Investigating Virus Entry using Cell-Culture Adapted Hepatitis C Virus

Simrat Dhillon

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Faculty of Biomedical and Life Sciences

University of Glasgow

Centre for Virus Research

Institute of Infection Immunity and Inflammation

Church Street

Glasgow

G11 5JR

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Abstract

Hepatitis C virus (HCV) is a major cause of chronic hepatitis worldwide. Present estimates predict that between 120-130 million people worldwide are infected with HCV with the majority of all infections progressing to chronicity, ultimately leading to fibrosis, cirrhosis and hepatocellular carcinoma. The virus, which belongs to the family Flaviviridae, has a single-stranded RNA genome of positive polarity that codes for a unique polyprotein of approximately 3000 amino acids. The structural proteins E1 and E2 constitute the viral envelope glycoproteins. These glycoproteins have multiple functions in the viral life cycle such as promoting viral entry and fusion, assembly of infectious virions and aid in viral persistence through immune escape. Numerous cell culture-adaptive mutations have been reported within the HCV glycoproteins. The value of such mutations in understanding the virus interaction(s) with cellular receptors and neutralizing antibodies was first recognised from studies characterizing the E2 cell culture adaptive mutation G451R. This single mutation altered the affinity of HCV to the cell surface receptors CD81 and SRB1 as well as increasing its sensitivity to neutralizing antibodies targeting the viral glycoproteins. A striking observation from previously reported E2 cell culture adaptive mutations is their frequent occurrence within a highly conserved region of E2, spanning residues 412-423. Indeed, the long-term passaging of JFH1 infected cells here in this study also created an adapted virus with a substitution at residue 415. The aim of this study was to determine the phenotypic changes to viral entry caused by mutations in this region. To do this, four JFH1 viruses containing the mutations N415D, T416A, N417S and I422L were constructed and characterized. These mutant viruses were found to have very similar phenotypes to the G451R virus, suggesting all E2 adaptive mutations are selected to alter a specific function in viral entry. Residues 412-423 of HCV E2 also constitute the epitope of the in-house generated broadly neutralizing antibody AP33. ELISA binding and virus infection inhibition assays using AP33 with the E2 mutant viruses provided important information regarding the E2 contact residues of this antibody.

In a separate study, intergenotypic chimeric JFH1 viruses were generated and characterised. Viable intra- and intergenotypic JFH1 chimeric viruses have previously been generated by different research groups by replacing the core to NS2 genes of JFH1 with those from different genotypes. Many of these chimeric viruses required numerous cell culture adaptive mutations to permit efficient infectious virus production. In the present study, 5 intergenotypic viruses were constructed by replacing the JFH1 envelope genes

with those from other HCV genotypes. Despite these chimeric genomes replicating efficiently, none were capable of producing infectious virus. These viral genomes also failed to acquire infectivity during pro-longed cell passaging, suggesting that replacing the JFH1 envelope glycoproteins with those from other genotypes may confer total incompatibility for virus assembly. In addition to this work, the infectivity of a previously generated genotype 4a/JFH1 chimera was improved by repeatedly passaging the virus infected cells. The chimeric virus contained the core to NS2 genes of a genotype 4a strain in place of the those from the original JFH1 sequence. A total of six-adaptive mutations were identified throughout the adapted genome that enhanced infectivity by more than 100-fold. Achieving higher titers with this chimera permitted studies on its viral entry properties as well as its sensitivity to neutralizing antibodies. The ability of the adapted virus 4a/JFH1 virus to spread during multiple rounds of infection was greatly reduced compared to WT/JFH1 due to its inefficient cell-to-cell spread. The 4a/JFH1 virions were also highly sensitive to neutralizing antibodies targeting both linear and conformational E2 epitopes, suggesting that the glycoproteins are more exposed on the surface of this virus.

In its totality, this study has provided key insights into the viral entry and antibody-mediated neutralization properties of cell-culture adapted and intergenotypic chimeric forms of the JFH1 virus.

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Publications

Dhillon, S., Witteveldt, J., Gatherer, D., Owsianka, A.M., Zeisel, M.B., Zahid, M.N., Rychłowska, M., Foung, S.K., Baumert, T.F., Angus, A.G. & Patel, A.H (2010). Mutations within a conserved region of the hepatitis C virus E2 glycoprotein that influence virus-receptor interactions and sensitivity to neutralizing antibodies. *J Virol* **84**, 5494-5507.

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Abbreviations

°C	degrees celcius
%	percentage
µ	micro (10^{-6})
ALT	alanine aminotransferase
Apo	apolipoprotein
APS	ammonium persulphate
ARFP	alternative reading frame protein
bp	base pair(s)
BSA	bovine serum albumin
C-	carboxy-terminus
cDNA	complementary DNA
CLDN	claudin
CMFDA	5-chloromethylfluorescein diacetate
cp	cell passage
CPE	cytopathic effect
CREs	*cis*-acting replication elements
CTL	cytotoxic T lymphocyte
DAPI	4',6-diamidino-2-phenylindole
dH_2O	deionised molecular biology grade water
DMEM	Dulbecco's modified eagles media
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNTPs	deoxynucleotides triphosphates
E.coli	Escherichia coli
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra-acetic acid
eIF3	eukaryotic initiation factor 3
ELISA	enzyme-linked immunosorbant assay
ER	endoplasmic reticulum
FCS	foetal calf serum
FFU	focus-forming unit(s)
FMDV	foot and mouth disease virus
g	gram

GAG	glycosaminoglycan
GFP	green fluorescent protein
h	hour(s)
HAV	hepatitis A virus
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCl	hydrochloric acid
HCV	hepatitis C virus
HCVcc (virus)	HCV cell culture-derived virus
HCVpp	HCV pseudoparticles
HDL	high-density lipoprotein
HeBS	HEPES-Buffered Saline
HRP	horseradish peroxidase
Huh-7	human hepatoma 7 (cells)
HVR	hypervariable region
IF	indirect immunofluorescence
IFN	interferon
IgG	immunoglobulin G
IgVR	intergenotypic variable region
IL28	interleukin 28
IRES	internal ribosome entry site
IRF-3	interferon regulatory factor
ISG	IFN-stimulated gene
JFH	Japanese fulminant hepatitis
kbp	kilobase pair(s)
kDa	kilodalton(s)
LB	lysogeny Broth
LB2	cell lysis buffer
l	litre(s)
LDL	low-density lipoprotein
LEL	large extracellular loop
LVP	lipoviroparticle
m	milli (10^{-3})
M	molar
mAb	monoclonal antibody

MAF	membrane associated foci
min	minute
miR-122	microRNA-122
m.o.i	multiplicity of infection
mRNA	messenger RNA
nano	(10^{-9})
N-	amino terminus
nAbs	neutralizing antibodies
NANBH	non-A, non-B hepatitis
NH_4OAc	ammonium acetate
NK	natural killer
OCLN	occludin
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS(A)	phosphate-buffered saline
PBST	PBS(A)–Tween
PCR	polymerase chain reaction
pH	potential of hydrogen
PI	protease inhibitor
PKR	protein kinase R
PTB	polypyrimidine tract binding protein
RdRp	RNA dependent RNA polymerase
RIG-I	retinoic-acid-inducible gene I
RNA	ribonucleic acid
RNase	ribonuclease
r.p.m	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription PCR
RT-qPCR	reverse transcription-quantitative PCR
sec	second(s)
SCID	severe combined immunodeficiency
SDM	site directed mutagenesis
SDS	sodium dodecyl sulphate
sE2	soluble E2

SEAP	secreted alkaline phosphatase
SGR	subgenomic replicon
siRNA	small inhibitory RNA
SP	signal peptidase
SPP	signal peptide peptidase
SR-BI	scavenger receptor class B member I
STAT-C	specifically targeted antiviral therapy for hepatitis C
SVR	sustained virological response
$TCID_{50}$	50% tissue culture infectious dose
TEMED	N'N'N'N'-tetramethylethylene-diamine
TMD	transmembrane domain
TRIS	2-Amino-2-(hydroxymethyl)-1,3-propanediol
TV	trypsin/versene
U	unit
UTR	untranslated region
uv	ultraviolet
V	volts
VLDL	very-low density lipoprotein
wt	wild-type
w/v	weight/volume ratio
YTB	yeast tryptose broth

One and Three Letter Amino Acid Abbreviations

Amino acid	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Author's Declaration

This work was completed at the University of Glasgow between October 2006-2009 and has not been submitted for another degree. All work presented in this thesis was obtained by the author's own efforts, unless otherwise stated.

1. Introduction

1.1. An overview of Hepatitis C virus

1.1.1. Discovery of Hepatits C Virus

By the early 1970s serological tests were available that allowed the specific diagnosis of hepatitis A virus (HAV) and hepatitis B virus (HBV) in patients suffering of viral hepatitis. However, despite HAV and HBV screening, as many as 10% of blood transfusion cases still resulted in hepatitis, indicating the existence of a yet unknown agent (Alter *et al.*, 1975; Feinstone *et al.*, 1975). This unknown agent was termed non-A, non-B hepatitis (NANBH). By infecting chimpanzees with blood extracted from NANBH infected patient it was established that the agent was capable of causing chronic infection (Alter *et al.*, 1978; Hollinger *et al.*, 1978). Further work, including filtration experiment and sensitivity to organic solvents, suggested that the cause of NANBH was a small enveloped virus (Bradley *et al.*, 1985; Feinstone *et al.*, 1983; He *et al.*, 1987). Due to low concentration of NANBH serum in chimpanzees, conventional immunological methods failed to identify the etiologic agent. Also, further molecular characterisation of NANBH remained obscure for a long time mainly by the lack of cell culture and small animal model for the propagation of the virus.

In the late 1980s Michael Houghton's lab at Chiron eventually succeeded in identifying the causative agent of NANBH. The identification was facilitated by molecular biology techniques such as PCR and nucleic acid methods. Houston and colleagues used a 'blind immunoscreening approach', where they first reverse transcribed nucleic acid extracted from NANBH infected material, followed by generating a cDNA library in bacteriophage that expressed polypeptide encoded by cDNA. This cDNA library was then screened using serum derived from a patient diagnosed with NANBH infection. Finally, the group managed to isolate a single positive clone designated 5-1-1 that expressed polypeptide derived from NANBH viral genome (Choo *et al.*, 1989). Further experimentation revealed that the infectious agent was a single-stranded RNA (ssRNA) genome of ~ 10 kb and possessed 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).

1.1.2. HCV Properties and Classification

HCV shares great similarity with flaviviruses and pestiviruses in terms of genetic organisation and virion morphology and thus has been classified in the *Flaviviridae* family of the *Hepacivirus* genus (Choo *et al.*, 1991). Other member of the *Hepacivirus* genus includes the canine hepacivirus (CHV) that infect dogs (Kapoor *et al*., 2011) and nonprimate hepacivirus (NPHV) that infect horses (Burbelo *et al.*, 2012). Flaviviridae consists of positive-sense, single-stranded RNA genome encoding a polyprotein of more than 3000 amino acids. Based on filtration, rate zonal-ultracentrifugation and electron microscopy data, HCV particles in serum are estimated to be 50 nm in diameter with spherical outer structure and an inner 30 nm capsid (Bradley *et al.*, 1985; He *et al.*, 1987; Kaito *et al.*, 1994).

HCV displays high genetic variability and based on the nucleotide sequence recovered from infected individuals HCV is classified into seven different genotypes and numerous subtypes (Gottwein *et al.*, 2009; Simmonds, 2004; Simmonds *et al.*, 2005). Moreover, within a single individual the virus exists as constantly evolving quasispecies. This genetic variabilty is primarily triggered by the error-prone nature of the RNA dependent RNA polymerase (RdRp), amplified by the high viral production rate of 10^{12} particles/day (Neumann *et al.*, 1998) and further accelerated by the selective pressure exerted by the host immune response (Troesch *et al.*, 2006). The genotypes differ in their nucleotide sequence by 30-35% across the whole viral genome. The variation, however, is not evenly spread throughout the genome as the greatest diversity is found within the viral glycoproteins E1 and E2 (Simmonds, 2004). In particular, the variability is mostly confined to the so-called hypervariable region 1 (HVR1) of HCV E2 glycoprotein, an important target of the antibody response.

The circulating HCV particles in clinical isolates have shown to form complex with very low density lipoprotein (VLDL) and low density lipoprotein (LDL), hence the name lipoviroparticle (LVP). In addition to VLDL and LDL, the HCV particles also form complex with immunoglobulin (Nielsen *et al.*, 2006; Thomssen *et al.*, 1993). As a result of these virus-host complexes, the HCV serum particles are associated with wide range of buoyant density and sedimentation, which varies based on the method of analysis (Andre *et al.*, 2002; Choo *et al.*, 1995; Thomssen *et al.*, 1992). The infectious virions are found in the lowest density fractions (~ 1.06 g/ml), which are associated with LDL and VLDL, whereas the highest density particles (1.17-1.21 g/ml) display reduced infectivity (Choo *et al.*,

1995; Hijikata *et al.*, 1993b; Thomssen *et al.*, 1992). Interestingly, the intracellular HCV particles exhibit a higher buoyant density (1.15-1.20 g/ml) compared to the secreted virions (Gastaminza *et al.*, 2006), indicating a modification in the biochemical composition of HCV during viral egress.

1.1.3. HCV Transmission and Epidemiology

On a global scale more than 170 million people (~ 3%) are estimated to be infected with HCV according to the WHO report for 2011, making HCV the leading cause of chronic liver disease worldwide. In developing countries, HCV infection is largely transmitted by parenteral exposure to contaminated blood or blood products in the form of transfusion, unsafe therapeutic injections and intravenous drug use (Shepard *et al.*, 2005; Yang & Roberts, 2010). Since routine screening for HCV was implemented in 1990's, new cases of transfusion associated infections are nearly eliminated in economically developed countries and intravenous drug use accounts for the vast majority of the newly acquired infections (Thomson, 2009). Rare cases of perinatal transmission and sexual activity have also been documented however, data from different studies remain inconsistent (Alter, 2007). The incidence of HCV infection is difficult to determine as most acute infections are asymptomatic and therefore remain undetected. Furthermore, some countries lack adequate community-based disease reporting systems and studies are performed in selected groups and thus does not represent the general population such as blood donors or drug users (Alter, 2007; Lavanchy, 1999). Although endemic in most parts of the world, the prevalence of HCV infection is much higher (up to 15%) in some African and Asian countries as compared to America (1.7 %) and Europe (1.03 %) (Te & Jensen, 2010; Yang & Roberts, 2010) (Figure 1.1). Egypt, for example, has the highest reported seroprevalence rate for HCV, up to 20% in some cases. Discontinued since 1980s, this high prevalence is a result of the country's antischistosomal mass treatment campaign where many people acquired HCV infection due to re-use of contaminated syringes (Frank *et al.*, 2000). Moreover, the seven HCV genotypes vary in their geographical distribution. Genotypes 1-3 are very widely distributed while 4 is more prominent in Africa and Middle East, 5 in South Africa, genotype 6 is primarily found in South East Asia and genotype 7 in Central Africa (Gottwein *et al.*, 2009; Simmonds, 2004) (Figure 1.2). In addition to their geographical distribution, the HCV genotypes also differ in the severity of the disease and establishing persistent infection and also respond differently to therapy.

1.1.4. HCV Pathogenesis

In most cases HCV is a silent disease where the acute phase may be associated with jaundice but more often the infection remains asymptomatic. Unfortunately, in the majority of cases the infection becomes chronic with the most severe manifestations being liver cirrhosis and hepatocellular carcinoma (Hoofnagle, 1997).

1.1.4.1. Acute HCV

The majority of HCV infections are asymptomatic during the acute phase and therefore remain undiagnosed. Data available of acute phase infection comes from studies conducted on patients who acquired infection via needle stick injury or following transfusion. In very few cases patients develop non-specific symptoms including decreased appetite, fatigue, malaise, jaundice, anorexia and fever. The acute phase is considered the first 6 months after infection (Alter & Seeff, 2000; McCaughan *et al.*, 1992; Thimme *et al.*, 2001). The infection is marked by viral RNA detection within 1 to 2 weeks following exposure and elevated serum alanine aminotransferase (ALT) levels (Farci *et al.*, 1991; Thimme *et al.*, 2001). Resolution of acute infection occurs spontaneously in around 25% of the cases, which is marked by absence of HCV RNA and normal ALT levels (Hoofnagle, 1997). Cases of fulminant HCV causing liver failure during acute infection have been documented but are very rare (Farci *et al.*, 1996a). Fulminant hepatic failure is a dramatic clinical syndrome characterized by the massive necrosis of liver cells, which in the HCV-associated cases arises shortly following infection (2-8 weeks) (Farci *et al*., 1996; Kato *et al*., 2001). Such cases are though to be caused by highly virulent strains that circulate in the patients blood at high titers (10^5-10^8/ml) during the peak stages of infection (Farci *et al*., 1996; Kato *et al*., 2001). Interestingly, the only HCV strain (JFH1) to date that is capable of completing the full viral lifecycle in cell culture was isolated from a Japanese fulminant hepatitis patient (Wakita *et al*., 2005).

1.1.4.2. Chronic HCV

HCV infection persisting for more than six months is defined as chronic infection. In general around 60-80% of patients become chronically infected. The outcome of infection depends on a number of viral and host factors including the age of person at the time of exposure, ethnicity, gender, lifestyle, underlying disease, the viral genotype, viral quasispecies diversity and importantly the efficiency of infected individual's immune response. During the chronic stage patient may remain symptomless for decades, experiencing fatigue as their only symptom, but will eventually develop serious liver

conditions including hepatic steatosis, progressive fibrosis, compensated and later decompensated cirrhosis and ultimately hepatocellular carcinoma (HCC) (Poynard *et al.*, 1997). The level of liver enzymes ALT and viral load do not usually correlate with the amount of liver injury. The level of HCV viremia remains relatively constant with ~ 10^{12} virions produced daily (Neumann *et al.*, 1998). Around 20% of the chronically infected patient will develop liver cirrhosis within 20 years of infection. Once cirrhosis is established, the risk of developing HCC is 1-4% each year (Hoofnagle, 2002). How HCV induces cirrhosis and HCC is a matter of much debate. The majority of evidence indicates that HCV is not significantly cytopathic in the nonimmunosuppressed state and that hepatocellular damage likely reflects destruction of HCV infected cells by cytotoxic CD8+T cells (McGuinness *et al*.,1996; Brillanti *et al*., 1993). However, patients with impaired cellular responses suffer a more severe course of liver disease than immunocompetent patients (Einav & Koziel, 2002). Thus, these observations suggest that liver cirrhosis induced by HCV infection could be both immune-mediated and viral-derived. The mechanism through which HCV causes HCC is unknown however, there is compelling evidence regarding the potential oncogenic properties of core protein from studies in transgenic mice. Approximately 30 % of transgenic mice expressing core protein developed HCC between 16-19 months from birth, compared to a 0 % incidence in the control mice. Immunologic analysis found that the expression of core protein was higher in the tumours cells than in surrounding normal hepatocytes (Moriya *et al*., 1997).

1.1.5. Immunology

1.1.5.1. Innate Immunity

Both the innate and the adaptive immunity play a crucial role in clearing HCV infection. The innate immune response, which is the first line of defence, activates very early during the incubation phase of infection and slows down the progression of infection until the adaptive immune response gets activated. Main components of innate immunity include a network of cells including natural killer (NK) cells, monocytes, macrophages, leukocytes and dendritic cells together with production of type 1 interferons and other interferon stimulated genes (ISGs) by hepatocytes. Toll-like receptor 3 (TLR3) and retinoic-acid inducible gene I (RIG-I) are pattern recognition receptors involved in anti-HCV innate immunity (Saito *et al.*, 2008; Wang *et al.*, 2009). Detection of double stranded RNA and the polyuridine motif of HCV 3' untranslated region (UTR) triggers these two pathways, which leads to the phosphorylation and activation of interferon regulatory factor (IRF)-3. IRF-3 activation results in IFN- β production and secretion from the infected cell. Secreted IFN-β binds type 1 IFN-α /β receptors, resulting in autocrine/paracrine activation of JAK-STAT signalling pathway, which in turn results in the expression of IFN-α and many dozens of ISGs causing an antiviral state in infected and neighbouring hepatocytes. Expression of ISGs such as OAS1 degrades viral and cellular RNA, ADAR1 induces mutation in viral dsRNA and destabilizes secondary viral RNA structures and P56 and PKR inhibits translation of viral and host RNAs. ISG further results in amplification of the IFN response through the upregulation of IFN-β production (Sumpter *et al*., 2005).

Evidence suggests that HCV attenuates the IFN response at multiple levels and thereby may subvert the host innate immunity. In particular, the HCV non-structural protein NS3/4A protease disrupts RIG-I and TLR3 activation pathway by cleaving adaptor molecules TRIF and thereby blocking IRF-3 activation involved in IFN-β production (Foy *et al.*, 2005). Furthermore, the HCV core protein has been shown to interact with certain signalling pathways and impairs the induction of ISG, while the HCV E2 and NS5A proteins disrupt the downstream function of ISG (Rehermann, 2009). HCV core protein inhibits STAT1 activation and thereby interferes with JAK/STAT signalling and ISG expression. NS5A has attracted considerable interest because of its potential role in modulating the interferon response. The first data to support this hypothesis came from Japan where a correlation between the high rate of mutations in NS5A, especially in NS5A ISDR (IFN-α sensitivity determining region), was identified to be associated with resistance to IFN-α treatment (Enomoto *et al*., 1996). Furthermore, HCV NS5A and also

E2 bind to and antagonize the dsRNA-activated PKR suggesting a possible mechanism by which HCV may evade the antiviral effect of IFN (Francois *et al*., 2000). Binding of HCV E2 to the cellular receptor CD81 have also shown to suppress the function of NK cells. NK cells are involved in direct lysis of infected cells and produce IFN-γ which has shown to have direct antiviral effect. The attenuation of innate immune signalling profoundly affects the subsequent activation of adaptive immune response as cytokines produced during innate immune response trigger the activation of adaptive immunity.

1.1.5.2. Adaptive Immunity

The adaptive immune response is more specific and can take weeks to develop following infection. The two arms of the adaptive immune response are the cell-mediated immune response (comprised of T-lymphocytes) and the humoral immune response (comprised of B-lymphocytes).

1.1.5.2a. Cell-mediated Immunity

The main components in the cell-mediated immune response are CD4 helper and CD8 cytotoxic T lymphocytes (CTL), which appear 5-10 weeks, post infection. In both chimpanzees and human, a strong, broad and persistent HCV specific T-cell response during the acute phase of infection is associated with viral clearance, although the exact role of each subset of T-cells is not entirely clear. A broad response is defined as T-cells targeting multiple epitopes and most HCV proteins (Bowen & Walker, 2005; Gruner *et al.*, 2000; Lechner *et al.*, 2000; Thimme *et al.*, 2001; Thimme *et al.*, 2002). In particular, a vigorous and long lasting CD4 T cell response has shown to be important in resolving acute infection and recurrence of viremia has been associated with CD4 T cell loss (Gerlach *et al.*, 1999; Urbani *et al.*, 2006). CD8 T cells response can develop in the absence of CD4 T cell response without being able to control viremia, however, absence of CD8 T cells appears to lead to the establishment of chronic infection (Gruener *et al.*, 2004). CD8 T cells may eliminate virus infection by non-cytolytic mechanism involving cytokines such as IFN-γ or by direct cytotoxicity which is associated with rise in serum ALT levels and fall in viremia (Cooper *et al.*, 1999; Shoukry *et al.*, 2003; Thimme *et al.*, 2001).

The broad T cell response is often lost during chronic stage and virus-specific T cells are found in low frequencies in the blood targeting a few epitopes (Rosen *et al.*, 2002).

Although, HCV specific T cells can be derived from the liver of chronically infected patients, their role in controlling viral replication and contribution to the immunopathology are not fully clear. CD4 and CD8 memory T cells can be detected in individuals with resolved acute HCV infection, albeit at extremely low frequencies. Although immunological memory does not protect against re-infection, it does reduce the risk of developing chronic infection upon re-exposure (Farci *et al.*, 1992; Lai *et al.*, 1994; Mehta *et al.*, 2002). Furthermore, the immunological memory appears to be strain specific and therefore limited due to viral diversity (Sugimoto *et al.*, 2005).

1.1.5.2b. Humoral Immunity.

HCV specific antibodies are usually detectable 7 to 31 weeks post primary infection and persist for variable time. A large number of antibodies are produced by the B-lymphocytes during infection, however, data supporting their protective role in viral clearance is so far limited (Pawlotsky, 2004; Pawlotsky *et al.*, 1999). In some individuals responses are absent while in some cases responses are present but appear to be inadequate in resolving infection. Some studies reveal that naturally generated HCV specific antibodies are not necessarily required to resolve infection, as spontaneous resolution has been observed in chimpanzees without seroconverion (Cooper *et al.*, 1999). In addition, high-titer antibodies have been observed far more commonly in chronic patients than in those who clear infection (Bartosch *et al.*, 2003; Logvinoff *et al.*, 2004; Meunier *et al.*, 2005). Also antibodies against HCV seem to play a limited role in preventing secondary infection in both humans and chimpanzees (Farci *et al.*, 1992; Lai *et al.*, 1994). However, treatment of HCV with antibodies has proved to be effective in blocking the infection of target cells *in vitro* (Farci et al., 1994). Furthermore, administration of immune globulin associated with transfusion revealed to be protective in preventing NANB hepatitis if administered before exposure to the virus (Yu *et al.*, 2004). Also serum particles associated with immunoglobulins have proved to be less infectious than free virus particles (Hijikata *et al.*, 1993b).

In a clinical study Lavillette and colleagues characterised the neutralising immune response in a cohort of 17 patients acutely infected with single HCV viral strain. It was found that the emergence of strong neutralising immune response correlated with a substantial loss of viremia, while lack of neutralising response resulted in production of high level of viral replication (Lavillette *et al.*, 2005b). In a separate study, Pestka and colleagues studied a cohort of 49 women infected in a single-source outbreak of HCV

following administration of contaminated immunoglobulin preparations during pregnancy (Pestka *et al.*, 2007). The data revealed that a rapid neutralising antibody induction in the acute phase of HCV infection correlated with viral clearance as assessed with HCV pseudoparticles. However, soon after resolving infection this antibody response was lost. In contrast, absence of, reduced or delayed humoral response during acute phase resulted in a chronic state. Both these studies, however, lack the involvement of cellular immune response. Clearly both the cellular and the adaptive immune response play an equally important role in clearing viral infection.

Although presence of antibodies to all HCV antigens has been reported, the natural target of neutralising antibodies are the two envelope glycoproteins (E1/E2) which also happens to be the most variable region within the HCV genome. Interestingly, antibodies extracted from serum of HCV infected patients are usually directed against hypervariable region 1 (HVR1) in E2, which is under constant selective pressure and thus evolve constantly. Hence, these circulating antibodies exhibit strain specific neutralizing activity. However, studies using the HCV pseudoparticle system have shown that the presence of certain neutralising antibodies exhibiting cross-reactivity have the potential to prevent infection with pseudoparticles harbouring homologous and heterologous HCV glycoproteins (Bartosch *et al.*, 2003; Meunier *et al.*, 2005; Meunier *et al.*, 2008a).

1.1.6. Disease Progression

HCV is believed to be non-cytopathic virus that infects and persists in target cells without inducing inflammation or damage (Thimme *et al.*, 2002). This is evident by the fact that despite high level of viremia no increase in serum ALT has been seen in patients following liver transplantation or in cases of accidental infection with contaminated needles during health care practice (Thimme *et al.*, 2001). It is only HCV genotype 3 that has been associated with steatosis, the accumulation of lipids in hepatocytes (Pawlotsky, 2004). It is believed that liver injury and disease progression is mainly immune mediated as T-cell infiltration of the liver during acute infection correlates with elevated serum ALT level. However, rapid disease progression has been noted in immunodeficient and immunosuppressed patients, implying a possible role of viral factors in HCV pathogenesis. Indeed, cytopathic effects have been observed in cells infected with the JFH1 cell culture infectious virus (Rehermann, 2009). Together, the data suggest that liver damage following HCV infection is a consequence of both the host immune response and a direct viral effect.

1.1.7. Diagnosis of HCV Infection

HCV infection during the acute phase is rarely diagnosed as a majority of the people remain asymptomatic during this phase. When suspected, the diagnosis involves both serological and virological detection methods.

Serological tests detect presence of antibodies against different HCV epitopes in an enzyme immunoassay, given that seroconversion has occurred and sufficient antibodies are available for detection. This method can measure the level of antibody present in the serum/plasma but cannot determine whether the infection is ongoing (Chevaliez & Pawlotsky, 2007).

Virological assays rely on virus nucleic acid amplification and give both a qualitative and quantitative analysis of viral load. Following extraction from plasma/serum, HCV RNA is amplified using either the polymerase chain reaction (PCR), transcription mediated amplification (TMA) or branched DNA (b-DNA) assay (Chevaliez & Pawlotsky, 2007).

The serological or the virological method can be used to determine the specific HCV genotype a person is infected with, which is important since the genotype determines the dose and the duration of treatment. The HCV genome sequencing is particularly useful during patient follow up in order to identify resistance mutations to antiviral therapies. Lastly, a negative HCV RNA result should be demonstrated on multiple occasions to confirm the clearance of infection from a previously HCV positive patient (Chevaliez & Pawlotsky, 2007).

1.1.8. Treatment of HCV Infection

Although 20% to 25% of acute HCV infection is eradicated spontaneously by the host immune response, there is a very small chance of spontaneous resolution once infection becomes chronic. At present no vaccine is available for HCV and the standard treatment for chronic HCV is combination therapy of pegylated interferon alpha (IFN-α) and ribavirin. Several factors including patient gender and age, HCV genotype, viral load and stage of liver fibrossis are predictive of treatment outcome. For those who develop decompensated liver cirrhosis or HCC, liver transplantation is the only option available.

1.1.8.1. Current Treatment

At present, a combination of IFN-α injection and ribavirin pills are adminstered for a period of 24 (genotype 2 or 3) or 48 weeks (genotype 1, 4 or 6) depending on the HCV genotype (Di Bisceglie & Hoofnagle, 2002). Fewer data are available on the treatment outcome of genotype 4, 5 and 6. The combination therapy results in sustained virological response (SVR) in 40%-50% (genotype 1 or 4) to 80% (genotype 2 or 3) of patients. Notably, genotype 1 infections have the lowest SVR of between 25-20% (Soriano *et al.*, 2009; Tsubota *et al.*, 2011). SVR is defined as the loss of detectable HCV RNA during treatment and its continuing absence at least 6 months after stopping therapy. Unfortunately, apart from having limited response rate, the combination of IFN-α and ribavirin is highly costly and further contraindicated in many patients due to severe side effects including fever, headache, myalgia, haemolytic anaemia and severe depression (Fried *et al.*, 2002). Consequently, more effective and less toxic therapies to treat HCV are urgently required.

The mechanism by which IFN and ribavirin exert their therapeutic effect is currently not clear. IFNs are a group of endogenous glycoprotein that play crucial role in the innate antiviral immune response and have antiviral and immunomodulatory properties. IFN-α is thought to act as endogenous IFN and induces expression of numerous genes which trigger an antiviral innate response (Feld & Hoofnagle, 2005). Interestingly, non-responsive patients tend to have a high constitutive expression of ISGs that cannot be further stimulated by treatment (Sarasin-Filipowicz *et al.*, 2008). Moreover, several polymorphisms at the IL28 locus, which encodes a type of IFN-λ, have been reported to be associated with the therapy outcome (Ge *et al.*, 2009). Ribavirin is a guanosine analogue and interestingly is effective only when used in combination with IFN-α. Being a nucleoside analogue it is hypothesised that Ribavirin may inhibit HCV replication after being misincorporated by HCV polymerase during RNA synthesis (Feld & Hoofnagle, 2005). Another mechanism by which Ribavirin is thought to exert its anti-viral effect is by acting as a RNA mutagen causing an accumulation of mutations that may lead to collapse in viral fitness termed “error catastrophe” (Crotty *et al.*, 2001).

1.1.8.2. Novel Antivirals

A major challenge in developing new antiviral compounds is that they should be more effective, better tolerated and preferably allow shorter treatment periods than the current therapy. In addition, the risk of developing drug resistance due to vast numbers of

quasispecies present during the course of infection presents another major obstacle that needs to be considered. With better insight in HCV molecular virology and HCV replication, research is focused on the production of STAT-C (specifically targeted antiviral therapy for hepatitis C) compounds as well as improving the current therapy with pegIFNα/ribavirin. In addition, immunomodulatory agents other then IFN that stimulate host innate and adaptive immune response are also being tested. The role of neutralising antibodies as a potential therapeutic approach will be discussed in section 1.5.

In terms of modifying current treatment, Human Genome Sciences (HGS) in collaboration with Novartis AG developed Albuferon, a recombinant drug consisting of IFNα conjugated to human albumin. Conjugation to albumin prolongs the half life of the compound in the blood and thereby allowing a less frequent dosing (Qureshi *et al.*, 2009). After obtaining promising data from their phase III clinical trial HGC filed an application to the FDA (Food and Drug Administration) in 2009 for marketing approval.

STAT-C compounds specifically and directly target HCV life cycle. Table 1.1 lists the STAT-C drugs currently in clinical trial pipeline. The most popular targets are the NS3/4A serine protease and NS5B RdRp. Indeed, protease inhibitors (PI) (telaprevir and boceprevir) directly targeting NS3/4A serine protease has recently been approved by FDA. NS3/4A is the major HCV protease required for cleavage of viral polyprotein during HCV replication and is also believed to be the primary HCV protein responsible for evasion of IFN response. These PIs exert their effect by blocking the NS3/4A ability to cleave the HCV polyprotein and restore innate immune signalling (Rosen, 2011). Clinical data suggest that telaprevir or boceprevir combined with current pegIFNα/ribavirin therapy improve SVR by 79% and 63%, respectively, compared to 50% SVR with pegIFNα/ribavirin alone. The triple therapy has specifically shown improved SVR in difficult to treat or non-responder HCV genotype 1 infected patients (Jazwinski & Muir, 2011; Rosen, 2011; Tsubota *et al.*, 2011). Main adverse effects seen with telaprevir and boceprevir are skin rashes, nausea and anaemia.

NS5B polymerase inhibitors include nucleoside analogue (chain terminators) and non-nucleoside analogues (allosteric inhibitors). Among nucleoside analogue, R-1626 demonstrated potent antiviral activity against all genotypes in early *in vitro* experiments (Soriano *et al.*, 2009). However, during phase II clinical trials, patients suffered severe neutropenia and high rate of HCV infection relapse after completion of therapy and thus in

2008 its further development was halted (Pockros *et al.*, 2008). An additional nucleoside analogue that so far appears to be safe and well tolerated when combined with pegIFNα/ribavirin is R7128, which is currently being evaluated in clinical phase II trials. Lastly, BMS-824 potently inhibits HCV replication by binding NS5A protein and demonstrated promising efficacy in phase I clinical trial (Gao *et al*., 2010). Extensive research is being put into developing IFN-free regimen for treating HCV, however so far in most cases STAT-C drugs under trial would have to be combined with IFN treatment to prevent emergence of resistance strains.

Apart from directly targeting viral proteins, another approach is to target cellular factors involved in HCV life cycle. This method will eliminate the risk of developing drug resistance, however, major drawback of targeting cellular factors is the risk of cytotoxicty (Khattab, 2009).

Lastly, antiviral therapy or immunomodulatory drugs combined with therapeutic vaccine might prove efficient especially when considering the variability of HCV. Vaccine research is focused on generating cytotoxic T cell response as well antibody response. Types of vaccines under development include epitope vaccines, vector vaccines, recombinant protein vaccines and DNA vaccines (Halliday *et al.*, 2011). List and progress of vaccines currently in clinical trials is reviewed in table 1.2.

1.2. HCV Genome Organization and Function

HCV contains a small single stranded RNA genome (9.6kb) of positive polarity and consists of a single ORF that contains 9024 to 9111 nucleotides depending on the genotype. The ORF is flanked at the 5' end by an untranslated region (UTR) that functions as an internal ribosome entry site (IRES) and at the 3' end by a highly conserved sequence essential for genome replication. The polyprotein is cleaved by viral and cellular protease to yield structural proteins core, E1 and E2 and non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B. The p7 protein, currently unassigned to either category, separates the structural proteins from the nonstructural proteins (Figure 1.3).

1.2.1. The 5' UTR

The 5' UTR region of HCV comprising the first 341 nucleotides is a highly conserved sequence involved in replication and translation of viral genome (Bukh *et al.*, 1992; Friebe *et al.*, 2001; Honda *et al.*, 1999). The 5' UTR contains 4 highly structured domains (termed I-IV) consisting of numerous stem-loop motifs (Honda et al., 1996a). Domain II-IV together with 12-40 nucleotides of the core sequence forms the IRES that directly recruits 40s ribosomal unit to the AUG codon and initiates protein translation in a cap-independent manner involving eukaryotic translation factor elF3 (Honda *et al.*, 1999; Reynolds *et al.*, 1996). Domain IV is not required for ribosome binding, in fact the structural stability of this region is negatively correlated with the translation efficiency (Honda *et al.*, 1996b). Domain I seems to be dispensable for overall IRES activity but may have a regulatory role in translation efficiency (Honda *et al.*, 1996a). Apart from its importance in protein translation, the 5' UTR also serves an important role in RNA replication. Domain I and II are sufficient for viral RNA synthesis, although the efficiency of this process is enhanced by the presence of the complete 5' UTR (Friebe *et al.*, 2001; Kim *et al.*, 2002). Apart from translation factor elF3, the 5'UTR is also known to interact with a number of other cellular proteins (PTB, La, hnRNP L and PCBP) that are important in regulating both replication and translation of HCV viral genome (Ali & Siddiqui, 1995; 1997; Fukushi et al., 2001; Hahm et al., 1998; Jopling et al., 2005). Moreover, interactions between the liver-specific microRNA, miR-122, and domain I and II of the 5'UTR have shown to be essential for efficient HCV RNA replication both in infected chimpanzees and during infection in cultured cells (Jopling *et al*, 2005; Jopling *et al*, 2008; Lanford *et al*, 2010; Machlin *et al*, 2011). It is hypothesized that miR-122 may protect HCV RNA from nucleolytic degradation or may prevent the activation of enzymes that induce innate immune response (Machlin *et al*, 2011). Due to its considerable importance and conservation, the 5' UTR constitutes a promising target for RNA interference based therapy (Kanda et al., 2007).

1.2.2. The 3' UTR

3' UTR contains approximately 225 nucleotides and is organised into three domains consisting of a variable region of around 40 nucleotides, a poly (U/UC) stretch that regulates replication and a highly conserved 98-nucleotide X-tail (Kolykhalov *et al.*, 1996). Based on biochemical and structural studies, 3' UTR is proposed to harbour two stem loops in the variable region (VSL1 and VSL2) and three stem loops in the X-tail (SL1, SL2 and SL3) (Blight & Rice, 1997; Tanaka *et al.*, 1996). VSL1 and VSL2 are dispensable for

RNA replication in cell culture and for HCV infectivity in chimpanzees as small deletions within these regions significantly reduce the efficiency of viral replication but are not lethal (Friebe & Bartenschlager, 2002; Yanagi *et al.*, 1999). However, the U/UC region and X-tail are essential for HCV RNA replication as deletions or substitutions within any of these regions often prove lethal. At least 50 nucleotides of the U/UC stretch are required to allow viral replication (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003). Moreover, cis-acting replication elements (CREs) have been identified in the NS5B coding sequence that have shown to interacts with 3'UTR. The NS5B coding sequence contains a predicted cruciform structure (5BSL3) consisting of three stem loops, 5BSL3.1, 5BSL3.2 and 5BSL3.3. Among these 5BSL3.2 have shown to be essential for RNA replication (Friebe *et al*., 2005). Apart from its role in replication, 3'UTR is also thought to enhance IRES dependent translation of viral RNA by increasing the efficiency of termination (Bradrick *et al.*, 2006; Song *et al.*, 2006).

1.2.3. Core

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

HCV-related steatosis (McLauchlan, 2000; 2009; Roingeard & Hourioux, 2008). However, all these observations are derived from heterologous overexpression experiments and still need to be confirmed in an infectious system.

The translation of an alternative reading frame in the core coding sequence can also yield a small protein (~17 kDa), called ARFP or F protein. However, the F protein is dispensable for RNA replication in the HCVcc system and its possible function is unclear so far (McMullan *et al*., 2007; Vassilaki *et al*., 2008). However, specific immune responses targeting the F protein have been detected in some chronically infected patients (Xu *et al*., 2001).

1.2.4. p7

P7 is a small (7 kDa) intrinsic membrane spanning protein with a cytoplasmic loop and its N- and C- termini oriented towards the ER lumen (Carrere-Kremer *et al.*, 2002). It belongs to the viroporin family of proteins, which is able to oligomerise *in vitro* and form an hexameric cation-selective ion channel (Griffin *et al*., 2003; Luik *et al.*, 2009; StGelais *et al*., 2007), which constitutes a potential antiviral target. Indeed, this p7 function can be inhibited by amantadine, a compound that inhibits the influenza A encoded M2 ion channel activity (Griffin *et al.*, 2003). Furthermore, several inhibitory compounds known to interfere with p7 ion channel activity also repress the release of infectious virus particles in the HCVcc system (Griffin *et al.*, 2008; Steinmann *et al.*, 2007b). Indeed, p7 has been shown to be critical for the release of infectious HCVcc particles, but has no role in RNA replication (Jones *et al.*, 2007; Steinmann *et al.*, 2007a). Recently, it was demonstrated that p7 has a proton-selective ion-channel activity, the function of which is directly responsible for the enhancement of infectious virus production (Wozniak *et al.*, 2010).

1.2.5. NS2

NS2 is a hydrophobic integral membrane protein responsible for the autoproteolytic cleavage at the NS2/NS3 junction of the polyprotein. The 23 kDa protein is believed to harbour multiple membrane spanning domains that localise NS2 to the ER membrane. However, the exact membrane topology and mechanism of translocation remains controversial (Santolini *et al.*, 1995). The catalytic activity of the NS2-3 cysteine protease resides in the C-terminal half of NS2 and requires the N-terminal one-third of NS3, notably

through the zinc binding domain (Grakoui *et al.*, 1993; Hijikata *et al.*, 1993a). The Crystal structure of NS2/3 proposes three crucial residues (His143, Cys184 and Glu 163) forming the catalytic triad that together form an active site (Lorenz *et al.*, 2006). Subgenomic replicons not encoding the NS2 protein replicate efficiently in Huh-7 cells suggesting that NS2 is indispensible for RNA replication (Lohmann *et al.*, 1999). However, full length NS2 is critical for the assembly of infectious particles in HCVcc, independent of its catalytic activity (Jirasko *et al.*, 2008; Jones *et al.*, 2007). There is increasing evidence supporting a crucial role for NS2 in factoring the coordination of virus assembly through stable interactions with E1, E2, p7, NS2, NS3 and NS5A (Jirasko *et al.*, 2010; Ma *et al.*, 2011; Popescu *et al.*, 2011; Stapleford & Lindenbach, 2011). The use of chimeric construct consisting of NS3-NS5B region from HCV strain JFH1, fused with the structural genes from other genotypes, have shown that the position of the intra/inter-genotypic junction within NS2 affects infectious particle production (Pietschmann *et al*., 2006). For most chimeras, a junction between the first and second TMD is optimal, indicating that interactions between the N-terminus of NS2 and p7 or the structural protein is important for virus production (Pietschmann *et al.*, 2006). Additionally, cell culture adaptive mutations that enhance virus production have been found within the NS2 coding region (Russell *et al*., 2008; Yi *et al*., 2007).

1.2.6. NS3 and NS4A

NS3 is a multifunctional 70 kDa protein harbouring a serine-type protease within its N-terminal, while the two-third of C-terminal features a RNase helicase/NTPase motif. Both the NS3 serine protease and the helicase activities require NS4A as a cofactor. NS4A is the smallest HCV-encoded protein (6 kDa) and consists of an N-terminal hydrophobic region, a central domain and a C-terminal acidic region. The crystal structure of NS3/NS4A revealed that the central part of NS4A is mandatory for proper NS3 folding (Bartenschlager *et al.*, 1995; Lin *et al.*, 1995; Tai *et al.*, 1996) while its N-terminal transmembrane domain (TMD) allows stabilisation and localization of NS3 at the ER membrane (Wolk *et al.*, 2000). The C-terminal acidic region of NS4A influences NS5A phosphorylation and HCV RNA replication (Lindenbach et al., 2007). Upon association with the ER membrane, NS3 serine protease facilitates HCV polyprotein processing *in cis* at the NS3/NS4A and *in trans* at the NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions. The catalytic triad is formed by residues His 57, Asp 81 and Ser 139 and mutation of any of these amino acids abolishes NS3 mediated cleavage (Bartenschlager *et*

al., 1993; Grakoui *et al.*, 1993). In addition to processing viral proteins, NS3/4A protease also cleaves cellular targets, in particular, Cardif and TRIF and thereby blocking the antiviral innate immune response triggered by RIG-I and TLR-3 at the early stage of infection (Li *et al.*, 2005; Meylan *et al.*, 2005). The NS3 helicase-NTPase has several functions, including RNA-stimulated NTPase activity (Kim *et al.*, 1998; Yao *et al.*, 1997), RNA binding and unwinding of RNA regions of extensive secondary structure (Serebrov & Pyle, 2004). Although the structural data has been determined, the mode of action and precise role of the helicase in the virus life cycle remains unclear. Recent studies with intergenotypic chimeras have identified compensatory mutations within the NS3 helicase domain that rescue the assembly of virus particles of chimeras otherwise defective for virion production (Ma *et al.*, 2008; Phan *et al.*, 2009; Yi *et al.*, 2007).

