 was obtained for mutants G311A, G315A, and W320A. (B) Effect of E1 mutations on the incorporation of envelope proteins in HCVpp particles. Cells producing HCVpp were lysed and analyzed by Western blotting. HCVpps contained in the supernatants of transfected 293T cells were concentrated on a 20% sucrose cushion by ultracentrifugation and analyzed by Western blotting. E1, E2, and capsid were detected using MAbs A6, 3/11, and CRL1912, respectively.

Discussion

Although the precise role of E1 in the HCV life cycle has been poorly characterized until recently, the structure of E2 suggests that E1 might play a key role in the fusion process (10, 11). Moreover, the recent characterization of the role of conserved residues in the N-terminal part of the protein highlighted a role for E1 in the virus interplay with CLDN-1 and in the incorporation of viral RNA into the nucleocapsid (12). To further characterize the role played by the E1 protein during the HCV life cycle, we investigated the functional role of the C-terminal region of the ectodomain, which contains the putative fusion peptide and two alpha helices. Our results show that several residues in the PFP and the downstream region play a role in E1E2 heterodimerization, as well as in the assembly and release of infectious viral particles. Several mutations in the PFP and the α2 helix region decreased the sensitivity of the virus to neutralization by CLDN1 or SR-BI-specific antibodies, indicating a functional role for E1 in HCV-CLDN1 and HCV-SR-BI interaction. Furthermore, most mutations in the α2 region affected virus infectivity without any effect on virion assembly, suggesting that they led to the secretion of non-infectious or attenuated viruses, likely defective in virus entry.

Most mutations in the PFP region and the downstream region (G278A, D279A, L286A, Q302A, E303A, Y309A) affect the interaction between E1 and E2. These results confirm the involvement of E1 region aa290 to 306 in E1E2 interaction, as observed in the computational prediction of the E1E2 heterodimer structure proposed by Freedman (30). Thus, although interactions between E1 and E2 transmembrane domains are essential for E1E2 heterodimerization, several residues in their ectodomains also contribute to the interaction. Indeed, N-terminal residues 201 to 206 are essential for the structure of the AR5A and AR4A antibody epitope, which spans the E1 and E2 proteins (20). Recently, Gopal and collaborators generated a library of alanine scanning mutants covering the full E1E2 sequence of the genotype 1a H77 strain (31). Using a high throughput flow cytometry-based assay, they probed the mutants library for binding to a collection of antibodies. In agreement with our data, this approach revealed a decrease in the recognition of mutants G278A, D279A, Q302A and D303A by AR5A whereas mutants G282A, L286A, Q289A and G311A were unaffected. Moreover, the characterization of the functionality of chimeric heterodimers derived from different genotypes allowed the identification of residues at position 308, 330, and 345 as being involved in the functional interaction between E1 and E2 (32). In addition, we previously

identified three residues (W239, I262, D263) located in the β-sheet structure of the N-terminus part of E1 that are involved in E1E2 interaction (12). Among the mutants in the PFP region that presented a deficiency in infectivity, only G282A was not affected in its capacity to interact with E2. However, this mutation led to an impairment in virion assembly that might explain the loss of infectivity of this mutant. It is worth noting that the functionality of this residue was characterized in the HCVpp system of genotypes 2a and 1a (33, 34), and in a cell culture model allowing the trans-complementation of E1 in the HCV genome lacking the E1-coding sequence. In these contexts, the mutation led to a severe decrease in infectivity. Furthermore, in the E1-transcomplementation HCV system, lower levels of extracellular viral RNA were obtained for this mutant, which supports an effect of the mutation on virus assembly.

Several attenuated mutants in the PFP (L286A), in the downstream region (E303A), and in the α2 region (M323A, P328A) exhibited an increased sensitivity to neutralization of infectivity by CLDN1-specific antibodies, suggesting that these mutants are more dependent on CLDN1 for cellular entry. This result is in line with the potential involvement of E1 in HCV-CLDN1 interplay, as previously observed (12, 35). However, E1 mutations reported until now (T213A, I262A, H316N) had an opposite effect on CLDN1 dependence, since they led to a decreased sensitivity of the virus to the inhibitory effect of anti-CLDN1 antibodies while increasing its dependence on CLDN6 for entry. Thus, it seems that different regions of E1 have opposite effects on the requirement of CLDN1 for HCV entry. Moreover, these results strengthen the hypothesis of the involvement of E1 in HCV particle interaction with the CLDN1 coreceptor. Whereas some E1 mutations have been shown to affect the binding of HCVpp to CLDN1-expressing cells, no direct interaction between E1 and CLDN1 has been reported until now. Alternatively, E1 could modulate the affinity of E2 for CLDN1 as it was reported for the E2-CD81 interaction (12, 19).

Several mutations in the α2 region (M323A, W326A, P328A) affect the dependence of HCV on SR-BI for entry. Indeed, these mutants were found to be partially resistant to inhibition of the infection by an SR-BI-specific antibody or by siRNA treatment. Several mutations in E2 (G451R, V514A, and the murine CD81-adapted HCV mutant Jc1/mCD81), as well as the deletion of HVR1 have been previously shown to decrease the dependence of the virus on SR-BI (28) (7, 27). At the same time, these variants exhibited an increased affinity for CD81. In contrast, the M323A, W326A, and P328A E1 mutants had no significant effect on the dependence of the virus on CD81, but M323A and P328A presented an increased dependence on CLDN1. Therefore, it seems that the alteration of the interaction between the envelope

proteins and a receptor frequently impacts their affinity for other entry receptors. These findings are in agreement with the finely regulated process of HCV entry that sequentially involves SR-BI, CD81, CLDN1, and OCLN (reviewed in (32)). SR-BI plays a role at several steps of the entry process (28, 36). It is notably thought to contribute to virus attachment through interaction with virus-associated lipoproteins. Its lipid transfer activity has been also shown to be important for productive viral entry. In agreement with the functional interaction of SR-BI with HCV-associated lipoproteins, several E2 mutations that reduce SR-BI dependence were found to be associated with an increase in viral particle density (27). This density shift is supposed to reflect a change in the lipid content of HCV particles and has been suggested to explain the lower dependence of this mutant on SR-BI for entry. Interestingly, cysteine mutations in E1 (C207A and C272A) have also been shown to affect the density of infectious viral particles, suggesting that the E1E2 heterodimer influences the interplay between HCV and lipoproteins. In line with this result, E1 and E2 have been shown to interact with ApoE, whose expression is essential to the production of infectious viral particles (37–39). In the case of the W326A and P328A mutants, no change in the density of the particle could be observed, which suggests that other parameters might influence the interaction of the virus with SR-BI. Alternatively, E1 mutations might modulate the interaction of E2 with SR-BI, as previously reported for its interaction with CD81 (19).