1.2.7. NS4B

HCV RNA replication takes place in specialized membrane derived vesicles called membranous web or membrane associated foci (MAF) where all HCV non-structural proteins accumulate (Egger *et al.*, 2002; Gosert *et al.*, 2003; Gretton *et al.*, 2005). Formations of these membranous webs are induced by NS4B. NS4B is a highly hydrophobic 27 kDa integral membrane protein tightly associated with the ER membrane (Egger *et al.*, 2002; Hugle *et al.*, 2001). The central portion of NS4B is predicted to harbour four TMDs and a N-terminal amphipathic helix that are responsible for its association with membrane (Elazar *et al.*, 2004; Hugle *et al.*, 2001; Lundin *et al.*, 2003). Disrupting the ability of NS4B to bind to ER leads to loss of non-structural proteins at the MAF in addition to abolishing replication of the viral genome (Elazar *et al.*, 2004). Thus, these NS4B induced ER membrane alterations likely create an environment suitable for viral RNA replication.

1.2.8. NS5A

NS5A is a phosphoprotein that can be found in basally phosphorylated (56 kDa) and hyperphosphorylated (58 kDa) form and plays an important role in virus replication and regulation of cellular pathways (Macdonald & Harris, 2004). NS5A is divided into three domains that are separated by low complexity sequences (LCS) (Lemon *et al.*, 2010).The N-terminal region of NS5A contains an amphipathic alpha-helix that mediates membrane association and possibly localises the protein to replication complexes. Disruption of this

helix structure leads to a loss in membrane binding of NS5A and is lethal for HCV RNA replication. (Brass *et al.*, 2002; Elazar *et al.*, 2004; Penin *et al.*, 2004a). Downstream from the helix motif resides domain I which contains a zinc binding site that is required for HCV RNA replication (Tellinghuisen *et al.*, 2004). The crystal structure of domain I reveals that NS5A may dimerise to create a basic groove amenable for RNA binding. Indeed, NS5A is able to bind the poly (U/UC) stretch present in 3'UTR of HCV (Huang *et al.*, 2005). Moreover, domian I promotes NS5A association with lipid droplets (LDs), an event that is crucial for HCVcc assembly (Miyanari *et al*., 2007). Unlike domain I, domain II and III are poorly characterised. It has been noted that many cell culture adaptive mutations enhancing replication affect serine residues within NS5A and thus, reduce its hyperphosphorylation. This indicates that the phosphorylation state of NS5A modulates the efficiency of HCV RNA replication (Appel *et al.*, 2008; Tellinghuisen *et al.*, 2007; Tellinghuisen *et al.*, 2008).

NS5A is able to further interact with several other cellular signalling pathways such as mitogenic signalling, apoptosis, phosphatidylinositol 3-kinase-mediated signalling and calcium/reactive oxygen signalling pathways, which may be related to HCV pathogenesis (Macdonald *et al*., 2005; Macdonald *et al*., 2003).

1.2.9. NS5B

NS5B is the RdRp that promotes synthesis of both the positive strand RNA and the negative strand intermediate in the absence of other viral or cellular factors *in vitro* (Behrens *et al.*, 1996; Lohmann *et al.*, 1997). A specific interaction between NS5B and the 3'UTR has been reported (Cheng *et al.*, 1999). The enzyme lacks a proofreading function, which contributes to the high genetic variability of HCV (Domingo *et al.*, 1996). NS5B is an integral membrane protein with a cytosolic orientation and associates to the ER membrane via a highly conserved C-terminal TMD (Ivashkina *et al.*, 2002). The membrane association appears to be crucial for NS5B function in cells (Moradpour *et al.*, 2004). NS5B has the classical 'fingers, palm, thumb' polymerase structure (Ago *et al.*, 1999; Bressanelli *et al.*, 1999; Lesburg *et al.*, 1999; Poch *et al*., 1989), where the interaction between fingers and thumb form a tunnel for binding the RNA template and the central palm domain harbours the catalytic GDD motif.

NS5B has been shown to interact with cellular components that modulate its polymerase activity. For example, interaction with cyclophilin B seems to enhance viral RNA synthesis through modulation of the RNA binding capacity of NS5B (Watashi *et al.*, 2005).

Cyclophilin A is also required for HCV RNA replication and directly interacts with NS5A, which is speculated to induce a conformation necessary for replicase formation and activity (Kaul *et al*., 2009). Interestingly, drugs targeting cyclophilins are being developed as new promising HCV antiviral targets (Table 1.1). For example, cyclophilin A is targeted by the cyclosporine analogue DEBIO-025, which has shown potent anti-HCV activity (Kaul *et al*., 2009).

1.3. Models to study HCV

1.3.1. Animal models

Many features of the HCV infection are similar between human and chimpanzees and for more than a decade the chimpanzee was the only animal model available to study the course of HCV infection (reviewed in Bukh, 2004). Due to the silent nature of HCV disease, acute infections in human usually go unnoticed and the chimpanzee model provided the advantage of monitoring the progression of HCV disease from beginning to end. Studies in the chimpanzee shed further light on different aspects of the cellular immune responses and their role in disease outcome (Jo *et al.*, 2011). Although chimpanzees provide a well controlled clinically relevant model for the study of HCV, it is extremely limited in its availability, is highly expensive and due to ethical issues has not been used for anti-HCV drug discovery.

Many attempts have been made to establish a small animal model for HCV that is widely available, affordable and reproducible and one that represents most aspects of human HCV infection and disease. Chimeric (xenograft) mice harbouring human hepatocytes uPA/SCID (urokinase plaminogen activator/ severe combined immunodeficiency) is probably the most relevant small animal model set up so far (Meuleman & Leroux-Roels, 2008) Inoculation of SCID mice with HCV derived from human serum has shown to support prolonged HCV infections with clinically relevant titers (Mercer et al., 2001; Meuleman et al., 2005). Most importantly, SCID mice have proven useful in the evaluation of efficacy and toxicity of antiviral compounds (Kremsdorf & Brezillon, 2007).

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

1.3.2. Replicons

Detailed studies on the HCV life cycle were hampered, primarily due to the incapacity of HCV serum particles to establish infection in cell culture. Nevertheless a major breakthrough came in 1999 with the development of a selectable, subgenomic replicon (SGR) system that allowed HCV RNA replication in cell culture (Lohmann *et al.*, 1999). The first functional SGR, derived from a genotype 1b strain called Con1, was a bicistronic construct. In this system the first cistron encoded neomycin phophotransferase (neo^r) gene, translated under the control of the HCV IRES, resulting in G418 resistance. The second cistron encoded non-structural proteins (NS3-NS5B) directed by a heterologous EMCV IRES. Upon electroporation of Huh-7 cells with *in vitro* synthesized SGR RNA and selection with G418 resulted in low numbers of surviving cell colonies (Figure 1.6). The low frequency of cells supporting SGR RNA replication was partly because replicon RNAs had to acquire adaptive mutations for efficient replication in the Huh-7 cells and secondly due to limited host cell permissiveness. Adaptive mutations tend to cluster within the non-structural proteins NS3, NS4B, NS5A and NS5B. Several replication-enhancing mutations resulted in loss of NS5A hyperphophorylation (Blight *et al.*, 2000). Introducing adaptive mutations into the parental replicon significantly enhanced RNA replication level to various extents (Blight *et al.*, 2000; Krieger *et al.*, 2001; Lohmann *et al.*, 2001). Furthermore, curing Huh-7 cells supporting viral replication by IFN treatment identified cell clones that supported greater levels of RNA replication. For the Huh-7.5 cell clone, this phenotype was thought to be due to a defective IFN response in these cells caused by a mutation in the RIG-I gene (Sumpter *et al.*, 2005). The aforementioned advancements in

the understanding of the SGR system through viral adaptive mutations and highly permissive cell clones eventually led to the development of autonomously replicating full-length replicons harbouring the entire HCV ORF (Blight *et al.*, 2003; Ikeda *et al.*, 2002; Pietschmann *et al.*, 2002).

1.3.3. HCVpp

Availability of stable cell lines harbouring autonomously replicating SGR RNAs helped define the functional replication unit, as well as the viral and cellular determinants involved in replication complex and their subcellular localization. It was however, the development of HCV pseudoparticle system (HCVpp) that enabled the study of viral entry (Bartosch *et al.*, 2003; Drummer *et al.*, 2003; Hsu *et al.*, 2003) (Figure 1.6). HCVpp are engineered viral particles consisting of a retroviral or lentiviral core enveloped by a lipid bilayer. Present within the envelope are HCV E1 and E2 gene products, which confer entry of the pseudoparticle into target cells. To quantify the number of infected cells, a reporter gene, such as luciferase or green fluorescent protein (GFP), is also included. HCVpp harvested from transfected 293T cell supernatant can be used to infect naive Huh-7 cells, where entry is mediated by HCV glycoproteins and can be blocked by anti-E1 or anti-E2 specific antibodies (Bartosch *et al.*, 2003; Hsu *et al.*, 2003; Lavillette *et al.*, 2005b). Since HCVpp are replication deficient and support only a single infection event, the quantification directly reflects the HCVpp capacity to enter the cells. Studies have shown HCVpp and HCVcc virions share the same pH-dependent internalisation and fusion steps of the entry process (Tscherne *et al*., 2006; Sharma *et al*., 2011). More importantly, HCVpp representing glycoproteins of all major genotypes have been successfully generated (Lavillette *et al.*, 2005b; Owsianka *et al.*, 2005) and have allowed in depth studies of virus binding, attachment and internalization and also helped in identifing novel HCV receptors.

1.3.4. HCVcc

In 2005, three research groups published reports on the development of a cell culture system capable of producing infectious HCV particles (HCVcc) (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). Each system relied on the properties of a unique genotype 2a isolate, known as JFH1. SGRs derived from JFH1 were previously shown to be capable of efficient RNA replication in multiple liver and non-liver specific cell lines

without the need for adaptive mutations (Kato *et al.*, 2003; Kato *et al.*, 2005). Later, it was demonstrated that RNA transcripts from the full-length JFH1 genome transfected into Huh-7 cells produced infectious virus (Wakita *et al.*, 2005) (Figure 1.6). However, virus titers released from transfected or infected cells were moderate at best. Higher virus titers were shown to be achieved when JFH1 was propagated in the Huh-7.5.1 cell-line (Zhong *et al.*, 2005). Also, a chimeric JFH1 clone containing the core to NS2 region of HCV strain J6 fused to the JFH1 NS3-NS5B was found to be more infectious than full-length JFH1 (Lindenbach *et al.*, 2005). The virus release was further improved by altering the fusion junction from NS2/NS3 to a crossover point that resides after the first TM segment of NS2 (Pietschmann *et al.*, 2006). This cross-over point also improved the infectivity of other intergenotypic chimeras. Thus, virus release is more efficient when the first TM NS2 segment is from the same isolate as the core-to-p7 region and the remainder of NS3 is homologous to the replicase. It is believed that this enhancement may be related to interactions between the N-terminal NS2 region and the structural protein(s) or p7, alterations of cleavage at the p7–NS2 site that is processed with delayed kinetics, or effects on cleavage at the NS2–NS3 site. Since the development of the J6/JFH1 chimera, viable JFH1 chimeras representing all genotypes have been generated (Gottwein *et al.*, 2009).

1.4. HCV Life Cycle

The HCV life cycle is a highly complex multistep process (Figure 1.4) that begins with the viral glycoproteins interacting with several cellular factors, which mediate internalisation of the virus into the host via clathrin-mediated endocytosis (Figure 1.5). HCV replication occurs in association with cytoplasmic membranes and virion assembly and release are believed to occur via the ER membrane following the same secretion pathway as VLDL assembly. The following sections will detail the different stages of this process.

1.4.1. Virus Binding and Entry

1.4.1.1. Attachment factors

Prior to the interaction of HCV with specific cellular receptors, several attachment factors are first involved in bringing the virus into close proximity with the cell surface. These are believed to be glycosaminoglycans, low density lipoprotein receptors and C-type lectins such as Liver or dendritic cell specific intercellular adhesion molecule 3-grabbing nonintegrins (L/DC-SIGN).

Low Density Lipoprotein Receptors

HCV serum particles are suggested to complex with host lipoproteins LDL and VLDL and circulate as LVP (Andre *et al.*, 2002). The lipoprotein association is thought to facilitate the attachment of HCV to target cells by the LVPs interacting with the LDL receptor (Popescu & Dubuisson, 2009). In accordance with this notion, an increased accumulation of HCV viral RNA in cells was found to correlate with increased LDL receptor expression and LDL entry (Germi *et al.*, 2002; Molina *et al.*, 2007). In addition, HCV RNA accumulation in cells is inhibited with antibodies directed against LDL receptor (Agnello *et al.*, 1999). Furthermore, HCV infectivity is neutralised in a dose dependent manner by antibodies directed against ApoB and ApoE (major protein components of VLDLs) (Agnello *et al.*, 1999; Chang *et al.*, 2007). Collectively, these data support the essential role of lipoproteins at an early stage of HCV infection.

L/DC-SIGN

L-SIGN expressed on endothelial cells in liver sinusoids and DC-SIGN expressed on dendritic cell subsets have been proposed as tissue-specific capture receptors. L/DC-SIGN can bind HCV sE2 with high affinity and an interaction with HCVpp and serum particles has also been reported (Gardner *et al.*, 2003; Lozach *et al.*, 2004; Lozach *et al.*, 2003). L/DC-SIGN are believed to bind viral envelope glycoproteins via mannose-type oligosaccharides without mediating entry, thus serving as capture receptors that disseminate viral particles to target organs (Cormier *et al.*, 2004a).

Glycosaminoglycans

Glycosaminoglycans (GAGs) are long polysaccharides that may be covalently linked to a core protein to form proteoglycans. GAGs are negatively-charged structures ubiquitously expressed as cell surface molecules and are believed to serve as initial docking site for many viruses including several members of the Flaviviridae family (Barth *et al.*, 2003). HCV is believed to interact with GAG via positively charged residues within the N-terminus of E2 glycoprotein (Penin *et al.*, 2001). Although GAGs such as heparin and heparin sulphate efficiently binds to intracellular E1E2, no such interaction is detected between HCVpp derived envelope glycoproteins (Barth *et al.*, 2003). However, the treatment of cells with heparinase (enzyme degrading HS) or virus incubation with heparin, a HS analogue, inhibit HCV attachment to cells as well as inhibit HCVpp and

HCVcc infectivity (Barth *et al.*, 2006; Basu *et al.*, 2007; Koutsoudakis *et al.*, 2006; Morikawa *et al.*, 2007).

1.4.1.2. Specific Receptors

CD81

By screening a cDNA library, using soluble E2 (sE2) as a probe, CD81 was identified as the first putative HCV receptor (Pileri *et al.*, 1998). CD81 is widely expressed throughout the human body and is associated with several cellular functions such as cell-cell adhesion and immune cell differentiation (Levy *et al.*, 1998).

CD81 is a 26kDa surface protein and is a member of the tetraspanin family. It consists of a small and a large extracellular loop with four TMDs. The large extracellular loop (LEL) has been demonstrated to permit binding to the ectodomain of sE2, although the E1/E2 heterodimer exhibits binding implying that of E1 might modulate the binding process (Cocquerel *et al.*, 2003). As for E2, mutagenesis and alanine substitutions of conserved epitopes have revealed several discontinuous regions to be involved in CD81 binding (Drummer *et al.*, 2006; Owsianka *et al.*, 2006).

The importance of CD81 in both HCVcc and HCVpp infection has been demonstrated with the inhibitory effect of anti-CD81 antibodies, CD81-LEL and by downregulation of CD81 expression (Bartosch *et al.*, 2003; Cormier *et al.*, 2004b; Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhang *et al.*, 2004; Zhong *et al.*, 2005). Inhibition assays using anti-CD81 revealed that CD81 acts after virion binding to target cells, suggesting that CD81 is not the only HCV receptor (Cormier *et al.*, 2004b). Although CD81 is important for HCV entry the fact that a wide range of cells expressing CD81 are non-permissive for HCV indicates that other liver-specific molecules are involved in facilitating viral entry.

SR-BI

SR-B1 is a multiligand receptor expressed on liver cells and on steroidogenic tissue. It binds to numerous lipoproteins such as HDL, LDL, and VLDL. SR-BI is a 509 amino acid long protein with two cytoplasmic domains, two TMDs and a large extracellular loop responsible for ligand binding (Acton *et al.*, 1996; Acton *et al.*, 1994). SR-BI was initially identified as the major receptor for HDL, facilitating selective cholesterol uptake in the liver (Acton *et al.*, 1996).

SR-BI as a putative HCV receptor was described by cross-linking sE2 to biotinylated cell-surface proteins followed by immunoprecipitation using an anti-E2 antibody. This interaction was dependent on HVR1 of E2 since deletion of HVR1 was shown to impair the interaction between SR-BI and sE2 and reduces HCVpp infectivity. Antibodies against SR-BI and knockdown of SR-BI expression inhibits HCVpp and HCVcc infectivity confirming the importance of this receptor in HCV entry (Bartosch *et al.*, 2003; Lavillette *et al.*, 2005b; Scarselli *et al.*, 2002). However, since anti-E2 or anti-HVR1 antibodies do not inhibit the HCV-SR-BI interaction, it has been proposed that lipoproteins attached to virion may also interact with the receptor (Maillard *et al.*, 2006). In support of this theory, various SR-BI ligands such as VLDL and oxidized LDL exhibit inhibitory effects on HCV entry (Maillard *et al.*, 2006; von Hahn *et al.*, 2006).

Interestingly, the major SR-BI ligand HDL facilitates HCVpp and HCVcc entry, although no direct interaction between HDL and virus particles has been found. It is hypothesized that HDL-mediated enhancement of HCVpp and HCVcc entry relies on the lipid transfer function of SR-BI (Dreux *et al.*, 2006).

Tight Junction proteins

Recently, members of the claudin (CLDN) protein family, involved in the formation of tight junctions, have been implicated in the entry of HCV. Evans *et al.* showed that ectopic expression of CLDN-1 in the HCVpp entry resistant non-hepatic cells lines, HEK 293T and SW13, rendered them susceptible to HCV infection (Evans *et al.*, 2007). CLDN-1 is a cell surface protein with four TMDs, two extracellular loops and intracellular N- and C-termini. CLDN-1 is most highly expressed in the liver, which may determine the tissue tropism of HCV. Residues within the first extracellular loop appear to be critical for mediating HCV entry, although no interaction between the HCV glycoproteins and CLDN-1 has been demonstrated (Evans *et al.*, 2007). Initial studies indicated that CLDN-1 acts at a step after virus attachment to cells and its subsequent interaction with CD81 and SR-BI but prior to membrane fusion (Evans *et al.*, 2007; Zheng *et al.*, 2007). More recent studies have shown an association between CD81 and CLDN-1, implying that these two entry factors act in a cooperative manner during HCV entry (Harris *et al.*, 2008; Krieger *et al.*, 2010). Therefore, it is possible that CLDN-1 may potentiate CD81 association with HCV particles by way of E2 interactions. In addition to CLDN-1, CLDN-6 and CLDN-9 are also able to mediate HCV entry into target cells (Meertens *et al.*, 2008; Zheng *et al.*, 2007).

More recently, another tight junction protein, known as occludin (OCLN), has been implicated in HCV cell entry. OCLN is a 60 kDa protein with four TMDs, two extracellular loops and N- and C- terminal cytoplasmic domains (Furuse & Tsukita, 2006). OCLN-E2 binding in HCVcc infection has been confirmed by co-immunoprecipitation assays. Strikingly, HCV infection induces re-localization of tight junction proteins to the ER, reducing their expression on HCV infected cells. Knockdown of CLDN-1 and OCLN expression on cell surface inhibited HCVpp and HCVcc cell entry (Benedicto *et al.*, 2009; Liu *et al.*, 2009; Ploss *et al.*, 2009).

Importantly, expression of CD81, SR-BI, CLDN-1 and OCLN renders murine and hamster cells infectable with HCVpp, suggesting that these four receptors complete the set of cell surface factors required for HCV entry (Dorner *et al.*, 2011; Ploss *et al.*, 2009).

1.4.1.3. HCV Internalisation

HCV entry is pH dependent (optimal pH 4.5) and thus requires the formation of vesicles in order to be directed to the site of replication. siRNA knockdown of clathrin as well as inhibition of clathrin-coated pit assembly using chloropromazine reduced HCV entry confirming that HCV utilizes the clathrin-mediated pathway for internalisation. Following its interaction with the panel of receptors, HCV gets internalised into clathrin-coated pits, which fuse with early endosomes where a drop in pH triggers the fusion process by inducing conformational changes in the envelope glycoproteins. Indeed, endosomal acidification inhibitors have been shown to significantly reduce both HCVpp and HCVcc infectivity (Blanchard *et al.*, 2006; Hsu *et al.*, 2003; Koutsoudakis *et al.*, 2006; Meertens *et al.*, 2006). Fusion has further shown to be temperature-dependent and enhanced by cholesterol (Lavillette *et al.*, 2006). A very recent study showed that HCV-CD81 binding could confer fusion competence upon encountering low pH. The results from this study provide strong evidence that the CD81 receptor may be both necessary and sufficient to render E1E2 competent for fusion with acidic intracellular compartments (Sharma *et al.*, 2011).

1.4.2. Genome Translation and RNA replication

1.4.2.1. Genome Translation

HCV RNA released into the cytoplasm is immediately translated into the viral polyprotein by the host ribosomal machinery via the HCV IRES located within the 5' UTR (section

1.2.1). The viral genome serves as a messenger RNA for synthesis of the HCV polyprotein precursor. IRES-mediated translation is initiated by direct binding of a vacant 40S ribosomal subunit to the IRES and positioning of the polyprotein start codon into the 40S P site (Kieft *et al.*, 2001; Otto & Puglisi, 2004). The IRES can capture the 40S ribosomal subunit in the absence of other initiation factors known to be required for ribosome binding to an mRNA. Following its formation, the IRES-40S complex then binds to the eukaryotic initiation factor 3 (eIF3), followed by recruitment of the eIF2:Met-tRNAi:GTP complex to generate a 48S-like complex (Pestova *et al.*, 1998; Ji *et al.*, 2004). Upon hydrolysis of GTP, the initiator factors are released and the 60S ribosomal subunit can then attach to the 48S complex to form a functional IRES-80S complex, which can initiate viral protein synthesis. Translation occurs in close association with ER membrane where the polyprotein is cleaved co- and post-translationally by viral and cellular proteases into the 10 mature proteins.

1.4.2.2. RNA Replication

Like other positive-strand viruses, HCV infection induces rearrangement of intracellular membranes to form replication complexes where RNA synthesis takes place. Replication complexes (membranous web) are ER-derived membranes that are rich in cholesterol and fatty acids. Replication complexes contain positive- and negative-strand viral RNA and the HCV non-strutural proteins, as well as cellular components involved in genome replication (Egger *et al.*, 2002; Gosert *et al.*, 2003; Waris *et al.*, 2004). Replication complexes are enclosed structures where HCV RNA and proteins are resistant to ribonucleases and proteases and thus protected from the intracellular environment (Quinkert *et al.*, 2005; Waris *et al.*, 2004). Numerous cellular factors have been identified with potential roles in HCV RNA replication (Ploss & Rice, 2010). The best characterized of these include the DEAD-box RNA helicase DDX3 and GBF1 - a guanine nucleotide exchange factor for small GTPases of the ARF family. DDX3 is crucial for virus RNA replication and is believed to incorporate into replication complexes to facilitate genome translation (Ariumi *et al.*, 2007; Geissler *et al.*, 2012). The presence of GBF1 in the cells is critical for replication complex activity, although its exact mechanism of action remains to be determined (Gouslain *et al.*, 2010).

Within these active complexes viral RNA is amplified by the NS5B RdRp where replication proceeds via synthesis of a negative-strand intermediate that serves as a template to generate multiple nascent HCV genomes. Replication complexes are estimated

to contain one minus-strand RNA template, up to ten positive-strand RNA copies and several hundred non-structural proteins (Quinkert *et al.*, 2005). HCV replication is thought to occur rapidly after virus entry as negative-strand templates are detectable at 2-4 hours after introduction of RNA into cells (Binder *et al.*, 2007).

1.4.3. Assembly and Release

1.4.3.1. HCV Assembly

It has been suggested that the core-LD association functions to concentrate core protein near the ER-located replication complexes (Boulant *et al.*, 2008; Miyanari *et al.*, 2007). This close proximity likely facilitates the formation of genome containing nucleocapsids and their subsequent usage towards assembly of virions at ER sites harbouring the E1E2 proteins. Evidence within recent studies suggests that NS2 provides the link between replication complexes and the structural proteins by directly interacting with E1, E2, p7, NS3 and NS5A (Jirasko *et al.*, 2010; Ma *et al.*, 2011; Popescu *et al.*, 2011; Stapleford & Lindenbach, 2011). Indeed, IF studies have shown that NS2 recruits these factors to assembly sites at the LD-ER interface (Boson *et al.*, 2011; Jirasko *et al.*, 2010; Popescu *et al.*, 2011). Although no specific interaction has been identified between core and NS2 (Jirasko *et al.*, 2010; Ma *et al.*, 2011; Stapleford & Lindenbach, 2011), it is possible that the NS2-NS5A interaction acts in concert with the core-NS5A interaction (Masaki et al., 2008) during the assembly process. However, results within very recent studies suggest that p7 is involved in recruiting core (Boson *et al.*, 2011) and NS2 (Tedbury *et al.*, 2011) to sites adjacent to replication complexes. The exact function of NS2 during assembly remains to be determined but one possible role may be to release the viral RNA from the replication complexes and allow its encapsidation into nascent HCV particles. Presumably, the recruitment of the glycoproteins by NS2 to these cites results in the envelopment of these genome containing nucleocapids. In addition to these theories, the results from another study suggested that NS2 functions at a late-post assembly maturation step, after the interaction of core and NS5A with host cell lipid droplets and the formation of core-protein containing particles (Yi *et al*., 2009).

1.4.3.2. HCV Release

HCV is suggested to utilise the LDL/VLDL assembly and secretion pathway for egress. VLDLs are produced in hepatocytes and play an important role in maintaining lipid homeostasis in the body. Interestingly, LDs are the intracellular storage compartments for

cholesterol and triglyceride ester and VLDLs are responsible for exporting these into the extracellular environment. Apolipoprotein B (apoB) and apoE are main components of VLDL and microsomal triglyceride transfer protein (MTP) transports triglyceride from LDs to the apoB and apoE for VLDL assembly (Gibbons *et al.*, 2004; Shelness & Sellers, 2001). siRNAs targeting apo B and apo E and an MTP inhibitor are capable of reducing the release of infectious virions (Chang *et al.*, 2007; Gastaminza *et al.*, 2008; Huang *et al.*, 2007). Antibodies against apo B and apo E also inhibit serum derived HCV and anti-apo E antibodies inhibit HCVcc infectivity (Chang *et al.*, 2007; Maillard *et al.*, 2006). Intracellular virions have a reduced lipoprotein content suggesting that lipoproteins are complexed to particles post-envelopement (Gastaminza *et al.*, 2006; Tao *et al.*, 2009). In contrast to released virions, the infectivity of intracellular particles are sensitive to low pH treatments. Recent evidence suggests that the proton-selective ion-channel activity of p7 prevents the acidification of intracellular virions during their transit through otherwise acidic intracellular compartments (Wozniak *et al.*, 2010).

1.5. HCV envelope glycoprotiens; synthesis, maturation and function

The envelope glycoprotein E1 and E2 have shown to mediate HCV binding to the host cell and initiate fusion between the viral envelope and the cellular membrane at the initial stage of virus replication. Furthermore, the glycoproteins play an important role in virus particle assembly and also in modulating antibody-mediated neutralization response during HCV infection. E1E2 are class I *trans*-membrane proteins, located at the endoplasmic reticulum (ER). E1 (~30 kDa) and E2 (~70 kDa) (Dubuisson, 2000) form non-covalent heterodimers that are attached to the viral lipid surface and consist of a N-terminal ectodomain and a hydrophobic C-terminal domain. The ectodomains are highly modified through N-linked glycosylation at the ER (Goffard & Dubuisson, 2003). The development of the HCVpp and HCVcc systems has mostly confirmed previous findings however, differences in glycoprotein maturation have been observed between these two systems. At the surface of HCVpp, functional E1 and E2 have been shown to form noncovalent heterodimers, as with their intracellular forms (Op De Beeck *et al.*, 2004). In contrast, it was recently shown that in the HCVcc system, virion-associated E1 and E2 form large covalent complexes stabilized by disulfide bridges, whereas the intracellular forms of these proteins assemble as noncovalent herterodimers (Vieyres *et al.*, 2010). These differences observed in the biochemical composition of virion-associated E1E2 may account for the differences

observed in some of the HCV envelope protein functions when the HCVpp and HCVcc systems were compared (Russell *et al*., 2008).

1.5.1. Synthesis of E1 and E2 Glycoproteins

HCV polyprotein processing occurs at the ER membrane, where structural proteins are cleaved by the ER SP during and immediately after translation. However, a delayed cleavage at the E2/P7 and P7/NS2 site results in the production of an uncleaved E2/P7/NS2 precursor. Although NS2 is progressively released from E2/P7/NS2 precursor, the cleavage between E2/P7 remains uncleaved over time resulting in the detection of E2, E2/P7, P7 and NS2 (Dubuisson *et al.*, 1994; Dubuisson & Rice, 1996; Lin *et al.*, 1994). Both E1 and E2 proteins harbour a single C-terminal TMD that anchors them into the ER membrane while the N-terminal ectodomain is oriented towards the ER lumen (Cocquerel *et al.*, 2002). Upon E1E2 heterodimerization, these TMDs have shown to favour a single membrane-spanning topology (Op De Beeck *et al.*, 2000). Sequence analysis has shown that E1 and E2 TMDs are composed of two hydrophobic stretches connected by a short hydrophilic segment that contains at least one fully conserved charged residue responsible for their ER retention (Cocquerel *et al.*, 2000). The second hydrophobic stretch functions as an ER signal sequence for the downstream protein. Before signalase cleavage, the TMDs form a hairpin loop allowing E1 and E2 ectodomains to face the ER lumen. However, after cleavage between E1 and E2 the second hydrophobic stretch reorients towards the cytosol, forming a complete TMD (Cocquerel *et al.*, 2002). E1E2 TMD is a multifunctional sequence that mediates ER retention, serve as ER signal sequence and further play a major role in E1E2 heterodimer formation.

1.5.2. Folding of E1 and E2 Glycoproteins

E1 and E2 glycoproteins interact to form noncovalent heterodimers that constitute the functional subunit of HCV envelope. However, in heterologous expression systems majority of these proteins also form misfolded aggregates stabilized by disulfide bridging (Dubuisson *et al*., 1994). E1 and E2 ectodomains contain numerous conserved cystein residues that form intramolecular disulfide bonds in the oxidizing environment in the ER. The overall conformation of E1E2 depends on the formation of these bonds. Kinetic studies on disulfide bond formation have revealed that E1 and E2 fold slowly and the

correct conformational folding of either protein is dependent on the expression of the other (Lavie *et al.*, 2007; Op De Beeck *et al.*, 2001). Presumably the slow folding may increase the tendancy of non-productive misfolded aggregate formation. The exact role of aggregates is unclear but may play a role in downregulating protein synthesis in order to avoid immune surveillance (Deleersnyder *et al.*, 1997). Additionally, several host ER chaperones such as calnexin, calreticulin and BiP have been shown to interact with E1 and E2, suggesting that HCV exploits cellular factors during protein maturation (Dubuisson & Rice, 1996). Interaction of E1E2 with calnexin result in properly folded glycoprotiens, whereas calreticulin and BiP have shown to interact with non-productive aggregates (Choukhi *et al.*, 1999).

1.5.3. Glycosylation of E1 and E2 glycoprotiens

Both E1 and E2 ectodomains are highly modified by N-linked glycosylation with E1 and E2 containing up to five and eleven potential glycosylation sites, respectively. Sequence analyses indicate that most of these sites are fully conserved among genotypes (Goffard & Dubuisson, 2003). N-glycosylation occurs by the transfer of an oligosaccharide from a lipid intermediate to an Asn-X-Ser/Thr motifs onto the nascent polypeptide. N-glycosylation starts in the ER, followed by glycan trimming, protein folding and glycan modification that continues in the golgi apparatus. Glycans are a signature of protein trafficiking. Glycans play an important role in protein folding, E1E2 interaction with cellular receptors and modulate sensitivity to neutralising antibodies (Goffard *et al.*, 2005).

1.5.4. Functional regions of E1 and E2 Glycoproteins

The E1E2 heterodimer is essential for HCV entry where E2 is involved in binding to cellular receptors CD81 and SRBI. CD81 binding has been mapped to distinct regions on E2 while HVR1 is responsible for E2-SRBI binding. Viral attachment to receptors triggers a conformational change within the glycoprotein, which may expose the fusion peptide. Fusion peptides are divided into two different structural classes, designated as class I and class II. It has been proposed that HCV E2 is a class II fusion protein with a β-sheet organisation and an internal fusion peptide (Penin *et al.*, 2004b). Class II fusion proteins alter their conformation in response to a cellular trigger such as an acidic milieu, resulting in the exposure of the fusion peptide. Indeed, studies based on HCVpp and HCVcc have demonstrated that HCV entry occurs via clathrin mediated pathway, which fuses with early

endosomes. The low pH in early endosomes triggers the fusion of the viral envelope with the endosomal membrane, which leads to the liberation of the HCV genome (Blanchard *et al.*, 2006; Meertens *et al.*, 2006). However, acidic pH alone is not sufficient to induce fusion, suggesting the involvement of additional but yet unknown factors (Meertens *et al.*, 2006; Tscherne *et al.*, 2006).

Sequence analyses propose that E1 may contain a putative fusion peptide in its ectodomain (Flint & McKeating, 1999; Garry & Dash, 2003), while structural homology with other class II fusion proteins suggest that E2 could be the fusion peptide (Yagnik *et al.*, 2000). Recently, Krey *et al*, (2010) proposed a tertiary structure model of sE2 based on the disulfide connectivity pattern, CD81 binding data, secondary structure prediction and class II fusion protein model (Figure 1.8). This model divides E2 into three domains where domain I constitutes the central position and harbours most of the two regions involved in CD81 interaction. Domain II is proposed to contain the putative fusion peptide and is connected to domain III via the intergenotypic variable region (IgVR) located at its C-terminal. Upon fusion this flexible linker region may allow the translocation of domain III, which also contains some of the residues important for CD81 binding, to the side of fusion complex (Krey *et al.*, 2010).

1.6. HCV Envelope Glycoproteins and Virus Neutralization

Broadly neutralizing antibodies (nAbs) targeting conserved epitope present attractive therapeutic candidates to block infection at an early stage. Using HCV specific antibodies combined with antiviral drugs targeting different steps of the viral lifecycle will be particularly useful in controlling re-infection of the graft following liver transplantation or accidental exposure to HCV (Angus & Patel, 2011). However a deeper understanding of the evasion mechanisms and factors leading to viral escape from nAbs resulting in chronic infection needs to be addressed for potential therapeutic development (Dilorenzo et al, 2011).

1.6.1. Antibodies to Linear Epitopes

Being part of the virion particle, the HCV glycoproteins are the natural target for the host neutralizing antibody response. HCV infection is specifically neutralized with sera derived from HCV infected patients (Bartosch *et al.*, 2003). These antibodies recognise conformational epitopes on E2 and generally act by blocking the interaction of E2 with the CD81 receptor. However, the glycoproteins account for the large sequence variability among HCV genotypes and subtypes. This together with the error prone nature of the RNA polymerase and high replication rate of the genome (Neumann *et al.*, 1998) results in the production of escape mutants that dominate the quasispecies population during chronic infection (Bukh *et al.*, 1995). The E2 glycoprotein contains three HVRs that harbour positively selected sites and account for greater variability at the quasispecies level than E1. HVR1 is located at the N-terminus of E2 and (amino acids 384-410) is the best characterised HCV HVR due to its role in virus entry and its capacity to elicit nAb responses. HVR1 mediates sE2 binding to SR-BI (Scarselli *et al*., 2002) and attenuates sE2 and HCVcc binding to CD81-LEL (Bankwitz *et al.*, 2010). Virions lacking HVR1 have been shown to revert back to WT characteristics through the emergence of compensatory mutations elsewhere in E2. Similarly, deletion of HVR1 in the HCVpp system results in a 40-fold decrease in infectivity (Bartosch *et al*., 2003c), indicating the defect occurs at the entry level. These results were recently confirmed in the HCVcc system (Bankwitz *et al*., 2010) where deletion of HVR1 also impaired infectivity but not viral replication. Less is known about HVR2 (amino acids 474-482) and HVR3 also known as IgVR (amino acids 570-580) (Hijikata *et al.*, 1991; McCaffrey *et al.*, 2007; Weiner *et al.*, 1991). According to the recent sE2 model by Krey *et al*, (2010) the HVRs form unstructured domains on E2 where HVR1 protrude from N-terminal extension, HVR2 from domain II and HVR3 forms the linker sequence between domain I and domain III (Krey *et al.*, 2010). HVR1 is highly immunogenic and is the main target for the humoral immune response during the natural course of infection both in humans (Kato *et al.*, 1993; Scarselli *et al.*, 1995) and in chimpanzees (Meunier *et al.*, 2005). However, HVR1 specific antibodies display limited cross reactivity and during chronic infection error prone RNA replication produces HVR1 variability, which results in the accumulation of immune escape variants and thus represents a poor vaccine candidate (Farci *et al.*, 1996b; Ray *et al.*, 1999). Strikingly, despite being highly tolerant to amino acid substitution the overall conformation of HVR1 is well conserved and contains basic residues which, in theory may be required for interaction with negatively charged molecules at the cell surface (Penin *et al.*, 2001).

The E2 protein contains several discontinuous regions conserved across different genotypes that are involved in binding to the CD81 receptor (Owsianka *et al.*, 2006) and thus are essential for viral fitness. The neutralizing antibody responses specific to these important conserved regions will minimise the likelihood of escape mutants. Mouse monoclonal antibody (mAb) AP33 is one such antibody that recognizes a highly conserved linear epitope spanning residue 412-423 located immediately downstream of HVR1 (Owsianka *et al.*, 2006). MAb AP33 has been shown to potently neutralize HCVpp incorporating E1E2 from all major genotypes and subtypes (Owsianka *et al.*, 2005) and also inhibits HCVcc infectivity (Tarr *et al.*, 2006). Several other broadly nAbs have also been described that recognise this region, including 3/11 (Hsu *et al.*, 2003), HCV-1 and 95-2 (Broering *et al.*, 2009). *In vivo*, the prevalence of antibodies reactive to this conserved E2 region is <2.5 % (Tarr *et al.*, 2007) suggesting a poor ability of this epitope to elicit antibody responses. Amino acid substitutions within this epitope have been shown to either decrease viral fitness (Owsianka *et al.*, 2006; Tarr *et al.*, 2006) or increase particle vulnerability to nAbs (Dhillon *et al.*, 2010; Tao *et al.*, 2009) highlighting the importance of epitope conservation in virus entry. Consequently, a successfull vaccine candidate would shift immunodominace towards more conserved neutralising epitopes, rather than non-specific or highly variable regions.

1.6.2. Antibodies to Conformational Epitopes

To date, there have been numerous reports describing the generation of patient-derived mAb targeting either linear or conformational epitopes on E2 (Allander *et al.*, 2000; Hadlock *et al.*, 2000; Johansson *et al.*, 2007; Keck *et al.*, 2008; Keck *et al.*, 2004; Keck *et al.*, 2007; Mancini *et al.*, 2009; Perotti *et al.*, 2008). Among these, a subset of antibodies displayed cross neutralizing activity against different genotypes. Competition assay of human mAbs derived from peripheral B-cells of HCV infected patients, the epitopes of which are shown in figure 1.7, revealed the presence of three immunogenic domains on E2 called A, B and C (Allander *et al.*, 2000; Hadlock *et al.*, 2000; Keck *et al.*, 2004; Keck *et al.*, 2007). MAbs recognising domain B and C exhibit cross-neutralizing response while domain A antibodies demonstrate non-neutralizing characteristics. In particular, the CBH-5 human mAb harbours cross neutralising activity against all HCV genotypes and alanine substitution revealed that it recognises conserved amino acids important for E2-CD81 binding encompassing residue 523-535 (Owsianka *et al.*, 2008).

1.6.3. Antibodies targeting E1 Glycoprotein

An E1-specific antibody response is reported less frequently, possibly because E1 appears to be less immunogenic than E2. However, E1 does contain neutralizing epitopes and studies have shown that E1-specific sera are capable of neutralizing both HCVpp as well as HCVcc infectivity (Dreux *et al.*, 2006; Pietschmann *et al.*, 2006). Furthermore, a highly conserved E1 epitope has been identified between amino acids 313–327 and mAbs targetting to this epitope strongly neutralise genotypes 1a, 1b 4a, 5a and 6a HCVpp as well as WT/JFH1 and the chimeric 1a/JFH1 HCVcc (Meunier *et al.*, 2008b). Less is known about the role of the E1 glycoprotein and development of E1-specific antibodies will assist in delineating the role of this protein in HCV entry.

1.7. Mechanism of Immune Escape

Together with cellular immune response, nAbs play an important role in controlling HCV infection. Anti-HCV nAbs targeting the glycoproteins can be detected during the very early phase of infection (Lavillette *et al.*, 2005b; Pestka *et al.*, 2007). Despite a strong immune response HCV establishes persistent infection in majority of the infected patients. Antibodies may mediate neutralisation by either causing aggregation of virus particles, by inhibiting virus-host interaction or preventing viral envelope fusion with cellular membrane. In any case, neutralization requires the availability of critical epitopes on the virion surface and an efficient binding of antibody to these epitopes. HCV utilizes several effective nAb escape strategies, which are addressed in the following section.

1.7.1. Genetic Diversity within HVR1

As a response to host immune pressure HCV evolves rapidly with new mutations arising regularly resulting in genetic diversity within the infected individual. HCV glycoproteins display the highest degree of genetic heterogeneity between genotypes and subtypes (Simmonds, 2004) and the constant production of viral variants may result in the emergence of more pathogenic strains and the subsequent development of chronic infection (Farci & Purcell, 2000). HVR1 is implicated in facilitating E2/SR-BI mediated entry where HDL has been shown to influence HCVpp entry (section 1.7.1.2) and resistance to nAbs (Bartosch *et al.*, 2005; Scarselli *et al.*, 2002). Intriguingly, positive

selection within HVR1 occurs at residues involved in HCV entry without altering the overall conformation of the segment, resulting in nAb escape but at the cost of efficient cell entry (Brown *et al.*, 2007). In addition to HVR1, other studies have reported mutations elsewhere in E2 that function to escape from the humoral immune response (Duan *et al.*, 2010; Keck *et al.*, 2009).

1.7.2. The Glycan Shield

The HCV envelope proteins are highly glycosylated with up to 5 potential N-linked glycosylation in E1 (E1N1-E1N5) and up to 11 potential sites in E2 (E2N1-E2N11). The glycosylation of envelope proteins is essential to ensure correct protein processing, folding and fascilitating cellular entry (Goffard *et al.*, 2005). Particular N-glycans located in close proximity to the E2-CD81 binding residues are believed to mask important epitopes from nAbs as removal of these glycans enhances the sensitivity of HCVpp and HCVcc to neutralization by nAbs and molecules that block CD81 binding (Falkowska *et al.*, 2007; Fukushi *et al.*, 2001; Helle *et al.*, 2007; Helle *et al.*, 2010).

1.7.3. Interfering Antibodies

The continuous evolution of HCV may result in the emergence of previously hidden glycoprotein epitopes (Zhang *et al.*, 2004). This may elicit antibody responses targeting epitopes that are not necessarily involved in virus entry. Indeed, the presence of non-neutralising anti-E2 antibodies in patient sera has been identified that can compete with nAbs targeting the 412-423 conserved epitope (Zhang *et al.*, 2007; Zhang *et al.*, 2009). According to these studies a small segment of E2 could be divided into two epitopes where non-nAbs binding to epitope II (432-447) may mask the nAbs binding site in epitope I (412-419). Accordingly, depletion of interfering antibodies resulted in enhanced neutralization. The presence of interfering antibodies may possibly lead to establishment of persistent infection despite a strong host immune response during infection.

1.7.4. Cell-to-cell transmission

Recently it has been shown that HCVcc is able to spread from infected cell to the neighbouring uninfected cells via direct cell-cell contact without being secreted in the

extracellular environment (Timpe *et al.*, 2008; Witteveldt *et al.*, 2009). Numerous viruses are reported to utilise the cell-to-cell transmission mode, which enables the viruses to circumvent host humoral immune factors such as nAbs and thus enables the rapid spread of infection (Mothes *et al.*, 2010). Consequently, it has been shown that HCV is able to spread in the presence of polyclonal and monoclonal anti-HCV glycoprotein antibodies (Timpe *et al.*, 2008; Witteveldt *et al.*, 2009). It is yet not clear whether the cell-to-cell transmission utilises the same entry mechanism as the entry by extracellular virus. However, recent studies report that SR-BI and tight junction proteins claudin-1 (CLDN) and occludin (OCLN) seem to be required for entry (Brimacombe *et al.*, 2011; Ciesek *et al.*, 2011). As for CD81 there is conflicting data as to whether cell-to-cell entry is CD81 independent or not (Brimacombe *et al.*, 2011; Jones *et al.*, 2010; Timpe *et al.*, 2008; Witteveldt *et al.*, 2009). However, the CD81-independent mechanism observed by the first 3 studies may have been due to an unreliable flow cytometry method for measuring cell-to-cell transfer that produces false positive results. This was shown by the most recent study (Brimacombe *et al*, 2011), where they modified the flow cytometry method to eliminate these false positives. Using the new system they found that CD81 was essential for cell-to-cell transfer. Collectively, the available data support a role for cell-to-cell transmission which may partially explain how viruses are able to evade the host nAb response during chronic infection.

1.7.5. Lipoproteins

HCV serum particles have shown to exhibit highly heterogenous density profiles (ranging from <1.06 to >1.20 g/mL) because of the viral particle association with distinct cellular components (Nielsen *et al.*, 2006; Thomssen *et al.*, 1993). The high density HCV particles which exhibit poor infectivity are shown to be linked with immunoglobulins, whereas highly infectious particles are shown to be associated with lipoprotein such as LDL and VLDL (Hijikata *et al.*, 1993b). Apart from facilitating the virion entry into host cell via LDL receptor, the HCV-lipoprotein association may also protect the virus from the humoral immune response possibly by shielding the virus surface glycoproteins from circulating nAbs. In support of this, it has been shown that intracellular immature HCVcc virions are more sensitive to neutralization with anti-E2 antibodies and less sensitive to anti-lipoprotein antibodies compared to the secreted virion (Tao *et al.*, 2009). Furthermore, the neutralization sensitivity of extracellular virions with anti-glycoprotein nAbs has been shown to increase with HCV particle density, suggesting that the lipoprotein content

affects the neutralization efficiency (Grove *et al.*, 2008). Confirming this hypothesis, it has been shown that a single mutation in E2 (I414T) (Tao *et al.*, 2009) reduced the HCVcc lipoprotein content and thereby enhanced its neutralization efficiency by anti-E2 antibodies. Thus, lipoproteins may help the virus evade recognition by the host immune response by masking the essential epitopes targeted by anti-glycoprotein nAbs.

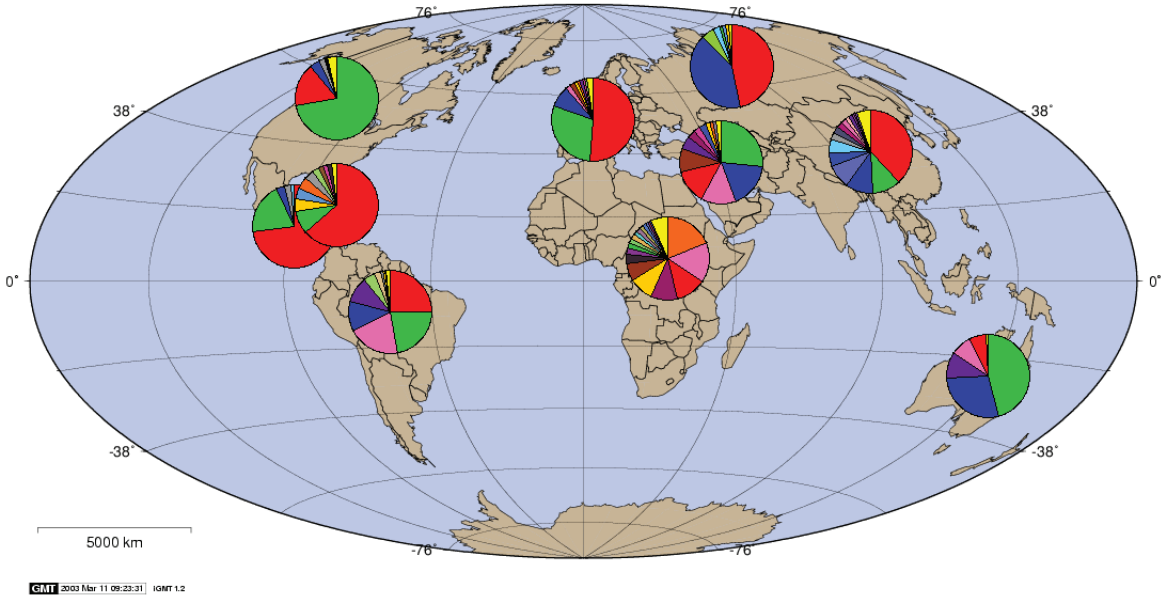
The high-density lipoproteins (HDL) found in human serum have demonstrated the ability to attenuate the antibody-mediated neutralization of HCV by stimulating viral entry at a post-binding step (Bartosch *et al.*, 2005; Lavillette *et al.*, 2005a; Voisset *et al.*, 2005). The enhancement of HCV entry decreases the time window through which nAbs can bind and neutralize the virus (Dreux *et al.*, 2006). This process is facilitated by HVR1 and also depends on the expression of SR-BI and its selective lipid-uptake function (Bartosch *et al.*, 2005; Voisset *et al.*, 2005). ApoC-I was demonstrated to be the HDL component responsible for enhancing HCV infection (Meunier *et al.*, 2005). It is believed to be recruited from HDL to influence the infectivity of HCV particles at a postbinding stage by promoting their membrane fusion properties (Dreux *et al.*, 2007).

Molecules targeting the lipoproteins associated with virus particles or those stimulating virus entry could affect HCV infectivity and, as thus, could have implications for future therapeutic approaches based on the inhibition of HCV infection. Indeed, HCV-lipid interactions may be attractive targets for the development of antiviral drugs, because the targeting of essential host cell factors could limit the development of escape mutations effective against drugs directly targeting virus components.

1.8. Aims

The aim of this project was divided into two lines of investigation, both of which focused on the HCV envelope glycoproteins, namely E1 and E2. The first aim was to investigate the importance of a highly conserved region in E2, spanning amino acids 412-423, to the viral entry process and antibody-mediated neutralization. This was achieved by analysing the viral phenotypes created by four cell culture adapted mutations located within this E2 region. The second objective was to generate and study the entry properties of infectious HCVcc particles harbouring the glycoproteins from a diverse range of genotypes.

Figure 1.1. Global Variation in Prevalence of Chronic HCV Infection. Extracted from Yang and Lewis, (2010).



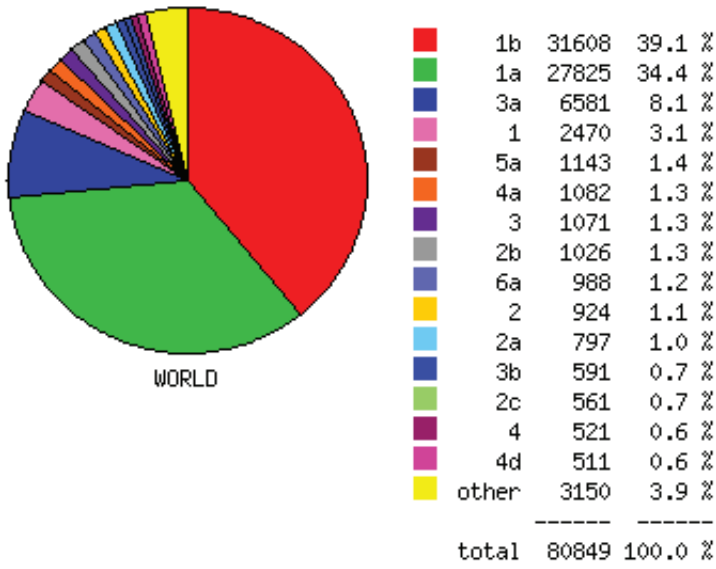


Figure 1.2. HCV Subtype Distribution Worldwide. The geographic and subtype distribution is shown for the 80849 sequences available online at http://hcv.lanl.gov (accessed October 28, 2011).

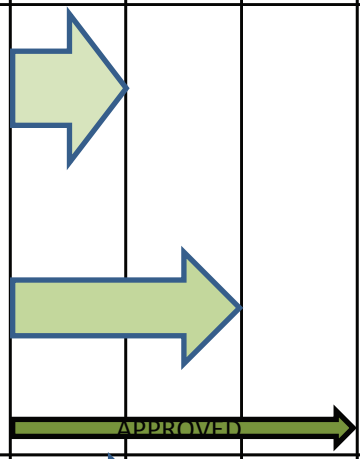
Drug Name	Phase of Clinical Trial			Mode of Action
	I	II	III	
BMS-850032 ACH-1625 GS-9256 ABT-450 IDX320 GS-9451 ACH-2684 MK-6172 TMC435 BI 201335 Vaniprevir (MK-7009) Narlaprevir (SCH-900518) Danoprevir (ITMN-191, RG7227) Telaprevir Boceprevir				NS3/4A protease inhibitors
INX-184 R7128 IDX184 PSI-7977 PSI-938				NS5B polymerase nucleoside inhibitors
IDX375 ABT-333 GS-9190 Filibuvir (PF868554) ANA-598				NS5B polymerase nonnucleoside inhibitors
PPI-461 GS-5885 BMS-824393				NS5A inhibitors
SCY-635 Debio 025				Cyclophilin inhibitors

Table 1.1. STAT-C molecules in clinical trials. Adapted from Jazwinski & Muir, (2011).

Type	Vaccine	Phase of Clinical Trial			Outcome
		I	II	III	
Recombinant Protein	E1/E2/MF59C adjuvant	→			Induce antibody and E1/E2 specific T-cell response
	E1 absorbed on alum	→	→		Induce both Humoral and Cellular immune response
	Yeast expressing NS3-core protein (GI-5005)	→	→		Elicit antigen specific T cell response
	Core in ISCOMATRIX adjuvant	→	→		Induced humoral response in all volunteers
Peptide based Vaccine	Core, NS3 and NS4 peptides with poly-L-arginine adjuvant (IC41)	→	→		Strong T-cell response but transient effect on viral load.
	Peptide derived from Core protein (C35-44)	→			Induced peptide specific CD8 T cell response in some patients
	HCV NS3 formulated with influenza virosomes	→			Result pending
DNA based Vaccine	ChonVac-C; NS3-4a DNA Vaccine	→	→		Transient effect on viral load
	CICGB-230: core/E1/E2+recombinant core protein	→			Weak cellular and humoral response
Recombinant Viral Vectored Vaccine	MVA expressing NS3/4/5B (TG4040)	→			Decrease in viral load in some patients
	Adenovirus expressing NS3-5B (Ad6, AdCh3)	→			Highly Immunogenic

Table 1.2. HCV vaccine candidates in clinical trials. Adapted from Halliday *et al*, (2011).

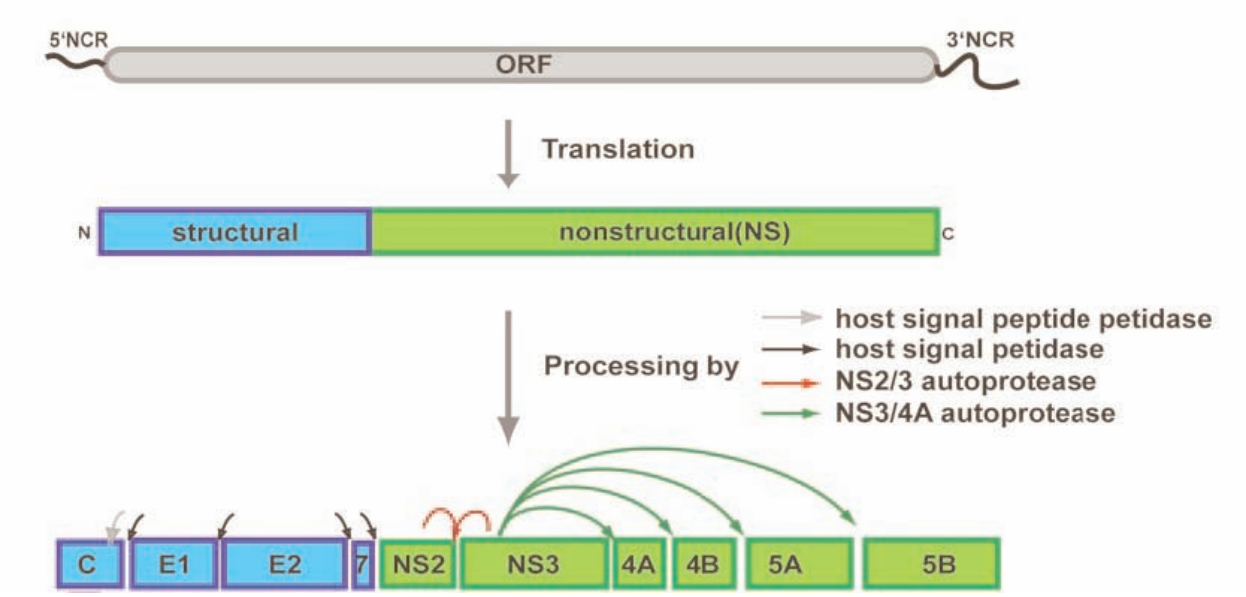


Figure 1.3. The Positive Strand RNA Genome of HCV. It contains 5' and 3' non-coding regions (NCRs) and a single large open reading frame (ORF) that is translated into a polyprotein containing the structural and nonstructural (NS) proteins. The polyprotein is co-and post-translationally cleaved by both host and viral proteases to produced the 10 individual HCV proteins. Extracted from Tellinghuisen *et al*. (2007).

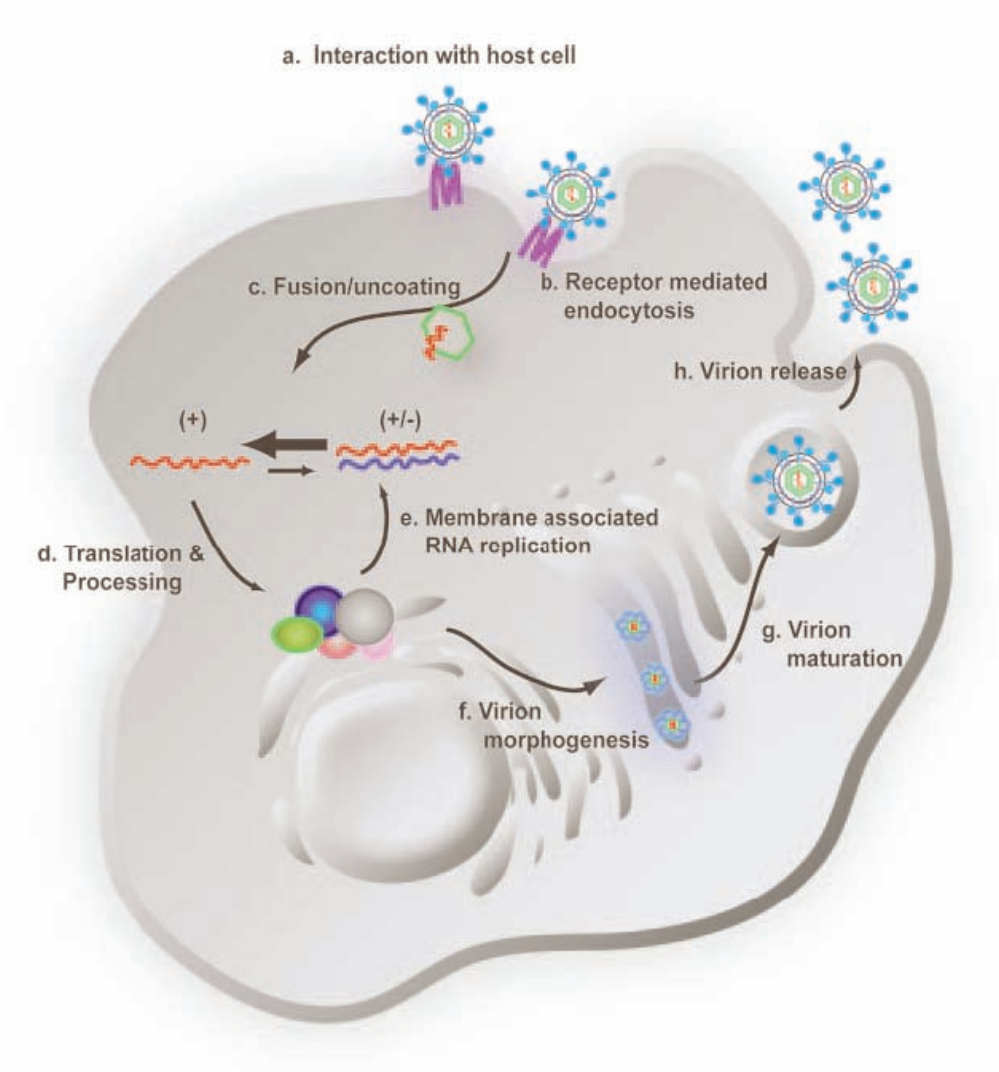


Figure 1.4. HCV Lifecycle. Extracellular virus particles enter hepatocytes by interacting with cell surface receptors (a), followed by receptor-dependent endocytosis. Upon arrival in early endosomes the HCV glycoproteins mediate fusion of the viral envelope with the endosome and the viral genome is released into the cytoplasm (c). The viral RNA is then translated to generate the large polyprotein that is processed into the 10 mature HCV proteins (d). Replication complexes are formed through the association of the NS proteins with ER-derived membranes and they replicate the genome (e). A portion of the newly synthesized positive-sense RNA is packaged into nucleocapsids that then associate with the HCV glycoproteins, leading to budding into the ER (f). Maturation of the virions then occurs during their transit through the cellular secretory pathway (g). Mature virions are then secreted from the cell, completing the life cycle (h). Extracted from Tellinghuisen *et al*. (2007).

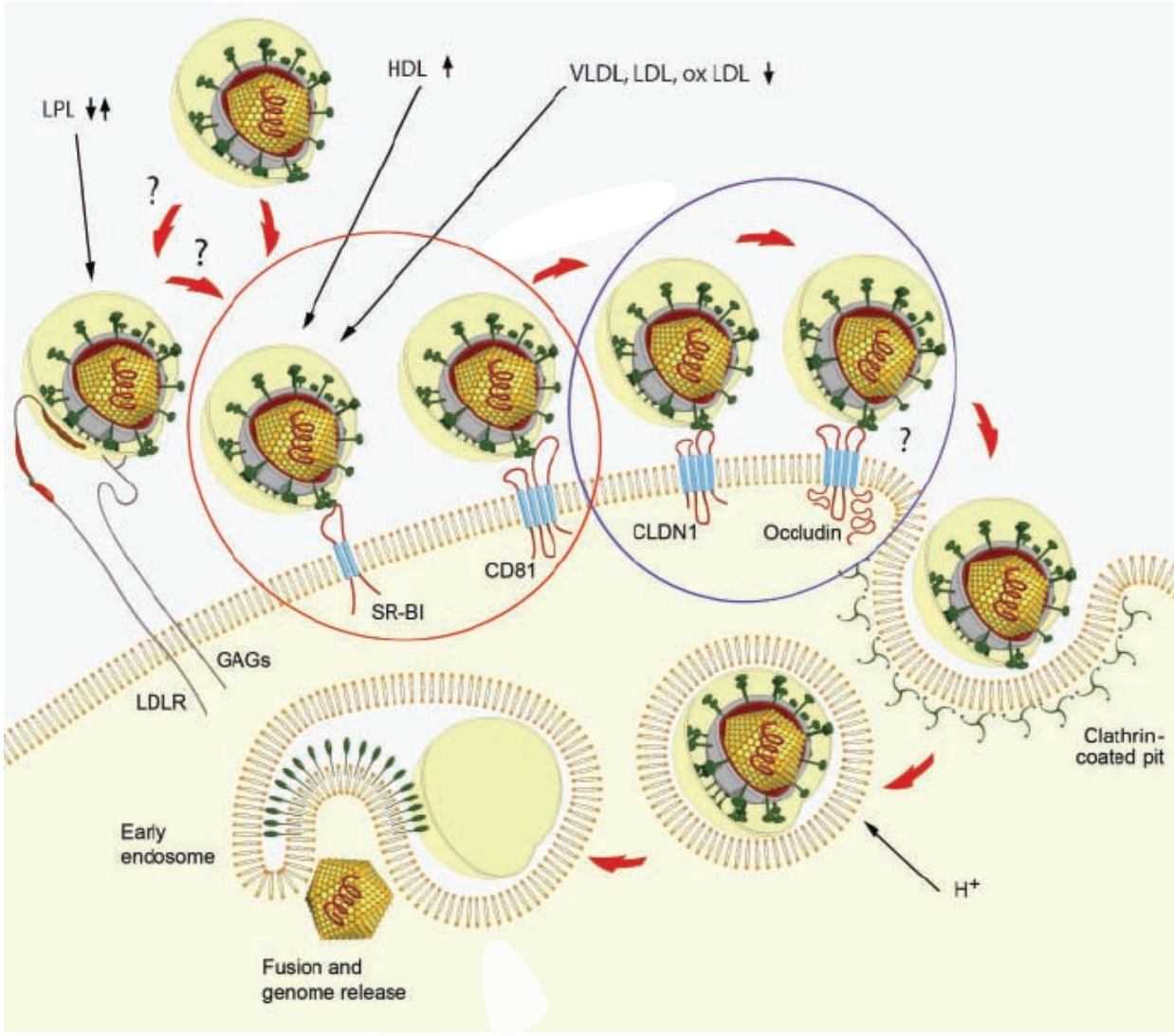


Figure 1.5. Cell Entry of Lipoprotein Associated HCV. Several cell surface molecules are involved in mediating HCV binding and internalization into host cells. LDL-R and GAGs may support the initial attachment by interacting with the viral glycoprotein and virion-associated lipoproteins, respectively. Virus particles then interact with the entry receptors SR-BI and CD81, followed by the tight junction proteins claudin-1 (CLDN-1) and occludin (OCLDN). HCV enters the cell by clathrin-dependent endocytosis and fusion is mediated by the envelope glycoproteins upon acidification of early endosomes. Lipoprotein-mediated HCV cell entry is inhibited by natural ligands of lipoprotein receptors such as LDL, VLDL and oxidized LDL, whereas HDL indirectly enhances HCV entry in a SR-BI dependent manner. Adapted from Burlone et al, (2009).

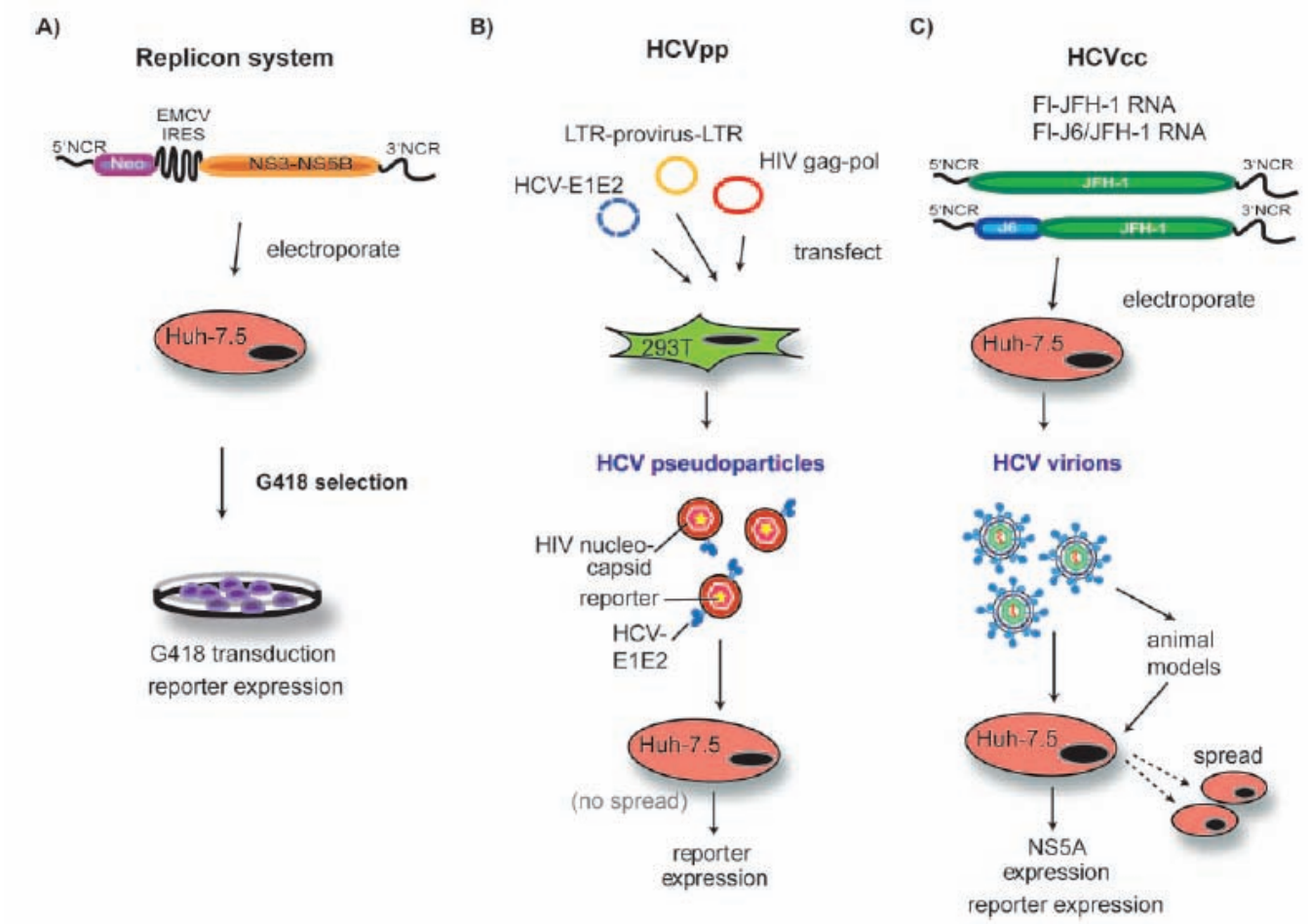
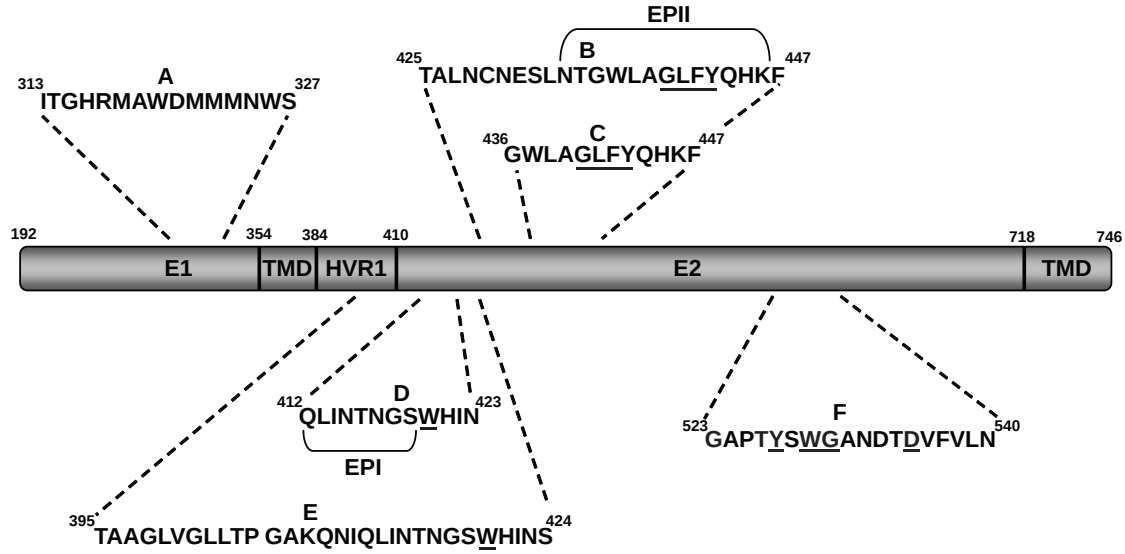


Figure 1.6. ***In vitro*** **systems available for the study of HCV replication, entry and infectivity.** (A) HCV replicon system allows for the study of viral RNA replication in cell culture. Bicistronic replicon RNAs, encoding a selectable marker (Neor) under control of the HCV IRES in the first cistron and the HCV non-structural (NS3-NS5B) proteins under control of a heterologous IRES from encephalomyocarditis selectable marker in the second cistron, are electroporated into Huh-7 based cells lines. RNA replication results in the production of the selectable marker and allows for selection of cell colonies that support active viral RNA replication. (B) HCVpp allows for the study of HCV glycoproteins mediated events in the HCV life cycle. Recombinant retrovirus expressing HCV envelope glycoproteins on their surface are generated by co-transfecting HEK-293T cells with plasmids encoding HCV glycoproteins, retroviral core and polymerase proteins and a proviral genome harbouring a reporter gene. Following infection of permissive cell lines, the retrovirus genomes express a reporter gene allowing for a quantitative measure of cell entry. (C) The HCVcc system provide a method to study the entire HCV lifecycle. This system uses either JFH1 HCV genomic RNA or chimeras of this genome with heterologous sequence. Electroporation of these RNAs into permissive cell lines yield infectious HCV virions that can be used to infect naïve cells or animal models. Productive infection can be monitored by several methods allowing the detection of intracellular viral RNA or protein levels. Extracted from Tellinghuisen *et al*., (2007).

A



B

Antibody	Epitope	Reference
IGH505	A (Linear)	Meiner *et al*., 2008b
IGH526	A (Linear)	Meiner *et al*., 2008b
95-2	D (Linear)	Broering *et al*., 2009
HCV-1	D (Linear)	Broering *et al*., 2009
AP33	D (Linear)	Owsianka *et al*., 2005
3/11	D (Linear)	Tarr et al, 2005
CBH-5	F (Conformational)	Keck *et al*., 2004
A8	F (Conformational)	Johansson *et al*., 2007
1:7	F (Conformational)	Johansson *et al*., 2007
AR3A-D	E,C,F (Conformational)	Law *et al*., 2008
e137	C,E (Conformational)	Perotti *et al*., 2008
e20	F (Conformational)	Mancini *et al*., 2010
CBH-2	B,F (Conformational)	Keck *et al*., 2004
HC-1	F (Conformational)	Keck *et al*., 2008
HC-11	B,F (Conformational)	Keck *et al*., 2008

Figure 1.7. Organization of Functional Domains and Conserved epitopes recognized by broadly nAbs in E1 and E2. Underlined letters in (a) indicate residues critical for E2-CD81 binding. Letters A to F correspond to the antibody binding epitope shown in (b). HVR1: Hypervariable region; TMD: Trans-membrane domain.

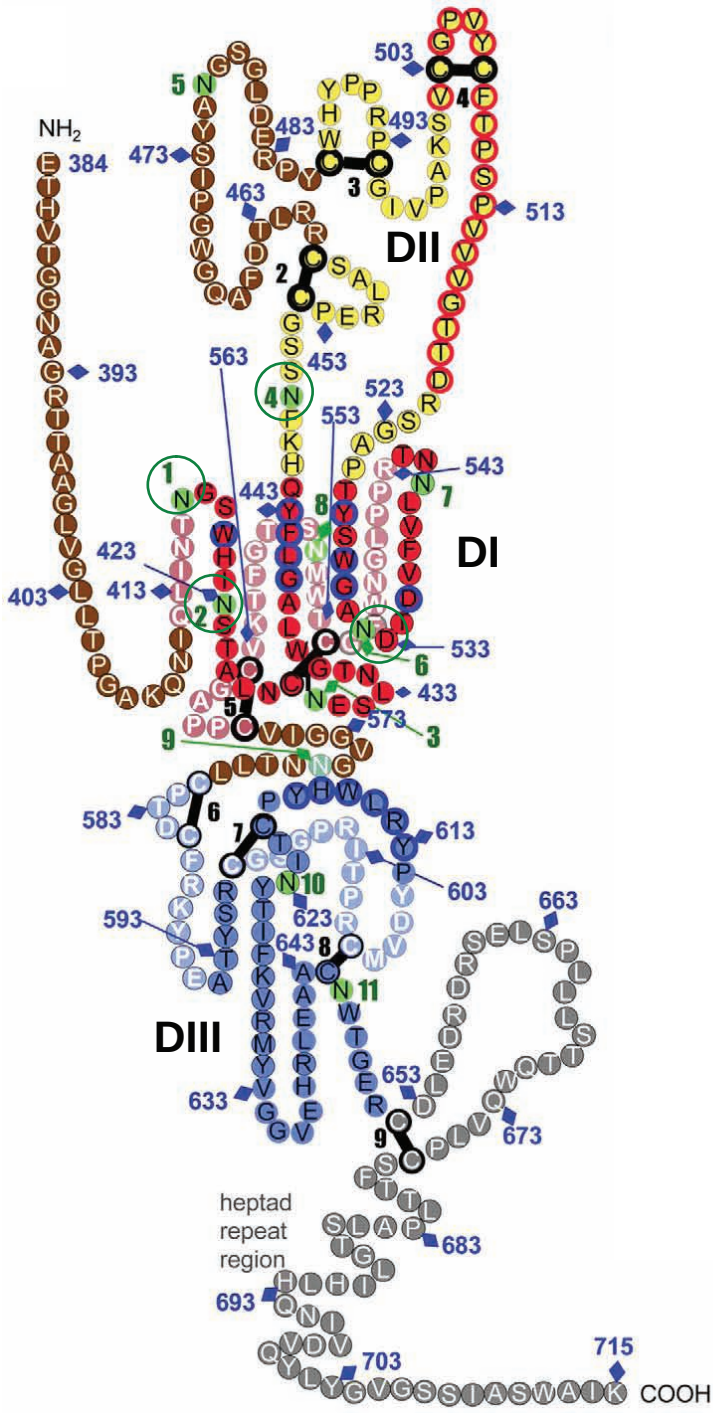


Figure 1.8. Model of HCV E2 Glycoprotein

Based on class II fusion proteins, E2 consists of three separate domains and a stem region connected to the transmembrane domain. The linear sequence of HCV H77-E2 ectodomain is represented as a chain of beads (coloured circles) labelled with the corresponding amino acid. The pale and bright coloured circles represent residues in the background and foreground of the domains, respectively labelled in white and black fonts. Disulfide bonds and glycosylation sites are indicated by thick black bars and green circles, respectively and numbered sequentially. Unstructured segments are in while font on a brown background. Residues that participate in CD81 binding are contoured in blue, and those from the putative fusion loop region in red. The four glycosylation sites (E2N1, E2N2, E2N4 and E2N6) that modulate CD81 binding are indicated by green circles. Adapted from Krey *et al*, 2010.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

Chemical / Reagent	Supplier
2-Amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)	BDH
2-Mercaptoethanol	Sigma
3,3'5,5'-tetramethylbenzidine (TMB) substrate	Sigma
30 % Acrylamide / bis solution 37.5:1	Bio-Rad laboratories
4',6-diamidino-2-phenylindole (DAPI)	Promega
5-chloromethylfluorescein diacetate (CMFDA)	Invitrogen
Absolute ethanol	Bamford Laboratories, UK
Agarose	Melford
Ammonium persulphate (APS)	Bio-Rad Laboratories
Ampicillin	Melford
Bromophenol Blue	BDH
Butanol	Fisher Scientific
Chloroform	Sigma
Ethanol	Fisher Scientific
Ethidium Bromide	Sigma
Glucose	BDH
Glycine	BDH
Iodixanol	Axis-Shield
Isopropanol	Fisher Scientific
Methanol	Fisher Scientific
N,N,N',N'-Tetramethylethylene-diamine (TEMED)	Sigma
Phenol	Sigma
Puromycin	Sigma
Sodium Chloride (NaCl)	BDH
Sodium dodecyl sulphate (SDS)	BDH
Sucrose	BDH
Triton X-100	Sigma
TRIzol	Invitrogen
Tween-20	Bio-Rad Laboratories

2.1.2. Kits

Kit	Source
Advantage® cDNA polymerase Kit	Clonetech
Luciferase Assay System	Promega
MEGAclear Purification Kit	Ambion
MEGAscript High Yield Transcription Kit	Ambion
QIAamp Viral RNA Kit	Qiagen
Qiagen Plasmid Maxi Kit	Qiagen
QIAprep Spin Miniprep Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
QuickChange Site-Directed Mutagenesis Kit	Stratagene
RNeasy Plus Mini Kit	Qiagen
TaqMan Reverse Transcription Kit	Applied Biosystems
SEAP Chemiluminescence Assay kit, Phospha light™	Applied Biosystems

2.1.3. Clones

Name	Details	Source
pcDNA3.1-E1E2	Encoding E1E2 gps of diverse HCV genotypes representing amino acids residues 176 to746 (referenced to strain H77) cloned downstream from a human CMV promoter.	Lavillette *et al.* (2005)
MLV-Gag/Pol	Encoding MLV gag and pol genes cloned downstream of a human CMV promoter.	Bartosch et al., 2003
MLV-Luciferase	MLV based luciferase transfer construct under control of a human CMV promoter.	Bartosch et al., 2003
pJFH1	Full length JFH1 cDNA downstream of the T7 RNA polymerase promoter.	Wakita *et al.* (2005)
$pJFH1_{\Delta E1E2}$	As JFH1, except carries a deletion in the envelope glycoprotein sequences.	Wakita *et al.* (2005)
$pJFH1_{GND}$	As JFH1, except carries a mutation in the NS5B GDD motif.	Wakita *et al.* (2005)
$pJFH1_{N415D}$	As JFH1, except carries a mutation in E2 (N415D).	Dhillon *et al.* (2010)

pJFH1$_{T416A}$	As JFH1, except carries a mutation in E2 (T416A).	Dhillon *et al.* (2010)
pJFH1$_{N417S}$	As JFH1, except carries a mutation in E2 (N417S).	Dhillon *et al.* (2010)
pJFH1$_{I422L}$	As JFH1, except carries a mutation in E2 (I422L).	Dhillon *et al.* (2010)
pJFH1$_{1B12.6}$	As JFH1, except envelope glycoprotein region is replaced by genotype 1 (1B12.6) sequence.	S. Dhillon, Unpublished
pJFH1$_{2A2.5}$	As JFH1, except envelope glycoprotein region is replaced by genotype 2 (2A2.5) sequence.	S. Dhillon, Unpublished
pJFH1$_{3A1.28}$	As JFH1, except envelope glycoprotein region is replaced by genotype 3 (3A1.28) sequence.	S. Dhillon, Unpublished
pJFH1$_{5.15.7}$	As JFH1, except envelope glycoprotein region is replaced by genotype 5 (5.15.7) sequence.	S. Dhillon, Unpublished
pJFH1$_{6.5.340}$	As JFH1, except envelope glycoprotein region is replaced by genotype 6(6.5.340) sequence.	S. Dhillon, Unpublished
p4a/JFH1	As JFH1, except core to NS2 region is replaced by genotype 4a ED43 sequence.	M. Iro Unpublished
pAd4a/JFH1	As 4a/JFH1, except contains six cell-culture adaptive mutations in E2, NS2, NS3 and NS5A.	S. Dhillon Unpublished
phCMV E1E2	Encoding JFH1 sequence representing amino acids 132-746 cloned downstream of a human CMV promoter.	Witteveldt et al., 2009
phCMV E1E2$_{N415D}$	As phCMV E1E2, except carries a mutation in E2 (N415D), downstream of a human CMV promoter.	Dhillon *et al.* (2010)
phCMV E1E2$_{T416A}$	As phCMV E1E2, except carries a mutation in E2 (T416A), downstream of a human CMV promoter.	Dhillon *et al.* (2010)

phCMV $E1E2_{N417S}$	As phCMV E1E2, except carries a mutation in E2 (N417S), downstream of a human CMV promoter.	Dhillon *et al.* (2010)
phCMV $E1E2_{I422L}$	As phCMV E1E2, except carries a mutation in E2 (I422L), downstream of a human CMV promoter.	Dhillon *et al.* (2010)

2.1.4. Cell lines

Cells	Description	Source
Huh7	Human Hepatoma cell line	Jean Dubuisson (CNRS, Institut de Biologie de Lille, Lille, France)
Huh-7 J20	Human Hepatoma reporter cell line	Arvind Patel (Centre for Virus Research, Glasgow, UK)
HEK-293T	Human Embryonic Kidney cell line	American Type Culture Collection

2.1.5. Antibodies

2.1.5.1. Primary Antibodies

Antibody	Name	Type	Raised in	Source
Anti-core	C7-50	Monoclonal	Mouse	Bioreagents
Anti-NS5A	9E10	Monoclonal	Mouse	Lindenbach *et al*. (2005)
Anti-NS5A	Anti-NS5A	Polyclonal	Sheep	Macdonald *et al.* (2003)
Anti-E2	AP33	Monoclonal	Mouse	Clayton *et al*. (2002)
Anit-E2	D3.7	Monoclonal	Mouse	Dhillon *et al*. (2010)
Anti-E2	3/11	Monoclonal	Rat	Flint *et al*. (1999)

Anti-E2	CBH-4B	Monoclonal	Human	Keck *et al*. (2003)
Anti-E2	CBH-5	Monoclonal	Human	Keck *et al*. (2003)
Anti-E2	HC-11	Monoclonal	Human	Keck *et al*. (2008)
Anti-HCV	IgG7	Purified polyclonal IgG	Human	Haberstroh *et al.* (2008)
Anti-HCV	IgG17	Purified polyclonal IgG	Human	Haberstroh *et al.* (2008)
Anti-HCV	IgG19	Purified polyclonal IgG	Human	Haberstroh *et al.* (2008)
Anti-Tubulin	Anti-Tubulin	Monoclonal	Mouse	Sigma
Anti-CD81	Anti-CD81	Monoclonal	Mouse	BD Biosciences
Anti-SR-BI	CLA-1	Monoclonal	Human	BD Biosciences
Anti-SR-BI	Anti-SR-BI	Polyclonal sera	Rat	Zeisel *et al.* (2007)
Anti-MLV gag	CRL-1912	Monoclonal	Rat	American Type Culture Collection

2.1.5.2. Secondary Antibodies

Antibody	Source
FITC-conjugated donkey anti-mouse IgG	Invitrogen
FITC-conjugated goat anti-human IgG	Invitrogen
FITC-conjugated goat anti-rabbit IgG	Invitrogen
TRITC-conjugated goat anti-mouse IgG	Invitrogen
TRITC-conjugated donkey anti-sheep IgG	Invitrogen
Cy5-conjugated donkey anti-rabbit IgG	Invitrogen
PE-conjugated anti-mouse IgG	BD Biosciences
PE-conjugated anti-mouse IgG 2a specific	BD Biosciences

Anti-streptavidin-HRP conjugate	Sigma
Anti-mouse-HRP conjugate	Sigma
Anti-rabbit-HRP conjugate	Sigma
Anti-human-HRP conjugate	Sigma

2.1.6. Solutions

2.1.6.1. Bacterial Expression

Solution	Components
Lysogeny Broth (LB)*	170 mM NaCl, 10 g/l Bactopeptone, 5 g/l yeast extract
LB-agar*	LB plus 1.5 % (w/v) agar
Yeast tryptose broth (YTB)*	85 mM NaCl, 16 g/l Bactopeptone, 10 g/l yeast extract

* Prepared in-house by the media department

2.1.6.2. DNA Manipulation

Solution	Components
DNA loading dye	30 % glycerol, 0.25 % bromophenol blue, 0.25 % xylene blue
TBE (10x)	8.9 M Tris-borate, 8.9 M boric acid, 0.02 M EDTA (pH 8.0)

2.1.6.3. SDS-PAGE

Solution	Components
Running Gel Buffer	40 mM Tris, 185 mM Glycine, 0.1 % SDS
Resolving Gel Buffer	0.5 M Tris-HCl pH 8.9, 0.4 % SDS
Stacking Gel Buffer	0.5 M Tris-HCl pH 6.9, 0.4 % SDS
Sample loading buffer 2X (Reducing)	100 mM Tris-HCl pH 6.9, 2 % SDS, 10 % glycerol, 5 % β-mercaptoethanol, 1 µg/ml bromophenol blue

2.1.6.4. Western Blot Analysis

Solution	Components
Towbin Buffer	25 mM Tris-HCL (pH 8.0), 192 mM glycine, 20 % (v/v) methanol
PBSA*	170 mM NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 25 mM Tris-HCl (pH 7.2)
PBST	PBSA, plus 0.05 % (v/v) Tween-20

*Prepared in-house by the media department

2.1.6.5. Cell Lysis

Solution	Components
Cell Lysis Buffer (LB2)	20 mM Tris-HCl pH 7.4, 20 mM iodoacetamide, 150 mM NaCl, 1 mM EDTA, 0.5 % Triton X-100

2.1.6.6. Tissue Culture

Solution	Components
Trypsin solution	0.25 % (w/v) Difco trypsin dissolved in PBSA, 0.002 % (w/v) phenol red
Versene	0.6 mM EDTA in PBSA, 0.002 % (w/v) phenol red

2.1.7. Oligonucleotide Synthesis

Oligonucleotides were purchased from Sigma.

2.2. Methods

2.2.1. Basic Technique

2.2.1.1. Tissue culture

All components of cell culture media were supplied by Invitrogen. Huh7 and HEK-293T cells were grown in $DMEM_{complete}$ (Dulbecco's modified Eagle's medium [DMEM] supplemented with 10 % fetal calf serum [FCS] (heat inactivated at 56^{o}C for 30 min), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM non-essential amino acids and 2 mM glutamine) and maintained at 37 ^{o}C in an atmosphere of 5 % CO_2. Huh-7 J20 cells were cultured in $DMEM_{complete}$ in the presence of puromycin at a concentration of 2µg/ml. This Huh-7 reporter cell-line stably expresses a fusion protein composed of the enhanced green fluorescence protein and secreted alkaline phosphatase linked by an octapeptide spacer and the HCV NS4A/4B cleavage site that acts as a substrate for the viral serine protease NS3/4A. The role of EGFP is to retain the entire fusion protein within the cell. During HCV replication the NS3/4A protease releases SEAP from the fusion protein, thus enabling its N-terminal signal peptide to direct its secretion into the extracellular culture medium. During infection the SEAP levels directly reflect the levels of intracellular HCV RNA replication (Iro *et al*., 2009). Cell lines were typically grown in 80 cm^2 or 175 cm^2 tissue culture flasks (Nunc). At reaching about 90 % confluency cells were washed gently with PBSA followed by their removal with trypsin (Sigma) diluted 1:100 in versene (TV). Cells were then resuspended in 10 ml of $DMEM_{complete}$ before re-seeding or use in experiments.