TABLE 1 Summary of the phenotypes of E1 mutants

Wild type or mutant	E1 region	Infectivity[a]	Core secretion[b]	E1E2 heterodimerization and folding[c]				Infection inhibition assays[d]					
				CD81 PD				hCD81 LEL	Anti-E1E2		Anti-CD81	Anti-CLDN1	Anti-SRBI
				E1	E2	IP AR5A	IP AR4A		AR5A	AR4A			
Wild type		+++	++	++	++	++	++	++	++	++	++	++	++
G278A	FP	–	–	–	++	–	ND[e]	ND	ND	ND	ND	ND	ND
D279A	FP	–	–	–	++	+	ND	ND	ND	ND	ND	ND	ND
G282A	FP	–	–	++	++	++	ND	ND	ND	ND	ND	ND	ND
L286A	FP	++	–	+	++	++	++	++	++	++	++	+++	++
Q289A	FP	+++	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
Q302A		–	–	–	++	–	ND	ND	ND	ND	ND	ND	ND
E303A		++	–	+	++	+	+	++	++	++	++	+++	++
Y309A		–	–	–	++	–	ND	ND	ND	ND	ND	ND	ND
G311A		+	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
T314A		+	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
G315A	α2	+	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
H316A	α2	+	++	+	++	+	ND	ND	ND	ND	ND	ND	ND
R317A	α2	+++	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
M318A	α2	–	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
W320A	α2	–	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
D321A	α2	–	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
M322A	α2	–	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
M323A	α2	++	++	++	++	++	++	++	+++	+++	++	+++	–
W326A		++	++	++	++	++	++	++	+++	+++	++	++	–
P328A		+++	++	++	++	++	++	++	+++	+++	++	+++	–
R339A		++	++	++	++	++	++	+	+++	+++	++	++	–
P341A		–	+	++	++	++	ND	ND	ND	ND	ND	ND	ND

[a]The infectivity of HCVcc harboring the different E1E2 glycoproteins in the supernatant of electroporated Huh-7 cells was quantified 96 h postelectroporation (Fig. 3). +++, infectious titers higher than 10^4 FFU/ml; ++, infectious titers higher than 10^3 FFU/ml; +, infectious titers higher than 10^2 FFU/ml; −, titers between 0 and 50 FFU/ml.

[b]Secretion of core Ag in the supernatant quantified at 48 h postelectroporation of Huh-7 cells (Fig. 4). ++, concentration greater than or equal to the wild type; +, concentration reduced by <1 log.; −, concentration reduced by ≥1 log.

[c]The recognition of E1E2 proteins by AR4A and AR5A conformational antibodies and their interaction with hCD81 LEL were determined by precipitation experiments (Fig. 5). ++, similar amount of E1E2 precipitated to that of the wild type; +, lower amount of E1E2 precipitated; −, no E1E2 precipitated.

[d]The sensitivity of the mutants to inhibition of infectivity by different antibodies or the hCD81 LEL was assessed (Fig. 6 and 7). ++, wild-type sensitivity to neutralization; +++, higher sensitivity to inhibition than the wild type; +, lower sensitivity to inhibition than the wild type.

[e]ND, not determined.

Most mutations in the α2 helix region of E1 led to a severe decrease or a loss of infectivity without affecting E1E2 heterodimerization nor the viral particle assembly. It is worth noting that the absence of effect of most mutations in α2 helix region on E1E2 conformation had been previously observed in the characterization of the binding of E1E2-specific antibodies to an alanine scanning E1E2 mutants library (31). Further characterization of the attenuated mutants (M323A, W326A, P328A) revealed a change in their dependence on CLDN1 and SR-BI for entry. These results suggest that the E1 α2 helix region is important for the interplay of HCV with SR-BI and CLDN1 receptors during the entry process. For the severely attenuated mutants G311A and G315A, we observed a deficiency in the incorporation of the envelope proteins in the HCVpp model that could explain the effect of these mutations on infectivity. Due to the difficulties of producing non-infectious HCVcc particles in great amounts, we could not determine the level of E1E2 incorporation in HCVcc particles for these mutants. On the opposite, the characterization of the non-infectious W320A mutant did not reveal any deficiency that could explain its loss of infectivity. Indeed, this mutation had no effect on E1E2 heterodimerization, particle assembly, E1 trimerization, or viral RNA encapsidation. These results and the absence of infectivity observed for this

mutant in the HCVpp system support an effect of the mutation on the virus's entry step. Unfortunately, our attempts to further determine the step of entry that was impaired were unsuccessful due to the low production of particles. Finally, our data would be in agreement with the direct or indirect involvement of the α2 region in the fusion step of the entry, as suggested by the membranotropic properties of this region (15).

Interestingly, the functional characterization of IGH526 epitope (16) revealed that, similarly to what we found in HCVcc, G315A, M318A, D321A and M322A mutations dramatically affected the infectivity of genotype 1a HCVpp. However, introduction of W320A mutation in HCVpp of genotype 1a only resulted in a 30% decrease of infectivity. The more pronounced decrease of infectivity observed for this mutant in HCVcc and HCVpp 2a could be due to the difference of sequences between genotypes 1a and 2a envelope proteins that may modulate the functional impact of the mutations. The potential involvement of α2 helix region in fusion would suggest that IGH526 inhibits HCV infection by targeting the fusion step as it has been reported for some HIV and influenza viruses neutralizing antibodies (16, 40).

On the other hand, our data revealed that the PFP region is important for E1 and E2 interaction, as well as for virus assembly, which does not exclude an involvement of this region in the fusion step between the viral and cell membrane. Indeed, while mutations in the fusion peptide of the viral fusion protein lead to a loss of activity and a consequent loss of virus entry, they can also affect additional steps of the viral life cycle. For instance, mutations in the fusion peptide of the Semliki Forest virus have been shown to affect the interaction between the two envelope proteins, as well as the assembly of the virus (41, 42). In addition, mutation of one glycine residue in the fusion peptide of the influenza virus can result in a variety of phenotypes, depending on the nature of the substituted residue (43). However, the fact that mutations in the PFP region affect different steps of the HCV life cycle complicates the characterization of the role of this region in the entry and fusion steps. Thus, in addition to the potential involvement of PFP in fusion suggested in previous studies (13, 14), our results suggest that the E1 α2 helix also participates in this entry step. The contribution of several E1 regions to fusion is in line with the fact that fusion is a complex process that involves several membranotopic segments of the envelope proteins. While the fusion peptide is responsible for the first step of fusion, additional envelope proteins segments have been shown to be involved at later stages of fusion, such as pore formation or enlargement (44–46). As found for other enveloped viruses, the resolution of the E1 crystal structure would provide crucial elements to definitively identify the fusion peptide of HCV.

To conclude, our results exemplify the important roles played by E1 at different stages of the HCV life cycle, including involvement in E1E2 assembly, in virus morphogenesis, in the interplay with HCV receptors, and potentially in the fusion step.

MATERIALS AND METHODS

Cell culture. Huh-7 human hepatoma cells (47) were grown in Dulbecco's modified essential medium (ThermoFisher), supplemented with GlutaMAX, 10% fetal calf serum and nonessential amino acids.