2.2.1.2. Indirect Immunofluorescence

Immunofluorescence (IF) was used to visualise the intracellular expression of HCV proteins following electroporation or infection. Cells seeded onto coverslips in a 24-well plate were first washed with PBSA and then fixed in 100 % methanol at -20^{o}C for a minimum of 2 h. Subsequently cells were washed 3 times with PBSA, blocked for 10 min in blocking buffer (PBSA containing 2 % FCS) and incubated at room temperature (RT) for 1 h with primary antibody diluted at appropriate concentration in the blocking buffer. Cells were then washed 3 times with PBSA and stained with secondary antibody conjugated with FITC, TRITC, or Cy5 for 1 h at room temperature. Finally cells were washed 3 times with PBSA and then dH_2O and the coverslips were mounted on a glass slide. Mounting media used contained DAPI (Biochemika) for nuclei staining. The cells

were examined with a Zeiss Laser Scanning LSM510 META inverted confocal microscope (Carl Zeiss Ltd., UK). The images were analyzed using LSM510 software.

2.2.1.3. ELISA

Cells were lysed directly in LB2 and the lysates were clarified by briefly spinning at 13,000 r.p.m. for 1 min. ELISA was performed to determine the reactivity of the intracellular HCV E2 glycoproteins to different anti-E2 antibodies or to the soluble CD81-LEL (hCD81-LEL). Immulon 2HB flat bottom plates were either coated with *Galanthus nivalis* (GNA) lectin (5 µg/ml) or hCD81-LEL (5 µg/ml). 100 µl of capture agent diluted in PBSA was added to each well and incubated overnight at 4 ^{o}C under gentle shaking. Plates were washed 3 times in PBST and blocked for 2 h at RT in 200 µl PBST with 2 % dried milk. Plates were washed as above. 100 µl of neat and 3-fold serially diluted cell lysate in PBST was captured onto the GNA and hCD81-LEL coated plates and incubated overnight at 4 ^{o}C. Cell lysate from naive uninfected cells diluted in PBST served as negative control. Plates were washed as above and incubated for 1 h at RT with 100 µl/well anti-E2 specific antibodies diluted at appropriate concentration in PBST. Plates were washed as above and incubated for 1 h at RT with 100 µl/well of appropriate anti-species HRP conjugated secondary antibody. Following incubation plates were washed 6 times in PBST and binding was realised by addition of 100 µl/well TMB substrate. Reactions were stopped with 0.5M H_2SO_4 and absorbance value was measured at 450 nm**.**

2.2.1.4. SDS-PAGE

2.2.1.4a. Preparation of cell extract for SDS-PAGE analysis

For SDS-PAGE cells were either lysed directly in 1x sample loading buffer (reducing) or LB2. Cells lysed in sample buffer were homogenised by passing through a 22-gauge needle. Following lysis in LB2, the lysates were spun briefly (13,000 r.p.m. for 1 min) to remove nuclei and the clarified lysates were mixed 1:1 with 2 x sample loading buffer. All samples were denatured by boiling at 100 ºC for 5 min before use.

2.2.1.4b. SDS-PAGE analysis

Resolving gels were prepared using acrylamide solution at a final concentration of 10-14 % in 1 x resolving gel buffer. Stacking gels were prepared using acrylamide at a final concentration of 5 % in 1 x stacking gel buffer. Polymerisation of gels was initiated by the addition of APS (to 0.1 %) and TEMED (to 0.08 %). A 10-tooth Teflon comb was typically used to form wells in the stacking gel. Denatured proteins were loaded onto the

gel alongside a protein marker (Amersham) for size determination. Fractionation was performed at 100 V in gel running buffer. Upon separation gels were removed from the apparatus and transferred onto nitrocellulose membrane.

2.2.1.5. Western Immunoblotting

Proteins separated on polyacrylamide gels were transferred to HybondTM-ECL^{TM} nitrocellulose membranes using a BioRad transblot semi-dry blotting device. Transfer was carried out at 25 V for 15 min. Following transfer membranes were incubated for 1 h at RT or overnight at 4 ^{o}C in PBST containing 5 % dried milk powder to block non-specific binding of antibody. Membranes were washed 3 times in PBST and probed with the appropriate antibody (diluted in PBST) for 1 h at RT. The membranes were washed as above and incubated with the appropriate HRP conjugated secondary antibody (diluted in PBST) for 1 h at RT. Finally, the membranes were washed as above and bound antibody was detected using Enhanced Chemiluminescence Reagents I and II (ECL I & II) (Amersham) in equal ratio. Bands were visualized by autoradiography using Kodak X-OMAT film and a Konica SRX-101-A film processor.

2.2.1.6. Flow Cytometry

Flow cytometry was used for counting and sorting of cells resuspended in a stream of fluid. Cells of interest were washed in PBSA and either trypsinised for 5 min or de-attached from the surface using cell dissociation buffer (Sigma). Cells were then washed in PBSA, pelleted at 1000 rpm for 5 min, resuspended in PBSA and counted using a haemocytometer. When necessary cells were fixed in 2 % paraformaldehyde (PFA) for 15 min at RT. Cells were then washed once in PBSA followed by a wash in PBSA + 2 % FCS + 0.01 % sodium azide (FPBSA). Subsequent steps, including blocking, washes and incubation with antibodies, were carried out in this buffer. When necessary fixed cells were permeabilised using FPBSA + 0.1 % saponin and subsequent steps were carried out in this buffer. Prior to staining cells were blocked at RT for 30 min. Following incubations cells were pelleted and $2x10^5$ cells were incubated for 45 min at RT in 100 µl primary antibody or an irrelevant IgG control diluted in FPBSA (+/- 0.01 % saponin). Unbound antibody was removed by washing and the cells were further incubated for 45 min at RT with fluorescently conjugated secondary antibody. Finally cells were washed, resuspended in FPBSA and analysed on BD FACSCalibur using CellQuest Pro software (BD Biosciences).

2.2.2. Molecular Cloning

2.2.2.1. PCR Amplification

PCRs were performed in a GeneAmp PCR machine (Applied Biosystems). PCR amplification was used to introduce point mutations, generate cDNA from total RNA, amplify cDNA for sequencing and fuse PCR products together to produce JFH1 chimeric constructs.

2.2.2.1a. PCR amplification of cDNA

For direct sequencing, viral cDNA generated from total cellular RNA was amplified using Advantage cDNA polyermase kit (Clonetech). Each reaction mix had a final volume of 50 µl and consisted of 1x reaction buffer, 5 µl cDNA, 1 µl each of 10 µM forward and reverse primer, 1 µl 10 mM dNTPs and 1 µl DNA polymerase. Each reaction was amplified at

1 min	95 °C	
30 sec	94 °C	} 35 cycles
3 min	68 °C	
3 min	68 °C	
∞	15 °C	

Following amplification the entire reaction was subjected to agarose gel electrophoresis (section 2.2.2.3.) and the band of interest purified by gel extraction (section 2.2.2.4.) using QIAquick gel extraction kit (Qiagen).

2.2.2.1b. Fusion PCR (to generate E1E2/JFH1 Chimeric viruses)

Fusion PCR was performed in three stages using the Herculase II fusion DNA polymerase (Agilent Technologies). Primers were designed to generate fragments with overlapping overhangs and additional primers to fuse the fragments together (Appendix A). In the first PCR step pUC JFH1 and pcDNA3.1-E1E2 were used as templates to generate three PCR fragments with overlapping overhangs. Reaction mix consisted of 30 ng DNA template, 1 µl each of 10 µM forward and reverse primers, 1x reaction buffer, 1 µl dNTPs and 0.5 µl herculase polymerase in a total of 30 µl volume. For each fragment PCR programme consisted of:

2 min 94 °C
15 sec 94 °C ⎫
20 sec 56 °C ⎬ 10 cycles
60 sec 72 °C ⎭
∞ 4 °C

The PCR product was separated on 1 % agarose gel (section 2.2.2.3.) and the gel purified (2.2.2.4.) fragments were fused together in a second PCR step. An equal amount of each fragment was mixed in the absence of primers with 1x reaction buffer, 1 µl dNTPs and 0.5 µl herculase polymerase in a total of 30 µl volume. The PCR program consisted of:

2 min 94 °C
15 sec 94 °C ⎫
20 sec 56 °C ⎬ 10 cycles
60 sec 72 °C ⎭
∞ 4 °C

2 µl of the fusion product was subsequently amplified in the presence of 1µl each of the 10 µM forward and reverse primers, 1x reaction buffer, 1 µl dNTPs and 0.5 µl herculase polymerase in a total of 30 µl volume. The PCR program consisted of:

2 min 94 °C
15 sec 94 °C ⎫
20 sec 65 °C ⎬ 10 cycles
2,30 min 72 °C ⎭
3 min 72 °C
∞ 4 °C

The final PCR product was run on 1 % agarose gel (section 2.2.2.3.) and the gel purified (section 2.2.2.4.) fragment was digested with either *FspAI/NotI* or *KpnI/AgeI* restriction enzyme. Finally, the digested fragments were cloned into pJFH1 backbone digested with the appropriate restriction enzyme (section 4.2). The proper incorporation of inserts into the pJFH1 template was verified by nucleotide sequencing (Appendix B).

2.2.2.1c. Site Directed Mutagenesis

Mutagenesis reactions were performed using the QuickChange II XL Site-Directed Mutagenesis kit (Stratagene). Forward and reverse primers (Appendix C) were designed

(according to the manufacturer's instructions) with the desired mutation(s) incorporated in the middle of the primer sequence of length between 25 and 45 bases. PCR mix consisted of 50 ng pUCJFH1 DNA template, 1x reaction buffer, 1.25 µl each of 10 µM forward and reverse mutagenic primers, 1 µl dNTPs, 3 µl Quick solution (added to improve linear amplification) and 1 µl pfuUltra HF DNA polymerase in a total of 50 µl reaction. The PCR program consisted of

1 min	95 °C	
50 sec	95 °C	⎫
50 sec	60 °C	⎬ 18 cycles
12 min	68 °C	⎭
7 min	68 °C	
∞	4 °C	

Following amplification, each reaction was chilled on ice for 2 min followed by the addition of DpnI (10 units) to digest the non-mutated dam-methylated parental DNA. The reactions were gently mixed by pipetting and then centrifuged at 13,000 r.p.m. for 1 min, followed by incubation at 37 ºC for 1 h. 2 µl of the DpnI-treated DNA was then transformed into 45 µl of XL10-Gold Ultracompetent cells mixed with 2 µl of β-ME according to the manufacturer's instructions.

2.2.2.1d. RT-qPCR

RT-qPCR was a two-step protocol. The first step involved reverse transcription of viral and cellular RNA in a GeneAmp PCR machine (Applied Biosystems) using the TaqMan Reverse Transcription Reagents Kit. Each reaction contained 1 µl purified cellular RNA mixed with 1x RT buffer, 4.4 µl $MgCl_2$, 4.0 µl dNTPs, 1 µl random hexamers, 0.4 µl RNase inhibitor, 0.5 µl multiscribe reverse transcriptase in a total volume of 20 µl. Reaction cycle consisted of

10 min	25 °C
60 min	37 °C
5 min	95 °C
∞	4 °C

The second step involved either relative or absolute quantification of RNA where each sample was analysed in triplicate as a singleplex reaction. For relative quantification of HCV RNA, cDNA obtained from first step was amplified using both HCV -specific and GAPDH-specific primers (Applied Biosystems) in the presence of FAM™ (HCV-specific) and VIC® (GAPDH-specific) labelled probes. The JFH1 probe and primer sequences are located in the 5' UTR of the viral genome (Witteveldt *et al*., 2009). To determine mRNA transcript of SR-BI and CD81 genes, cDNA obtained from first step was amplified using TaqMan probes (ABI) specific for SR-BI (Hs00969819) and CD81 (Hs00174717). Each sample was normalised to the endogenous GAPDH control gene. The Real-Time PCR reaction mix for each sample contained

JFH1 RNA Reaction Mix	**Volume (µl)**
18 µm Forward Primer (Final 900 nm)	1.0
18 µm Reverse Primer (Final 900 nm)	1.0
5 µm FAM JFH1 Probe (250 nm)	1.0
TaqMan Fast Universal Mix (2x)	10.0
dH_2O	5.0
cDNA	2.0

GAPDH, SR-BI and CD81 RNA Reaction Mix	**Volume (µl)**
Probe	1.0
TaqMan Fast Universal Mix (2 x)	10.0
dH_2O	7.0
cDNA	2.0

The amplification was performed on an Applied Biosystems 7500 Fast Real-Time PCR System using Fast Universal PCR conditions and the data analysed using Applied Biosystems software (SDS version 1.3.1), according to the manufacturer's instructions.

20 sec	95 °C	
3 sec	95 °C	} 40 cycles
30 sec	60 °C	

In order to determine the absolute quantification of purified HCV RNA, *in vitro*-transcribed JFH1 genomic RNA of known concentration was used as a standard. Serially diluted standard cDNA and the undiluted sample cDNA obtained in first step was amplified and analysed using the same fast universal conditions as for relative quantification using HCV- specific primer and FAM probe.

2.2.2.2. Restriction Enzyme Digestion

Restriction enzyme digests were performed at 37 °C for at least 1 h unless otherwise specified by the manufacturer. Reactions were typically carried out in 50 µl volume using 10 units of each enzyme per µg DNA. All reactions were performed using the appropriate enzyme buffers and BSA if necessary.

2.2.2.3. Agarose Gel Electrophoresis

This method was used to isolate and purify DNA fragments amplified by PCR or DNA fragments produced by restriction enzyme digestion. Typically, larger fragments (>500 bp) were separated on 1 % agarose gel and smaller fragments (<500 bp) on 2 % agarose gel. The agarose gels were prepared in 1x TBE containing 1 µg/ml ethidium bromide. DNA fragments to be loaded on the gel were first mixed with 0.1 volume of 10x DNA loading dye and gels were typically run at 100 V in 0.5 x TBE buffer. DNA fragments were run alongside 1 kb or 100 bp DNA ladders (NEB). To excise digested DNA fragments and PCR product, fragments were visualised using long wave UV light followed by purification using the QIAQuick gel extraction kit (Qiagen) (section 2.2.2.4.).

2.2.2.4. Purification of DNA from Agarose Gels

Excised gel slices containing digested DNA fragments were dissolved in buffer QG (solubilisation buffer) at a ratio of 1:3 and incubated at 50 ^{o}C. Dissolved DNA fragment was then mixed with 1 gel volume of isopropanol to increase DNA yield. DNA in the gel solution was then bound to a QIAquick spin column by centrifugation at 13, 000 r.p.m. for 1 min. The column was washed once with 750 µl buffer PE (washing buffer) and centrifugation was repeated at 13, 000 r.p.m. for 1 min. DNA was eluted from the column by addition of 30-50 µl of buffer EB (elution buffer) followed by further centrifugation at 13, 000 r.p.m. for 1 min.

2.2.2.5. DNA fragment Ligation

Gel purified DNA fragments following restriction enzyme digestion were ligated for 16 h at 16 °C in 10 µl reactions containing 1x ligase buffer and 2 units of T4 DNA ligase. Following ligation, 2 µl DNA was used for electroporation into competent *E. coli* bacteria (section 2.2.2.7.).

2.2.2.6. Preparation of electrocompetent bacterial cells

NEB DH5α competent *E. coli* culture was grown overnight on a shaker at 37 ^{o}C. 10 ml of the overnight culture was added to 1 L pre warmed LB and incubated at 37 °C to an OD_{600} reached around 0.8. The culture was chilled on ice for 30 min followed by centrifugation at 4000 rpm for 10 min at 4 °C. The formed pellet was resuspended in 1 L ice-cold deionised molecular biology grade water (dH_2O). Cells were re-centrifuged as before and resuspended in 500 ml ice-cold dH_2O with 10 % glycerol. Again cells were centrifuged as before and resuspended in 2-3 ml ice-cold dH_2O with 10 % glycerol. The suspension was aliquoted into 70 µl aliquots and stored for up to 6 months at -70 °C.

2.2.2.7. Transformation of Electrocompetent *E. coli* Cells

2 µl of plasmid or ligated DNA was added to 70 µl electrocompetent *E. coli* in a pre-chilled 1 mm gap cuvette (Apollo) and electroporated (1.8 kV, 25 µF, 200 Ω) using a BioRad GenePulser Xcell. Electroporated cells were then resuspended in 0.5 ml YTB and incubated at 37 °C with shaking at 180 rpm for 1 h before being plated out on LB-agar plates with ampicillin (100 µg/ml). Plates were then incubated overnight at 37 °C.

2.2.2.8. Small Scale Plasmid Preparation (Minipreps)

Single colonies were picked from a freshly streaked selective agar plate to inoculate 5 ml cultures of LB with ampicillin (100 µg/ml), followed by overnight incubation at 37 ºC with vigorous shaking at 180 r.p.m. 1.5 ml of cultures was then centrifuged at 13,000 r.p.m. for 5 min and the bacterial pellets were resuspended in 250 µl buffer P1 (cell resuspension buffer). The cells were then lysed by mixing equal volume of buffer P2, vortexed briefly and then incubated at RT for 5 min. 350 µl of buffer N3 (precipitation buffer) was added to the cells and the samples were vortexed briefly, followed by centrifugation at 13, 000 r.p.m. for 10 min in order to pellet cellular debris. The supernatants were then applied to the QIAprep spin columns, followed by centrifugation at 13, 000 r.p.m. for 1 min. The spin columns containing the bound DNA were washed once with 750 µl buffer PE (washing

buffer) and centrifugation was repeated at 13, 000 r.p.m. for 1 min. DNA was eluted from the column by addition of 30-50 µl of buffer EB (elution buffer) followed by further centrifugation at 13, 000 r.p.m. for 1 min.

2.2.2.9. Large Scale Plasmid Preparation from Transformed Bacteria

A single colony was picked from a freshly streaked selective agar plate to inoculate a 5 ml starter culture of LB with ampicillin (100 µg/ml). Following 8 h incubation, the starter culture was diluted 1:500 into 200 ml of LB with ampicillin as above and cultured overnight at 37 ºC with vigorous shaking (180 r.p.m.). A large scale DNA preparation was then made from the bacterial pellet harvested by centrifugation at 3000 r.p.m. for 10 min at 4 ºC, using the Qiagen HiSpeed plasmid Maxi kit. The pellet was resuspended in 10 ml buffer P1 (cell resuspension buffer) by pipetting. 10 ml of buffer P2 (Lysis buffer) was added to the suspension and mixed by vigorously inverting the tube few times. The suspension was incubated at RT for 5 min before adding 10 ml of buffer P3 (precipitation buffer) and further mixing by inversion. The lysate was then poured into the barrel of the QIAfilter cartridge and incubated at RT for 10 min. During the incubation period a high speed maxi tip was equilibrated using 10 ml buffer QBT and the column was allowed to empty by gravity flow, followed by filtering the lysate into the equilibrated tip and again allowing the column to empty by gravity. Once the lysate had filtered through and plasmid DNA had bound, the column was washed with 60 ml buffer QC (wash buffer) and allowed to empty by gravity. Plasmid DNA was then eluted by adding 15 ml of buffer QF (elution buffer) and the DNA was precipitated by adding 10.5 ml isopropanol to the eluate followed by incubation at RT for 5 min. The eluate/isopropanol mixture was then transferred into the 30 ml syringe attached to the QIAprecipitator maxi module and the mixture was filtered by inserting the plunger using constant pressure. The DNA was then washed by adding 2 ml 70 % ethanol to the syringe and filtered as above. The plasmid DNA bound to membrane was allowed to dry by pressing air through the QIAprecipitator quickly and forcefully by inserting the plunger a couple of times. In the final step the QIAprecipitator was attached to a 5 ml syringe and the plasmid DNA eluted by adding 1 ml of buffer TE (elution buffer) to the syringe and eluting DNA by inserting the plunger.

2.2.2.10. DNA Quantification

DNA aliquots were diluted 1:50 in dH_2O and the OD_{260} measured using a BioPhotometer (Eppendorf).

2.2.2.11. Nucleotide Sequencing

Sequencing of plasmid and amplified cDNA was performed by GATC biotech, Germany. A minimum of 30 µl of DNA (100 ng/µl) and primers (10 µM) were required for each reaction. Completed sequences were analyzed using Chromas (ABI) and NCBI alignment software.

2.2.3. HCVcc Based Work

HCVcc based work was performed using $JFH1_{WT}$, $JFH1_{\Delta E1E2}$, $JFH1_{GND}$, JFH1 containing E2 point mutations and intergenotypic chimeric JFH1 viruses.

2.2.3.1. Generating E1E2/JFH1 chimeras

E1E2/JFH1 chimeras were designed as described in section 2.2.2.1b. Primers used for PCR amplification and for sequencing the final product are listed in Appendix A and B.

2.2.3.2. Introducing E2 Mutations into pJFH1

All single mutations were introduced individually into pUC-JFH1 by site-directed mutagenesis using primers listed in Appendix C. Several colonies from each agar plate were selected and plasmids extracted using mini-preps were sequenced using sequencing primers NA17 and NA24 (Appendix D). Positive clones were then restriction digested with BsaBI and BsiWI and the desired fragments ligated into the parental pJFH1 vector backbone digested with the same enzymes. The ligation mix was then transformed into competent DH5α cells and plasmid maxi-prep was prepared. To verify the presence of desired mutations and to confirm the correct ligation of the fragments, the plasmids were sequenced again using the same primers as above. Following sequencing the correct plasmids were linearised and used to prepare *in vitro* transcribed viral RNA.

2.2.3.3. Introducing Cell Culture Identified Mutations into 4a/JFH1 vector

A total of six mutations selected during long term passaging of 4a/JFH1 infected cells were introduced into the parental 4a/JFH1 plasmid by site directed mutagenesis (SDM). Mutations I690M, T827A and T889I were introduced into the same plasmid by three separate SDM reactions. To do this, an SDM reaction was first used to introduce the I690M mutation using the appropriate primers. Bacterial colonies obtained from the

transformation of this reaction were used to generate minipreps of plasmid DNA. Sequencing was then performed on each miniprep using primers to confirm the presence of the desired mutation and the absence of any unwanted PCR mutations between the *BglII* (nt 3123) and *BsrGI* (nt 7793) restriction enzymes sites. A correct plasmid was then used in a second SDM reaction to introduce the T827A reaction using the same method as above. A third SDM was then performed on the plasmid containing the I690M and T827A changes to then finally introduce the T8891 mutation. In parallel, mutations M956L, A1271V and Y2289H were incorporated into the 4a/JFH1 using the same method described above for introducing the I690M, T827A and T889I mutations. The two clones each containing the three respective mutations were digested with *BglII* and *BsrGI* and subsequently ligated together. Proper ligation of the two fragments and presence of mutations was confirmed by nucleotide sequencing (Appendix D) prior to linearizing the plasmid and preparing *in vitro* transcribed viral RNA.

2.2.3.4. Preparation of template DNA for *In Vitro* Transcription

Briefly, 50 µg of plasmid DNA was linearised by *XbaI* digestion in a 200 µl reaction in a 1.5 ml RNase free tube (Ambion). The linearised template was then treated with Mung Bean Nuclease for 30 min at 30 ^{o}C to allow complete removal of the sticky ends generated by *XbaI*. To clean the template of proteins, Proteinase K (final concentration 100 µg/ml) and SDS (final concentration 0.5 %) were added and the mixture incubated for 30 min at 50 °C. The linearised DNA was then recovered using the phenol/ chloroform purification. 200 µl of neutral phenol-chloroform (25 parts saturated neutral phenol: 24 parts chloroform: 1 part isoamylalcholol) was added and the mixture was vigorously vortexed. The solution was then centrifuged at 13,000 r.p.m. for 2 min to separate the aqueous layer from organic layer. The aqueous layer was placed in a fresh RNase-free tube and mixed with 0.1 volumes of 5 M NH_4OAc and 3 volumes 100 % ethanol. The sample was stored at -20 °C for a minimum of 30 min before being centrifuged at 13,000 r.p.m. for 15 min to pellet the precipitated DNA. The supernatant was carefully removed, pellet washed with 70 % ethanol, centrifuged again at 13,000 r.p.m. for 15 min and the washed pellet allowed to dry at RT before being resuspended in 30 µl nuclease-free dH_2O.

2.2.3.5. *In Vitro* Transcription

RNA transcripts were generated using the T7 Megascript Kit (Ambion) according to the manufacturer's instructions using 1 µg of linearised DNA template. The reaction mixture was incubated at 37 ^{o}C overnight followed by DNase-I treatment for 15 min at 37 ^{o}C and

the RNA purified using the MEGAclear kit (Ambion) according to remove nucleotides, proteins and salt from the RNA. 1 µl of purified RNA was mixed with 49 µl H_2O and the RNA concentration was measured by reading the OD_{260} using a BioPhotometer (Eppendorf). Typically, this kit yielded RNA concentrations of 70-100 µg.

2.2.3.6. RNA Electroporation of Huh-7 cells

Cells at 60-80 % confluency were trypsinised, washed in $DMEM_{complete}$ and counted using a haemocytometer. For each electroporation, aliquots of 4 x 10^6 cells were washed 2 times in PBSA and pelleted by centrifugation at 1000 r.p.m. for 5 min. The pellet was then resuspended in 400 µl PBSA and added to a 4 mm gap cuvette (Apollo) along with 10 µg of *in vitro* transcribed viral RNA. Electroporation was performed using a BioRad GenePulser Xcell (250 V, 950 µF), following the manufacturer's instructions. The transfected cells were then allowed to rest for 10 min prior to being resuspended in the appropriate amount of $DMEM_{complete}$ and seeded into the appropriate tissue culture flask or plate. Typically electroporated cells were resuspended in 15 ml $DMEM_{complete}$ and plated in 90 mm culture dish.

2.2.3.7. Generation of HCVcc Virus

Huh-7 cells electroporated with *in vitro* transcribed RNA were transferred to category 3 containment laboratory and the virus was harvested at 24 h, 48 h, 72 h and 96 h post electroporation by filtering the culture media through a 0.45 µm pore-sized membrane.

2.2.3.8. Measuring HCVcc Infectivity

Virus infectivity was determined using either the 50 % Tissue Culture Infectious Dose ($TCID_{50}$) assay as described by Lindenbach *et al*. (2005) or the focus forming unit (FFU) assay, as described by Zhong *et al*. (2005). Naive Huh-7 cells were seeded at 1 x 10^3 cells/ per well in 200 µl $DMEM_{complete}$ in a flat bottomed 96-well plate (Nunc). Six wells per dilution were used with the $TCID_{50}$ method and three wells per dilution with the FFU method. Cells were allowed to settle at 37 ^oC for 24 h before being inoculated with 200 µl of serial 5-fold or 10-fold dilutions of harvested culture medium. 48 h post-infection the medium was removed, cells washed with PBSA and fixed with ice-cold methanol at -20 °C for a minimum of 2 h. The cells were then washed three times in PBSA and probed with anti-NS5A mAb 9E10 diluted 1:20,000 in PBST and incubated for 1 h at RT. Cells were washed again as above and incubated for 1 h at RT with FITC conjugated secondary antibody diluted 1:500 in PBST. The cells were then washed 3 times in PBSA before being

overlaid with 100 µl of dH_2O, followed by visualization under a fluorescent microscope (Nikon Eclipse TS100).

Infectivity of cell-associated virus was determined using previously established methods (Gastaminza *et al*., 2006; Shavinskya *et al*., 2008). Electroporated cells seeded into a 90 mm tissue culture dish were washed once in PBSA at 48 h post incubation. The cells were then removed from the culture dish using a cell scraper in the presence of 10 ml PBSA followed by centrifugation at 1000 r.p.m. for 5 min. The pellet was then resuspended in 1 ml $DMEM_{complete}$, and freeze-thawed rapidly three times using dry ice/ethanol and a 37 ^oC water bath. Finally, the samples were centrifuged at 4000 r.p.m. for 5 min to remove cell debris, and the infectivity was determined using the $TCID_{50}$ method.

2.2.3.9. Preparation of cells for HCVcc replication and protein Expression Analysis

The viral replication was visualised by either IF and western immunoblotting or determined by RT-qPCR. For IF analysis, a few drops of electroporated cells were seeded onto coverslips in a 24 well plate and incubated for 48 h at 37 ^oC before being fixed, stained and visualised. For IF analysis following infection, naive Huh-7 target cells were seeded onto coverslips and 24 h later infected with 200 µl harvested culture media. At 48 h post infection cells were fixed, stained and visualised as before. For western immunoblotting, electroporated cells seeded onto 90 mm culture dishes were washed once in PBSA at 72 h post incubation and lysed either directly in sample loading buffer or in LB2 and analysed as described in section 2.2.1.5. To quantify virus replication by RT-qPCR cells were typically washed in PBSA before being lysed in RLT buffer (RNeasy Kit) and total RNA was extracted according to the manufacturer's instructions. Briefly 1 volume of 70 % ethanol was added to the lysate and mixed thoroughly by pipetting. Samples were then transferred to an RNAeasy spin column and centrifuged at 10, 000 r.p.m. for 30 sec. 700 µl buffer RW1 (wash buffer) was added to the spin column and the centrifugation was repeated at 10, 000 r.p.m. for 30 sec. Spin column was then washed twice with 500 µl buffer RPE (wash buffer with ethanol) and centrifuged at 10, 000 r.p.m. for 2 min. At this point the spin column was placed into a fresh collection tube and the RNA was eluted by adding 30-50 µl RNase-free water and spinning the sample at 10, 000 r.p.m. for 1 min. Following electroporation, cells were seeded onto 90 mm culture dishes and incubated at 37 ^oC. At 4 h post-incubation cells were washed trypsinised and resuspended in $DMEM_{complete}$. Next, the resuspended cells were divided equally into three

25 cm^2 flasks and at appropriate time-points the cell-lysates (for RT-qPCR) and culture supernatant (for $TCID_{50}$) were harvested. To determine virus replication kinetics after infection, 53,000 naïve Huh-7 cells were seeded into three 6-well culture dishes. For each virus, cells were infected at the indicated multiplicity of infection (m.o.i.) for 6 h in a total volume of 900 µl before supplementing the cells with 2 ml of fresh $DMEM_{complete}$. At 24, 48 and 72 h cell lysates and culture supernatants were harvested.

2.2.3.10. Passaging of HCVcc infected cells

Cells infected with $JFH1_{WT}$ were passaged every 3-5 days at the time of subconfluency. At each passage cell culture supernatants were harvested and infectivity determined by $TCID_{50}$. The cells were washed, trypsinised, resuspended in fresh $DMEM_{complete}$ and seeded onto coverslips for viral protein expression analysis by IF. Cells electroporated with 4a/JFH1 viral RNA or infected with adapted 4a/JFH1 virus were passaged as described above. However, in addition to analysing the infectivity of the culture supernatant and the intracellular viral protein expression by IF, 1 ml of cell resuspension was pelleted and lysed in RLT to generate cDNA for sequencing or to determine RNA replication by RT-qPCR.

2.2.3.11. Iodixanol gradient

Huh-7 cells were electroporated in duplicate with the relevant viral RNAs and seeded into 90 mm culture dishes in 15 ml $DMEM_{complete}$. The culture medium from each dish was harvested, pooled and purified through 20 % (w/v) sucrose cushions in PBS(A) by ultracentrifugation at 25,000 r.p.m. for 4 h at 4^{o}C using a Sorvall Discovery 90SE ultracentrifuge. Viral pellets were then re-suspended overnight in 500 µl PBSA. Linear iodixanol gradients were prepared according to a pre-established protocol described by Lindenbach *et al*., 2005. Equal volumes of 10-40 % Optiprep (Iodixanol; Axis-Shield) solutions in PBS were layered in 5 % increments and incubated at 4^{o}C for 16 h. Next, the concentrated virus was layered on top of the gradients and equilibrium was reached by ultracentrifugation at 36, 000 rpm for 16 h at 4^{o}C using a using a Sorvall Discovery 90SE ultracentrifuge. Following centrifugation 1 ml fractions were harvested from the top of the tube and 1/10 of each fraction was weighed to calculate fraction density. 100 µl aliquots of each fraction were diluted 10 times in $DMEM_{complete}$ before the infectivity was determined by FFU assay.

2.2.3.12. Determining Virus Neutralisation

HCVcc inhibition assays were performed using either Huh-7 or HuhJ-20 cells and infectivity levels were determined by FFU or secreted alkaline phosphatase (SEAP) reporter assay, respectively. With FFU, Huh-7 cells were seeded at $3x10^3$ cells/ per well in 200 µl $DMEM_{complete}$ in a 96-well plate and were allowed to settle at 37 ^oC for 24 before infection. For HCVcc inhibition with anti-E2 antibody, 100 FFU of virus was pre-incubated for 1 h at 37 ^oC with the appropriate antibody or control antibody diluted in $DMEM_{complete}$ prior to infecting the cells with the antibody/virus mixture. To treat the target cells by anti-receptor antibodies, target cells were incubated with the appropriate antibody diluted in $DMEM_{complete}$ for 1 h at 37 ^oC. Next the antibody was removed and cells were infected with 100 FFU of virus per well. The infectivity was determined by staining cells with anti-NS5A mAb 9E10.

To measure HCVcc neutralisation by SEAP reporter assay, naive Huh-7 J20 cells were seeded as above in 96 well plate. The next day cells were inoculated with 100 µl of virus at an m.o.i of 0.5 and incubated for 3 h after which the inoculum was replaced with fresh medium and cells incubated at 37 ^oC. At 72 h post incubation 90 µl of culture medium was lysed in 10 µl of 10x concentrated LB2 to inactivate the virus and the SEAP activity was measured using SEAP Chemiluminescence Assay kit, Phospha light^{TM} (ABI) following manufacturer's instructions (section 2.2.3.13.).

2.2.3.13. SEAP Assay

In a 96-well plate 30 µl of lysed cell medium was mixed 1:1 with 1x dilution buffer and incubated at 65 ^oC for 30 min. The plates were then cooled on ice to reach room temperature. 50 µl of the cooled reaction was then mixed with 50 µl assay buffer in a black 96-well microplate. Before measuring the luminescence, 50 µl of reaction buffer was added to the plate. Reaction buffer was prepared by diluting 1,2 dioxetane phosphate (CSPD) substrate 1:20 with reaction buffer diluents. The SEAP acitivity was measured by using a Hidex Chameleon plate reader and expressed as relative light units (RLU)

2.2.3.14. Analysing Effect of HDL on HCVcc Infectivity

$JFH1_{WT}$ and JFH1 E2 mutant virus inoculum was used to infect naive Huh-7 cells at an m.o.i. of 1.0. At 3 h post incubation cells were washed 3 times in PBSA and supplemented with cell culture medium containing 3 % lipoprotein-deficient fetal calf serum. Released virus in the culture medium was harvested at 6 days post-infection. To test the effect of

HDL on virus infectivity, naive Huh-7 J20 cells were seeded in a 96-well plate and incubated overnight. The following day cells were treated with cell culture medium containing 3 % lipoprotein-deficient fetal calf serum for 3 h at 37 ^{o}C prior to infection with virus in the presence or absence of HDL (20 µg/ml). At 72 h post-infection the SEAP activity was measured to determine infectivity.

2.2.3.15. RNA Interference

The expression of CD81 and SR-BI protein was down-regulated by reverse transfecting gene specific siRNA into naive Huh-7 cells. Two siRNAs targeting two different regions of CD81 (14501 and 146379) and SR-BI (s2650 and s2649) were obtained from Ambion. Each siRNA, supplied as a 5 nmol stock, was diluted in dH_2O to obtain a stock concentration of 100 µM. Transfections were performed in 24-well plates in triplicates. For each well, 100 µl Optimem-I was mixed with 1 µl lipofectamine RNAiMAX reagent (Invitrogen) and 0.5 µl each of the two siRNAs targeting either CD81 or SR-BI gene or 1 µl scrambled siRNA . The plates containing the siRNA and lipofectamine mix were then incubated for 20 min at RT. Following incubation the wells were seeded with $1x10^4$ cells/well in 900 µl $DMEM_{complete}$, giving a total volume of 1 ml/well and a final concentration of 100 nM of siRNAs. At 48 h post-incubation the viability of cells and mRNA expression levels were determined by ELISA and RT-qPCR, respectively. To determine the level of cells surface expressed CD81 and SR-BI protein, FACS analysis was performed using anti-CD81 and anti-CLA-I antibody, respectively. In parallel, siRNA treated cells were infected at 48 h post-incubation with $JFH1_{WT}$, $JFH1_{N415D}$, $JFH1_{T416A}$, $JFH1_{N417S}$ and $JFH1_{I422L}$ virus for 4 h before being supplemented with fresh $DMEM_{complete}$. At 24 h post infection the intracellular HCV RNA levels were measured by qRT-PCR.

2.2.3.16. Cell Viability Assay

Cell viability of Huh-7 cells following reverse transfection with siRNAs was determined using the colorimetric WST-1 assay (Roche) according to the manufacturer's instructions. siRNA reverse transfected cells were seeded in a 96-well plate in a total volume of 180 µl. At 48 h post-incubation 20 µl (1/10 dilution) of WST-1 was added to the cell medium and the cells were incubated for 3-4 h at 37 ^{o}C. Following incubation the cell metabolism was measured by ELISA.

2.2.3.17. Cell-to-Cell Transmission Assay

The cell-to-cell transmission efficiency of $JFH1_{WT}$ and gt4a/JFH1 virus was tested using the previously established co-culture assay (Brimacombe *et al.*, 2010) (Appendix 6). This

study showed this system to be more reliable than the previously reported HCV cell-to-cell transfer techniques. This assay requires donor and target cells to be cultured together at high levels of confluency to maximize the occurrence of viral spread. To generate the donor cells, Huh-7 cells were transfected with *in vitro* transcribed HCV RNA and incubated at 37^{o}C. At 72 h post-incubation these donor cells were labelled with CMFDA dye by incubating the cells at 37^{o}C with 5 μM of CMFDA dye for 30 min. In parallel, 9 x 10^4 naive Huh-7 cells (Target cells) were seeded in a 24-well cell culture plate and allowed to rest for 1 h at 37^{o}C in the presence of 100 μg nAb AP33 or no antibody control (PBSA). Upon CMFDA labelling, donor cells were trypsinized, washed and counted and equal amount of cells were then mixed with the target cells and incubated for 48 h. The additions of nAb AP33 in the test samples prevented cell free virus from infecting the target cells. At 48 h post co-culture incubation the cell medium was harvested to test the level of neutralization of infection with AP33 nAb. This was done by infecting naive Huh-7 J20 cells followed by measuring SEAP activity at 48 h post-infection. In parallel, the level of cell-to-cell transfer from donor to target cells was determined by performing FACS analysis on the co-cultured cells to quantify NS5A positive cells in the presence or absence of nAb AP33.

2.2.4. HCVpp Based Work

HCVpp assay was used to compare infectivity and neutralisation profile of AP33 mutants. pJFH1 containing the individual AP33 mutations were digested with BsiWI and BsaBI restriction enzymes that have recognition sites located at the N-terminus of E1 and C-terminus of E2, respectively. The resulting fragments containing the mutations were ligated into phCMV vector digested with the same restriction enzymes. The proper incorporation of inserts into phCMV template was confirmed by sequence analysis using primers NA15, NA17 and NA19 (Appendix D).

2.2.4.1. Generation of HCVpp (Transfection of DNA)

HCVpp were generated as decribed previously (Bartosch et al., 2003; Hsu et al., 2003; Owsianka et al., 2005). Typically one million HEK-293T cells were seeded into a 90mm tissue culture dish in 20 ml $DMEM_{complete}$ 24 h before transfection. The following day the cells were co-transfected with three different plasmids expressing (i) replication deficient MLV gag/pol core (8 μg), (ii) HCV E1E2 glycoprotein (3 μg) and (iii) luciferase reporter gene (8 μg) using the Sigma Calcium Phosphate Transfection Kit. The plasmid DNA were

mixed with molecular biology grade water and 40 µl 2.5 M $CaCl_2$ in a total volume of 200 µl in a sterile eppendorf. In a second 1.5 ml eppendorf tube, 200 µl of 2 x HEPES-Buffered saline (HeBS), pH 7.05 was added. To prepare the precipitate, the HeBS solution containing sodium phosphate was gently bubbled using a sterile pasteur pipette while the DNA/$CaCl_2$ solution was gently added dropwise. The precipitate was then incubated for 20 min at RT before being gently distributed over the cells. At 24 h post transfection the medium was replaced with 5 ml fresh $DMEM_{complete}$ replenished with 10 mM Hepes. At 48 h post-transfection the culture media containing HCVpp was harvested by filtering through a 0.45 µm pore-sized membrane. The transfected cells were washed once in PBSA and later lysed in 500 µl 1 x lysis buffer (Promega) for ELISA assay or lysed directly in sample buffer for WB. Lysed cells were centrifuged 1000 r.p.m. for 10 min and the clarified supernatant was stored at -2 0^oC and used in ELISA assay.

2.2.4.2. Luciferase Infection assay

Target cells (Huh-7) were seeded 24 h before infection at a concentration of 2.5 $x10^4$ cells per well of a 24 well tissue culture plate in a total volume of 1 ml. To infect, cells were incubated for 3 h with 200 µl harvested HCVpp culture media, after which the media was replaced with fresh $DMEM_{complete}$ and cells were incubated for 48 h. Infection was assessed with a luminometer using Luciferase Assay System (Promega) according to the manufacturer's instructions. Briefly, cells were washed with excess PBSA and cell lysates were prepared by adding 100 µl of 1 x lysis buffer to each well and the mix incubated on gentle shaking for 5 min at RT. Meanwhile, luciferase assay reagent was prepared by dissolving 17.1 mg lyophilized luciferase assay substrate into 1 ml luciferase assay buffer. Cell lysate was then mixed with equal amount of luciferase assay reagent in 1.5 ml eppendorf and luminescence measured immediately.

3. Characterisation of E2 Cell Culture Adaptive Mutations

3.1. Introduction

The first groups to establish the HCVcc system described the ability of JFH1 and J6/JFH1 to persistently infect Huh-7 cells and the derived sublines Huh-7.5 and Huh-7.5.1 cells (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). Since then numerous studies have documented that the long-term culturing of infected cells results in the virus acquiring cell culture adaptive mutations throughout the genome, some of which enhance infectivity. An interesting observation from these studies is that nonsynonymous mutations frequently arise within the E2 glycoprotein. Furthermore, these mutations tend to cluster within the highly conserved region spanning amino acids 412-423 (QLINTNGSWHIN). Given its high level of conservation, it is possible that this region is crucial for the structural integrity of the glycoproteins. In line with this theory, a comprehensive mutagenesis study reported that several of the residues within this region were crucial for HCVpp infectivity (Owsianka *et al.*, 2006). This site also harbours one of the critical E2 residues, at position W420, required for CD81 binding (Owsianka *et al.*, 2006). This region is also of great interest with regards to immunotherapy and vaccine development as several broadly neutralising mAbs bind to this epitope. These include mouse mAb AP33, rat mAb 3/11 and human mAbs e137, HCV1, 95-2, AR3A, AR3B, AR3C and AR3D (Broering *et al.*, 2009; Flint *et al.*, 1999; Law *et al.*, 2008; Owsianka *et al.*, 2005; Perotti *et al.*, 2008). Of these, mAbs AP33, 3/11 and e137 are known to block binding of E2 to CD81. The in house generated mAb AP33 has been extensively characterized. AP33 has been shown to potently neutralize HCVpp bearing glycoproteins representative of the major HCV genotypes (Owsianka *et al.*, 2005). The development of the HCVcc system has now afforded us the opportunity to examine the importance of the conserved 412-423 region in E2 for virus infection and AP33-mediated neutralization using authentic HCV virions. Alanine substitution of these residues is not a suitable approach for such HCVcc studies as these mutations could affect different stages of the viral lifecycle other than cell binding and entry, such as RNA replication, virus assembly and egress. In contrast, cell culture adaptive mutations in this site of E2 are valuable for such studies as they have been shown not to alter virus replication levels.

In this study, an asparagine-to-aspartic acid mutation at residue 415 (N415D) in HCV strain JFH1 E2 was first identified following the long-term passaging of infected Huh-7 cells. Alongside N415D, three adjacent cell culture adaptive mutations (T416A, N417S

and I422L) reported previously (Bungyoku *et al.*, 2009; Kaul *et al.*, 2007; Russell *et al.*, 2008) were then characterized, to gain further insight into the function of this E2 region in viral infection and antibody mediated neutralization.

3.2. Identification of a Cell Culture Adaptive Mutation in E2

With the aim of generating our own cell culture adapted virus, naïve Huh-7 cells were infected at a low m.o.i. with the $JFH1_{WT}$ virus harvested from cells electroporated with viral RNA and serially passaged over a period of 6 weeks (9 passages). The infectious virus yields in the culture supernatants increased up to cell passage (cp) 6 at which time the titers peaked at 1 x 10^5 $TCID_{50}$/ml (Figure 3.1A). The infectivity then decreased in the subsequent passages and then settled at 1 x 10^4 $TCID_{50}$/ml at cp 8 and 9. Interestingly, no correlation was found between the number of HCV infected cells and the levels of virus released at each cell passage (Figure 3.1B). Furthermore, virus infection between cp 5 and 6 induced clear cytopathic effects (CPE) as characterized by rounded cells floating in the culture medium. Zhong and colleagues reported similar observations during the adaptation of JFH1 in serially passaged cells (Zhong *et al.*, 2006). This study also showed that the adapted virus obtained from the late cell passages could achieve superior viral titers following its infection of naïve cells. To determine if our late passaged virus displayed a similar phenotype, naïve Huh-7 cells were infected with virus collected from cp 9. At 3 days post-infection, the viral titers were approximately 100-fold higher than those recorded at cp 9, indicating that this virus had acquired infectivity enhancing mutations. As this high tittered virus was generated after one supernatant passage, this preparation will now be referred to as supernatant passage 1 (sp1) virus. To identify the mutation(s) responsible for this changed phenotype, RNA harvested from sp1 infected cells was RT-PCR amplified, and the population was sequenced over the core to NS5B region. In total 3 nonsynonymous mutations were detected in NS5A (A2322G), NS4A (C1678S), and E2 (N415D). The latter mutation is located within the highly conserved region between residues 412-423. Interestingly, three other studies also reported adaptive E2 mutations within this epitope, at positions T416 (Bungyoku *et al.*, 2009), N417 (Russell *et al.*, 2008) and I422 (Kaul *et al.*, 2007) (Figure 3.1C). These were generated in Huh-7.5 cells using the chimeric J6/JFH1 (T416A) or the $JFH1_{WT}$ (N417S and I422L) HCVcc. To study what effect these aforementioned mutations have on virus infectivity, receptor affinity and antibody-

mediated neutralization, each mutation was first introduced separately into the parental genome by site directed mutagenesis as described in materials and methods. These four viruses were then characterized alongside the $JFH1_{WT}$.

3.3. Effects of E2 Mutations on Virus Infection

First, the effect of these E2 mutations on viral RNA replication and infectious particle release was determined following electroporation of the in vitro transcribed RNAs into Huh-7 cells. In contrast to $JFH1_{GND}$, which served as a negative control, both the $JFH1_{WT}$ and the four E2 mutant virus RNAs were capable of releasing similar levels of infectious particles into the medium (Figure 3.2A) and showed comparable levels of intracellular replication at all the time points tested (Figure 3.2B). To further determine the effect of the introduced mutations on virus assembly, the intracellular versus extracellular infectivity in Huh-7 cells was titrated at 72 h post electroporation. Again no major difference was noticeable in the infectivity of intra- and extracellular virus (Figure 3.2 C). Concomitantly this data suggest that each individual mutation has no major impact on virus replication, virus assembly or virus release following electroporation with the viral RNA.

Next, a short term infection kinetics experiment was performed. Extracellular virus collected at 72 h post-electroporation was used to inoculate naïve Huh-7 cells at an m.o.i of 0.1 and 1.0. At each m.o.i tested, no major differences were observed in the infectivity and intracellular RNA replication of each mutant compared to $JFH1_{WT}$ throughout the time course (Figure 3.3A-D). Interestingly, the $JFH1_{sp1}$ virus, which was included for comparison, produced infectious titers ~1 log greater than $JFH1_{WT}$ and the E2 mutants. Furthermore, the intracellular RNA replication levels of $JFH1_{sp1}$ were greatly reduced compared to the other viruses, particularly at the 24 and 48 h time points. These results suggest that more $JFH1_{sp1}$ virus particles are produced per intracellular RNA molecule. The contrasting phenotype of $JFH1_{sp1}$ next to $JFH1_{N415D}$ is likely due to the mutations in the non-structural regions of the genome. Next, the specific infectivity (defined as infectious titer relative to the HCV RNA titer) of $JFH1_{N415D}$, $JFH1_{T416A}$, $JFH1_{N417S}$ and $JFH1_{I422L}$ was determined using a pre-established protocol (Zhong *et al.*, 2006). To do this, naïve Huh-7 cells were inoculated at an m.o.i. of 1.0 and at 72 h post-incubation, the level of virus infectivity and HCV RNA in the culture medium was determined by $TCID_{50}$ and RT-qPCR. Interestingly, compared to $JFH1_{WT}$, the specific infectivity of each mutant were

much lower, with values 5.7-, 21.1-, 4.3- and 2.2-fold less for $JFH1_{N415D}$, $JFH1_{T416A}$, $JFH1_{N417S}$ and $JFH1_{I422L}$, respectively (Fig. 3.3E). This result indicates that the E2 mutant viruses release fewer non-infectious particles than $JFH1_{WT}$, which could be explained by these mutants being more efficient at packaging the viral genome during virus assembly.

3.4. E2 Mutations Alter Sensitivity to Neutralizing Antibodies

Next it was determined whether these single changes alter the affinity of E2 to the broadly neutralizing mAbs AP33 and 3/11. Both these mAbs recognize distinct but overlapping epitopes within the highly conserved region of E2 spanning residues 412 to 423 (QLINTNGSWHIN), where these four mutations are located. To test the binding efficiency of E2 with AP33, cells were fixed and probed for IF analyses at 48 h post electroporation. While a clear E2-AP33 interaction was visible for $JFH1_{WT}$, $JFH1_{T416A}$ and $JFH1_{I422L}$, AP33 binding to E2 from $JFH1_{N415D}$ and $JFH1_{N417S}$ appeared to be completely abrogated in IF imaging (Figure 3.4A). The conformational sensitive anti-E2 human mAb CBH-5 was used as a control antibody. Immunoblotting of infected cell lysates confirmed the IF data, showing no binding of mAb AP33 to E2 from $JFH1_{N415D}$ and $JFH1_{N417S}$ mutant viruses. Immunoblotting with the anti-NS5A mAb 9E10 and the anti-E2 mAb D3.7 confirmed that there was a similar level of viral protein expression in each infected cell lysate (Figure 3.4B).

Next the reactivity of mAb AP33 or 3/11 to the glycoproteins of each E2 mutant virus was quantified by ELISA. The levels of $JFH1_{WT}$ and mutant E2 in Huh-7 cells electroporated with appropriate viral RNAs was first normalized by measuring binding to the conformation sensitive anti-E2 human mAb CBH-4B (Appendix 1A). Notably, the various E2s also bound to the mouse mAb D3.7, which recognizes a linear epitope in E2, with comparable efficiency (Appendix 1B). Consistent with IF and immunoblotting data, AP33 showed very weak binding to the E2 from $JFH1_{N415D}$ and $JFH1_{N417S}$ captured onto GNA ELISA (Figure 3.5B and Appendix 2). Similar binding efficiency in ELISA was observed when probing GNA captured E2 with mAb 3/11 (Figure 3.6B and Appendix 3). Furthermore, the efficiency of these anti-E2 antibodies to block infection of $JFH1_{WT}$ and E2 mutant viruses was also tested. Virus harvested at 72 h post-electroporation was incubated with mAb AP33 or 3/11 prior to infecting target cells. It was found that $JFH1_{T416A}$ and $JFH1_{I422L}$ were highly sensitive to neutralization by both mAbs, whereas

$JFH1_{N415D}$ and $JFH1_{N417S}$ were completely resistant (Figure 3.5A and 3.6A). Together, the neutralization and ELISA data show that the $JFH1_{N415D}$ and $JFH1_{N417S}$ mutations disrupt the binding of mAbs AP33 and 3/11 to E2. This data is further supported by the IF and immunobloting results shown in figure 3.4A and 3.4B. However, while the reactivity of the intracellular glycoproteins from $JFH1_{T416A}$ and $JFH1_{I422L}$ was unaltered for both mAbs, the virions were more sensitive to neutralization by these antibodies. This is indicated by the IC_{50} values ranging between 25- and 12.5-fold lower for mAb AP33 and 11- and 6- fold lower for mAb 3/11 (Table 3.1).

A range of polyclonal anti-HCV IgGs purified from HCV-infected patients that inhibit infection after virus attachment to the cell have been reported previously (Haberstroh *et al.*, 2008). The sensitivity of $JFH1_{WT}$ and the E2 mutant viruses to neutralization by three of these IgG preparations was tested. Virus harvested at 72 h post-electroporation was incubated with different amounts of patient IgGs; IgG7, IgG17 or IgG19 prior to infecting target cells. The degree of inhibition of the $JFH1_{WT}$ virus afforded by these IgGs was in accordance with the previous published data (Haberstroh *et al.*, 2008). However, the mutant viruses were more sensitive to neutralization, with IC_{50} values 18- to 60-fold lower for IgG17, 9- to 20-fold lower for IgG19 and >25 fold lower for IgG7 (Figure 3.7C, D and E and Table 3.1). As expected, antibodies purified from an uninfected individual had no effect on virus infectivity at the highest concentration tested for each IgG (20 μg/ml) (Figure 3.7F). Next the efficiency of neutralization of all viruses was tested using the conformation-sensitive anti-E2 hmAbs, CBH-5 and HC-11. Both of these hmAbs recognize discontinuous overlapping epitopes within the domain B of E2 and like CBH-5, HC-11 also inhibits viral entry into cells by blocking the E2-CD81 interaction (Keck *et al.*, 2008). With both the antibodies it was found that each mutant virus was more sensitive to neutralization compared to $JFH1_{WT}$, with the IC_{50} values ranging from 12- to 30- fold lower for CBH-5 and strikingly 3 to 4 log lower for HC-11 (Figure 3.7A and B; Table 3.1). Together, these findings indicate that these E2 mutations enhance the exposure of neutralizing epitopes on the virion glycoproteins.

3.5. E2 Mutations Alter Virus-Receptor Interactions

Grove and colleagues reported that the E2 cell culture adaptive mutation G451R altered the affinity of virions to CD81 and SRB1. A similar phenotype was also observed for delta

HVR1 virions (Bankwitz et al., 2010). To establish whether the N415D, T416A, N417S and I422L mutations altered the affinity of glycoproteins for the virus receptor CD81, a competition assay using the hCD81-LEL was performed. This protein has been shown to interact with the E2 glycoprotein and inhibit HCV infection (Bartosch *et al.*, 2003). Viruses were incubated with various concentrations of hCD81-LEL for 1 h prior to infecting target cells. All four mutant viruses showed increased sensitivity to neutralization by hCD81-LEL (Figure 3.8A). To investigate if this was due to a change in the binding of the E2 mutant glycoproteins to CD81, their reactivity to hCD81-LEL was determined by ELISA. The viral glycoproteins used in this assay were obtained from cell lysates harvested at 72 h post-electroporation. Interestingly, despite the similar inhibition pattern of each E2 mutant virus by hCD81-LEL, their intracellular glycoproteins showed dissimilar levels of binding to hCD81-LEL by ELISA. Mutations N415D, T416A and N417S enhanced hCD81-LEL binding in a dose-dependent fashion (Appendix 4), by 38%, 106% and 64%, respectively (Figure 3.8B). In contrast, the I422L mutation bound hCD81-LEL with comparable affinity to $JFH1_{WT}$ E2 (Figure 3.8B and Appendix 4). A potential explanation for this difference between hCD81-LEL neutralization and binding is that each E2 mutation causes a local change to occur during the virion assembly process leading to a better exposure of the CD81-binding region and therefore enhanced neutralization. Given the increased sensitivity of the E2 mutant viruses to neutralization by the soluble form of CD81, one would expect these viruses to be less prone to inhibition by antibodies blocking this receptor. To test this, naïve cells were incubated with anti-CD81 mAb prior to infection with each virus. Surprisingly, no differences in inhibition were observed for the E2 mutant viruses compared to $JFH1_{WT}$ (Figure 3.8C). This may be explained by the high affinity of the anti-CD81 mAb simply masking any interaction differences that exist between the $JFH1_{WT}$ and E2 mutant virions with cellular CD81.

Having established that these mutations influence the HCV-CD81 interaction, it was next investigated whether these mutations have an effect on SR-BI-dependent entry. Naïve cells were pre-incubated with different concentrations of a neutralizing anti-SRBI rat serum (Zeisel *et al.*, 2007) prior to infection with each virus. Interestingly, all mutants were less sensitive than $JFH1_{WT}$ to neutralization by this antiserum with IC_{50} values 5- to 28-fold higher (Figure 3.9A and Table 3.1). It has been shown that HDL, which is a SR-BI ligand, enhances HCV entry through a process that requires the lipid transfer function of SR-BI (Bartosch *et al.*, 2005; Voisset *et al.*, 2005). To follow up on this observation, the effect of HDL on mutant virus infection was tested. Naïve Huh-7 J20 cells were pre-incubated with

lipoprotein-deficient fetal calf serum, prior to infection in the presence or absence of HDL. As shown in Figure 3.9B, while the infectivity of $JFH1_{WT}$ was significantly enhanced, the E2 mutants appeared insensitive to HDL treatment. Together, these data suggest that each adaptive mutation alters HDL/SR-BI-mediated uptake of the virus during entry. One explanation for these results is that these E2 mutant viruses have a reduced SRB1 dependency for viral entry. To test this, two siRNAs targeting different regions of SR-BI mRNA were transfected into Huh-7 cells to silence its expression. At the time of infection, these cells expressed 99% less SR-BI mRNA whilst maintaining 80% of the control cell viability (Figure 3.10A). The knockdown of cell-surface expressed SR-BI was also confirmed by FACS analysis (Figure 3.10C). Under these conditions, the infectivity of all viruses was inhibited by 94%-98%, showing the E2 mutant viruses still require sufficient expression of SR-BI for infection (Figure 3.10B). Similarly, efficient knock down of CD81 (Figure 3.11A and C) reduced the infectivity of all viruses (Figure 3.11B). These results indicate that despite the E2 mutations modulating the interactions of the virus with CD81 and SRB1, the expression of these receptors on the cell surface is still necessary for the mutant virions to infect target cells.

3.6. Bouyant Density of Infectious $JFH1_{N415D}$ Virions

Numerous reports have shown that both serum- and cell-culture-derived HCV is tightly associated with LDL and VLDL. *In vivo*, these associations protect HCV from the humoral immune response by shielding the glycoproteins from circulating nAbs (Bartenschlager *et al.*, 2011). In line with this, low-density HCVcc shows reduced sensitivity to neutralization by HCV patient IgG (Grove *et al.*, 2008). Thus, particle density can be used as an indirect measure of lipoprotein interaction. Previously, it was shown that the G451R mutation altered the relationship between buoyant density and infectivity, with high density mutant virus demonstrating greater infectivity than WT (Grove *et al.*, 2008; Zhong *et al.*, 2006). To investigate whether cell culture adaptive mutations within the conserved 412-423 E2 region caused similar alterations to infectious virion density, iodixanol density gradient ultracentrifugation was performed on $JFH1_{N415D}$ alongside $JFH1_{WT}$. Iodixanol was employed to form the gradients as it has been proven to preserve HCV-LDL/VLDL associations (Nielsen *et al.*, 2006). Consistent with previous reports (Gastaminza *et al.*, 2006) the buoyant density of $JFH1_{WT}$ was distributed over a broad range with the peak infectivity between fractions 5 and 7 (1.06-1.11 g/ml) (Figure 3.12). In contrast, the peak

infectivity of $JFH1_{N415D}$ was located between fractions 6 and 8 resulting in a rightward shift in the mean density of the virus. These results may suggest that the mutant virions contain less lipoproteins.

3.7. Infectivity and Neutralization Profiling in HCVpp System

Next, the effect of these E2 mutations on virus infectivity and neutralization was tested in the HCVpp system. To do this, HCVpps carrying the JFH1 glycoproteins with and without the E2 mutations were generated (see materials and methods). In contrast to the JFH1 HCVcc results, a notable reduction was observed in the infectivity of HCVpp carrying the same mutations (Figure 3.13A). Nevertheless, neutralization assays using mAbs AP33 (Figure 3.14A) and CBH-5 (Figure 3.14B) showed IC_{50} values very similar to those obtained in the HCVcc system (Table 3.1). These data further support the hypothesis that the mutations located between residues 412-423 of E2 may alter its conformation on the virus particle. E2 GNA-capture ELISA confirmed that the WT and the mutant E2 were expressed in comparable quantities (Appendix 5), and the mutations had no affect on the E2 incorporation into HCVpps as shown by immunoblot performed on pelleted supernatant from HCVpp infected cells (Figure 3.13B). Thus, the reasons for the lower infectivity of mutant HCVpps are not clear. Other studies have demonstrated differences in some HCV envelope protein functions when the HCVpp and HCVcc systems were compared (Grove *et al.*, 2008; Johansson *et al.*, 2007; Sainz *et al.*, 2009). It is conceivable that the former being a surrogate system may not always mimic the authentic virus in terms of glycoprotein presentation and function. Furthermore, HCVpp purely measure virus entry, excluding complications such as virus spread and RNA replication that exist within the HCVcc system when measuring virus infectivity. Therefore, direct comparisons in infectivity between these two systems are not always appropriate.

3.8. Discussion

In this section a number of key residues were identified in a conserved region of the E2 glycoprotein that determines the viral glycoprotein interaction(s) with cellular receptors

and nAbs. This region, located between amino acids 412-423, carries residues that are critical for recognition of two broadly nAbs used in this study, mAbs AP33 and 3/11. Two of the four JFH1 E2 mutations (N415D and N417S) described in this study abrogated E2 reactivity to, and virus neutralization by, mAbs AP33 and 3/11, while T416A and I422L displayed enhanced neutralization sensitivity.

The AP33 escape mutant N415D arose spontaneously during cell culture passaging. In the HCVpp system, alanine replacement of the residue N415 in the HCV genotype 1a H77 E2 drastically reduced mAb AP33 and 3/11 binding and completely abrogated infectivity (Owsianka *et al.*, 2006; Tarr *et al.*, 2006). In addition Gal-Tanamy and colleagues described an AP33 escape mutant, N415Y, selected in genotype 1a/2a chimeric HCVcc following repetitive rounds of antibody neutralization and amplification in cell culture (Gal-Tanamy *et al.*, 2008). The N415Y mutation alone severely attenuated mAb AP33 and 3/11 recognition and neutralization, but it did not enhance sensitivity to neutralization by other human anti-E2 mAbs. Furthermore the N415Y substantially reduced viral fitness, while having no major effect on CD81 binding. In contrast the N415D mutation showed enhanced affinity to hCD81-LEL, maintained viral fitness and displayed enhanced sensitivity to other human anti E2 mAbs. Interestingly, a recent study identified the N415D as a compensatory mutation that restored the infectivity of Jc1 virus lacking the HVR1 region (McCaffrey *et al.*, 2011). However, it remains to be determined whether this mutation rescues infectious particle assembly or entry of the $JFH1_{\Delta HVR1}$ virus.

Bungyoku and colleagues previously showed that the E2 T416A mutation in a chimeric J6/JFH1 HCVcc background does not alter virus infectivity in Huh-7.5 cells (Bungyoku *et al.*, 2009). In accordance with these data, no significant affect to $JFH1_{T416A}$ infectivity was observed in the present study. It was previously shown that the T416A mutation in the genotype 1a HCVpp system moderately reduced mAb AP33 and 3/11 recognition (Tarr *et al.*, 2006), enhanced CD81-binding, and abrogated virus infectivity (Owsianka *et al.*, 2006). In contrast, the present study found that this same mutation in HCVcc enhanced E2 reactivity to mAbs AP33 and 3/11 and did not alter virus infectivity. Together, the different infection system, viral isolates and/or cell lines used in each study likely account for these inconsistencies.

Russell and colleagues identified the N417S mutation during cell culture passaging of JFH1 virus in Huh-7.5 cells (Russell *et al.*, 2008). This single mutation did not alter

HCVcc infectivity but reduced HCVpp infectivity by 90% (Russell *et al.*, 2008). These results are consistent with the HCVcc and HCVpp infectivity data obtained for this mutation in the present study. The reasons for the lower infectivity of N417S mutant in HCVpps are not clear. Several studies have demonstrated functional differences between HCVpp and HCVcc. It is conceivable that the former being a surrogate system maynot always mimic the authentic virus in terms of glycoprotein presentation and function. Furthermore, HCVpp purely measure virus entry, excluding complications such as virus spread and RNA replication that exist within the HCVcc system when measuring virus infectivity. Therefore, direct comparisons in infectivity between these two systems are not always appropriate. This point is further strengthened by the observation that the G451R adaptive mutation, which enhances HCVcc infection, renders HCVpps noninfectious (Grove *et al*., 2008). Previous reports have also shown that alanine replacement of the residue N417 in the HCV genotype 1a H77 E2 reduced HCVpp infectivity by ~90% (Owsianka *et al.*, 2006). Furthermore, this mutation reduced the E2 binding of mAbs AP33 and 3/11 by ~60% and 40%, respectively (Tarr *et al.*, 2006). In line with these results, the N417S mutation in this study was found substantially reduce AP33 and 3/11 binding, rendering $JFH1_{N417S}$ HCVcc resistant to neutralization by either antibody and highlighting the contribution of N417 to their binding sites on E2.

The I422L mutation was first isolated alongside other structural and nonstructural mutations after several rounds of JFH1 passaging in Huh-7.5 cells and was shown not to alter virus infectivity (Kaul *et al.*, 2007), which is in agreement with the present findings in Huh-7 cells. Consistent with previous findings for I422A in the genotype 1a HCVpp system (Tarr *et al.*, 2006), this mutation did not affect E2 recognition by mAbs AP33 and 3/11, confirming that I422 is not a critical contact residue in E2 for either antibody.

In the present study substitutions N415D, T416A and N417S resulted in increased E2-CD81 binding, whereas the I422L mutation caused no alteration to CD81 binding. However, each mutant virus exhibited a similar heightened sensitivity to neutralization by hCD81-LEL, suggesting an increased affinity of the mutated glycoproteins for CD81. One theory that may explain these discrepancies is that each mutation causes structural alterations to the virion glycoproteins that result in the enhanced exposure of the CD81 binding epitope. The heightened inhibition of these mutants by a range of human anti-HCV glycoprotein antibodies (and by the anti-E2 rodent mAbs AP33 and 3/11 in the case of mutants T416A and I422L) supports this theory. Under these circumstances, the

differences in E2-CD81 binding observed by ELISA may be simply be masked by an improved ratio of virion E2 to hCD81-LEL in the neutralization assay.

The reduced sensitivity of these mutants to inhibition by the anti-SR-BI antibody was an unexpected result. In keeping with these observations, we found that all the mutants studied here were insensitive to HDL-mediated enhancement of virus infection. The exact mechanism by which the HDL-SR-BI association facilitates HCV entry is currently unknown. Although no interaction between HDL and HCVpp particles has been demonstrated in culture medium, the possibility of an association occurring at a post-binding stage cannot be discounted (Voisset *et al.*, 2005). More importantly, the binding of HDL to HCVcc virions has yet to be investigated. Also, it has been postulated that the lipid transfer events resulting from HDL-SR-BI binding, known to be essential for regulating the properties of cells membranes, may affect the fusion efficiency of the HCV envelope with cell membranes (Voisset *et al.*, 2005). SR-BI was first identified as a putative HCV receptor based on its ability to bind soluble, truncated E2 (sE2) via HVR1 (Scarselli *et al.*, 2002). However, sE2 may not fully mimic E2 structures on the HCV virion (Clayton *et al.*, 2002) and an interaction between SR-BI and the E1E2 heterodimers has yet to be confirmed. In addition, the initial binding of serum HCV to SR-BI was found not to be mediated by HVR-1 or indeed other regions of the E2 glycoprotein. Instead, the association of VLDL to virus particles appeared to play a critical role in the primary interaction with SR-BI (Maillard *et al.*, 2006). Thus, there is much uncertainty as to how HCV utilizes this receptor during virus entry. In the absence of definitive assays that can measure an interaction between SR-BI with full-length E1E2 or indeed HCVcc virions, it is difficult to decipher the effects caused by these E2 mutations to the entry process via this receptor. However, the siRNA knockdown experiment shows that SR-BI is not dispensable for the mutant virus entry.

The heightened sensitivity observed to several anti-HCV envelope glycoprotein nAbs could be due to several reasons. The mutant viruses secrete fewer non infectious particles and thereby may increase the ratio of nAbs to infectious virus compared to $JFH1_{WT}$. However all mutants display similar level of neutralization profile with different nAbs while a dissimilarity exists in specific infectivity of mutants. Therefore increased specific infectivity is unlikely to be the cause of enhanced neutralization sensitivity. The HCV envelope glycoproteins are highly N-glycosylated. These N-linked glycans are believed to mask the important antibody binding epitopes on the virion surface and removal of some of

these glycans have shown to enhance HCVpp and HCVcc antibody-mediated neutralization (Falkowska *et al.*, 2007; Helle *et al.*, 2007; Helle *et al.*, 2010). The residue N417 is part of an N-linked glycosylation site, the removal of which from genotype 1a E2 (N417Q) has been shown to increase the sensitivity of HCVpp to antibody neutralization and to increase CD81 binding (Helle *et al.*, 2007; Owsianka *et al.*, 2006). The latter observations are also in keeping with our findings. Our data show that the molecular weight of the genotype 2a E2 N417S mutant is identical to the WT glycoprotein; however, it would be inappropriate to conclude on this basis that this site is not used for glycosylation. This is because the N417S change potentially creates a new N-glycosylation site over positions 415-417 (i.e. a change from NTN to NTS), which, if utilized, will not alter the migration of the mutant E2 in SDS-PAGE. Therefore, it is unlikely that the effect of the N417S mutation on the exposure of the virion glycoproteins is due to a glycan loss.

The most likely explanation is that these mutations may induce global conformational changes on to the E2 glycoprotein and as a result the antibody binding epitopes on the virion surface may be more exposed. Gradient density data performed on $JFH1_{N415D}$ suggest that this could be likely due to reduced lipoprotein content associated with viral particle. In line with this theory, the E2 cell culture adaptive mutation I414T reduced the lipoprotein content of HCVcc virions that were more sensitivity to neutralization by anti-E2 antibodies (Tao *et al.*, 2009). The authors speculated that the reduced lipoprotein content of the virions could result in the increased exposure of the glycoproteins, making them more accessible for binding by anti-E2 nAbs. Further experiments are necessary to determine if the E2 mutant virions examined in the present study have less lipoproteins.

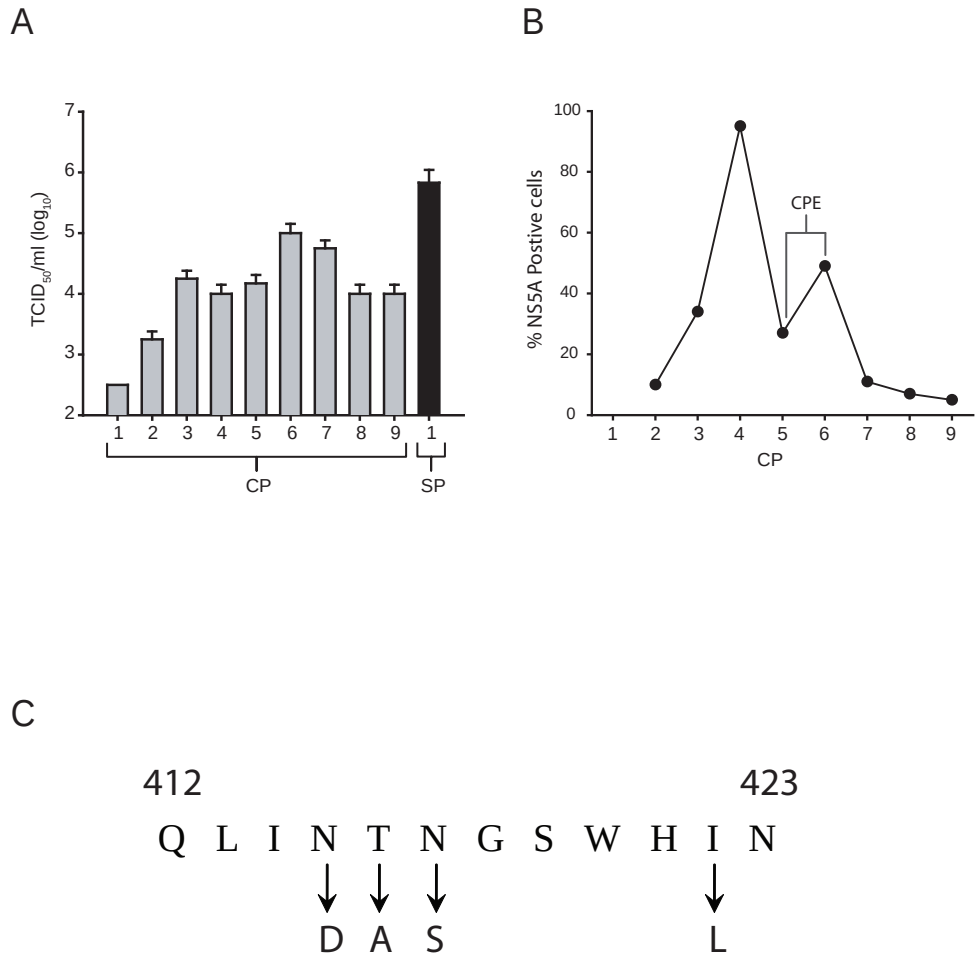


Figure 3.1. Cell Culture Adaptive Mutations Located in the E2 Glycoprotein

(A) Identification of a spontaneous mutation within E2. Huh-7 cells were infected with the $JFH1_{WT}$ virus at an m.o.i of 0.05 and serially passaged nine times. At each cell passage (CP) virus released into the medium was titrated by $TCID_{50}$ assay. Similarly, virus generated at CP 9 was used to infect naïve Huh-7 cells and the resulting infectious yield in the medium was measured as represented by the black bar (SP). (B) Percentage of NS5A positive cells were counted between CP 2-9. The passages where cytopathic effects (CPE) were observed are indicated. (C) The location of spontaneous mutations within the E2 glycoprotein residue 412-423 identified here and in other studies. The arrows denote amino acid substitution.

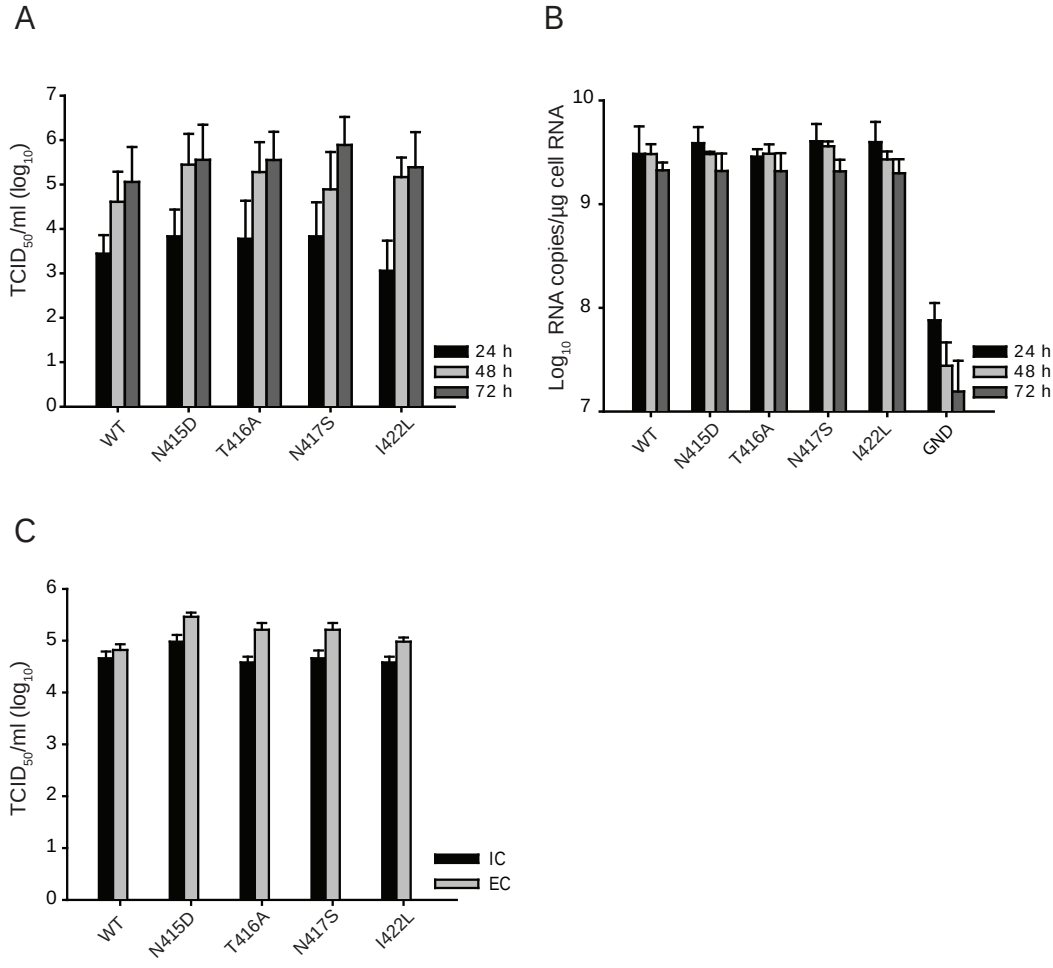
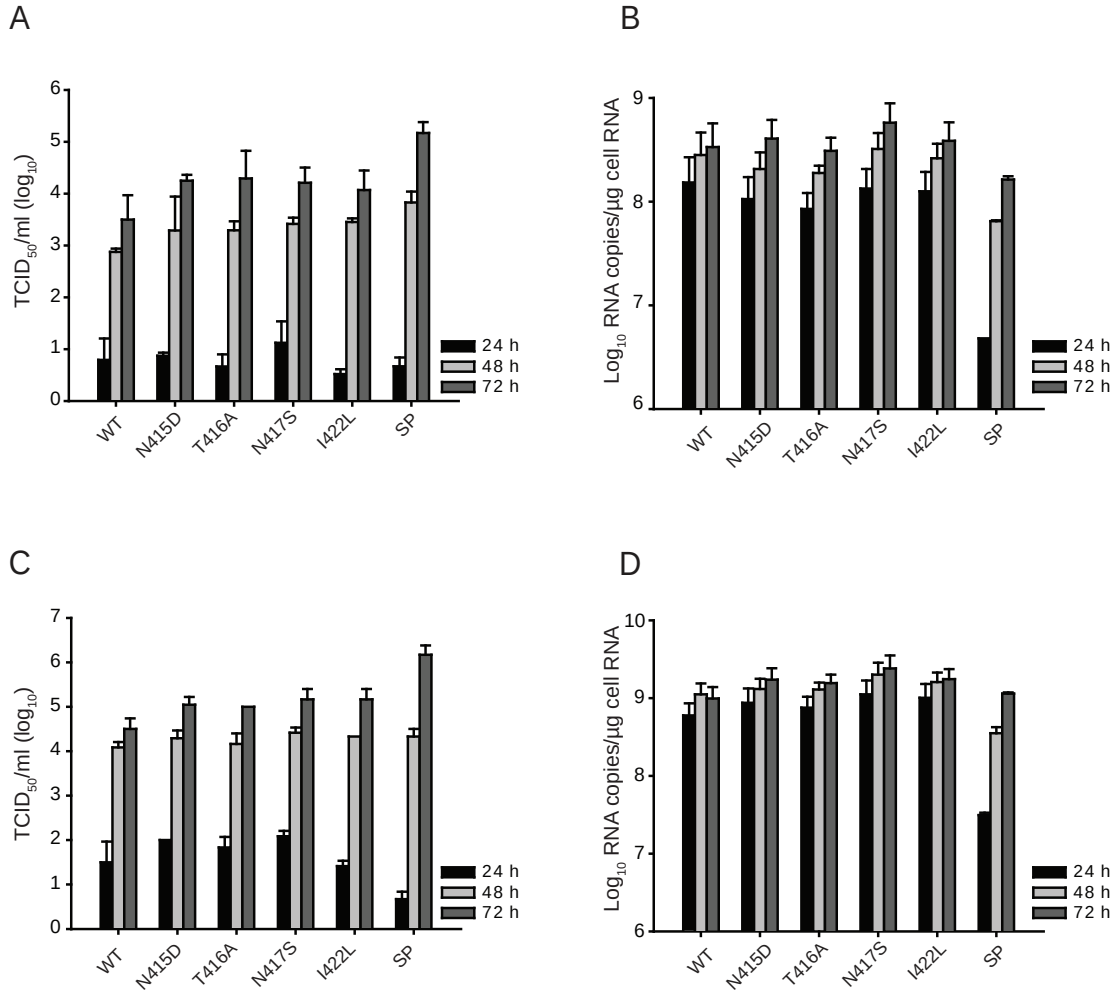


Figure 3.2. Replication Levels of E2 Mutants

Huh-7 cells were electroporated with $JFH1_{WT}$ and E2 mutant virus RNAs. At 24, 48 and 72 h post-incubation the released infectious virus in the medium (A) and the intracellular RNA (B) were quantified by $TCID_{50}$ and RT-qPCR, respectively. Means and error ranges of two independent experiments are given. (C) Huh-7 cells were electroporated with viral RNA and at 72 h post-incubation the intracellular (IC) and extracellular (EC) virions were harvested and their infectivity quantified by $TCID_{50}$.



E

Viruses	HCV RNA copies GE/ml	Infectious titer TCID/ml	Specific Infectivity
WT	1.1×10^7	2.3×10^4	1:485
N415D	8.2×10^6	9.6×10^4	1:85
T416A	2.4×10^6	1.0×10^5	1:23
N417S	1.1×10^7	1.0×10^5	1:114
I422L	2.2×10^7	1.0×10^5	1:221

Figure 3.3. Infection Kinetics of E2 Mutant Viruses

Huh-7 cells were infected at an m.o.i of 0.1 (A and B) and 1.0 (C and D) with $JFH1_{WT}$ and E2 mutant viruses harvested at 72 h post-electroporation. Virus from passage 9 (sp1), which was used to originally identify the N415D mutation (refer to Fig 1A) was also included in the experiment. At 24, 48 and 72 h post-infection virus released in the medium (A and C) and intracellular RNA (B and D) was measured by $TCID_{50}$ and RT-qPCR, respectively. Means and error ranges of two independent experiments are given. (E) Specific infectivity's of E2 mutants. Huh-7 cells were infected at an m.o.i of 1.0 with the viruses harvested at 72 h post-electroporation. At 72 h post-infection, the levels of extracellular infectivity and HCV RNA were quantified by $TCID_{50}$ and RT-qPCR, respectively.

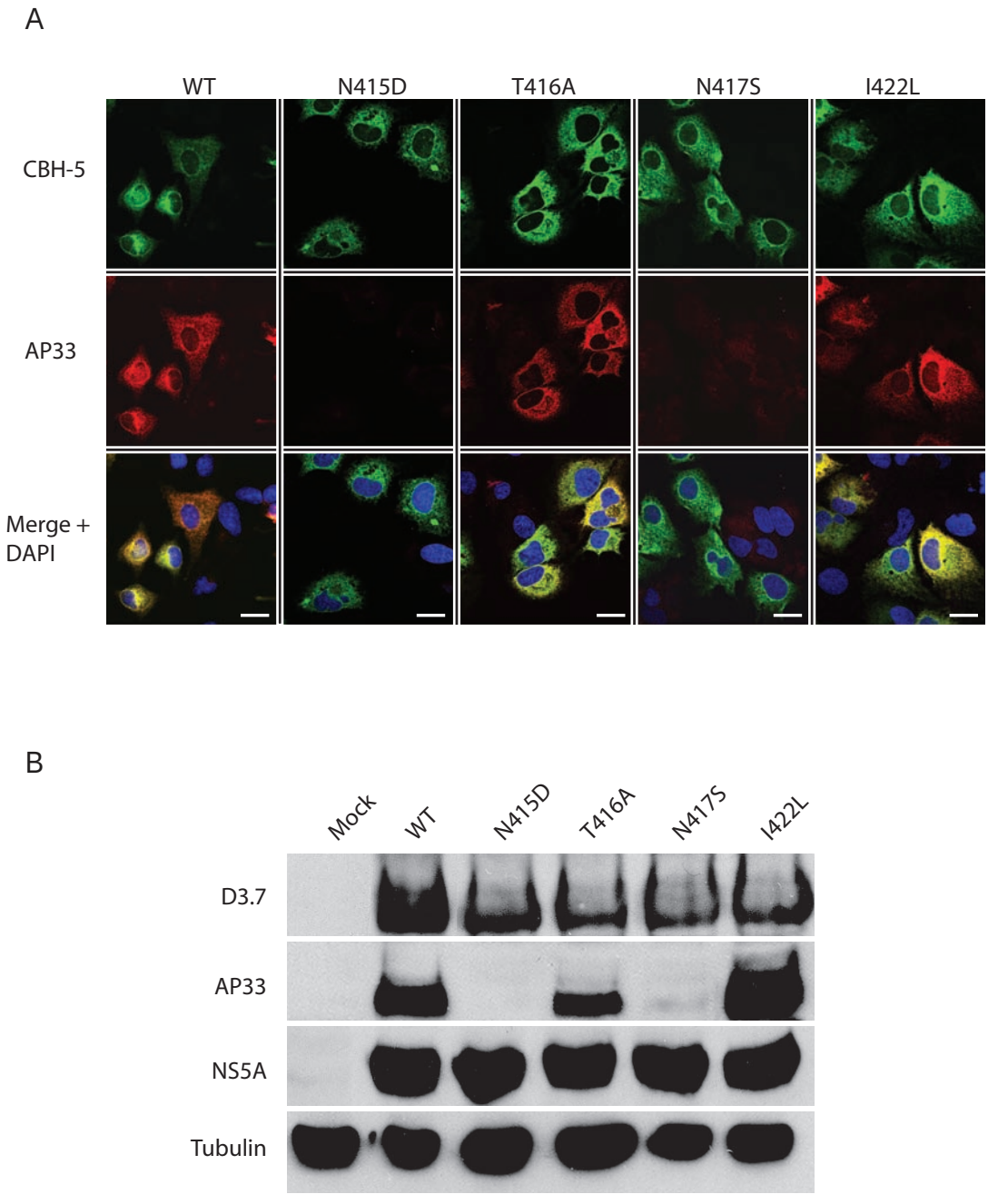


Figure 3.4. Intracellular Viral Protein Expression of E2 Mutants

(A) Huh-7 cells were electroporated with $JFH1_{WT}$ and E2 mutant viral RNAs. At 48 h post-incubation cells were fixed and probed with anti E2 antibodies CBH-5 and AP33. (B) Cell lysates harvested at 72 h post-electroporation were subjected to immunoblotting using antibodies against E2 (D3.7 and AP33), NS5A (9E10) and tubulin.

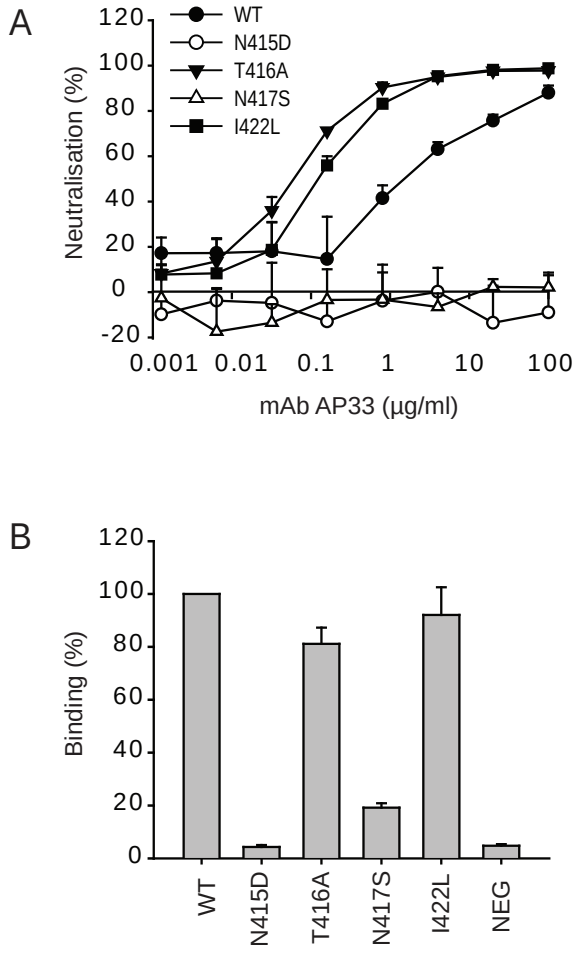


Figure 3.5. E2 Mutant Binding and Neutralization by mAb AP33

(A) $JFH1_{WT}$ and E2 mutant viruses were pre-incubated for 1 h with different concentrations of mAb AP33 before infecting Huh-7 J20 target cells. The level of virus inhibition was assayed by quantifying the SEAP activity in the medium. Percent neutralization was calculated by quantifying viral infectivity in the presence of mAb AP33 relative to infection in the absence of antibody. Error bars represent variability of the assay. (B) The levels of $JFH1_{WT}$ or mutant E2 in Huh-7 cells electroporated with appropriate viral RNAs were first normalized by measuring their binding to CBH-4B (Appendix 1). Normalised lysates were then tested for their reactivity to mAb AP33 by GNA capture ELISA and the data is presented as an average of two independent experiments (Appendix 2). Reactivity is expressed as percentage of binding relative to the $JFH1_{WT}$.

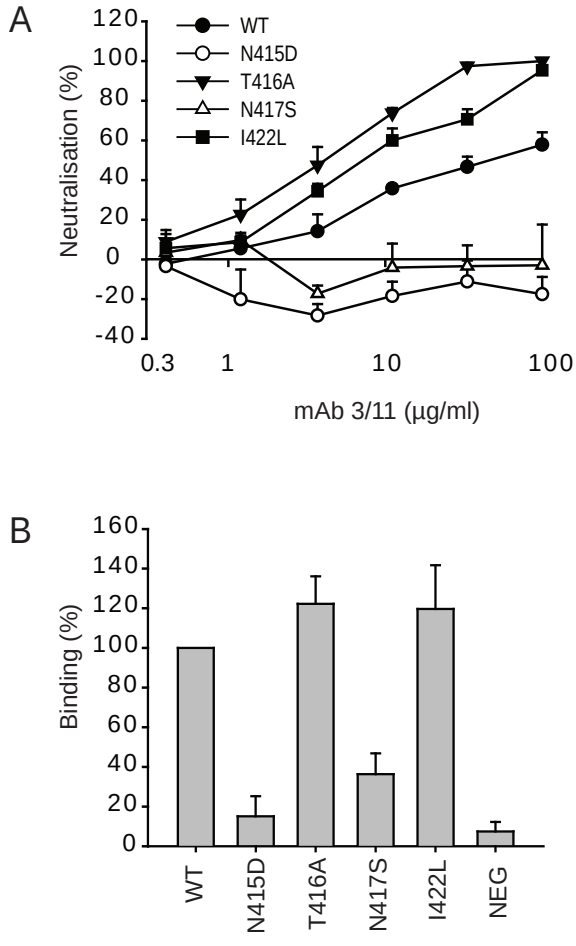


Figure 3.6. E2 Mutant Binding and Neutralization by mAb 3/11

(A) $JFH1_{WT}$ and E2 mutant viruses were pre-incubated for 1 h with different dilution of mAb 3/11 before infecting Huh-7 J20 cells. The level of virus inhibition was assayed by quantifying the SEAP activity in the medium. Percent neutralization was calculated by quantifying viral infectivity in the presence of mAb 3/11 relative to infection in the absence of antibody. Error bars represent variability of the assay. (B) The levels of $JFH1_{WT}$ or mutant E2 in Huh-7 cells electroporated with appropriate viral RNAs were first normalized by measuring their binding to CBH-4B (Appendix 1). Normalised lysates were then tested for their reactivity to mAb 3/11 by GNA capture ELISA and the data is presented as an average of two independent experiments (Appendix 3). Reactivity is expressed as percentage of binding relative to the $JFH1_{WT}$.