Antibodies. Anti-HCV monoclonal antibodies (mAbs) A4 (anti-E1) (48) and 3/11 (anti-E2; kindly provided by J. A. McKeating, University of Birmingham, Birmingham, United Kingdom)(49) were generated *in vitro* by using a MiniPerm apparatus (Heraeus) as recommended by the manufacturer. Anti-E1E2 mAbs AR4A and AR5A (20) and anti-E1 mAb IGH526 (16) were kindly provided by M. Law (Scripps Research Institute, La Jolla, CA, USA). The anti-NS5A mAb 9E10 (50) was a gift from C. M. Rice (Rockefeller University, New York, NY, USA) and a polyclonal antibody raised against NS5A (51) was kindly provided by M. Harris (University of Leeds, United Kingdom). The mAb A6 (anti-E1) has been previously described (52). Anti-CLDN1 mAb OM8A9-A3 has been described before (53). Anti-CD81 mAb JS81 (BD Pharmingen), anti-SR-BI mAb Cla-I (BD Biosciences) and anti-tubulin (Sigma) are commercially available, as well as the secondary antibodies used for immunofluorescence, which were purchased from Jackson ImmunoResearch. Anti-capsid of murine leukemia virus (MLV, ATCC CRL1912) was produced in vitro by using a MiniPerm apparatus (Heraeus) as recommended by the manufacturer.

Mutagenesis and virus production. The virus used in this work is a modified version of the JFH1 isolate (genotype 2a; GenBank accession number AB237837) (54), kindly provided by T. Wakita (National Institute of Infectious Diseases, Tokyo, Japan). It was engineered to reconstitute the A4 epitope in E1 (pJFH1-CS-A4) (17) and titer-enhancing mutations (55). Mutants were generated by site-directed mutagenesis and the selected conserved residues were replaced by alanine. Viral RNAs were produced by *in vitro* transcription as previously reported (56). Viruses were produced by electroporation of viral RNA into Huh7 cells as previously described (12). The controls used in this study are the GND mutant, a non-replicative control of the HCV genome containing a GND mutation in the NS5B active site

(54) and the ΔE1E2 mutant, an assembly deficient control, which contains an in-frame deletion introduced into the E1E2 regions (54).

Infectivity assays. Intra- and extra-cellular infectivities were determined as described (56). In brief, viral RNAs were electroporated into Huh-7 cells. Supernatants containing extracellular virus were collected at different time points (48, 72, 96h) after electroporation, and cell debris was eliminated by centrifugation for 5 min at 10,000 *g*. To obtain intracellular viral particles, infected cells were washed with phosphate-buffered saline (PBS) and harvested after trypsinization, which was then followed by four freeze-thaw cycles. Cell lysates were then clarified by centrifugation at 10,000 *g* for 7 min. The clarified supernatants containing extracellular virus or intracellular virus were used for infection of naive Huh-7 cells. Infected cells were later fixed with ice-cold methanol (100%) and immunostained with A4 anti-E1 antibody.

Immunofluorescence. Immunofluorescence analyses were performed as previously described (57). Briefly, after fixation of Huh-7 infected cells with cold methanol (100%) for 10 min, cells were washed twice with PBS and incubated in 10% goat serum for 10 min. The primary anti-E1 antibody A4 was diluted in 10% goat serum and the cover slips were incubated with the antibody for 25 min at room temperature. The cells were then washed three times with PBS. The secondary Cy3-conjugated antibody diluted in goat serum (1/500) was incubated with the cells for 20 min. The cells were washed again with PBS. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole). Images were processed using ImageJ software.

Equilibrium density gradient analysis. Equilibrium density gradient analyses were performed as described (19) after polyethylene glycol (PEG) precipitation of viral preparations (58). Briefly, supernatants were collected at 48h following electroporation of Huh7 cells. Approximately 80 ml of viral supernatant were precipitated using PEG 6000 at a final concentration of 8%. The mixture was incubated overnight at 4°C, centrifuged for 25 min at 8,000 rpm (Beckman JLA-10.5 rotor), and the pellet was resuspended in 1 ml sterile PBS. Then the concentrated viral solution was loaded on a 10 to 50% continuous iodixanol gradient. The gradients were spun for 16h at 36,000 rpm in a SW41 rotor (Beckman). Fractions of 1 ml were collected from the top of each tube and analyzed for their infectivity and density.

HCV core protein quantification. HCV core protein was quantified by a fully automated chemiluminescent microparticle immunoassay according to the manufacturer's instructions (Architect HCVAg; Abbott, Germany)(59).

Western blotting. Western blotting experiments were performed as previously described (12). Cells were lysed in PBS lysis buffer (1% Triton X-100, protease inhibitor cocktail [Roche]). Cell lysates were then precleared by centrifugation at 14,000 *g* for 10 min at 4°C. Protein samples were heated for 7 min at 70°C in Laemmli sample buffer, followed by separation by SDS-PAGE. The proteins were then transferred onto nitrocellulose membranes (Hybond-ECL; Amersham) and detected with specific primary antibodies, which was followed by incubation with the corresponding peroxidase-conjugated anti-rat (Jackson), anti-sheep (Amersham), anti-human or anti-mouse (Dako) antibodies. Detection of proteins was done using enhanced chemiluminescence (ECL) (Amersham) as recommended by the manufacturer.

CD81 interaction and immunoprecipitation assays. CD81 pulldown and immunoprecipitation experiments were performed as previously described (7). Cells were lysed in lysis buffer (1% Triton X-100, protease inhibitor cocktail (Roche) in PBS). Cell lysates were then cleared by centrifugation at 14,000 *g* for 15 min at 4°C. For CD81 pulldown, glutathione-Sepharose beads (glutathione-Sepharose 4B; Amersham Bioscience) were washed twice with cold PBS to remove the storage buffer. For each cell lysate sample, 50 µl of glutathione beads was incubated with 10 µg of human CD81 (hCD81) large extracellular loop (LEL) glutathione *S*-transferase (GST) recombinant protein in 1 ml of cold PBS containing 1% Triton X-100 for 2h at 4°C. After incubation, glutathione-Sepharose beads were washed with cold PBS. Cell lysates containing E1E2 proteins were then incubated with the glutathione beads-CD81-LEL complex overnight at 4°C. The next day, beads were washed five times with cold PBS 1% Triton X-100, then finally resuspended in 30µl of Laemmli buffer and heated at 70°C for 10 minutes. Samples were loaded onto 10% SDS-PAGE gels and HCV envelope glycoproteins were revealed by Western blotting. For immunoprecipitation assays, 70 µl of protein A-agarose beads were incubated with 10 µg of rabbit anti-human IgG (Dako) in 1 ml of cold PBS 1% Triton X-100 for 2h at 4°C. Meanwhile, 100 µl of cell lysates were incubated with 2 µg of mAb AR5A (anti-E1E2) or mAb IGH526 (anti-E1) in 400 µl of cold PBS 1% Triton X-100 for 2h at 4°C. After incubation, the agarose beads were washed twice with cold PBS 1% Triton X-100 and added to cell lysates. The mixture was incubated for 90 min at 4°C, which was followed by washing

the beads five times with cold PBS 1% Triton X-100. Finally, the beads were resuspended in 30 µl of Laemmli buffer. The presence of HCV envelope glycoproteins was then detected by Western blotting.

Entry inhibition assays and neutralization assays. Viruses or cells were preincubated with human CD81-LEL, mAb AR5A, or anti-receptor antibody for 2 h at 37°C. The viruses were then inoculated onto Huh-7 cells. At 6 h post-infection, the inoculum was removed, and the cells were further incubated for 72 h with complete medium. The cells were then processed for immunofluorescence to measure residual infectivity.

HCVpp assay. HCVpp were produced as described previously (6). Briefly, 293T cells seeded for 1 day in 6 well plates were co-transfected with 300ng/well of a pcDNA plasmid expressing HCV envelope glycoproteins (JFH1), 300ng of a murine leukemia virus (MLV) Gag-Pol expression packaging vector, and 400ng of a Firefly luciferase reporter transfer vector. Plasmid containing no envelope protein sequence was used as a negative control. After transfection, cells were incubated for 48h at 37°C. Supernatants containing the pseudoparticles were then harvested and filtered through 0.45-µm pore-sized membranes to be used as HCVpps in infection assays or pelleted by ultracentrifugation through a 20% sucrose cushion at 27000rpm (Beckman Type SW 41 rotor) for 4h at 4°C and analyzed by Western blotting. To minimize artifacts that might be caused by differences in the quality of preparations, each experiment was performed using concurrently produced pseudoparticles (60). Infectivity of HCVpp on target Huh-7 cells was assessed after 72 h by using a firefly luciferase reporter gene activity kit (Promega), as recommended by the manufacturer. Results are presented as the means ± standard deviations of results of three independent experiments.

Graphs and statistics. Prism, version 5.0c (GraphPad Software, Inc., La Jolla, CA), software was used for creating graphs and to determine statistical significance of differences between data sets using a Mann-Whitney test.

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Function of the hepatitis C virus E1 envelope glycoprotein in viral entry and assembly

Summary

Hepatitis C virus (HCV) envelope glycoproteins, E1 and E2, are multifunctional proteins. Until recently, E2 glycoprotein was thought to be the fusion protein and was the focus of investigations. However, the recently obtained partial structure of E2 and E1 rather support a role for E1 alone or in association with E2 in HCV fusion. Moreover they suggest that HCV harbours a new fusion mechanism, distinct from that of other members of the *Flaviviridae* family. In this context, E1 aroused a renewed interest. Recent functional characterizations of E1 revealed a more important role than previously thought in entry and assembly. Thus, E1 is involved in the viral genome encapsidation step and influences the association of the virus with lipoprotein components. Moreover, E1 modulates HCV-receptor interaction and participates in a late entry step potentially fusion. In this review, we outline our current knowledge on E1 functions in HCV assembly and entry.

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Keywords

Hepatitis C virus, envelope glycoproteins, glycoprotein structure, virus entry, virus assembly

Introduction

Hepatitis C virus (HCV) infects 70 million people worldwide, thus constituting a major health problem [1]. In most cases, HCV establishes chronic infection that can evolve into cirrhosis and hepatocellular carcinoma. The recent development of direct acting antivirals (DAA) has been a breakthrough in the treatment of hepatitis C, showing potent efficacy against all HCV genotypes and being associated with elevated HCV clearance rates [2,3]. However, eliminating HCV by 2030, as proposed by the WHO, will be difficult to achieve without the use of a preventive vaccine.

HCV belongs to the Hepacivirus genus of the *Flaviviridae* family. It is an enveloped virus that contains a positive stranded RNA genome [4]. Following the entry of the virus into host cell, the genome is translated in a single polyprotein that is processed into seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) and three structural proteins (the core and the two envelope glycoproteins E1 and E2) [5]. The viral particle is constituted of a nucleocapsid composed of the genomic RNA and the core protein, which is surrounded by a lipid membrane in which the two envelope glycoproteins E1 and E2 are anchored [6]. E1 and E2 associate into heterodimers and play key roles in viral entry and assembly [7,8]. One of the specificities of HCV resides in its association with host lipoproteins to form lipo-viro-particles. Thus, host lipoproteins participate in HCV particles composition, entry and assembly [9]. Studies have for a long time focused on E2 protein, which mediates the binding to the receptors and constitutes the main target of neutralizing antibodies. By analogy with the situation found in flaviviruses, E2 was initially thought to be responsible for the fusion step between the viral envelope and a host cell membrane. However the resolution of the structure of E2 core domain did not support this hypothesis, leading to the proposal that E1 alone or in combination with E2 was responsible for the fusion process [10,11]. As a consequence, there has been a surge of interest in studying E1 envelope glycoprotein in order to decipher its contribution to the different steps of the HCV life cycle. In this review, we summarize the recent advances made in the knowledge of the functions of HCV E1 glycoprotein during HCV entry and morphogenesis.

1. E1 synthesis and determinants for E1-E2 interactions

Similarly to E2, E1 is a type 1 transmembrane protein but its N-terminal ectodomain corresponds to approximately half of E2 ectodomain length (160 and 330 residues

respectively). E1 is addressed to the ER thanks to the signal sequences present in the C-terminal region of the Core protein that is encoded upstream of E1 on HCV polyprotein. Following polyprotein translation, E1 is cleaved from the polyprotein by a cellular signal peptidase. During the synthesis, E1 and E2 ectodomains are translocated in the lumen of the endoplasmic reticulum (ER), while their 30 residues transmembrane domains (TMD) are anchored in the membrane. E1 and E2 have been shown to assemble as noncovalent heterodimers [12] and to cooperate for their folding [13]. Whereas E2 can be expressed alone with a functional folding recognized by conformational antibodies [1,14], the folding of E1 depends on the co-expression of E2 [2,3,15,16]. Nevertheless, several studies reported that E1 could also modulate the folding of E2 ([4,17] [5,18] [6,19,20]). The TMDs of HCV envelope proteins are responsible for their ER retention as well as their heterodimerization [6-8,21]. In addition to the TMDs, several regions in E1 and E2 ectodomains have been shown to contribute to the interaction between the two proteins [9,20,22-25]. Indeed, several residues in E1 region aa278 to 309 have been shown to be involved in E1E2 interaction [10,11,24,25]. Residues 308, 330 and 345 have also been shown to be important for the functional interaction between E1 and E2 [23]. In addition, several reports support a role for E1 N-terminal region in E1E2 interaction [13,20,26]. Finally, the characterization of cell culture-derived HCV (HCVcc) virus harbouring chimeric heterodimers revealed that certain genotype combinations are not functional for virus entry [22,23,27,28].

Altogether these data underline a functional complementarity between E1 and E2 during their synthesis and in several steps of the virus life cycle.

2. Folding, glycosylation and disulphide bonds formation

The maturation of E1 and E2 takes place in the ER and involves the formation of disulphide bonds with the help of the Protein Disulphide Isomerase (PDI) as well as their glycosylation by the N-glycosylation machinery [29,30].

Glycosylation

HCV E1 and E2 are highly glycosylated with N-linked glycans contributing to one third of the mass of the heterodimer. Glycans are linked to aspargine (Asn) within the Asn–X–Thr/Ser motif where X corresponds to any residue except Proline. E1 harbours 4 N-linked glycans (reviewed in [31]) at amino acid positions 196, 209, 234 and 305 of genotype 1a

(strain H77). A fifth glycosylation site can be found in genotypes 1b and 6 at position 250, or in genotype 2b at position 299 [8]. Although HCV genome sequence is highly heterogenic, most N-glycosylation sites are conserved among the various genotypes, indicating that occupation of these sites by glycans is crucial for the HCV life cycle. Interestingly, the complete E1 glycosylation requires the co-expression of E2 [30,32]. E1 N-glycans have been shown to influence its folding as well as its functions during the HCV life cycle. Indeed, N-linked glycans at positions 196 and 305 are required for E1E2 proper folding and heterodimerization [33]. Moreover, N196 and N305 sites have been shown to be crucial for E1 folding and its incorporation in retroviral particles harbouring HCV envelope glycoproteins (HCVpp) [34]. In the HCVcc system, N196 is the most critical glycan for infectivity and assembly [35]. Moreover, the glycosylation of E1 may modulate intramolecular disulphide bond formation. Thus, due to steric hindrance, N305 glycosylation site could hamper the formation of disulphide bonds involving C306 [33].