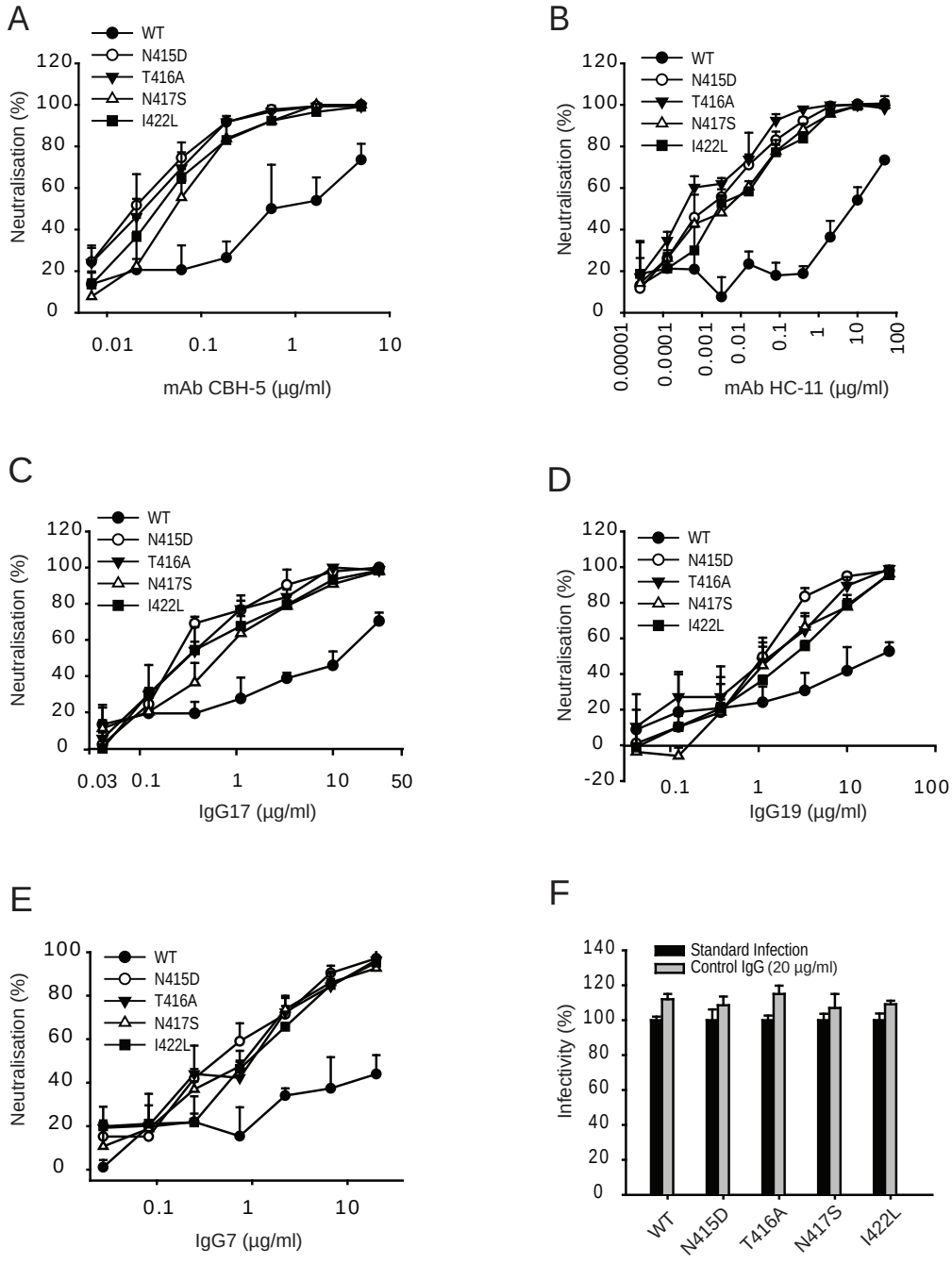


Figure 3.7. Neutralization of E2 Mutant Viruses by Human Anti-Envelope Antibodies

$JFH1_{WT}$ and E2 mutant viruses were incubated for 1 h with different amounts of human mAbs (A) CBH-5 or (B) HC-11, the HCV-infected patient derived IgGs (C) IgG17, (D) IgG19 or (E) IgG 7 or control IgG (F) purified from an uninfected individual prior to infecting Huh-7 J20 target cells. The level of virus inhibition was measured by quantifying the SEAP activity in the medium. Percent neutralization (A-E) or percent infectivity (F) was calculated by quantifying viral infectivity in the presence of anti-HCV E2 specific antibodies or control IgG relative to infection in the absence of antibodies. Error bars represent variability of the assays.

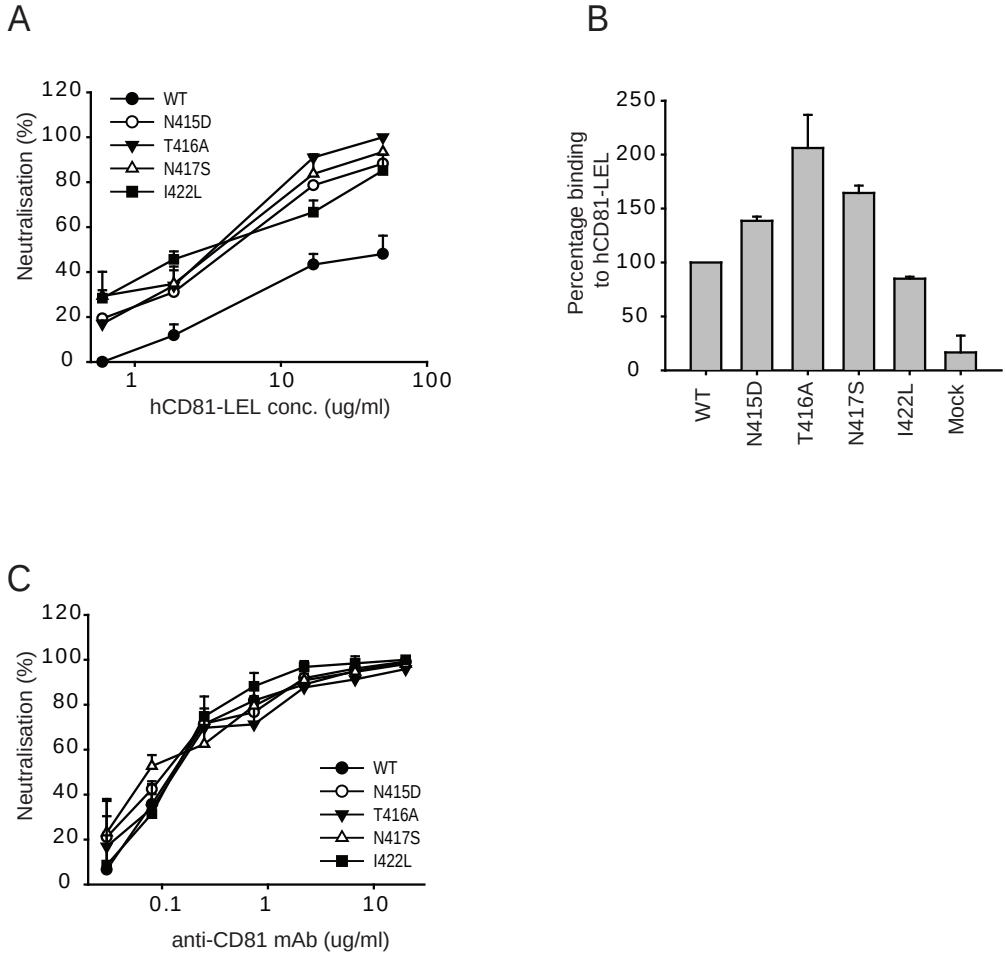


Figure 3.8. Effect of E2 Mutations on CD81 Binding

(A) $JFH1_{WT}$ and JFH1 E2 mutant viruses harvested at 72 h post-electroporation were incubated with different dilutions of hCD81-LEL for 1 h prior to infecting target cells. At 2 days post-infection, virus infectivity was determined by FFU assay. Percent neutralisation was calculated by quantifying viral infectivity in the presence of hCD81-LEL relative to standard infection. (B) The levels of $JFH1_{WT}$ or mutant E2 in Huh-7 cells electroporated with appropriate viral RNAs were first normalized by measuring their binding to CBH-4B (Appendix 1). Lysates containing equivalent E2 levels were assessed for binding to hCD81-LEL by ELISA and the data presented as average of two independent experiments (Appendix 4), each performed in triplicate. Reactivity is expressed as percentage of binding relative to the $JFH1_{WT}$. (C) Naïve Huh-7 cells were pre-incubated for 1 h with different concentration of anti-CD81 antibody before infection. Cells were then infected with $JFH1_{WT}$ or mutant viruses for 48 h and the infectivity levels determined by FFU assay. Percent neutralization was calculated by quantifying viral infectivity in the presence of inhibitory antibodies relative to standard infection. Error bars indicate variability of the assays.

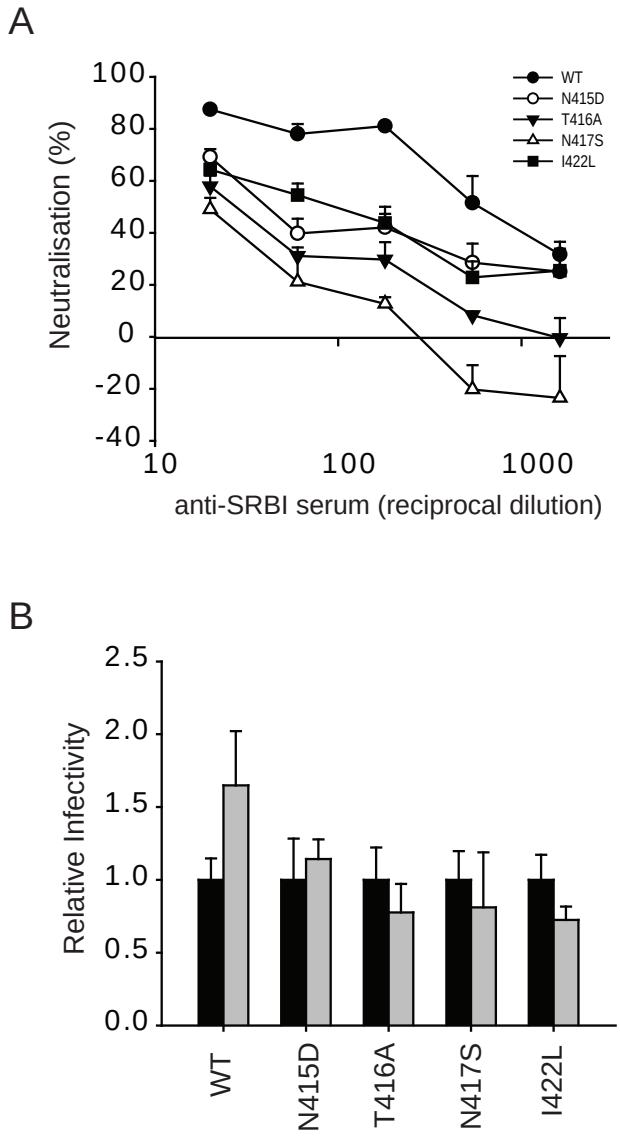


Figure 3.9. E2 Mutants Have an Altered Dependence on SR-BI

(A) Naïve Huh-7 cells were pre-incubated for 1 h with different dilution of anti SRBI serum before infection. Cells were then infected with $JFH1_{WT}$ or mutant viruses for 48 h and the infectivity levels determined by FFU assay. The data are expressed as percent neutralization relative to infection with the control rat serum. Error bars indicate variability of the assays. (B) Huh7-J20 cells were infected with $JFH1_{WT}$ or mutant viruses in the presence (gray bars) or absence of HDL (black bars) and virus infectivity determined by SEAP assay as described in Methods. Error bars represent variability of the assays.

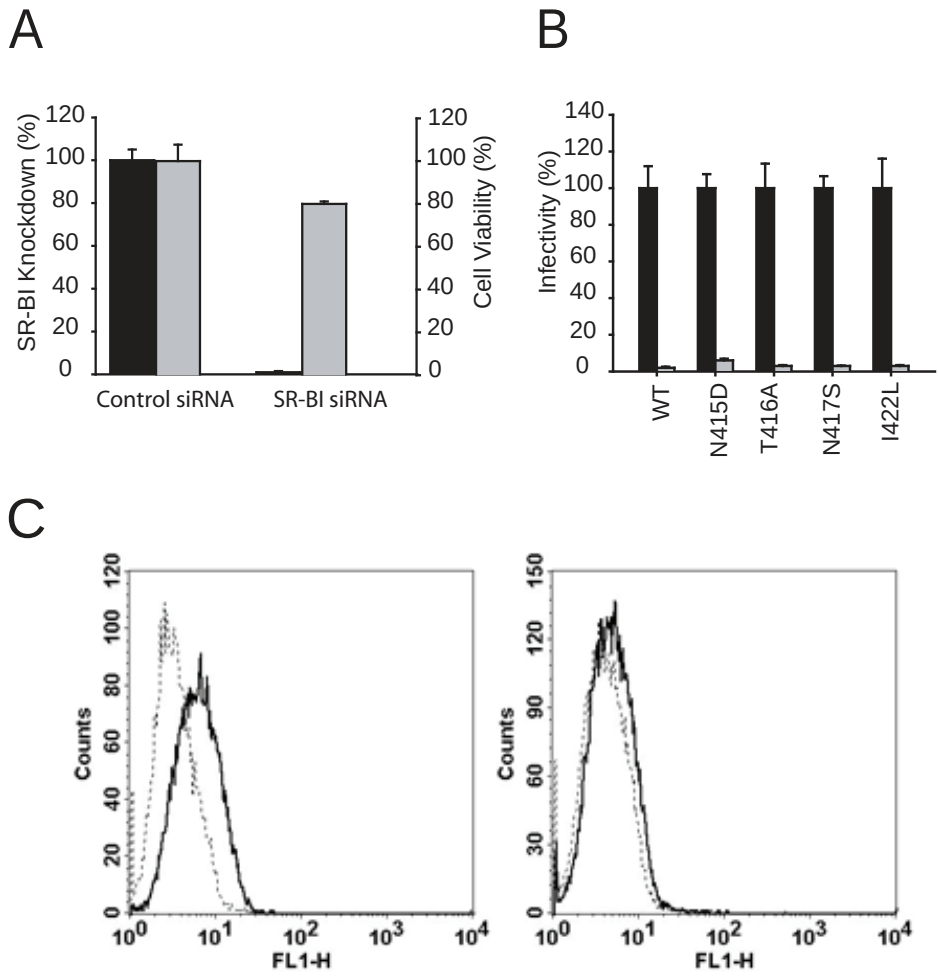


Figure 3.10. Effect of SR-BI Gene Silencing on E2 Mutant Virus Infection

Huh-7 cells were transfected with control siRNAs or siRNAs targeting SR-BI gene. (A) At 48 h post-transfection, the cell viability (grey bar) and mRNA expression levels (black bar) of SR-BI were measured by ELISA and RT-qPCR, respectively. (C) The expression of SR-BI on the surface of Huh-7 cells transfected with control siRNAs (left panel) or receptor-specific siRNAs (right panel) was determined by FACS. Solid and broken lines represent cells stained with anti-SR-BI antibody and IgG subtype control, respectively. (B) In parallel, the control siRNA-transfected Huh-7 cells (black bars) or the SR-BI knockout cells (grey bars) were infected with $JFH1_{WT}$ or mutant viruses and the intracellular HCV RNA levels measured by qRT-PCR to quantify infectivity. Error bars represent variability of the assays.

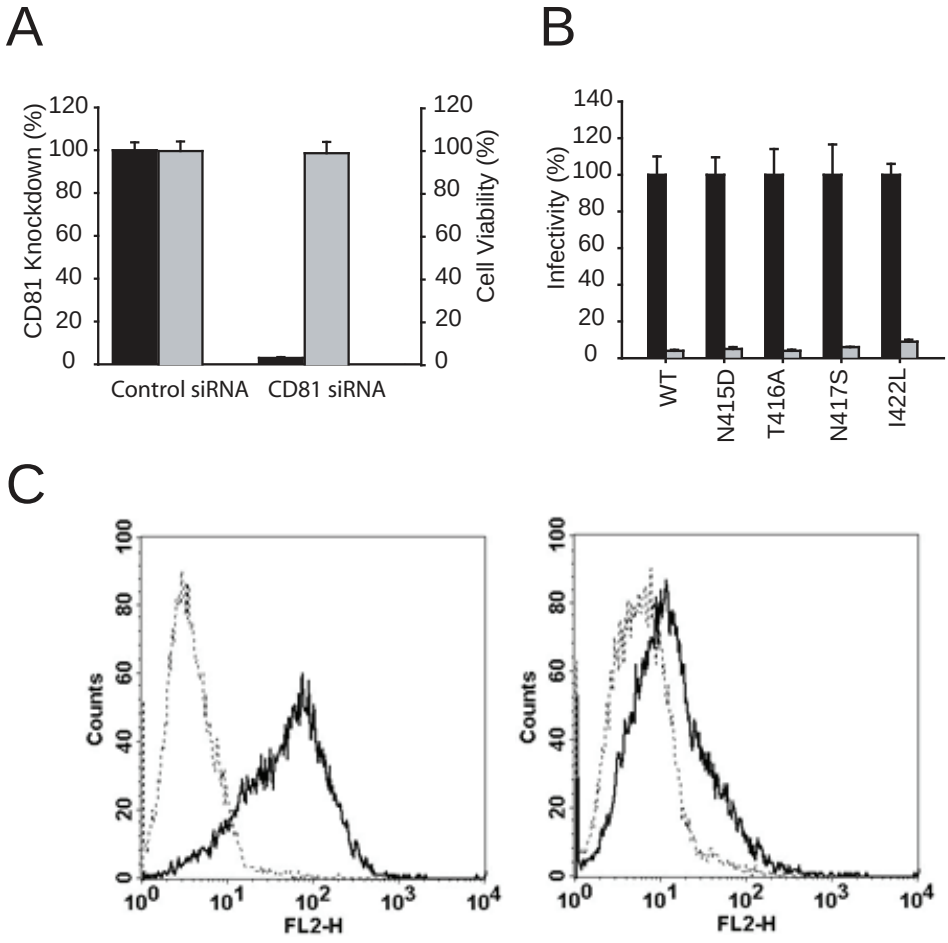


Figure 3.11. Effect of CD81 Gene Silencing on E2 Mutant Virus Infection

Huh-7 cells were transfected with control siRNAs or siRNAs targeting CD81 gene. (A) At 48 h post-transfection, the cell viability (grey bar) and mRNA expression levels (black bar) of CD81 were measured by ELISA and RT-qPCR, respectively. (C) The expression of CD81 on the surface of Huh-7 cells transfected with control siRNAs (left panel) or receptor-specific siRNAs (right panel) was determined by FACS. Solid and broken lines represent cells stained with anti-CD81 antibody and IgG subtype control, respectively. (B) In parallel, the control siRNA-transfected Huh-7 cells (black bars) or the CD81 knockout cells (grey bars) were infected with $JFH1_{WT}$ or mutant viruses and the intracellular HCV RNA levels measured by qRT-PCR to quantify infectivity. Error bars represent variability of the assays.

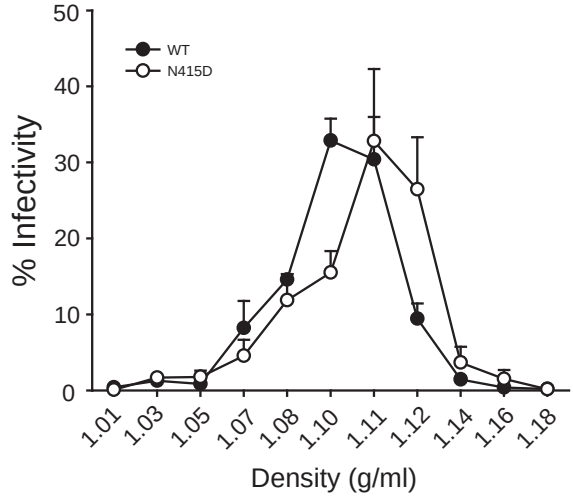


Figure 3.12. Buoyant Density of JFH1$_{N415D}$

Concentrated culture medium from JFH1$_{WT}$ and JFH1$_{N415D}$ electroporated cells was fractionated using 10-40% iodixanol density-gradient centrifugation. For each fraction, the amount of infectivity was determined by FFU assay. The average density measured at each fraction is plotted on the X-axis.

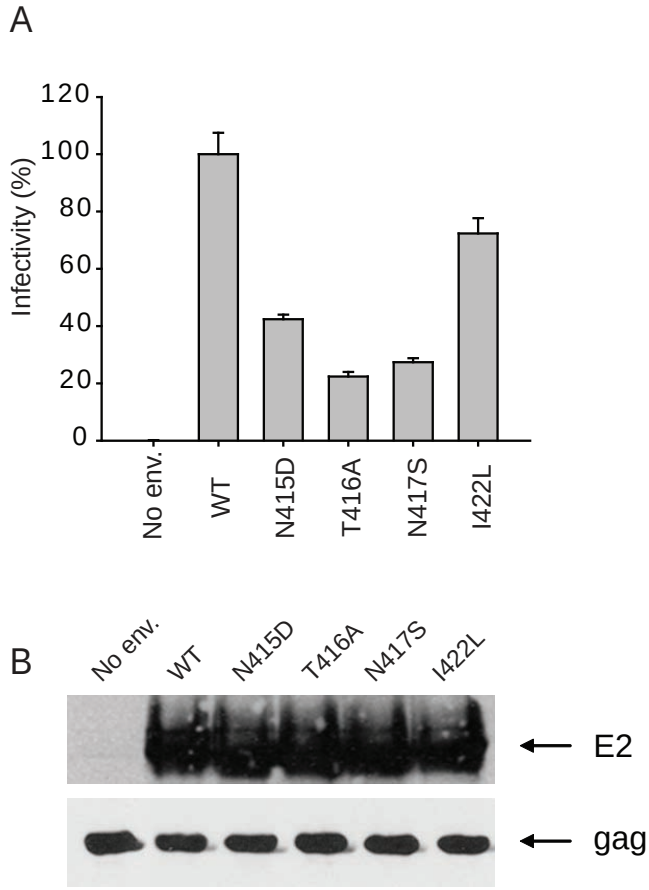


Figure 3.13. E2 Mutants Characterization in the HCVpp System

(A) HCVpp bearing $JFH1_{WT}$ E2 or mutant JFH1 E2 was generated in HEK-293T cells co-transfected with HIV gag-pol and luciferase reporter gene. At 48 h post-incubation the medium containing HCVpp was harvested and used to infect naïve Huh-7 cells. At 48 h post-infection the luciferase activity in the cell lysates was measured and the values are expressed as percentage infectivity relative to the $JFH1_{WT}$ E2 levels. Means and error range represents average of two independent experiments. (B) HCVpp harvested at 48 h post transfection was pelleted through a 20 % sucrose cushion, lysed and used for immunoblotting to detect virion E2 using an anti-E2 mAb D3.7 and MLV gag proteins using a gag-specific mAb.

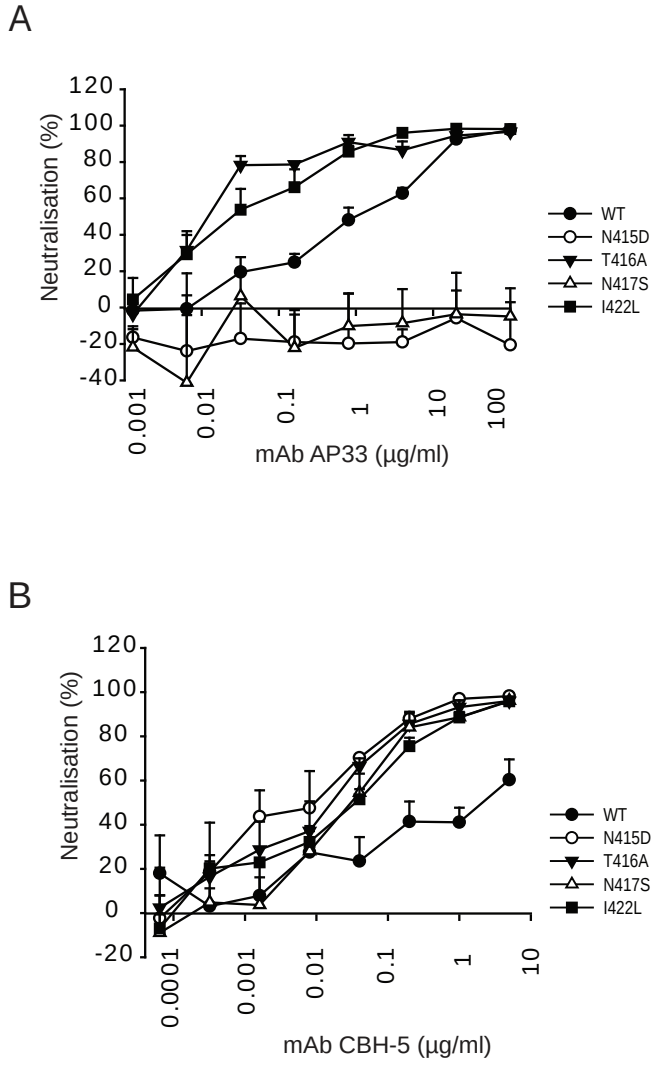


Figure 3.14. Further Characterization of E2 Mutant in the HCVpp System

HCVpp bearing $JFH1_{WT}$ E2 or JFH1 mutant E2 were first normalised with respect to their infectivity (luciferase reading) values and then mixed with mAbs AP33 (A) or CBH-5 (B) 1 h prior to infecting Huh-7 cells. Virus infectivity was measured 3 d post-infection by quantifying luciferase activity. Percent neutralization was calculated by quantifying viral infectivity in the presence of anti-E2 specific mAbs relative to infection in the absence of mAbs. Error bars represent variability of the assays.

Table 3.1. IC_{50} values (μg/ml) of hCD81-LEL and inhibitory antibodies for each virus.

Inhibitors	**WT**	**N415D**	**T416A**	**N417S**	**I422L**
hCD81-LEL	>50	4.4	3.4	3.7	3
anti-CD81	0.12	0.1	0.13	0.07	0.13
anti-SR-BI	1:560	1:40	1:25	<1:20	1:100
IgG7	>20	0.39	0.96	0.81	0.9
IgG17	12.11	0.2	0.3	0.66	0.3
IgG19	22	1.1	1.3	1.4	2.5
CBH-5	0.6	0.02	0.02	0.05	0.035
AP33	1.5	NN	0.06	NN	0.12
HC11	7.0	0.001	0.0003	0.004	0.002
3/11	44.9	NN	4.1	NN	7.12
AP33 (HCVpp)	1.0	NN	0.01	NN	0.02
CBH-5 (HCVpp)	2.3	0.01	0.016	0.03	0.036

NN = Non-neutralizable

ND = Not done

Anti-SR-BI IC_{50} values are represented as serum dilutions

4. Creation and Characterisation of JFH1 chimeras

4.1. Introduction

Until recently, studies on the HCV lifecycle have been limited due to the lack of a robust cell culture system for propagating the virus. Before HCVcc was available, the HCVpp system was the primary model used for characterizing the early HCV infection steps, involving virus binding and utilisation of cellular receptors for entry into host cells. The HCVpp system has been an invaluable tool for dissecting the role of the HCV E1E2 glycoproteins in viral entry and furthermore in measuring the neutralizing capacity of anti-E1E2 antibodies to prevent infection of target cells (Bartosch *et al.*, 2003; Drummer *et al.*, 2003; Hsu *et al.*, 2003). HCV exhibits high genetic variability, particularly in the E1 and E2 glycoprotein region of the HCV genome. Infectious HCVpp bearing the envelope glycoproteins of all HCV genotypes have been generated (Broering *et al.*, 2009; Lavillette *et al.*, 2005b; Law *et al.*, 2008). Studies using HCVpp of different genotypes have confirmed that all viruses require the expression of cell surface receptors CD81, SR-BI (Lavillette *et al.*, 2005b) and tight junction proteins CLDN-1 (Evans *et al.*, 2007; Fofana *et al.*, 2010; Meertens *et al.*, 2008) and OCLN (Ploss *et al.*, 2009) for entry. In addition, the HCVpp from all genotypes can be neutralized by patient serum and a panel of anti-E1E2 antibodies (Johansson *et al.*, 2007; Keck *et al.*, 2009; Keck *et al.*, 2007; Lavillette *et al.*, 2005b; Law *et al.*, 2008; Owsianka *et al.*, 2005; Perotti *et al.*, 2008). HCVcc is a more desirable system to study the different aspects of virus entry and antibody-mediated neutralization given that it produces authentic infectious virus particles. The HCVcc system is based on the genotype 2a HCV strain JFH1, cloned from an individual with fulminant hepatitis. The key limitation with this system is that the JFH1 clone only represents one of the seven HCV genotypes. Therefore, comparative studies on the effect of genetic variability on different stages of the life cycle, as well as the, development and evaluation of anti-viral strategies is not possible with this system.

With the aim of studying HCVcc entry from different HCV genotypes, 5 chimeric genomes were constructed by substituting the JFH1 E1E2 sequences with those from different patient isolates that represented genotypes 1, 2, 3, 5 and 6. While all chimeric genomes could replicate efficiently post-electroporation, none were able to assemble and release infectious virus particles. In addition, work was continued on a previously generated chimeric clone harbouring the JFH1 NS3-NS5B fused to the core-NS2 region of a genotype 4 isolate, ED43. The ED43/JFH1 viral RNA replicated efficiently post-electroporation, but was unable to produce sufficient infectious virus titers for

experimental purposes. The long-term culture of cells replicating ED43/JFH1 substantially improved the infectivity of this virus due to the selection of several cell-culture adaptive mutations in various regions of the genome. Introducing these mutations into the original ED43/JFH1 genome dramatically improved infectious virus production to levels comparable to $JFH1_{WT}$, post-electroporation. The cell-culture adapted ED43/JFH1 was then characterized alongside WT virus to compare its CD81 receptor dependency during virus entry, the ability of several anti-E2 nAbs to inhibit its infection and the ability of this virus to spread via cell-to-cell transfer.

4.2. Generation of E1E2/JFH1 Chimeric Genomes

Full length E1E2 sequences extracted from patients infected with diverse HCV genotypes and subtypes were cloned into the pcDNA3.1 V5-DTOPO mammalian expression vector. Due to the absence of unique enzyme recognition sites around the region of interest within the pcDNA3.1 vector, it was not feasible to use the simple digestion and ligation method for cloning. Therefore, an overlap extension strategy was applied to generate the chimeric viruses. Primers were first designed to generate three fragments with overlapping overhangs (Figure 4.1). Two fragments in pUC JFH1 (fragment 1 and 3) and one fragment (fragment 2) in the pcDNA3.1 was then PCR amplified and fused together. Subsequently, the fusion product was amplified by PCR (Figure 4.2). The sequences of fragments 1 and 3 contain unique *FspAI* and *NotI* enzyme recognition sites located in the JFH1 core and NS2 genes, respectively. For constructing the 2a2.5, 3A1.28 and 6.5.340 clones, the amplified fusion product was digested with *FspAI* and *NotI*. The 1B12.6 and 5.15.7 fusion product sequences contained additional *FspAI* and *NotI* restriction sites. Therefore, these products were digested with the *AgeI* and *KpnI* enzymes, which have unique cutting sites located in the JFH1 5'UTR and NS2, respectively. Each digested fragment was then subcloned into the pJFH1 backbone cleaved with the appropriate restriction enzymes. The proper incorporation of inserts into the pJFH1 template was verified by diagnostic digestion and further confirmed by sequence analysis.

4.3. Characterisation of E1E2/JFH1 Chimeras

All the chosen E1E2 sequences were fully functional in the HCVpp system (Owsianka *et al.*, 2005), except 2A2.5 (Dr Ania Owsianka, personnal communication). To determine the viability of the generated E1E2/JFH1 chimeras in the HCVcc system, Huh-7 cells were first electroporated with $JFH1_{WT}$ and the E1E2/JFH1 chimeric *in vitro* transcribed viral RNAs. $JFH1_{\Delta E1E2}$ was included as a replication competent but non-infectious virus control (Wakita *et al.*, 2005) and $JFH1_{GND}$, as the replication-deficient control. At 72 h post-incubation, the protein expression and virus release levels of each chimera was then tested. To do this, cells were fixed for IF analysis and lysed for immunoblotting, whereas the cell medium was titrated for infectivity and lysed for qRT-PCR assay. All E1E2 chimeras were replication competent, like $JFH1_{WT}$, as seen by the intracellular expression of E2 and NS5A protein by IF (Figure 4.3A) and core protein by immunoblotting (Figure 4.3B). As expected no E2 protein expression was observed for $JFH1_{\Delta E1E2}$ and $JFH1_{GND}$ was completely incapable of replication. However, apart from $JFH1_{WT,}$ none of the clones tested were capable of producing extracellular or intracellular infectious particles. Concomitantly, the relative extracellular HCV RNA levels for all chimeras were lower than the levels of $JFH1_{\Delta E1E2}$ virus (Figure 4.3B).

To further establish if functional clones could be selected for during long term passaging, electroporated cells were passaged serially every 5-6 days for a total of 4 months. At every passage cells were tested by IF for NS5A expression and the cell medium was titrated for infectious particles. Although positive cells supporting replication could be detected up to passage number 4, none of the viruses were able to release infectious particles into the medium. Collectively these results suggest that replacing the E1E2 proteins in JFH1 may not be suffice to generate fully functional chimeric clones. It is possible that the presence of the foreign glycoprotein sequences may prevent proper E2/p7 and p7/NS2 cleavage during the processing of the HCV polyprotein, which in turn prevents virion assembly occurring. It would be interesting to test if supplying the JFH1 glycoproteins into the cells replicating the E1/E2 chimeric genomes could rescue their infectivity. Indeed, such an approach proved effective for virus harboring a defective NS2 protein (Yi *et al.*, 2009). Recently published papers on chimeric viruses viable in the HCVcc system demonstrate that cloning the entire structural region from core-p7 together with the homologoues NS2 protein is necessary to obtain infectious chimeric viruses (Gottwein *et al.*, 2009; Pietschmann *et al.*, 2006; Scheel *et al.*, 2008). Some studies have gone a step further and have pinned down the exact NS2 junction essential to generate fully viable JFH1 based

chimeric viruses (Pietschmann *et al.*, 2006; Scheel *et al.*, 2008). Due to lack of access to the rest of the genome from these clones it was not possible to apply the aforementioned strategy to generate chimeras incorporating the core-NS2 region in the JFH1 backbone from these particular genotypes.

4.4. Generation of an Infectious Genotype 4a/JFH1 Chimeric virus

Sequences obtained from the genotype 4a reference strain, ED43 (Chamberlain *et al.*, 1997) were used to generate a genotype 4a/JFH1 chimera by replacing the core to NS2 region of JFH1 with the analogous region of ED43 (Figure 4.4A). The cloning procedure used to generate this chimeric virus was performed by Dr Michaela Iro at the MRC Virology Unit. Preliminary studies indicated that post-electroporation, this chimeric genome was replication competent but generated very low quantities of infectious virus. The following section extended the characterization of this previously generated chimera.

Following electroporation, the 4a/JFH1 viral RNA was replication competent, as seen by the intracellular expression of NS5A at 72 h post-incubation (Figure 4.5A). However, its ability to produce infectious virus particles was greatly reduced (6.81 $TCID_{50}$/ml) as compared to $JFH1_{WT}$ (4.64 x 10^5 $TCID_{50}$/ml) (Figure 4.5A). Previous studies have reported that for efficient virus production, certain chimeras depend on several cell-culture adaptive mutations that are selected during long-term culture (Gottwein *et al.*, 2007; Gottwein *et al.*, 2009; Scheel *et al.*, 2008; Yi *et al.*, 2007). In an attempt to adapt the 4a/JFH1 chimera and improve its infectivity, Huh-7 cells electroporated with viral RNA were subjected to several cell passages (P_1, P_2, P_3 etc.) and supernatant passages (I_1P_X and I_2P_X) (Figure 4.4B). The first supernatant passage or infection is noted I_1 with the corresponding passages and the second infection I_2. The level of replication was determined at each passage by staining cells for NS5A as well as measuring the levels of infectivity in the culture medium by limiting dilution assay. As depicted in figure 4.5A, the long-term passaging of 4a/JFH1 resulted in a ~100-fold increase in virus production. Based on the previous studies, the large increase in 4a/JFH1 infectivity was likely due to the selection of cell culture adaptive mutations. To identify the mutation(s) responsible for this changed phenotype, RNA harvested from I_2P_3 and I_2P_5 was RT-PCR amplified, and the population was sequenced over the core to NS5A region. In total, six nonsynonymous mutations were

detected at both passages, three of which were clustered within the NS2 region and one each within the E2, NS3 and NS5A proteins (Figure 4.5B). All the six changes were introduced into the parental 4a/JFH1 genome and the viability of the resulting virus, designated Ad4a/JFH1, was tested in Huh-7 cells.

4.5. Replication Properties of Ad4a/JFH1

The intracellular RNA and virus infectivity of $JFH1_{WT}$ and Ad4a/JFH1 was measured from electroporated cells at 24, 48 and 72 h post-incubation. In parallel, cells were lysed at 72 h post-electroporation for immunoblotting against the viral core and E2 proteins. The Ad4a/JFH1 displayed virus production level 32-, 7- and 6-fold lower than $JFH1_{WT}$ at 24, 48 and 72 h, respectively (Figure 4.6A). In contrast, the intracellular HCV RNA levels observed for Ad4a/JFH1 were very similar to WT at each time point (Figure 4.6B). In line with this, similar levels of intracellular core and E2 protein expression were observed for Ad4a/JFH1 and $JFH1_{WT}$, at 72 h post-electroporation (Figure 4.6C). Next, the infectivity and replication efficiency of Ad4a/JFH1 virus particles released from electroporated cells was determined. To do this, naive Huh-7 cells were inoculated at an equal multiplicity of infection (m.o.i) with virus obtained at 72 h post-electroporation. The released virus production in the medium and the level of intracellular replication were determined at 24, 48 and 72 h post-infection. As determined by the $TCID_{50}$ assay, the infectivity titers of Ad4a/JFH1 were lower than $JFH1_{WT}$ at each time point with values 2-, 3- and 20-fold lower at 24 and 48 and 72 h, respectively (Figure 4.7A). Also, the intracellular RNA levels of Ad4a/JFH1 were 9-, 3- and 2-fold lower at 24, 48 and 72 h compared to $JFH1_{WT}$ (Figure 4.7B). It is interesting to note that at the 24 h timepoint Ad4a/JFH1 virus production is similar to $JFH1_{WT}$, yet its intracellular RNA levels are much lower. This could indicate that Ad4a/JFH1 is more efficient than $JFH1_{WT}$ at producing virions at early timepoints following infection. A useful future experiment would be to test the specific infectivity of Ad4a/JFH1 virions at these early time points in order to gain better insight into this phenotype. Nevertheless, it is fair to say that overall these data suggest Ad4/JFH1 has lower replication levels compared to $JFH1_{WT.}$

4.6. Ad4a/JFH1 has Reduced CD81 Binding

To test the dependency of the Ad4a/JFH1 virus for the CD81 receptor, a competition assay using the hCD81-LEL was performed. To do this, virus was incubated with hCD81-LEL prior to infecting naive Huh-7 cells with virus harvested at 72 h post electroporation. The Ada/JFH1 virus showed a slightly decreased sensitivity to neutralization by hCD81-LEL with an IC_{50} value 2-fold higher than $JFH1_{WT}$ (Figure 4.8A). Next, the ability of a neutralizing CD81 antibody to inhibit Ad4a/JFH1 infection was tested. Naive Huh-7 cells were incubated with CD81 MAb prior to infection with virus harvested at 72 h post electroporation. As shown in figure 4.8B the Ad4a/JFH1 virus was more effectively neutralized compared to $JFH1_{WT}$ with an IC_{50} value 2-fold lower. Together, these results indicate that the E2-CD81 interaction is slightly lower for Ad4a/JFH1 than $JFH1_{WT}$.

4.7. Ad4a/JFH1 demonstrates an increased sensitivity to anti-E2 nAbs

Next the efficiency of several broadly neutralizing anti-E2 MAbs to block Ad4a/JFH1 infection was evaluated. Virus harvested at 72 h post-electroporation was incubated with Mab AP33, 3/11, CBH-5 or HC-11 prior to infecting target cells. All these antibodies have shown to possess broad cross-reactive activity and are thought to inhibit virus infection by blocking the E2-CD81 interaction (Keck *et al.*, 2009; Keck *et al.*, 2004; Keck *et al.*, 2007; Owsianka *et al.*, 2005; Owsianka *et al.*, 2008; Tarr *et al.*, 2006). The Ad4a/JFH1 chimeric virus found to be more sensitive to neutralization by all these antibodies compared to $JFH1_{WT}$ with IC_{50} values 6-, 8-, >8-, and 14-fold lower for AP33, 3/11, CBH-5 and HC11, respectively (Figure 4.9). These data indicate that the glycoproteins presented on the surface of Ad4a/JFH1 have altered affinities for nAbs targeting conserved linear and conformational epitopes on E2.

4.8. Cell-to-Cell Transmission of Ad4a/JFH1

It has been recently demonstrated that JFH1 as well as a number of intergenotypic and intragenotypic chimerics viruses are able to transmit via cell-to-cell, in addition to the cell-free route (Brimacombe *et al.*, 2011; Timpe *et al.*, 2008; Witteveldt *et al.*, 2009). The cell-

to-cell transfer efficiency of Ad4a/JFH1 was tested alongside $JFH1_{WT}$ using a previously described infectious co-culture assay (Appendix 6) (Brimacombe *et al.*, 2011). To do this, Huh-7 cells were electroporated with viral RNA and then labelled with CMFDA diffusion dye at 72 h post-incubation. These cells, known as donor cells, were then trypsinized and co-cultured with an equal ratio of naïve Huh-7 cells (target cells) in the presence of high concentrations of Mab AP33. At 48 h post-incubation, the $JFH1_{WT}$ extracellular infectious virus had been efficiently neutralized by AP33 (Figure 4.10A). Nevertheless, FACS analysis performed on the cultured cells revealed only a moderate reduction in the number of NS5A positive target cells in the presence of AP33 (33.4%) compared to the no antibody control (54.3%). This indicates that the majority of $JFH1_{WT}$ virus spreads via cell-to-cell transmission in cultured cells (Figure 4.10B). The majority of the Ad4a/JFH1 virus was also neutralized by MAb AP33 (Figure 4.10A). However, in great contrast to the $JFH1_{WT}$, there were far fewer NS5A positive target cells in the presence of nAb (2.9%) compared to the no nAb control (23.5%) (Figure 4.10B). This suggests that this chimeric virus is poor at cell-to-cell transfer and relies on cell-free transmission as its predominant mode of spread.

4.9. Discussion

The data from this section indicate that viable intergentoypic HCVcc chimeras cannot be generated by replacing the envelope glycoprotein coding sequences of JFH1 with those from other genotypes. The E1E2 chimeric genomes could replicate efficiently, however no infectious virus production could be detected following electroporation or after several cell passages. The process of passaging electroporated cells for long time periods was performed in the hope that spontaneous mutations would arise in the viral genomes and permit infectious virus production. While this lengthy process failed to adapt the E1E2 chimeric genomes, it was successful in improving the infectivity of the previously constructed 4a/JFH1 chimera. This viral genome was created by replacing the core-NS2 genes of JFH1 with those from the ED43 sequence and was based on the strategy used by Lindenbach and colleagues to generate the highly infectious J6/JFH1 intragenotypic chimera (Lindenbach *et al.*, 2005). However, unlike J6/JFH1, the 4a/JFH1 chimera required the selection of several nonsynonymous mutations before efficient growth could be achieved. Ad4a/JFH1 harbouring the six mutations identified in this study could assemble and release moderate quantities of infectious virus over 3 days following

electroporation, albeit at lower levels to $JFH1_{WT}$. As the RNA replication of Ad4a/JFH1 was comparable to $JFH1_{WT}$ within these cells, this indicates that the lower viral titers of this chimera is likely caused by a defect during infectious virus assembly and/or release. In contrast, in the infection experiment it was found that Ad4a/JFH1 had lower virus release and intracellular RNA replication kinetics. This could be explained by the Ad4a/JFH1 virions being slower at binding and/or entering target cells via the normal infection route. Furthermore, the cell-to-cell transfer assay found that Ad4a/JFH1 was poor at spreading via this mode of transmission, which would almost certainly contribute to the reduced infection kinetics of this virus. Interestingly, it was found that Ad4a/JFH1 virions had a reduced affinity for the CD81 receptor. This may be one reason that might account for the lower spreading capacity of this virus as this receptor is required for virus entry via the normal infection route as well as cell-to-cell transfer. However, future experiments are necessary to determine if the poor cell-to-cell transfer of Ad4a/JFH1 is primarily responsible for its poor infection kinetics. One way to confirm this theory would be to infect sparsely seeded target cells to limit the cell-to-cell spread of the virus and thereby ensuring that the infectious progeny measured in the culture medium would be derived from virus spreading mainly via cell-free infection. The Ad4a/JFH1 virions showed a heightened sensitivity to neutralization by the four nAbs AP33, 3/11, CBH-5 and HC-11, as indicated by the lower IC_{50} values. Therefore, it is possible that the glycoprotein sequence of this particular genotype has a stronger binding affinity for the nAbs compared to the $JFH1_{WT}$ sequence. Alternatively, the Ad4a/JFH1 glycoprotein conformation on the virion surface may differ somewhat to $JFH1_{WT}$, such that the neutralizing epitopes are more accessible to these nAbs. Further studies will be necessary to distinguish between these two possibilities.