Importantly, envelope proteins associated glycans have been shown to modulate their immunogenicity by masking epitopes targeted by antibodies (reviewed in [31]). Thus, removal of E1 N305 glycosylation site has a positive effect on the anti-E1 humoral immune response [36,37].

Disulphide Bonds

The ectodomains of HCV E1 and E2 contain several cysteine residues that form disulphide bridges in the oxidative environment of the ER. In heterologous expression systems, a large proportion of E1 and E2 proteins follows a non-productive folding and forms misfolded aggregates stabilized by intermolecular disulphide bonds connecting E1 and E2 [38,39]. The process leading to the formation of a functional heterodimer is slow and assisted by the calnexin chaperone. In this context, E1 and E2 form non-covalent heterodimers and the cysteine residues are involved in intramolecular disulphide bridges within E1 and E2 [13]. Interestingly, HCVpp particles mainly harbour non-covalent heterodimers, whereas the envelope proteins associated with HCVcc particles form large covalent complexes stabilized by intermolecular disulphide bonds [40-42]. These covalent bonds are thought to contribute to the resistance of the viral particles to low pH. As a consequence, a rearrangement of the disulphide bonds might be required for low pH induced fusion during entry [43]. However, HCV entry weakly depends on its redox status [44]. Unexpectedly, individual mutation of E1

cysteines only attenuated virus infectivity, while greatly increasing the sensitivity of the virus to freeze-thaw treatment [19]. This result suggests that disulphide bonds contribute to virion stability.

Finally, E1 and E2 envelope glycoproteins are subjected to important posttranslational modifications that are of crucial importance for their contribution to virus entry and morphogenesis.

3. Global organization and structure of E1 glycoprotein

For decades, characterization of the structure E1 and E2 has been hampered by the difficulties encountered to express and purify the proteins in their native form. Despite these difficulties, the crystal structure of the N-terminal region of E1 protein (residues 192 to 270) could be solved in 2014 [45]. The overall fold of the N-terminal E1 monomer consists of a β-hairpin followed by a segment constituted of a 16 amino-acid long α-helix flanking a 3-strand antiparallel β-sheet. In this β-sheet, the loop between β4 and β5 contains 10 disordered residues. The crystal structure revealed complex network of intertwined E1 homodimers that associate through covalent bonds. Interestingly, the N-terminus of E1 presents some structural homology with a phosphatidylcholine transfer protein, which would support the ability of E1 N-terminus to interact with hydrophobic ligands [45]. Thus, this domain may mediate the association of HCV with lipoproteins. Nevertheless, since it has been previously shown that proper folding of E1 requires the co-expression of E2 [15,16], the relevance of this truncated E1 structure will have to be further validated experimentally.

In addition to the structural characterization of the N-terminal region of E1, NMR studies have also been performed on a peptide located in the C-terminal region of E1 ectodomain (aa 314-342). This study revealed the presence of two other α-helices (α2 and α3, Figure 1) at residues 319 to 323 and 329 to 338 [46]. In agreement with this finding, the co-crystallization of the Fab of the human monoclonal antibody IGH526 with a major component of its E1 epitope (aa314-324) confirmed that this peptide adopts a helical structure when stabilized with an antibody[47]. This peptide was also shown to interact with membranes suggesting that it can either interact with the envelope membrane during assembly or with the host membrane during fusion [46].

Finally, NMR studies of E1 TMD have shown that this domain adopts a helical conformation with helical stretches at residues 354-363 (α4) and 371-379 (α5) separated by a more flexible segment of residues 364-370 [48,49].

In the absence of further structural data, some *in silico* models have been developed. Indeed, starting from the partial structure of E1 and E2 and experimental data, Freedman and collaborators used computational methods to develop a model of the structure of the remaining parts of E1 and E2. In this model, residues 275 to 286 form an α-helix that adjoins E1 core at its C-terminus. Two more β-strands spanning residues 290 to 303 follow this helix [50]. Moreover, the stem region of E1 model harbours three α-helices, the first two overlapping with α2 and α3 at residues 315 to 324, 333 to 338 and the last one from 348 to 352.

Castelli and collaborators combined computational analysis of E1E2 structure with functional characterization of a series of E1E2 mutants to propose an *in silico* model for the ectodomain of E1E2 [51]. In their model, E1 ectodomain is composed of three α-helices spanning residues 256-266, 269-291 and 317-324 surrounded by short β-strands of 3 to 5 residues.

Several studies demonstrated the functional importance of the conserved residues of the structured regions identified in E1 at different stages of the virus life cycle [20,52]. Overall, the determination of the structure of E1E2 heterodimer would be of great interest to further dissect E1 role and notably its involvement in HCV fusion.

4. Oligomerization

The oligomerization status of E1 and E2 might vary according to the viral life cycle steps. Thus, in HCV infected cells, E1 and E2 form non-covalent heterodimers, whereas they associate in large covalent complexes stabilized by disulphide bridges at the surface of viral particles [41]. Recently, the oligomeric state of HCV virion-associated envelope proteins was further investigated by SDS-PAGE in the absence of thermal denaturation [53]. This experimental setting allowed for the identification of SDS-resistant trimers of E1 on HCVcc as well as on HCVpp. The formation of E1 trimers required the co-expression of E2 and was mediated by the transmembrane domains of the envelope proteins. The highly conserved N-terminal G354xxxG358 motif in the TMD of E1 was shown to be crucial for its trimerization as well as for the virus infectivity, indicating that the trimeric form of E1 is of great importance for the virus life cycle. The fact that no E2 homotrimers could be detected

supports the hypothesis that the TMD of three E1 monomers contribute to the trimer formation while interacting in periphery with E2 to form a heterodimer (Figure 2) [53]. However, thermal instable trimers could also be detected in the lysates of infected cells suggesting that trimers of E1E2 heterodimers are already generated during the virus assembly intracellularly.

The ability of E1 to trimerize and the importance of this feature for HCV infection support a role for E1 in viral fusion.