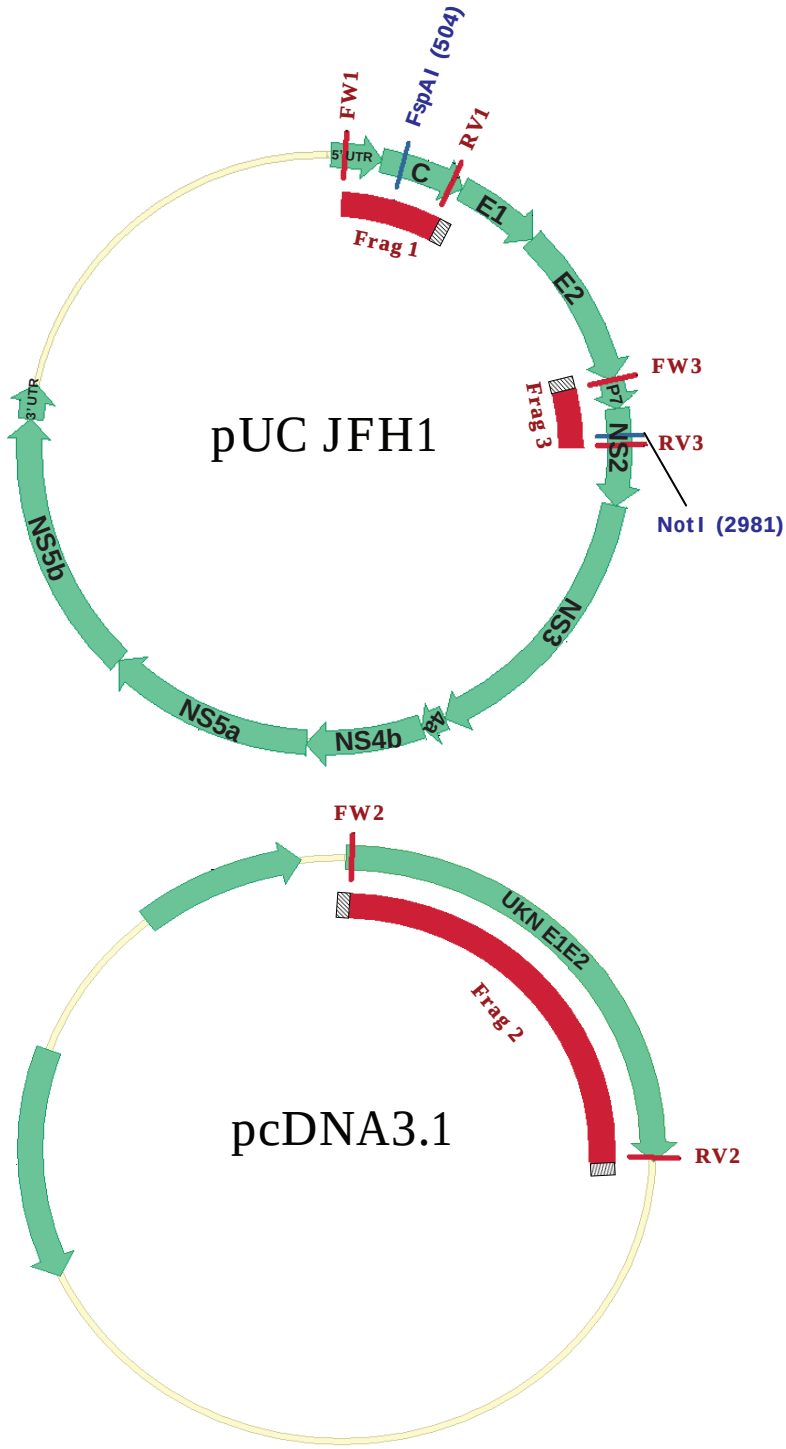


Figure 4.1. Cloning E1E2/JFH1 Chimeras

Schematic representation of the full length JFH1 plasmid (pUC JFH1) and the plasmid that encodes E1E2 of diverse genotypes (pcDNA3.1). To generate each chimeric virus two fragments in pUC JFH1 (frag 1 and 3) and one fragment in pcDNA3.1 (frag 2) were first PCR amplified and then fused together. The overlapping sequence in each fragment is represented by white shading. The location of sense (FW) and antisense (RV) primers for each fragment is indicated for each plasmid.

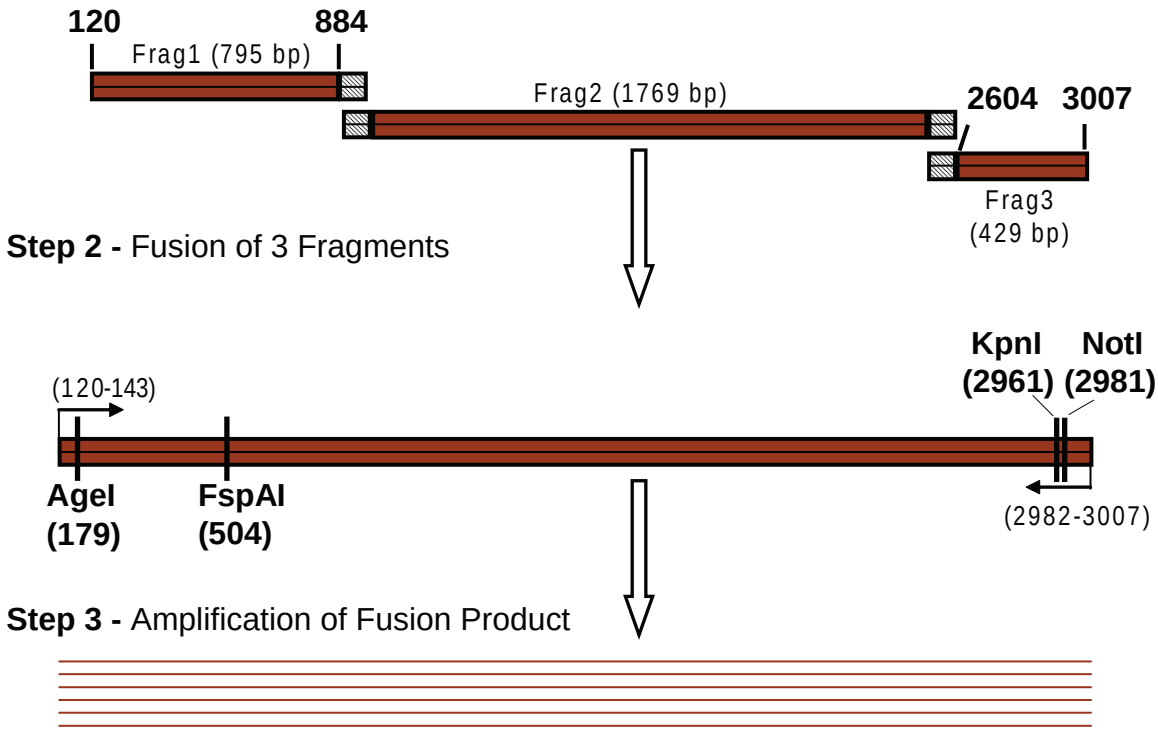


Figure 4.2. Illustration of the Fusion PCR Procedure

The 3 fragments were PCR amplified (step 1), fused together to form a fusion product (step 2), which was subsequently amplified by PCR (step 3). The nucleotide position of fragment 1 and 3 as well as the restriction enzyme used within pUC JFH1 are indicated in bold. White shading represents the overlapping sequence in each fragment. Black arrows represents the primer location used to PCR amplify the fusion product.

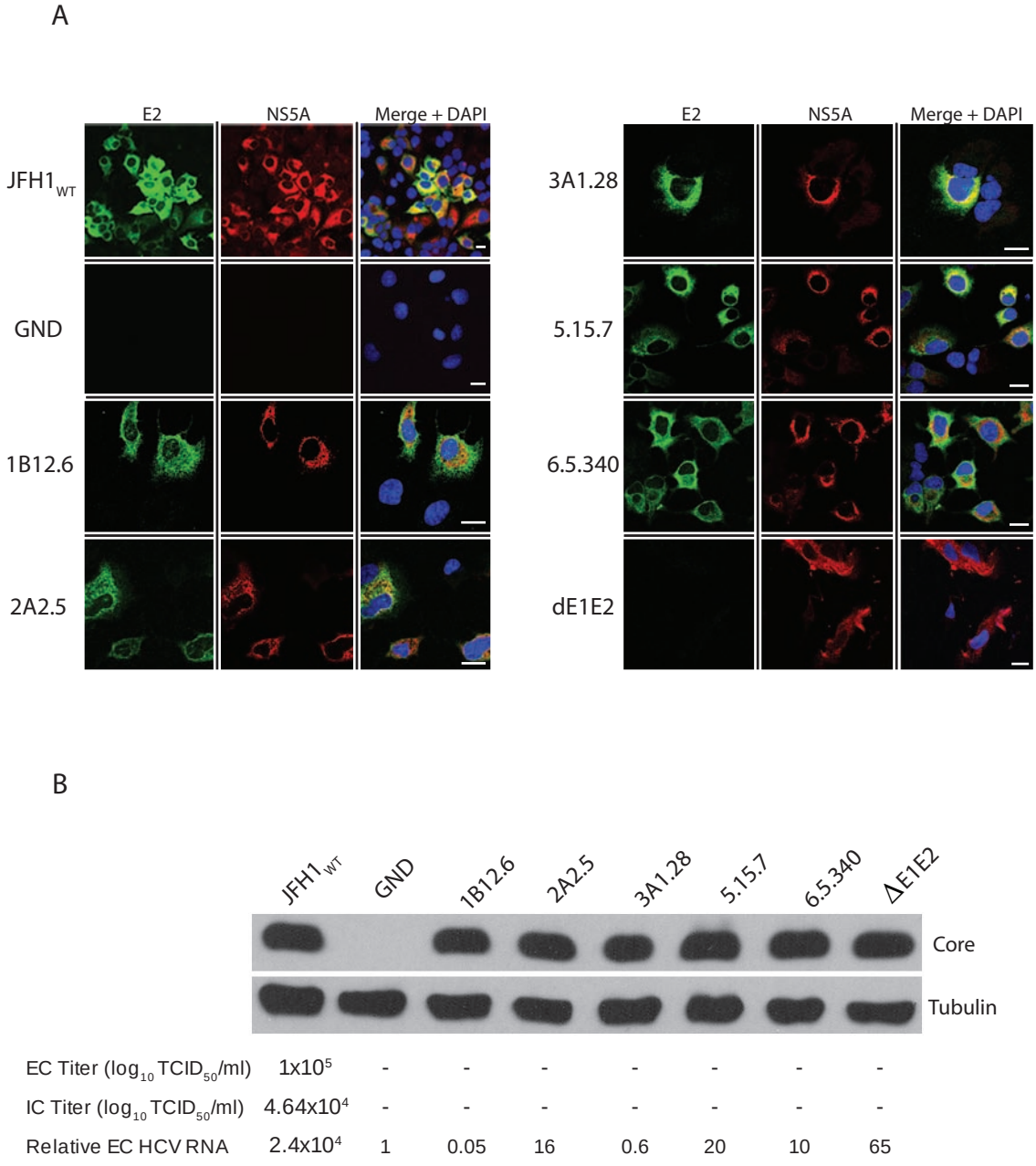
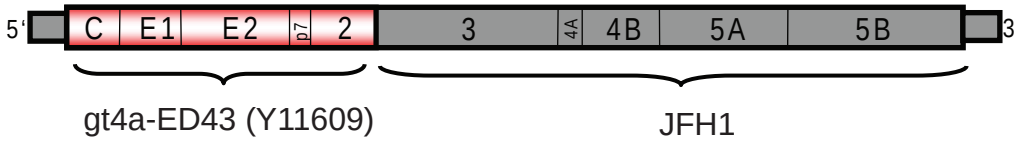


Figure 4.3. Replication Properties of E1E2/JFH1 Chimeric Genomes.

(A) Huh-7 cells electroporated with the indicated viral RNAs were fixed at 72 h post-incubation and analysed by confocal microscopy for the intracellular expression of E2 and NS5A using antibodies AP33 and anti-sheep NS5A, respectively. Bar, 20 µm. (B) Huh-7 cells electroporated with viral RNAs as shown were subjected to Western immunoblotting at 72 h post-incubation using anti-core mAb C7-50 and an anti-tubulin mAb. The infectious virus titers (intra- and extracellular) and the genomic HCV RNA levels from these samples were assayed by $TCID_{50}$ and RT-qPCR, respectively.

A



B

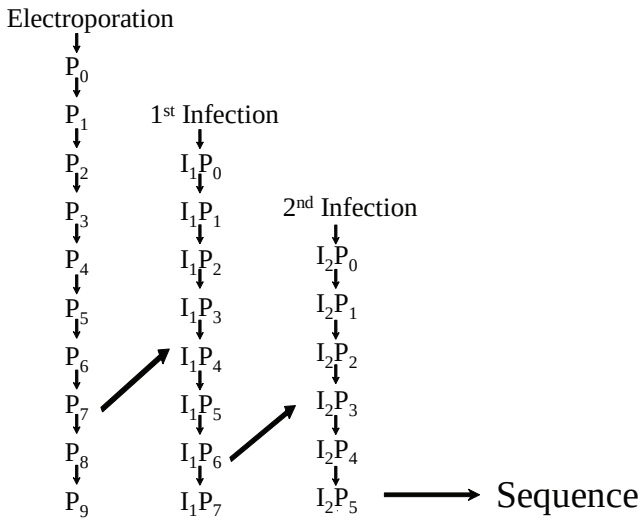


Figure 4.4. Organisation of the 4a/JFH1 Genome and its Long-Term Passaging in Cell-Culture.

(A) Diagram of the 4a/JFH1 chimeric genome design. (B) Schematic representation of the successive rounds of infection performed on 4a/JFH1 to improve its infectivity. Huh-7 cells electroporated with 4a/JFH1 viral RNA were subjected to several cell passages (P_1, P_2, P_3 etc.) and cell-free passages (I_1P_X and I_2P_X). The first cell-free passage or infection is noted I_1 with the corresponding passages and the second infection I_2.

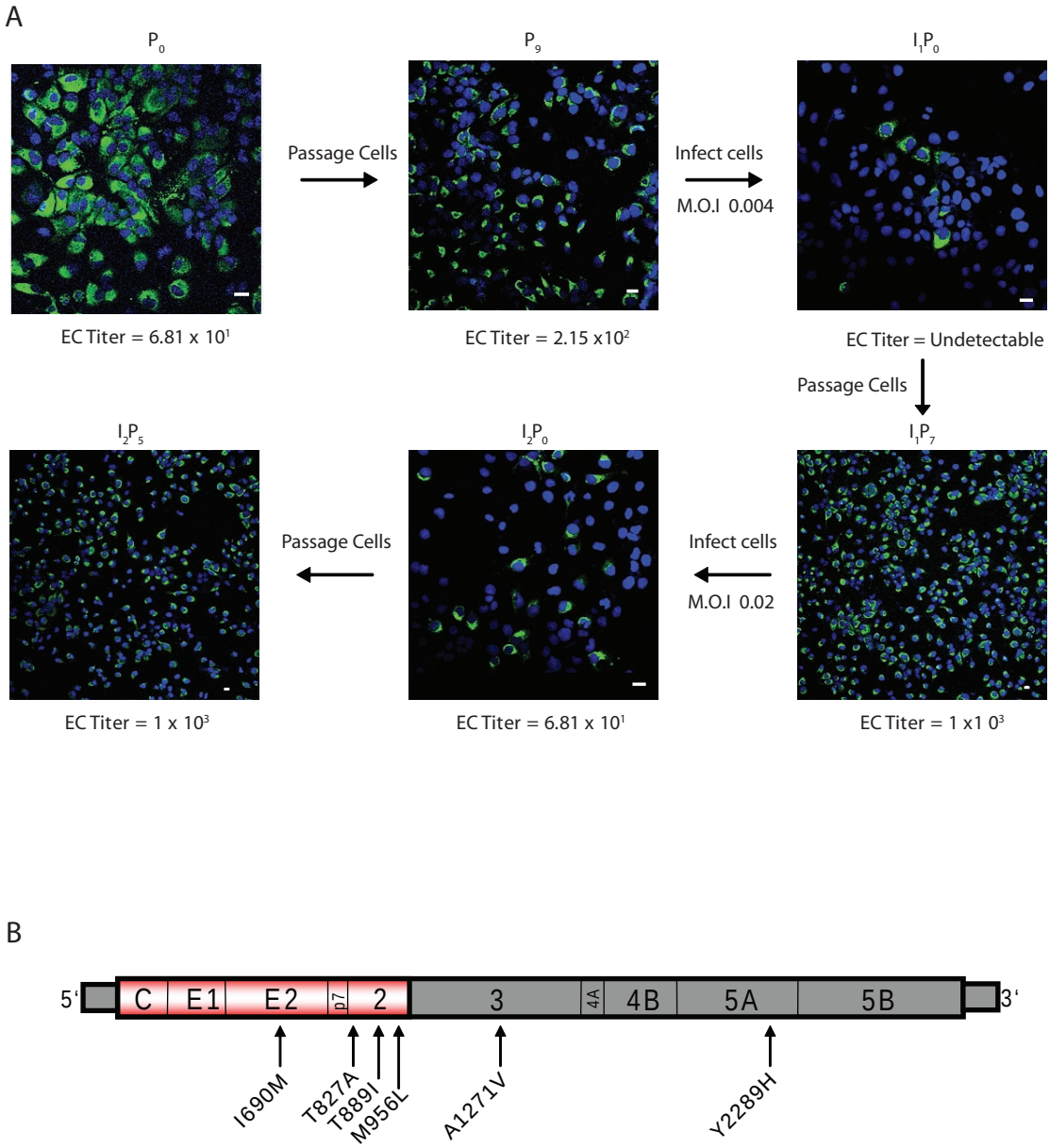


Figure 4.5. Passaging of 4a/JFH1 Chimeric Virus and Identification of Cell Culture Adaptive Mutations

(A) Huh-7 cells electroporated with 4a/JFH1 virus were subjected to several cell passages (P_0, P_1, and so on) and cell free passages (I_1 and I_2). At 72 h post- incubation cells were fixed and probed with anti-NS5A mAb, 9E10. Bar, 20 µm. At 72 h post-incubation virus released in the medium was titered using $TCID_{50}$ assay. (B) Cellular RNA from 2nd infection at P_3 and P_5 were used to amplify and sequence the viral genome and six mutations were identified.

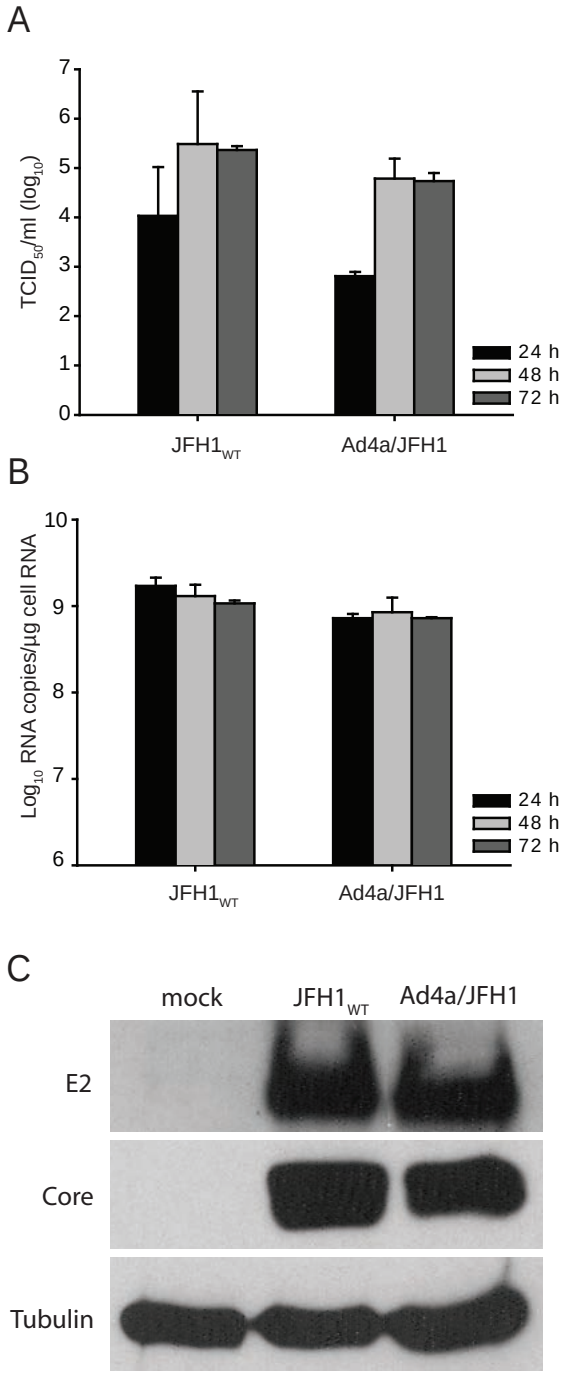


Figure 4.6. Characterisation of Ad4a/JFH1 Chimera.

(A) Infectious virus production and (B) intracellular HCV RNA replication of Ad4a/JFH1 at 24, 48 and 72 h post-electroporation into Huh-7 cells, as determined by $TCID_{50}$ and RT-qPCR, respectively. Means and error ranges from two independent experiments are given. (C) Huh-7 cells electroporated with indicated viral RNAs were subjected to western immunoblotting at 72 h post-incubation using anti-E2 mAb D3.7, anti-core mAb C7-50 and an anti-tubulin mAb.

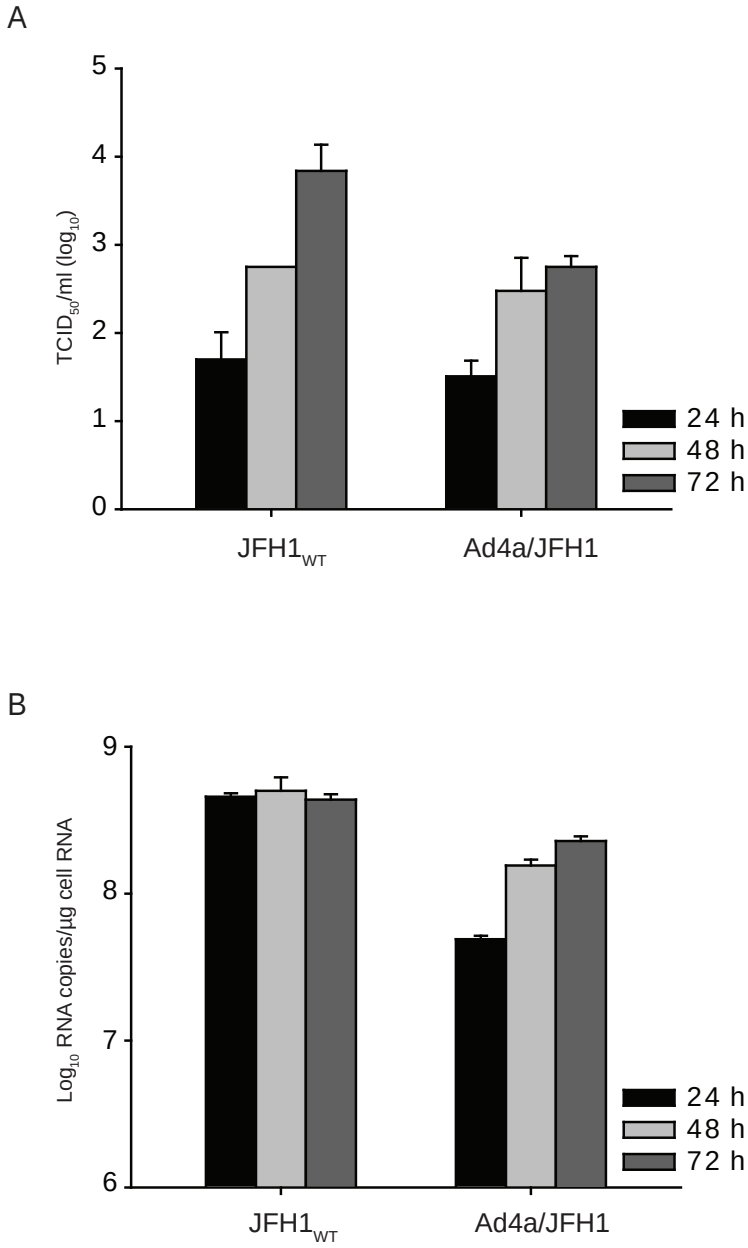


Figure 4.7. Infection Kinetics of Ad4a/JFH1.

Naïve Huh-7 cells were infected with $JFH1_{WT}$ and Ad4a/JFH1 at an m.o.i. of 0.2 using virus harvetsetd at 72 h post-electroporation. The infectious virus released into the medium (A) and the intracellular viral RNA (B) were quantified at 24, 48 and 72 h post-infection by $TCID_{50}$ and RT-qPCR, respectively. Error bars indicate variability of the assays.

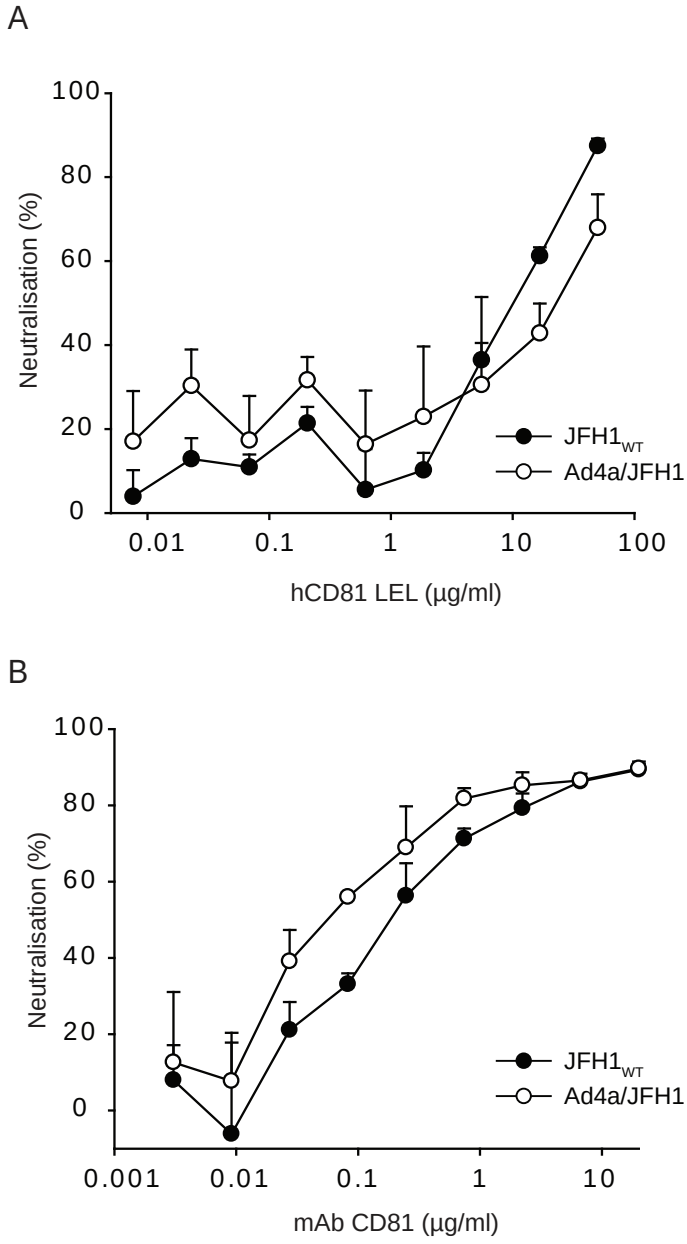


Figure 4.8. Inhibition of Ad4a/JFH1 Infection Using Soluble CD81 Molecule and Anti-CD81 Antibody

(A) Virus collected at 72 h post-electroporation was incubated with different amount of hCD81-LEL for 1 h prior to infecting Huh-7 J20 target cells. (B) Huh-7 J20 cells were preincubated for 1 h with different dilutions of anti-CD81 antibody prior to infection with virus. The extracellular SEAP activity was measured 3 days post-infection. The level of virus inhibition is expressed as percentage, normalized to the no-antibody control. Error bars indicate variability of the assays.

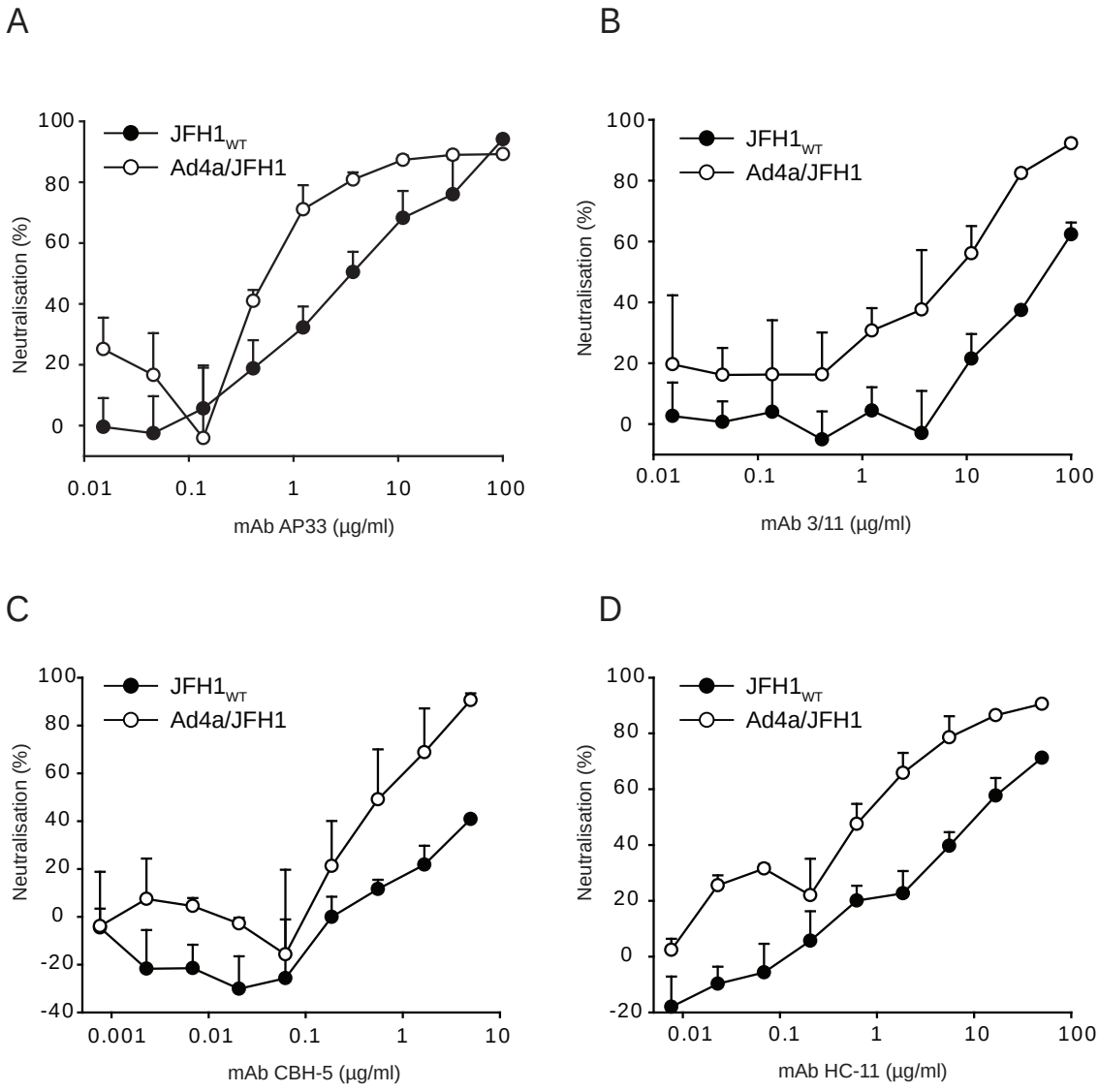


Figure 4.9. Inhibition of Ad4a/JFH1 Infection Using Anti-E2 Neutralising Antibodies

$JFH1_{WT}$ and Ad4a/JFH1 were incubated for 1 h with different dilutions of mouse mAb AP33 (A), rat mAb 3/11 (B) or human mAbs CBH-5 (C) and HC-11 (D) prior to infection of Huh-7 J20 cells using virus harvested at 72 h post-electroporation. The extracellular SEAP activity was measured 3 days post-infection. The level of virus inhibition is expressed as percentage, normalized to the no-antibody control. Error bars indicate variability of the assays.

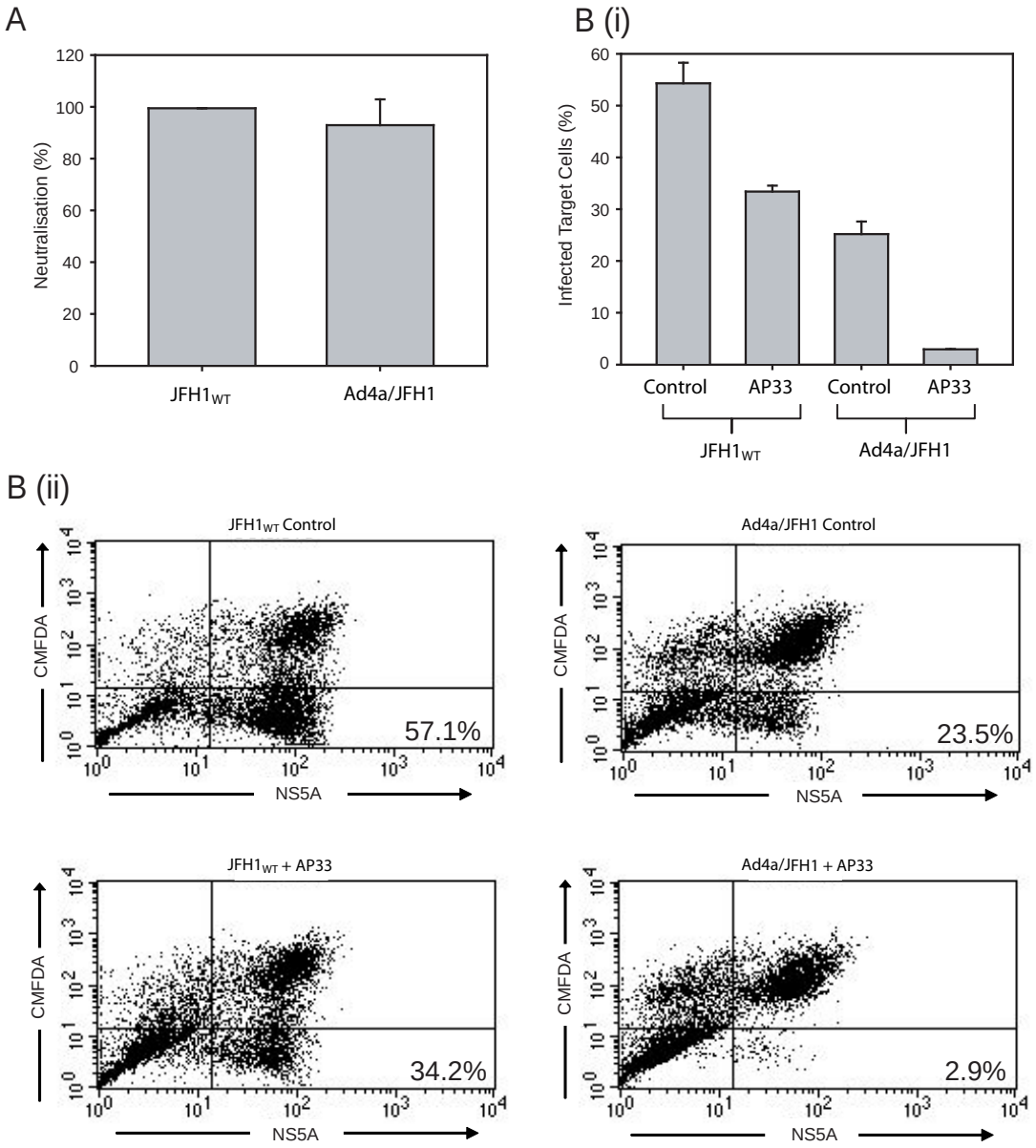


Figure 4.10. Cell-to-Cell Transmission of Ad4a/JFH1

(A) Percentage neutralization of extracellular virus in the co-culture medium in the presence of 100 μg of mAb AP33. Huh7-J20 cells were infected in triplicate with the medium obtained from the co-cultured cells at 48 h post-incubation. The SEAP activity present in the cell medium was then determined at 48 h post-infection and the percentage neutralization calculated. Values are relative to no nAb control. Means and error ranges from duplicate infections are shown. (B) Quantification of HCV transmission to co-cultured recipient cells by flow cytometry. (i) Bar graph plotting the percentage of infected target cells of all NS5A positive cells recorded for $JFH1_{WT}$ and Ad4a/JFH1 in the absence and presence of nAb. Means and error ranges from duplicate co-culture assays are shown. (ii) Dot plots representing one measurement from each of the duplicate co-culture assays. The lower left quadrant of each dot plot represents non-CMFDA labelled, uninfected cells, while the upper left quadrant shows uninfected CMFDA labelled cells. The upper right quadrant represents the CMFDA labelled donor cell population and the infected recipient target cells are shown in the lower right quadrant. The percentage of infected target cells of all NS5A positive cells is shown in the lower right quadrant of each dot plot.

5. Conclusions

5.1 Characterisation of E2 Cell Culture Adaptive Mutations

5.1.1. Summary

The two main studies described in the results section were each based on the adaptive properties of JFH1 that enables persistent viral infection to be established in cell culture. The adapted viruses from each section provided useful insights into the viral entry process as well as antibody-mediated inhibition of infection by broadly nAbs. The major findings from each of these sections are discussed separately below.

5.1.2. Characterization of E2 Cell Culture Adaptive Mutations

5.1.2.1. Viral Evolution during Persistent Infection

Cell culture adaptation that greatly enhance replication and infectivity of HCV during long term cell culture *in vitro* have been reported by several groups. As shown in Table 5.1, adaptive mutations appear to arise most frequently in E2, NS2 and NS5A. The use of different cell lines, different viruses and different methods of establishing persistent infection may explain the varied locations and number of adaptive mutations observed in these studies. Adapted variants have been generated from the serial passage of electroporated cells (Russell *et al.*, 2008; Zhong *et al.*, 2006) and infected cells (Bungyoku *et al.*, 2009) as well as additional passaging of the infected cell supernatants on naïve cells (Delgrange *et al.*, 2007; Kaul *et al.*, 2007; Pokrovskii *et al.*, 2011). Regardless of the process employed, these genetic studies have established that adaptive mutation(s) significantly enhance viral spread. However, the mechanism exploited by virus to achieve this efficiency seems to differ depending on where the mutation is located.

Zhong and colleagues were the first group to identify cell culture adaptations in JFH1 during persistent infection (Zhong *et al.*, 2006). In this study, the expansion kinetics and specific infectivity of virus taken from the day 183 (D183) time point in experiment 1 was superior to WT virus, which was due to the selection of the four mutations K74T, G451R, M1051T and C2219R. However, the E2 mutation G451R was primarily responsible for this phenotype. The infectivity of virus taken from the day 160 (D160) time point from experiment 2 was also higher than the WT virus. This virus contained a different set of mutations at positions V22A, K74Q, V388P, I414T and L644I. In a later report, the I414T

change was found to be the main mutation responsible for the improved infectivity of the D160 virus by improving virus secretion (Tao *et al.*, 2009). In contrast to the above studies, Delgrange and colleagues reported only a single mutation in E2 (N534K) after performing successive infections on naïve cells (Delgrange *et al.*, 2007). Nevertheless, the presence of this mutation alone greatly increased virus infectivity. Another study improved the infectivity of J6/JFH1 after 27 cell passages post-infection with the WT virus (Bungyoku *et al.*, 2009). The adapted virus contained the eight mutations T396A, N534H, A712V, Y852H, W879R, F2281L and M2876L. Interestingly, no individual mutation was capable of increasing virus infectivity. In a study by Russell and colleagues the effects of cell culture adaptive mutations in E2 (N417S), p7 (N765D), NS2 (Q1012R) and NS5A (L2175V) were characterized. The presence of all mutations significantly increased infectious virus production, for which the p7 and NS2 mutations were partly responsible for (Russell *et al.*, 2008). Kaul and colleagues passaged JFH1 in two separate experiments, both of which generated adaptive viruses that were more efficient at assembling of infectious particles than the WT (Kaul *et al.*, 2007). Similar to the study by Zhong and colleagues (Zhong *et al.*, 2006), the viral genomes in each experiment were mutated at different positions. Interestingly, the enhanced infectivity's of both adapted viruses were primarily due to one mutation; V2440L in NS5A for experiment 1 and N765D in p7 for experiment 2. Pokrovskii and collegues isolated 7 cell culture adaptive mutations following their passaging experiment of J6/JFH1 (Pokrovskii *et al.*, 2011). The increased infectivity of the adapted J6/JFH1 virus was explained by an improvement in infectious particle stability. This adaptive phenotype was conferred by the mutations core K78E, NS2 W879R, and NS4B V1761L.

5.1.2.2. Cellular Evolution during Persistent Infection

The exact reason for the emergence of the cell culture adaptive mutations in the above studies is unknown. However, Zhong and colleagues reported that during persistent infection cells that are resistant to HCV infection rapidly emerge and dominate the cell population (Zhong *et al.*, 2006). Examination of cell lines obtained from these populations identified two different mechanisms that explain such resistance to HCV infection:

1) Reduced CD81 cell surface expression that prevents virus entry.
2) A defect operating downstream of viral entry that prevents viral RNA replication.

Therefore, it seems that during persistent infection a complex virus-host relationship exists, which exerts selective pressure on both parties. It is likely that the death of permissive cells

caused by HCV infection is responsible for the outgrowth and dominance of these resistant cells. Therefore, the adaptive mutations selected throughout the genome may function to enhance viral fitness within this environment. In line with this theory, the G451R mutation allowed more efficient production of infectious virus particles and the entry properties of this mutant appeared to be less dependent upon CD81 molecules on the cell surface. Such properties would give this virus a selective advantage during the late period of persistent infection when resistant cells emerge. It is possible that the N415D mutation identified in the current study arose as a response to similar selective pressure. When passaging JFH1 infected cells in the current study, maximum number of NS5A positive cells were observed at cp 4, which was followed by maximum cell death visible at cp 5 and cp 6. This phase was followed by a drop in total number of NS5A positive cells as observed by IF, but an increase in the released infectivity was detected by $TCID_{50}$. Although cell surface CD81 expression was never investigated in the present study, the increased affinity of the adapted virus to CD81 may assist viral spread in cells presenting less CD81 during persistent infection. Similar circumstances may also account for the emergence of E2 cell culture adaptive mutations in the majority of the studies summarized in table 5.1.

Persistent infections in other virus cell culture systems have also reported co-evolution of both viruses and cells. Persistent infection with rotavirus and foot-and-mouth disease virus (FMDV) are good examples of these. During maintenance of persistent rotavirus infections of MA104 cells, mutations were selected in both viruses and cells. After an initial period of cell crisis the cultures stabilized and produced substantial titers of infectious virus for 12 months of continuous passage. The passaged viruses grew better than WT virus in cured cells, and the cured cells were less permissive for growth of WT virus than the parental cells. These observations suggest that cells manifesting resistance to viral replication are selected for their capacity to survive increasing viral titers. In addition mutant viruses were selected that exhibit a greater capacity to infect the resistant cells by bypassing the cellular block to viral replication. Therefore, it seems that for rotavirus persistent infections to survive in cell culture, an equilibrium must be reached between viral cytopathicity and cellular resistance in which ongoing viral replication is not sufficient to completely lyse the culture (Mrukowicz *et al.*, 1998). Virus and cells also evolve during the serial passage of cloned BHK-21 cells persistently infected with foot-and-mouth disease virus. The passaged cells become constitutively resistant to the parental FMDV, whereas the late passaged virus partly overcome the cellular block and were more cytolytic than WT virus

for BHK-21 cells. The results suggest that coevolution of BHK-21 cells and FMDV contributes to the maintenance of persistence in cell culture (De la Torre *et a.l*, 1988).