5. Neutralizing Epitopes of E1

The majority of identified HCV neutralizing antibodies target epitopes in the E2 glycoprotein. The difficulty to identify anti-E1 neutralizing antibodies could in part be due to the difficulty to express correctly folded E1 in the absence of E2 [54]. Nevertheless, several studies have demonstrated the capacity of E1 to induce neutralizing antibodies. Thus, neutralizing E1-specific polyclonal antibodies could be raised in mice immunized with E1-HCVpp or recombinant E1 protein [55,56]. Moreover, synthetic peptides derived from the C-terminal region of E1 could be recognized by immunoglobulins present in the sera from infected patients [57,58]. Two main regions of E1 have been shown to be targeted by anti-E1 antibodies. The first region is the N-terminal part of the protein, which is targeted by the human monoclonal antibody H111 (aa192-207) [59] and the murine monoclonal antibody A4 (aa197-207) [38]. While A4 is not neutralizing, H111 shows weak neutralizing activity. Recently, the A6 human monoclonal antibody was isolated from an HCV infected patient. This antibody recognizes an epitope located between residue 230 and 239 within the N-terminal region of E1. While recognizing envelope proteins from a broad range of genotypes, this antibody could not neutralize infection [60]. The second immunogenic region recognized by the broadly neutralizing monoclonal antibodies IGH505 and IGH526 is located at the C-terminus of E1 ectodomain, from residues 313 to 327 [47,61]. Recently the structure of the complex formed by IGH526 monoclonal antibody with a major component of its epitope (aa314-324) was reported [47]. This first antigenic epitope structure may be of great importance for future vaccine design.

Noteworthy, in addition to antibodies recognizing E1 alone, two human conformational neutralizing antibodies, AR4A and AR5A, recognize discontinuous epitopes on E1 and E2 [62] and are endowed with broad neutralization activity. Contrarily to most E2-specific

antibodies, they are not targeting HCV-CD81 interplay but might inhibit conformational changes of E1E2 heterodimer during virus entry.

Due to the relatively high level of conservation of E1 among genotypes, E1-specific antibodies might exert broad neutralization [19]. Moreover several studies suggested that immune response to E1 was impaired in chronically infected patients and was crucial for HCV clearance [63]. In agreement with this hypothesis, immunization of chimpanzees with E1 protected from the evolution of infection to chronicity [64]. These findings led to perform a phase 1 clinical study with a vaccine containing a recombinant truncated form of the E1 protein [65]. This vaccine reached the phase 3 clinical trials. Vaccination induced humoral and cellular immune responses to E1 but had no effect on the histological progression of liver disease [66].

These studies demonstrated the potential immunogenicity of E1 and supported its importance for HCV infection. However the use of a truncated form of E1 might limit immunogenicity to few epitopes and vaccine using both E1 and E2 might be more promising than vaccine using E1 alone. Supporting this hypothesis, vaccine strategy using recombinant HCV E1E2 provided protective immunity against HCV challenge in chimpanzees [67,68] and induced neutralizing antibodies as well as proliferative CD4 T cells responses in human volunteers in a phase 1 clinical trial [69,70]. Although this vaccine has been shown to be highly immunogenic in healthy volunteers and chimpanzees, its ability to protect from real-life exposures remains to be demonstrated.

6. Role of E1 in HCV entry

Viral envelope proteins are at the first line of the infection process by mediating virus entry into the host cell. HCV entry into target cells is a complex process that can be divided into several steps: attachment to the cell surface, interaction with specific receptors, internalization and fusion between viral and host cell membranes. Attachment of the virus to the hepatocyte is mediated by the negatively charged heparan sulphate proteoglycans that are plentiful on the liver surface and involves virion-associated ApoE [71-73]. The subsequent interaction of the particle with specific HCV receptors involves the envelope glycoproteins.

(a) E1 and cellular receptors

A surprisingly large number of cell factors have been reported to participate in virus entry

(reviewed in [74-77]). The contribution to HCV entry of four of them has been the most characterized. These are the scavenger receptor BI (SR-BI), the tetraspanin CD81, and the tight-junction proteins claudin-1 (CLDN1) and occludin (OCLN). Very recently, imaging of HCV entry in a three-dimensional polarized hepatoma system revealed a sequential interplay of the virus with SR-BI and CD81 at the basolateral membrane followed by the migration and association of the virus with OCLN and CLDN1 at the tight junctions [78]. Subsequently, the virus has been shown to be internalized via clathrin mediated endocytosis (reviewed in [76,79]).

Among HCV envelope glycoproteins, E2 is considered as the receptor binding protein. However, a direct interaction could only be shown between E2 and CD81 and between E2 and SR-BI [80,81]. Several data suggest that E1 maintains E2 in a functional conformation, modulating the interaction of E2 with cellular receptors. Thus, the mutation of each of the 8 conserved cysteines of E1 as well as some residues in the N-terminal part of E1 (JFH1 I212, T213, H222, W239) affects the interaction of E2 with CD81 [19,20]. Similarly, several mutations in the residues of E1 α2 region affected the dependence of HCV on SR-BI [24]. Furthermore, the characterization of the capacity of E1E2 chimera from different genotypes to interact with SR-BI and CD81 receptors revealed that the binding to these receptors requires a crosstalk between the two envelope proteins [23].

Interestingly, E1 seems to be involved in the interplay of HCV with CLDN1. Although there has been no evidence for direct interaction between E1 and CLDN1 until now, mutations in E1 can affect the binding of HCVpp to CLDN1- expressing cells [23]. Intriguingly, different residues of E1 have the opposite effect on the contribution of CLDN1 to HCV entry. Thus, T213A, I262A and H316N mutations in the N-terminal part of E1 and the α2 helix decrease the dependence of HCV on CLDN1 for entry while increasing its dependence on CLDN6 [20,82]. Conversely, replacement of residues L286, E303, M323 and P328 by alanine increases the sensitivity of the virus to neutralization by CLDN1-specific antibodies, suggesting that these mutants present a higher dependency on CLDN1 for cellular entry [24].

Supporting a role for E1 in HCV interplay with cellular receptors, E1 was recently shown to interact with the membrane protein related to lipid metabolism, CD36. Expression of this receptor was increased upon HCV infection. Moreover, CD36-specific antibodies inhibited virus entry and replication, suggesting that CD36 may constitute a new HCV co-receptor [83].

Thus, these data indicate that the interplay of HCV with cellular receptors is not only mediated by E2 but is also strongly modulated by E1.

b) E1 and membrane fusion

The fusion process is considered as the final step of HCV entry. Once the virus has entered the cells via clathrin-mediated endocytosis [84], fusion of the viral envelope with a host cell endosomal membrane occurs, leading to the release of the viral capsid into the cytosol. In endosomes, the fusion is induced by low pH, which causes conformational changes of the fusion protein. This has for consequence the exposure of the fusion peptide that can thus interact with cellular membranes [85]. Knowing that secreted HCV particles resist to acidic pH, it is believed that the interaction of CD81 with E2 is responsible for priming HCV glycoproteins to respond to low pH. This step would thus be required to induce the fusion between viral and endosomal membranes [86]. However, the precise molecular mechanism that drives HCV membrane fusion and the viral proteins involved remains unknown.

Fusion proteins are classified into three classes according to their structures and mechanism of fusion. In the *Flaviviridae* family, flaviviruses harbour class II fusion proteins [87]. Class II fusion proteins are also shared by viruses belonging to *Togaviridae* as well as *Bunyaviridae* families [85,88,89]. Class II fusion proteins are characterized by an elongated structure consisting predominantly of β-sheet. They are organized in three domains and form homo- or hetero-dimers at the surface of the particles. Domain II contains the fusion peptide, which is buried at the dimer interface in the pre-fusion conformation. Upon fusion induction at acidic pH, the fusion proteins rearrange into homotrimers that harbour a protruding trimeric spike that inserts into the endosomal membrane [90-93]. In flaviviruses, the fusion protein is also involved in the binding of the virus to the cellular receptors.

Due to the conservation of the genome organization in all members of the *Flaviviridae* family, it has been hypothesized that the viruses from the hepacivirus and pestivirus genera also encode class II fusion proteins [94]. Accordingly, HCV E2 was postulated to be the fusion protein [95]. However the resolution of the pestivirus E2 glycoprotein structure that shows no structural homology with class II fusion proteins did not support this hypothesis [96,97]. Similarly, the crystal structure of the core domain of E2 does not present the characteristics shared by fusion proteins [10,11]. Instead, E2 presents a compact globular shape including several regions with no regular secondary structure. In addition, the potential

fusion regions [95] are located in the hydrophobic core of the protein, which makes them unlikely to mediate fusion [10,11,98]. It was also reported that E2 does not undergo oligomeric or fold change at acidic pH. Altogether these findings suggest that E2 is not directly involved in the fusion process. This means that E1 alone or in combination with E2 mediates the fusion step. In agreement with this hypothesis, E1 presents the capacity to form trimers, which is a characteristic feature of viral fusion proteins [53]. Indeed, the post-fusion structures of class I, II and III viral envelope glycoproteins described so far are trimers [99]. Moreover, several regions of E1 present characteristics of fusion peptides. The first one corresponds to the highly conserved hydrophobic sequence from residues 272 to 291, which has been proposed to constitute a putative fusion peptide (pFP) [100,101]. This sequence is characterized by the presence of a highly conserved acidic residue (D279), which is present at a similar position in the fusion peptide of several flaviviruses. Moreover this sequence contains two cysteines and two glycine residues that are essential for the fusion in paramyxoviruses [102]. The peptides corresponding to this region induce the fusion and disruption of liposomes and hinder HCVcc infectivity [102,103]. Interestingly, several mutations conferring resistance to novel inhibitors of a late step of HCV entry arose in the C-terminal part of this pFP [104,105]. This finding re-enforced the hypothesis that this peptide is of crucial importance during the fusion process. However, recent studies of the function of the conserved residues of the pFP in the HCV life cycle by mutagenesis approaches revealed an important role of this region in E1-E2 interaction as well as in virus assembly [24,106]. These findings are not incompatible with a contribution of this region to the fusion step. Indeed, as found for the Semliki Forest virus, mutations in the fusion peptide can have an impact on the envelope proteins interactions and affect the virion assembly [107,108]. However, since mutations in the pFP region affect different steps of the HCV life cycle, the question of the specific involvement of the pFP in entry and fusion is difficult to address. The second region that is potentially involved in the fusion step is located in the C-terminal part of E1 ectodomain encompassing residues 314-342 which comprises the α2 and α3 helices. This region contains highly conserved residues. Mutations conferring HCV resistance to inhibitors of late entry steps have also been shown to arise in this region [109]. In addition, this peptide has been reported to interact with membranes [46]. Recently, we showed that several point mutations in the α2 region abolished infectivity with no impact on E1E2 folding nor on virus assembly [24]. Moreover, further characterization of some of these mutants in the HCVpp model revealed an effect of the mutations on viral entry. These findings support a

direct or indirect contribution of the α2 helix in the fusion process, which would be in agreement with the high membrane affinity of this region [46]. Altogether, these findings suggest that several regions of E1 contribute to the fusion step. This is in agreement with the fact that fusion is a sophisticated process involving numerous membranotropic segments of envelope proteins. Indeed, while the fusion peptide triggers the initial step of fusion, further membranotropic segments have been shown to contribute to subsequent stages [85,110,111]. Although E1 presents some of the characteristics of fusion proteins, the crystal structure of the N-terminal part of E1 is not comparable to any known class of fusion proteins [45]. Since, a similar situation was found for the envelope protein E2 from the pestivirus BVDV (bovine viral diarrhea virus), this suggests that viruses within the *Flaviviridae* family might employ quite different fusion mechanisms. Thus, HCV and pestiviruses fusion processes might differ from each other and from the better characterized mechanism employed by flaviviruses.

The strong cooperation between E1 and E2 during assembly and entry [15,16,112] suggests that the functional viral glycoprotein unit involved in fusion is the E1E2 complex. In line with this hypothesis, a computational method of coevolution prediction suggested that E1 co-evolved with the E2 back layer domain, and that this genetic association was of great importance for membrane fusion. This prediction could be supported experimentally since a soluble back layer-derived polypeptide was shown to inhibit HCV entry by acting on viral particle [113]. Thus, the characterization of E1E2 interplay together with the structure of the heterodimer might crucial to further dissect HCV fusion mechanism.

7. E1 and HCV morphogenesis

As a component of the virion, HCV envelope glycoproteins play a crucial role in virus assembly. Thus, the formation of E1E2 heterodimers seems to be a key step in HCV morphogenesis. Unfortunately, the precise characterization of HCV assembly is restricted by the weak yield of this step, which hampers the visualisation of assembly events in live cells by high-resolution microscopy. The assembly of the particle requires the gathering of the three structural proteins Core, E1 and E2 and the viral RNA. As found for other members of the *Flaviviridae* family, this step involves non-structural proteins, among which p7 and NS2 are the main coordinators. Once released from the virus polyprotein, the Core protein associates with lipid droplets (LD) whereas the viral genome replication takes place in the membranous web [114,115], derived from the ER. HCV envelope proteins reside mainly in the ER of infected cells. Subcellular fractionation studies have shown that RNA replication

and virion assembly occur in distinct membranous compartments. Indeed, assembly components have been shown to concentrate in detergent resistant LD-associated membranes from the ER [116,117]. At later time of infection, NS5A is recruited from the ER to the LD where it interacts with Core. This step might constitute the transition between replication and assembly. NS5A supports the delivery of HCV genome to the Core protein. Through its interactions with E1, E2, and non-structural proteins, NS2 has been proposed to play a critical role in the migration of E1E2 and Core to the assembly site [118-122]. Recently, the characterization of a mutant in the highly conserved D263 residues in the N-terminal part of E1 supported an involvement of E1 in the viral RNA encapsidation step. Indeed while affecting E1E2 interaction, D263A mutation led to the production of viral particles devoid of viral RNA and to a decrease in the co-localization of the viral RNA with E1 [20]. These findings support that through its interplay with Core, E1 participates to the genomic RNA encapsidation [123]. During or following viral RNA encapsidation, the envelopment of the nucleocapsid takes place at ER membrane. This process requires the envelope glycoproteins [124] and their interplay with other viral proteins, since chimeric viruses with glycoproteins from a different genotype than the rest of the viral proteins are impaired for the capsid envelopment [125]. Interestingly, several E1 mutations have been shown to affect virus assembly without any effect on E1E2 interaction, suggesting that E1 is endowed with specific assignments during that step [20]. In particular, two studies reported that mutations in several residues of the pFP region affect virus assembly, suggesting that this region is involved in HCV morphogenesis in addition to its potential role in entry [24,106].

HCV particles have a specific lipid composition that is similar to that of LDL and VLDL with an important proportion of cholesteryl ester [126]. Moreover they have been shown to incorporate a certain number of apolipoproteins [61,126,127]. The association of HCV with lipoproteins led to define the HCV particle as the lipo-viro-particle (LVP) [128]. The interplay of HCV with the host lipoprotein pathway that leads to the formation of LVP is poorly understood. The incorporation of lipoprotein components is thought to occur during the budding in the ER or after the budding into the lumen of the secretory pathway. E1 and E2 glycoproteins determinant modulate virion-lipoprotein association [19,129,130]. Hence, cysteine mutations in E1 resulted in a change of the density of infectious viral particles [19]. Several proteins of the VLDL pathway have been shown to contribute to the production of infectious HCV particles (reviewed in [9]). Among them, ApoE, which is incorporated in HCV particles, is crucial for HCV morphogenesis [131-133]. Thus, interaction of ApoE with

E1 and E2 is required at a HCV life cycle step between the nucleocapsid envelopment and the virions release from the cells [132,134]. Whereas a first study reported the interaction of E1 only with ApoE in enzyme-linked immunosorbent assay [135], further reports observed an intracellular interaction of ApoE with E1 and E2 glycoproteins [136,137]. Moreover Lee and colleagues established that the TMD of E2 was necessary for ApoE-E2 interactions. The different experimental approaches used in these studies might be responsible for these discrepancies.

Finally, recent characterization of E1 functions revealed a more important role than previously thought for E1 in the virus morphogenesis.

Conclusion and future perspective

HCV entry into host cell and assembly are two sophisticated steps in the HCV life cycle, involving an important number of cellular factors. E1 and E2 envelope proteins that play central roles in these two steps are thus involved in complex interplays with lipoproteins components and an extensive list of cell surface receptors, which remain to be further characterized. In the recent years, great progresses have been made on the characterization of E2 and E1 structure. However, far from confirming the working hypothesis placing E2 at the centre of the entry process, the results obtained raised new questions and revealed the underestimated diversity of viral fusion processes. Thus, HCV fusion might rely on a new type of membrane fusion machinery and E1 would play a central role during HCV fusion. Recent results obtained during the characterization studies of E1 support that it plays a more important role than previously thought in HCV entry and assembly. Moreover, an increasing number of evidence supports the functional interdependence of E1 and E2. Finally, additional structural studies aiming to resolve the full structure of the E1E2 heterodimer in the pre- and post-fusion conformation will be necessary to fully characterize HCV specific fusion process. Furthermore, a better understanding of E1 and E2 cross talks should greatly improve our understanding of HCV entry and assembly.

Executive Summary

HCV envelope glycoprotein E1 is a potential fusion protein candidate

-Until recently, E2 was the most studied HCV envelope glycoprotein

-E2 has been shown to interact with several HCV cellular receptors and is the main target of neutralizing antibodies

-The crystal structure of E2 core does not support a role for this protein in fusion, which suggests that E1 or E1E2 heterodimer is responsible for this step

-E1 contains several regions with fusion peptide properties

-Several mutations that confer resistance to inhibitors of late entry step are located in E1

-In a similar fashion to fusion proteins, E1 can associate in trimers on the viral particle

E1 contributes to the HCV life cycle

-E1 has been shown to modulate the interplay of the virus with several cell surface receptors

-E1 modulates the association of the virus with lipoproteins

-E1 participates in viral assembly, specifically in the encapsidation of the viral genome

HCV E1E2 heterodimers are potentially involved in a novel fusion mechanism

-The structure of the N-terminus part of E1 presents no homology with known fusion proteins

-Similarly, no homology with known class of fusion proteins was found for E2 protein from the Pestivirus BVDV, suggesting that *Flaviviridae* family gathers new classes of fusion proteins

-E1 and E2 interplay seems to be important for the fusion process in HCV entry

Future perspective

The resolution of the complete structure of the entire E1E2 heterodimer in pre- and post-fusion conformation will allow for the characterization of the fusion process defined by HCV.

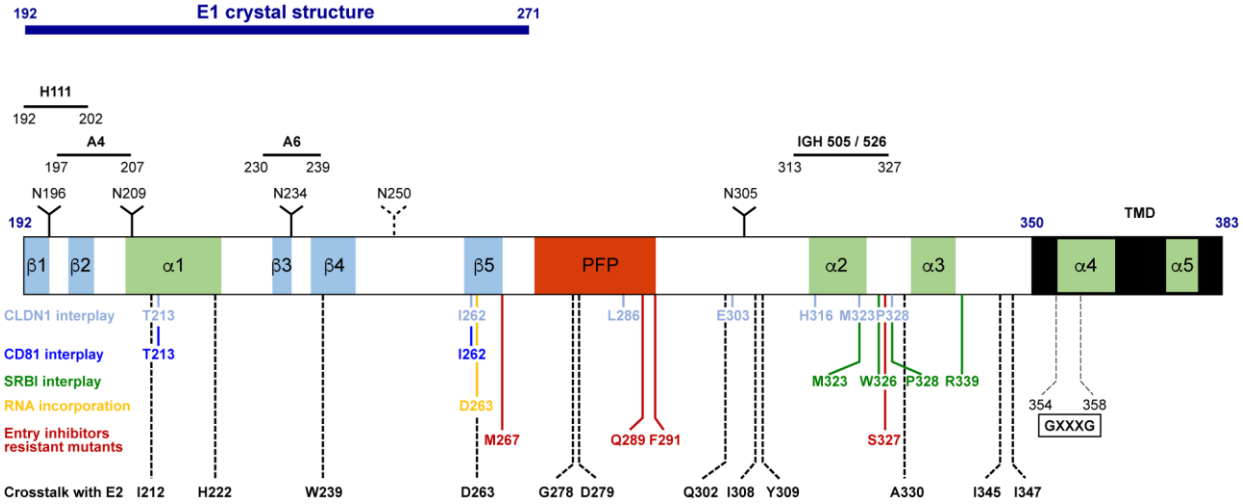

Figure 1: Schematic diagram of HCV E1

The transmembrane domain of E1 is represented by a black box containing the GxxxG motif [6,53]. Colored boxes indicate the different regions of E1 for which the secondary structure has been resolved (green boxes: alpha helices; blue boxes: beta strands)[45-49]. The putative fusion peptide region is indicated by a red box [102]. Residues that have been shown to be involved in the interplay of HCV with CLDN1, CD81 or SRBI receptors are indicated [20,24,82] as well as residues that are important for E1E2 cross-talk [20,23,24,129]. Positions identified with resistant mutations to entry inhibitors likely affecting fusion are shown in red [104,105,109]. Epitopes within E1 recognized by monoclonal antibodies are represented by black line segments [38,47,59-61].

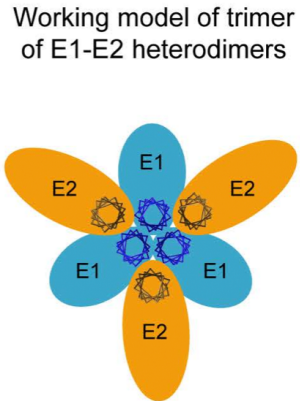

Figure 2: Theoretical arrangement of a trimer of E1E2 heterodimers on the viral surface

E1 proteins form trimers by interacting through their transmembrane domains at the center of the structure. Each E1 subunit binds to an E2 protein, thereby forming a trimer of heterodimers. Ectodomains of E1 and E2 are represented as oval shapes in blue and yellow, respectively, while TMDs are depicted as perpendicular spirals to the membrane surface [53].

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