5.1.2.3. E2 Cell Culture Adaptive Mutations

The two E2 cell culture adaptive mutations G451R and I414T have been well characterized. The presence of each mutation alone in the JFH1 genome improves virus spread as well as increasing the specific infectivity of secreted particles. The mechanism responsible for the enhanced infectivity of G451R was not determined whereas I414T was found to specifically increase the virus release stage of the lifecycle. In contrast, the $JFH1_{N415D}$, $-_{T416A}$, $-_{N417S}$ and $-_{I422L}$ E2 mutants described in this study, displayed no alteration to virus assembly/release or virus spread. Nevertheless, these four mutant viruses did share a number of phenotypic similarities to $JFH1_{G451R}$ and $JFH1_{I414T}$ such as:

-Altered CD81 and SRB1 receptor dependencies

-Heightened sensitivity to nAbs targeting HCV envelope glycoproteins

-Increased buoyant density of infectious virions

It is likely that the above characteristics are closely linked to one another. The shift in sedimentation rate of infectious virions does indicate reduced lipoprotein content. This could result in the virion glycoproteins being more exposed, which would explain the increased sensitivities of these mutants to anti-envelope antibodies as well as soluble CD81 molecule. In line with this theory, immature intracellular JFH1WT virions, which presumably have a lower lipoprotein content than released virions (based on density gradient analyses), are more sensitive to neutralization by anti-E2 antibodies and less sensitive to anti-ApoE and anti-ApoC1 antibodies than released virions. The I414T extracellular virions also shared similar inhibition profiles to intracellular $JFH1_{WT}$ virions using the anti-E2, anti-ApoE and anti-ApoC1 antibodies. It is interesting to speculate that these single amino acid changes in E2 may function to reduce the lipoprotein content of virions to better expose the E2-CD81 binding sites in order to enhance this virus-receptor interaction and maintain viral spread during persistent infection. The reduced lipoprotein content theory may also explain why these E2 mutants have an altered SRB1 dependency for virus infection. If the association of VLDL to virus particles does indeed play a critical role in the primary interaction with SR-BI, then virus containing less lipoproteins may have an altered requirement for this receptor (Maillard *et al.*, 2006). However, the altered SR-BI dependency is unlikely to improve HCVcc viral persistence during long term cell culture and may occur only as an indirect effect of the reduced lipoprotein content to

expose CD81 binding residues. In line with this, Zhong and colleagues found that the HCV resistant cells that emerge during persistent JFH1 infection had similar SR-BI expression levels as the parental cells (Zhong *et al.*, 2006).

On the other hand, the HCVpp results obtained in this study are at odds with the lipoprotein hypothesis. As described in the introduction, HCVcc assembles in an ER-derived compartment in association with very low density lipoproteins (Gastaminza *et al.*, 2008; Huang *et al.*, 2007) whereas HCVpp are assembled in a post-Golgi compartment and are not associated with lipoproteins (Meunier *et al.*, 2008a; Sandrin *et al.*, 2005). Tao and colleagues reported that the I414T mutation did not alter the sensitivity of HCVpp to anti-E2 nAbs, in contrast to the HCVcc findings (Tao *et al.*, 2009). These results should make sense as if the I414T change functions to delipidate virus particles and increase glycoprotein exposure, no such effect should occur to HCVpp which lack lipoproteins. In contrast, the present study found that HCVpp harbouring the four E2 mutations showed identical anti-E2 neutralization profiles as the HCVcc viruses. This suggests that their HCVcc phenotype may not be entirely lipoprotein related. Instead, these mutations may simply exert global conformational changes to the E2 glycoprotein that result in increased exposure of antibody binding epitopes presented on the virus particle. However, it still remains to be determined whether HCV virions associate with the low density lipoproteins present in the culture medium following their secretion from producer cells. Such information would aid in our understanding of the effect that these E2 mutations have on the virion glycoprotein conformation. Although the exact mechanism through which these E2 mutations enhance the exposure of the virion glycoproteins is not fully understood, their phenotypes have provided valuable information with regard to the antibody mediated neutralization of HCV.

The entry properties of the E2 mutant viruses also raise interesting questions regarding the current model for HCV entry (Figure 1.5), particularly regarding the SR-B1 receptor. As mentioned previously, HCV interacts with SR-B1 via the virion-associated lipoproteins as well as the HVR1 of E2. Indeed, delipidated virions as well as those lacking HVR1 have been shown to be less dependent on SR-B1 for entry (Grove *et al*., 2008; Tao *et al*., 2009). However, given that the E2 mutants in this study appear not to be delipidated and contain HVR1 suggests that the integrity of the conserved E2 sequence between residues 412-423 is also necessary for HCV-SR-B1 binding. It is possible that the glycoprotein conformational changes inflicted by these E2 mutations may have somehow reduced

HVR1-SR-B1 binding. The reduced SR-B1 dependence may explain why these E2 mutations reduce HCVpp but not HCVcc entry. In the HCVcc system, the virion lipoprotein-SR-B1 interaction of these E2 mutants is likely to still be functional, whereas it is seemingly lacking in the HCVpp system. Therefore, the HCVpp mutant virions may only be able to associate with SR-B1 via a weak E2 interaction, which could explain their impaired entry kinetics. Together, the entry properties of the E2 mutants analysed in this study allow certain revisions to be made to the current HCV entry model proposed in Fig. 1.5. Firstly, the attachment of HCV to cells via LDLR, GAGs and SR-B1 via its lipoproteins is not essential for virus entry as HCVpps can still infect cells. Furthermore, viruses can enter cells with no SR-B1-lipoprotein association and an impaired E2-SR-B1 interaction. It is possible that the enhanced E2-CD81 interaction compensates for the reduced E2-SR-B1 interaction. Clearly, these E2 mutations are extremely useful tools to dissect the complex entry pathway used by HCV at both the early and late stages of the process.

5.1.2.4. In vivo Significance of E2 Mutations

The four E2 mutations N415D, T416A, N417A and I422L were chosen for analysis due to their location within the conserved E2 region that is the epitope for nAb AP33. Two of these mutations (N415D and N417S) made virus resistant to AP33 inhibition. The selection of AP33 neutralization resistance viruses was recently described by propagating $JFH1_{WT}$ (Dhillon *et al.*, 2010) and chimeric H77/JFH1 (Gal-Tanamy *et al.*, 2008) in the presence of inhibitory concentrations of antibody. For each virus, mutations that permitted AP33 resistance occurred within residues critical for AP33-E2 binding. In the experiment using $JFH1_{WT}$, mutation G418D was responsible for the AP33 escape. Interestingly, characterization of this $JFH1_{G418D}$ found that it had an identical phenotype to the $JFH1_{N415D}$, $JFH1_{T416A}$, $JFH1_{N417S}$ and $JFH1_{I422L}$ in terms of virus infectivity, receptor utilization and sensitivity to human anti-envelope antibodies. In the study using H77/JFH1, mutation N415Y permitted AP33 escape however; the phenotypic properties of this virus were much different to the above JFH1 E2 mutants. The ability of $H77/JFH1_{N415Y}$ virus to spread over multiple rounds of infection was markedly reduced. The lower specific infectivity of the $H77/JFH1_{N415Y}$ suggested that its defects occur at the viral entry part of the lifecycle. Indeed, antibody binding studies suggested that this escape mutation may inflict global conformational changes to E2. Given the phenotypes of AP33 resistant mutants, it is fair to speculate that if escape variants to such antibodies do occur *in vivo* then their presence would be short-lived due to either reduced viral fitness or enhanced

inactivation by other circulating nAbs. Therefore, the existence of such variants *in vivo* is likely to be short-lived, which explains the low occurrence of T414, D415, Y415, A416, S417, L422 and the complete absence of D418 from 1311 HCV patient sequences (Tables 5.2 and 5.3) analysed within the Los Alamos National Laboratory sequence database (Derek Gatherer, Personnal Communication).

5.1.3. Creation and Characterisation of JFH1 chimeras

5.1.3.1. Generation of Intra- and Intergenotypic JFH1 Chimeric Viruses

With the development of HCVcc in 2005 (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005), it became possible for the first time to analyse full lifecycle of HCV infection and to develop and test antiviral drugs in a more authentic system. However, as JFH1 only represents one of the seven HCV genotypes it became desirable to generate recombinant viruses to represent all the genotypes. Since then several HCV groups have been successful in generating constructs using JFH1 clone as the backbone. The fully viable chimeric clones consist of 5' and 3' NCR, including NS3-NS5B region of JFH1 and the C-NS2 region replaced by genomes of different genotypes. These recombinant constructs have been useful in delineating the details in HCV entry and further highlighted the role of structural and non-structural proteins in the assembly process of HCV infection. JFH1 replicates to high levels in cell culture without the requirement of adaptive mutations (Wakita *et al.*, 2005; Zhong *et al.*, 2005). Likewise the J6/JFH1 chimera (2a/2a) has also shown to replicate and produce infectious particle without the need of cell culture adaptation (Gottwein *et al.*, 2009; Lindenbach *et al.*, 2005). The J6/JFH1 clone was generated by replacing C-NS2 region of JFH1 with the corresponding sequence of J6. Subsequently, all the chimeric constructs shown to be viable in cell culture have been generated using the same methodology (Gottwein *et al.*, 2007; Gottwein *et al.*, 2009; Jensen *et al.*, 2008; Pietschmann *et al.*, 2006; Scheel *et al.*, 2008; Yi *et al.*, 2007). Interestingly, naturally occurring intra- and intergenotypic HCV clones have been identified with the same recombination cross over point located within the NS2 region (Kalinina *et al.*, 2002; Legrand-Abravanel *et al.*, 2007). In addition to this strategy some groups have also focused on studying the role of specific non structural proteins in terms of their importance in replication and as a potential target for developing HCV infection inhibitors. Imhof and Simmonds, 2010, generated a panel of chimeras using the Jc1 genome as the backbone and replacing the NS3 protease with the homologous NS4A cofactor from different genotypes (Imhof & Simmonds). These HCV recombinants were

generated with the aim of evaluating the effect of different PIs on various NS3/4A genotypes. Similarly, Scheel and colleagues generated a panel of NS5A chimeras representing all the HCV genotypes 1-7 by replacing the entire NS5A region in J6/JFH1 genome (Scheel *et al.*, 2011). These recombinants were used to examine the antiviral effects of an NS5A inhibitor and interferon-α. Furthermore, to study the role of HVR1 in neutralisation, HVR1 was mutated or deleted from Core-NS2/JFH1 chimeras representing genotypes 1-6 (Prentoe *et al.*, 2011). Nevertheless, in order to obtain high efficiency in cell culture most of the recombinant viruses in the studies described above required cell culture adaptation. The inability of the E1E2/JFH1 chimeras generated in this study to adapt in cell culture suggests that replacing the JFH1 envelope glycoproteins with those from other genotypes may confer total incompatibility for virus assembly.

5.1.3.2. Compensatory Mutations Enhance Chimeric Virus Production

The successful generation of an infectious 4a/JFH1 chimera in this study relied on the selection of six compensatory mutations. All of the functional Core-NS2/JFH1 intergenotypic chimeric viruses described to date required compensatory mutations located at different sites throughout the genome. Table 5.4 lists the Core-NS2/JFH1 chimeras representing all genotypes and the mutations identified for their efficient replication. As is notable from the table the compensatory mutations appear in both the structural and the non structural proteins. In particular, mutations that improve virus production tend to be clustered within the NS2 region (Gottwein *et al.*, 2009; Jensen *et al.*, 2008; Scheel *et al.*, 2008; Yi *et al.*, 2007). One of the cell culture adaptive mutations identified in 4a/JFH1 at T827A within the N- terminal of NS2 protein has also been reported by Scheel and colleagues in their genotype 4a intergenotypic chimeric construct (Scheel *et al.*, 2008). In their study they demonstrated that introducing this single mutation into the parental genome significantly improved the virus infectivity. In addition Yi and colleagues identified a mutation at M827V which also enhanced the infectivity of their H-(NS2)-J (1a/2a) chimeric virus (Yi *et al.*, 2007). The latter study also identified a cell culture adaptive mutation at residue T889A, which was never characterised, while in this study a T889I change has been described. The effects of individual compensatory mutations or the effect of mutations in different combinations on 4a/JFH1 replication and infectivity was not tested due to time constraints. It may be possible that the T827A change alone could be responsible for increase in virus released observed with Ad4a/JFH1. Located in the TMD of NS2 this substitution is believed to possibly facilitate intramembranous interaction between HCV proteins (Jirasko *et al.*, 2008). Altogether three compensatory mutations

emerged in the NS2 region and one in NS3 during long term passaging of the 4a/JFH1 genome. These changes may be necessary to compensate for any incompatibilities between the two genotypes and confer essential protein-protein interactions necessary for virus assembly and release. In line with this, several recent studies have highlighted the role of NS2 as the key component in promoting virus assembly. Defects in infectious virus production caused by substitution, point mutations or deletion in the NS2 region could be restored by second site mutations emerging within E2, P7 and NS3 proteins (Dentzer *et al.*, 2009; Jirasko *et al.*, 2008; Phan *et al.*, 2009; Yi *et al.*, 2009). The locations of these mutations imply direct interactions between these proteins are required for virus assembly. Indeed, more recent studies have shown that NS2 directly interacts with E1/E2, P7 and NS3 and recruits the complex to the assembly site in near vicinity of LDs (Jirasko *et al.*, 2010; Ma *et al.*, 2011; Popescu *et al.*, 2011; Stapleford & Lindenbach, 2011).

5.1.3.3. Characteristics of Ad4a/JFH1

Following introduction of cell culture adapted mutations within the 4a/JFH1 genome, the Ad4a-ED43/JFH1 virus revealed following phenotype:-

- Comparable infectivity to $JFH1_{WT}$
- Reduced affinity to the CD81 receptor
- Enhanced sensitivity to antibody-mediated neutralization
- Reduced cell-to-cell transfer

The Ad4a/JFH1 virus displayed a heightened sensitivity to neutralisation against a panel of anti-E2 antibodies. As discussed in chapter 3, this could possibly be due to an enhanced exposure of antibody binding epitope on the virus particle as a result of the mutation within the E2 glycoprotein. Based on sequence comparison (Appendix 7) and migration of E2 on a polyacrylamide gel (Figure 4.6C) the heightened sensitivity observed is unlikely due to any difference in the glycosylation, as all the potential N-linked glycosylation sites in E1 and E2 proteins are preserved in the genotype 4a sequence (Goffard & Dubuisson, 2003). The threonine to alanine substitution at residue 416 (T416A) in the JFH1 genome resulted in heightened sensitivity of JFH1 virus to nAbs, presumably by enhancing exposure of the virion glycoproteins (Section 5.1.2.3). Within the 4a/JFH1 E2 sequence threonine at residue 416 is substituted to serine (T416S). Although speculative, this change may also enhance the exposure of the virion glycoprotein.

Inhibition of virus infectivity by anti-CD81 antibody confirms previous results that CD81 is an essential co-receptor required for virus entry into cells. However the difference in neutralisation observed with anti-CD81 MAb and soluble CD81 molecule suggest that the interaction of Ad4a/JFH1 with CD81 may be reduced. Given the enhanced sensitivity of Ad4a/JFH1 virions to nAbs, it is unlikely that the lower affinity to CD81 is caused by a reduced exposure of CD81 binding epitope(s) on virus particle. In contrast, based on the AP33 mutants data discussed in section 5.1.2.3, if the glycoproteins on Ad4a/JFH1 virion surface were more exposed then one would expect the virus to be more sensitive to inhibition by hCD81-LEL. Therefore, the altered CD81 interaction could be related to the two amino acid differences in the CD81 binding residues between JFH1 and ED43 sequence at A440S and Y622F (Appendix 7). In support of this argument, Drummer and colleagues reported a >50 % reduction in hCD81-LEL binding between A440 and S440 mutant in the HCVpp cell lysate derived E1E2 of the H77c (1a) strain (Drummer *et al.*, 2006). Furthermore the reduced dependency to the CD81 receptor might explain the reduced spreading of Ad4a/JFH1 in cells following infection and the poor cell-to-cell transfer, as both infection routes are dependent on CD81 usage.

5.1.4. Finishing Statement

The two major aims of this work were as follows:

a) Utilize E2 cell culture adaptive mutations to better understand why residues within the AP33 epitope are highly conserved.
b) Generate functional intergenotypic HCVcc chimeras and examine their viral entry properties.

Despite the different subject areas, both projects identified viruses that were more sensitive to nAb inhibition of infection. This phenotype is particularly common among the cell culture adaptive viruses harbouring E2 mutations. Evidence from the literature and this work suggest that these mutations lessen the interplay between virus particles and host lipoproteins. It is attractive to assume that the latter feature is directly related to the enhanced glycoprotein exposure. However, evidence from this work argues that these two features may be purely coincidental and not directly linked. Utilizing the virus generated in this study to understand the mechanisms that are responsible for this intriguing phenotype will form the basis of our future work.

Table 5.1. Mutations identified in JFH1 or J6/JFH1 genome during persistent infection

	Cells	Virus Passage	C	E1	E2	P7	NS2	NS3	NS4B	NS5A	NS5B	Ref
JFH1	Huh7.5.1	D119 P.I	K74T		G451R			M1051T		C2219R		Zhong et al., 2006
		D160 P.I	V22A K74Q		V388P I414T L644I							
JFH1	Huh7.5	Exp 1 (cp8/sp8)	N16D	I372V I374T	**I422L**					V2153A V2440L	V2941M	Kaul et al., 2007
		Exp 2 (cp6/sp4)				N765D		I1316V		Y2103H		
JFH1	Huh7.5	D20 PT			**N417S**	N765D	Q1012R			L2175V		Russell et al.,2007
J6/JFH1	Huh7.5	P27			T396A **T416A** N534H A712V		Y852H W879R			F2281L	M2876L	Bungyoku et al., 2009
		P47	K78E		T396A T416A N534H A712V		Y852H W879R			F2281L	M2876L T2925A	
J6/JFH1	Lunet-CD81	sp18	K78E				W879R		V1761L			Pokrovskii et al., 2011

List of genetic mutations identified during long term passaging of JFH1 or J6/JFH1 virus. Red highlights the mutations characterised in this study.

P.I is post infection; P.T is Post Transfection; P is passage; D is day; cp is cell passage; sp is cell free passage

Table 5.2. Naturally occurring substitutions in 1311 HCV E2 protein sequences.

Position	JFH1 Residue	Variant[a]	No.[b]	Total[c]	Prop.[d]	Accession number[e]
414	I	T	3	864	0.003	EU155238 (1a), FJ205869 (1a), GQ356306 (3a)
415	N	D	1	37	0.03	EU482838 (1a)
415	N	Y	10	37	0.27	AF207762 (1b), AY615798 (1a), AY878651 (6k), DQ278893 (6k), EF560519-21 (1a), EU155275 (1a). FJ025856 (4b), GQ356574 (3a)
416	T	A	13	182	0.07	AY956468 (1a), AY958005 (3a), EU155215 (1a), EU155249 (1a), EU155282 (1a), EU155285 (1a), EU155288 (1a), EU155379 (1a), EU255930 (1a), EU255980 (1a), EU482845 (1a), EU482836 (1a), EU643835 (6).
417	N	S	17	27	0.63	EU256046 (1a), EU256031 (1a), EU255964 (1a), EU255952 (1a), EU255943 (1a), EU155354 (1a), EU155347 (1a), EU155297 (1a), EU155274 (1a), EU155215 (1a), EF407468 (1b), EF407466 (1a), EF407477 (1a), EU407415 (1a), EF026073 (2/5 natural recombinant), AY957988 (3a), AM408911 (2/5)
418	G	D	0	2	0.00	
422	I	L	5	38	0.13	AB047643 (2a), AF271632 (1a), FJ828970 (1a), FJ828971 (1a), M62321 (1a)

[a]Substitutions described in this paper. [b]Total number of sequences in a sample of 1311 HCV E2 proteins with same substitution. [c]Total number of substituted residues in the sample of 1311 (see Table 3). [d]Proportion of naturally occurring substitutions that are identical to the substitutions produced in this paper. [e]Accession number of HCV sequences (genotypes shown in parenthesis) carrying the relevant variant[a]

Table 5.3. Total number of naturally occurring substitutions in 1311 HCV E2 protein sequences.

Positions	**414**	**415**	**416**	**417**	**418**	**422**
JFH1 Residues	I	N	T	N	G	I
Variants[a]	V M T	K Y H S T R D	N S A I K R	S D H G T	S	L V T
Total[b]	864	37	182	27	2	38

[a]Substitutions occurring in the sample of 1311 E2 protein sequences.

[b]Total number of sequences with a substitution. Position 416 is the most polymorphic in terms of total number of substitutions at 14% (182 in 1311 sequences). However, position 415 has a greater diversity of variants (8 amino acids used at least once, despite only 37 substitutions).

Table 5.4. List of adaptive mutations identified in different studies for all the Core-NS2/JFH1 intergenotypic chimeras

	Virus	Viral Passage	C	E1	E2	P7	NS2	NS3	NS5A	Ref.
1A	H-NS2/NS3-J	sp3		Y361H				Q1251L		Yi *et al.*, 2007
	H-(NS2)-J	Exp 1 (sp3)				Y777C	I839T			
		Exp 2 (P.T)				Y777C	M827V (T889A) T945A			
1B	J4/JFH1	cp1/cp2		N576S			F886L	Q1496L		Gottwein *et al.*, 2009
3A	S52/JFH1	Exp 1 (cp1)	F130L		M405V	I793S Y794C		Q1502L	S2274P	Gottwein *et al.*, 2007; 2009
		Exp 1 (cp2/cp3)	F130L		M405V K523Q	I793S Y794C		Q1502L	S2274P	
		Exp 2 (cp1)				I793T		K1404Q		
4A	ED43/JFH1-β	sp2/sp3					T827A E989K		T1989I V2436L	Scheel *et al.*, 2008
	ED43/JFH1-γ	sp3		A216T T329S			T827A T977S		C2270R	
	gt4a-ED43/JFH1	sp2 (cp4/cp5)			**I690M**		**T827A T889I M956L**	**A1271V**	**Y2289H**	This study
5A	SA13/JFH1						A1022G	K1119R		Jensen *et al.*, 2008
6A	HK6a/JFH1	Exp 1		F350S	N417T					Gottwein *et al.*, 2009
		Exp 2		F350S	I414T T416A				E2249K	
7A	QC69/JFH1	Exp 1	V157F		I414T					Gottwein *et al.*, 2009
		Exp 2			I414T		L882P			
		Exp 3			N428S					
		Exp 4			R391G					

Amino acid positions are numbered according to the HCV sequence of the isolate used to generate the recombinant clone. Mutations found in the current study in gt4a-ED43/JFH1 are highlighted in bold. cp is cell passage and sp is cell free passage.

Appendices

Appendix A. Primers used for generating E1E2/JFH1 chimeric clones. FW, sense; RV, anti-sense. Yellow highlights the overhang sequence in each primer.

	Primer	*Sequence (5'-3')*
Frag 1		
	FW-JFH1	TGAGTGTCGTACAGCCTCCAGGCC
	RV-JFH1-UKN1B.12.6	CAGGATAGCAAAGCCAAGAGGAAGATAGA GAAGGGGAAACCGGGTAGGTTCCCTGTTG
	RV-JFH1-UKN2A2.5	CAAGACAGAACGGCCAGTAAGAAGATAGA AAAGGGGAAACCGGGTAGGTTCCCTGTTG
	RV-JFH1-UKN3A1.28	GGCAAGAGAACAGAGCAAGAAGGAAGATA GAAAAGGGGAAACCGGGTAGGTTCCCTGTTG
	RV-JFH1-UKN5.15.7	CACGAGAGAAGTGCAAGGATGAAGATAGA GAAGGGGAAACCGGGTAGGTTCCCTGTTG
	RV-JFH1-UKN6.5.340	CACGAGAGTAGTGCCAAAAGGAAGATAGA GAAGGGGAAACCGGGTAGGTTCCCTGTTG
Frag 2		
	FW-UKN1B12.6-JFH1	ACAGGGAACCTACCCGGTTTCCCCTTCTCT ATCTTCCTCTTGGCTTTGCTATCCTGT
	FW-UKN2A2.5-JFH1	ACAGGGAACCTACCCGGTTTCCCCTTTTCT ATCTTCTTACTGGCCCAAGTGTGAACCAT
	FW-UKN3A1.28-JFH1	ACAGGGAACCTACCCGGTTTCCCCTTTTCT ATCTTCCTTCTTGCTCTGTTCTCTTGCC
	FW-UKN5.15.7-JFH1	ACAGGGAACCTACCCGGTTTCCCCTTCT CTATCTTCATCCTTGCACTTCTCTCGTG
	FW-UKN6.5.340-JFH1	ACAGGGAACCTACCCGGTTTCCCCTTCT CTATCTTCCTTTTGGCACTACTCTCGTG
	RV-UKN1B12.6-JFH1	CAAGACGACCAACTTCTCCAATGCTGCCT CAGCCTGGGCTACCAGCAGCATCATC
	RV-UKN2A2.5-JFH1	CAAGACGACCAACTTCTCCAATGCTGCTT CGGCTTGGCCCAATATGATGAGCATC
	RV-UKN3A1.28-JFH1	CAAGACGACCAACTTCTCCAATGCTGCTTC CGCCTGTGAAATCAGCAGCATCAG
	RV-UKN5.15.7-JFH1	CAAGACGACCAACTTCTCCAATGCTGCTTC AGCCTGGCAAACTAGGAGCATGATC
	RV-UKN6.5.340-JFH1	CAAGACGACCAACTTCTCCAATGCTGCTTC AACGTTGGTTATGAGCAGCATTAACCAG
Frag 3		

Fusion PCR Primers	FW-JFH1-UKN1B12.6	CTGCTGGTAGCCCAGGCTGAGGCAGCATTGGAGAAGTTGGTCGTCTTGCAC
	FW-JFH1-UKN2A2.5	GCTCATCATATTGGGCCAAGCCGAAGCAGCATTGGAGAAGTTGGTCGTCTTGCAC
	FW-JFH1-UKN3A1.28	CTGATGCTGATGATTTCACAGGCGGAAGCAGCATTGGAGAAGTTGGTCGTCTTGCAC
	FW-JFH1-UKN5.15.7	CATGCTCCTAGTTTGCCAGGCTGAAGCAGCATTGGAGAAGTTGGTCGTCTTGCAC
	FW-JFH1-UKN6.5.340	GGTTAATGCTGCTCATAACCAACGTTGAAGCAGCATTGGAGAAGTTGGTCGTCTTGCAC
	RV-JFH1	GAATATAGTGACGGCCCACGCGATGC
	FW-JFH1	TGAGTGTCGTACAGCCTCCAGGCC
	RV-JFH1	GAATATAGTGACGGCCCACGCGATGC

Appendix B. Sequencing Primers for E1E2/JFH1 Chimeric Product.

Primer	*Sequence (5'-3')*	*Description*
All UKN	CATCCCCGTCGTAGGCGCCC	Forward primer for JFH1 core
1B12.6 (143)	GTATGAGGCAGCGGACGTGATCAT	Forward primer for 1B12.6 E1
1B12.6 (684)	CAAACCACCTATGGGCTTACATCCCTCTTTAG	Forward primer for 1B12.6 E2
1B12.6 (1159)	GTACATGGATGAATGGCACCGGGTTTAC	Forward primer for 1B12.6 E2
1B12.6 (1655)	GTATATCGTGCTGCTCTTCCTCCTCCTG	Forward primer for 1B12.6 E2
2A2.5 (350)	TGTTCCATCTACGCGGGCCA	Forward primer for 2A2.5 E1
2A2.5 (851)	GGCGTTTTGACACATGAAGAGAACGT	Forward primer for 2A2.5 E2
2A2.5 (1351)	ACGTGGGAGGAGTCGAGCATAGGCT	Forward primer for 2A2.5 E2
3A1.28 (167)	GCACACACCTGGCTGTATACCTTGTG	Forward primer for 3A1.28 E1
3A1.28 (649)	ATACACCACCGGTGGCCAAGCAGCT	Forward primer for 3A1.28 E2
3A1.28 (1204)	ACGGGGTTTACCAAGACGTGCG	Forward primer for 3A1.28 E2
5.15.7 (170)	GGTTGTGTGCCTTGTGTTAGGACAGG	Forward primer for 5.15.7 E1
5.15.7 (649)	AACCCACTCAGTGGGCGGTGTTGTC	Forward primer for 5.15.7 E2
5.15.7 (1176)	GGTTTGTGAAGACTTGCGGAGCC	Forward primer for 5.15.7 E2
6.5.340 (174)	GCTTGCCTTGTGTGAGGGTCGATAA	Forward primer for 6.5.340 E1
6.5.340 (677)	GCTACTGCCGGCTTCGTTGGG	Forward primer for 6.5.340 E2
6.5.340 (1172)	ACGGGCTTTACCAAGACCTGTGG	Forward primer for 6.5.340 E2

Appendix C. Primer list for Site Directed Mutagenesis (SDM). FW, sense; RV, anti-sense.

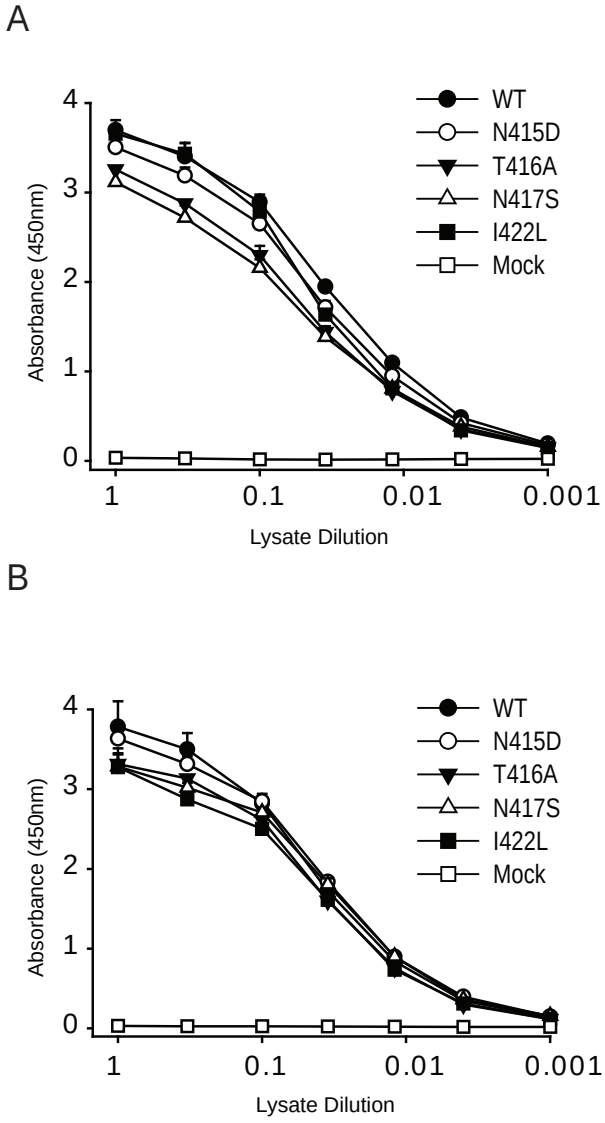
Primer	*Sequence (5'-3')*	*Description*
N415D-FW	ATTCAGCTCATT**G**ACACCAACGG CAGTTGG	**Forward primer for SDM N415D in JFH1 plasmid**
N415D-RV	CCAACTGCCGTTGGTGT**C**AATGA GCTGAAT	**Reverse primer for SDM N415D in JFH1 plasmid**
T416A-FW	ATTCAGCTCATTAAC**G**CCAACGG CAGTTGG	**Forward primer for SDM T416A in JFH1 plasmid**
T416A-RV	CCAACTGCCGTTGG**C**GTTAATGA GCTGAAT	**Reverse primer for SDM T416A in JFH1 plasmid**
N417S-FW	CATTCAGCTCATTAACACCA**G**CG GCAGTTGG	**Forward primer for SDM N417S in JFH1 plasmid**
N417S-RV	CCAACTGCCG**C**TGGTGTTAATGA GCTGAATG	**Reverse primer for SDM N417S in JFH1 plasmid**
I422L-FW	GGCAGTTGGCAC**C**TCAACCGTAC TGCC	**Forward primer for SDM I422L in JFH1 plasmid**
I422L-RV	GGCAGTACGGTTGA**G**GTGCCAAC TGCC	**Reverse primer for SDM I422L in JFH1 plasmid**
I690M-FW	TCCACCGGCTTGAT**T**CACCTCCA CCAAAATATCG	**Forward primer for SDM I690M in 4a/JFH1 plasmid**
I690M-RV	CGATATTTTGGTGGAGGTG**A**ATC AAGCCGGTGGA	**Reverse primer for SDM I690M in 4a/JFH1 plasmid**
T827A-FW	ATCGTTGTCATGCTG**G**CCATTCT GACACTGTCTCCGC	**Forward primer for SDM T827A in 4a/JFH1 plasmid**
T827A-RV	GCGGAGACAGTGTCAGAATGG**C**C AGCATGACAACGAT	**Reverse primer for SDM T827A in 4a/JFH1 plasmid**
T889I-FW	CCACACCTAGTATTTGACATCA**T**AAAAT ATCTTCTGGCCATCTTAGGG	**Forward primer for SDM T889I in 4a/JFH1 plasmid**
T889I-RV	CCCTAAGATGGCCAGAAGATATTTT**A**TG ATGTCAAATACTAGGTGTGG	**Reverse primer for SDM T889I in 4a/JFH1 plasmid**
M956L-FW	GACCACCTTACTCCC**C**TGTCAGA TTGGGCCGCTACGG	**Forward primer for SDM M956L in 4a/JFH1 plasmid**
M956L-RV	CCGTAGCGGCCCAATCTGACA**G**G GGAGTAAGGTGGTC	**Reverse primer for SDM M956L in 4a/JFH1 plasmid**
A1271V-FW	GGGGCGTACCTATCCAAGG**T**ACA TGGCATCAATCCCAACATTAGG	**Forward primer for SDM A1271V in 4a/JFH1 plasmid**
A1271V-RV	CCTAATGTTGGGATTGATGCCAT GT**A**CCTTGGATAG GTACGCCCC	**Reverse primer for SDM A1271V in 4a/JFH1 plasmid**
Y2289H-FW	ACGGCCTGAC**C**ACAACCCGCCGC TCGTG	**Forward primer for SDM Y2289H in 4a/JFH1 plasmid**
Y2289H-RV	CACGAGCGGCGGGTTGT**G**GTCAG GCCGT	**Reverse primer for SDM Y2289H in 4a/JFH1 plasmid**

Appendix D. Primers used for cDNA synthesis, amplification and Sequencing of JFH1 and 4a/JFH1 plasmid. FW, sense; RV, anti-sense.

Primer	*Sequence (5'-3')*	*Description*
JFH1 NegRT	TTGCGAGTGCCCCGGGA	Used to reverse transcribe negative strand HCV JFH1 AND ED43 core to NS5A sequence
SD1	TGTGGTACTGCCTGATAGGG	Used to reverse transcribe negative strand HCV ED43 core sequence
NA41	GGAGGCAGTTTTCACCG	Used to reverse transcribe negative strand HCV JFH1 NS4A to 3' NTR sequence
SD1-FW	TGTGGTACTGCCTGATAGGG	Used to sequence HCV ED43 core gene
SD2-FW	ATCCATGGCCTCTTTACGG	Used to sequence HCV ED43 core gene
SD3-RV	CCGTAAAGAGGCCATGGAT	Used to sequence HCV ED43 core gene
SD5-FW	GGCGGCTTGTTCCTAGTTGG	Used to sequence HCV ED43 E1 gene
SD7-FW	ATGACAGGCCTTATTGCTG	Used to sequence HCV ED43 E2 gene
SD8-FW	CCACCGATTGCTTCAGGAAG	Used to sequence HCV ED43 E2 gene
SD9-FW	TTTACCTGCCCTCTCCACCG	Used to sequence HCV ED43 E2 gene
SD10-FW	TTGTCTGGCATGTCAAGGGC	Used to sequence HCV ED43 P7 gene
SD11-RV	TGATGTCAAATACTAGGTGTG	Used to sequence HCV ED43 NS2 gene
SD13-FW	GACGTTAAGTTCCCGGGTGG	Used to amplify HCV ED43 core gene
SD14-RV	TGGATCAAGCCGGTGGAG	Used to amplify HCV ED43 E2 gene
SD15-FW	TGTGGATGAACAGTACCGGG	Used to amplify HCV ED43 E2 gene
SD16-RV	CCTGTTCTGTCCTGTCACGC	Used to amplify HCV ED43 NS2 gene
SD17-FW	AGGGGTGGAGACTCCTTGCC	Used to amplify HCV JFH1 NS3 gene
SD18-RV	ATCCAGTCGCCAGGCAATAT	Used to amplify HCV JFH1 NS4A gene
NA6-RV	GCAGAGAGACCAGTTACGGC	Used to amplify HCV JFH1 NS5A and 3'NTR gene
NA11-FW	GTCTCGTAGACCGTGC	Used to amplify and sequence HCV JFH1 core gene
NA12-RV	GCCAGTGGAGCGCCGATCTTTG	Used to sequence HCV JFH1 core gene

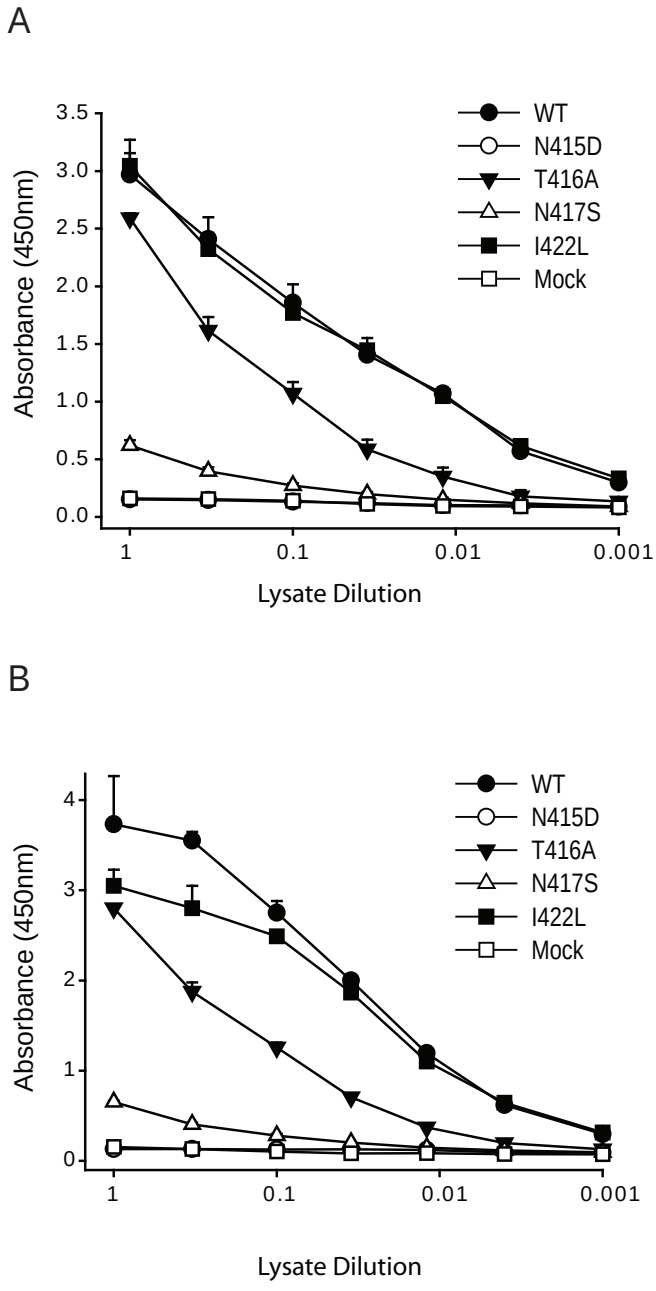
NA13-FW	CAAAGATCGGCGCTCCACTGGC	Used to sequence HCV JFH1 core gene
NA15-FW	CCTGTTGTCCTGCATCACCG	Used to sequence HCV JFH1 E1 gene
NA17-FW	CCAGGTGTTCATCGTCTCGC	Used to sequence HCV JFH1 E1 gene
NA19-FW	GTCATTGTCATCCTTCTGCTGGC	Used to sequence HCV JFH1 E2 gene
NA21-FW	GGATATGAGGCCGTACTGC	Used to sequence HCV JFH1 E2 gene
NA24-RV	CGTGGTAGAGTGCAACAGAG	Used to sequence HCV JFH1 E2 gene
NA25-FW	CTCTGTTGCACTCTACCACG	Used to sequence HCV JFH1 P7 gene
NA29-FW	CTTCTGCCTACTGCTCATGG	Used to amplify and sequence HCV JFH1 NS2 gene
NA30-RV	GCAACGCCAAAAGCCAT	Used to amplify HCV JFH1 NS2 gene
NA31-FW	ATGGCTTTTGGCGTTGC	Used to amplify HCV JFH1 NS2 gene
NA32-RV	GAGGCCTCGTGTTTGCTG	Used to sequence HCV JFH1 NS2 gene
NA33-FW	CAGCTGATGGCTACACCTC	Used to sequence HCV JFH1 NS3 gene
NA34-RV	CGTTCCGCGTGACCAGATATAG	Used to sequence HCV ED43 NS2 gene
NA35-FW	CTATATCTGGTCACGCGGAACG	Used to sequence HCV JFH1 NS3 gene
NA37-FW	GCCATCACGTACTCCACATATG	Used to amplify and sequence HCV JFH1 NS3 gene
NA39-FW	CGTGATCGACTGCAATGTAGC	Used to sequence HCV JFH1 NS3 gene
NA40-RV	CGGTGAAAACTGCCTCC	Used to amplify and sequence HCV JFH1 NS3 gene
NA41-FW	GGAGGCAGTTTTCACCG	Used to sequence HCV JFH1 NS4A and NS4B gene
NA43-FW	ACGTCAACCAGCGAGTCG	Used to amplify and sequence HCV JFH1 NS4B gene
NA44-RV	GGACTTCAACATCTCGGCTATC	Used to amplify HCV JFH1 NS3 and NS4A gene
NA47-RV	GGAAGCAAAGGCAATAAGC	Used to sequence HCV JFH1 NS4B gene
NA48-FW	GCTTATTGCCTTTGCTTCC	Used to sequence HCV JFH1 NS4B gene
NA49-RV	CTGTCAAGATGGTGCAAAC	Used to amplify HCV JFH1 NS4B gene
NA51-RV	CAGATGGCGGTCTTGTAGTTCG	Used to sequence HCV JFH1 NS5A gene
NA52-FW	CGAACTACAAGACCGCCATCTG	Used to sequence HCV JFH1 NS5A gene
NA55-RV	GAGAGCACAACCAGCAACG	Used to sequence HCV JFH1 NS5A gene
NA56-FW	CGTTGCTGGTTGTGCTCTC	Used to amplify and sequence HCV JFH1 NS5B gene

NA57-RV	CGGAGCAAGTAGACCAAGACC	Used to sequence HCV JFH1 NS5A gene
NA58-FW	GGTCTTGGTCTACTTGCTCCG	Used to sequence HCV JFH1 NS5B gene
NA59-RV	GCCGCTAGCTTGATGTCCT	Used to amplify HCV JFH1 NS5A gene
NA62-FW	GAAACCAGCTCGCCTCATCG	Used to sequence HCV JFH1 NS5B gene
NA66-FW	CTCAAATGTGTCTGTGGC	Used to sequence HCV JFH1 NS5B gene
NA68-FW	GCACACATACTCTCACCACG	Used to sequence HCV JFH1 NS5B gene



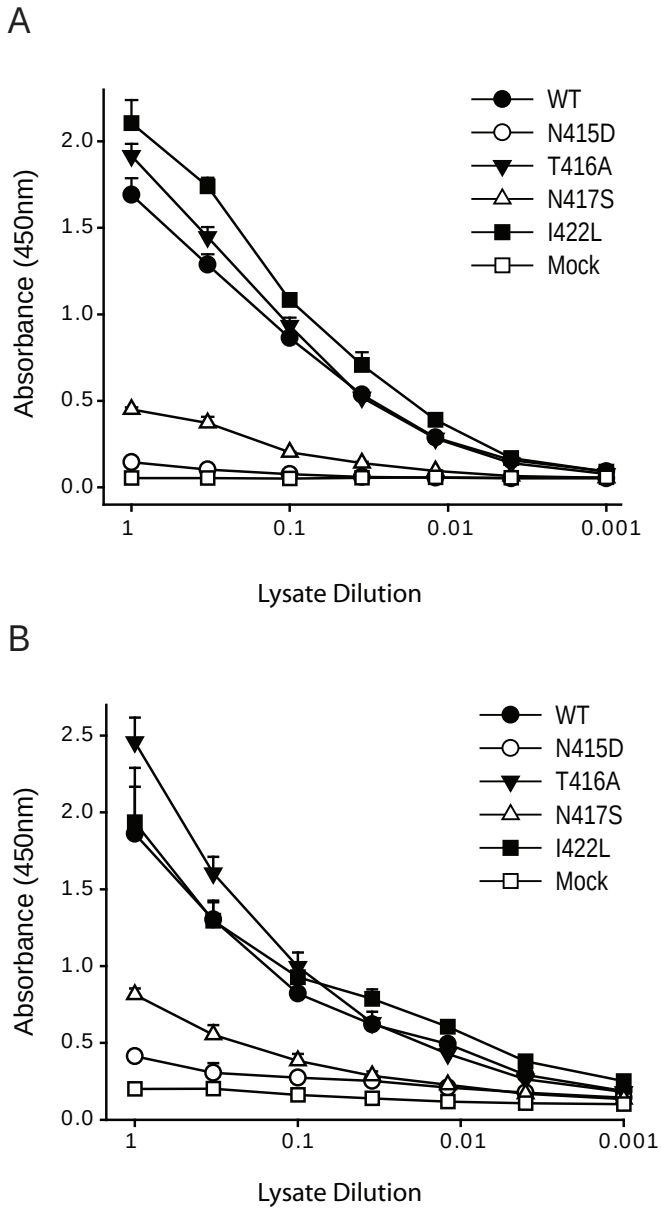
Appendix 1. Reactivity's of WT and Mutants Glycoproteins to Linear and Conformation Sensitive Anti-E2 Antibodies

Cell lysates harvested at 72 h post-electroporation with appropriate viral RNAs were captured onto GNA-coated plates and the bound E2 was detected using either (A) the conformation-sensitive human mAb CBH-4B or (B) the anti-E2 mouse mAb D3.7 that recognizes a linear epitope in E2. Error bars represent variability of the assays.



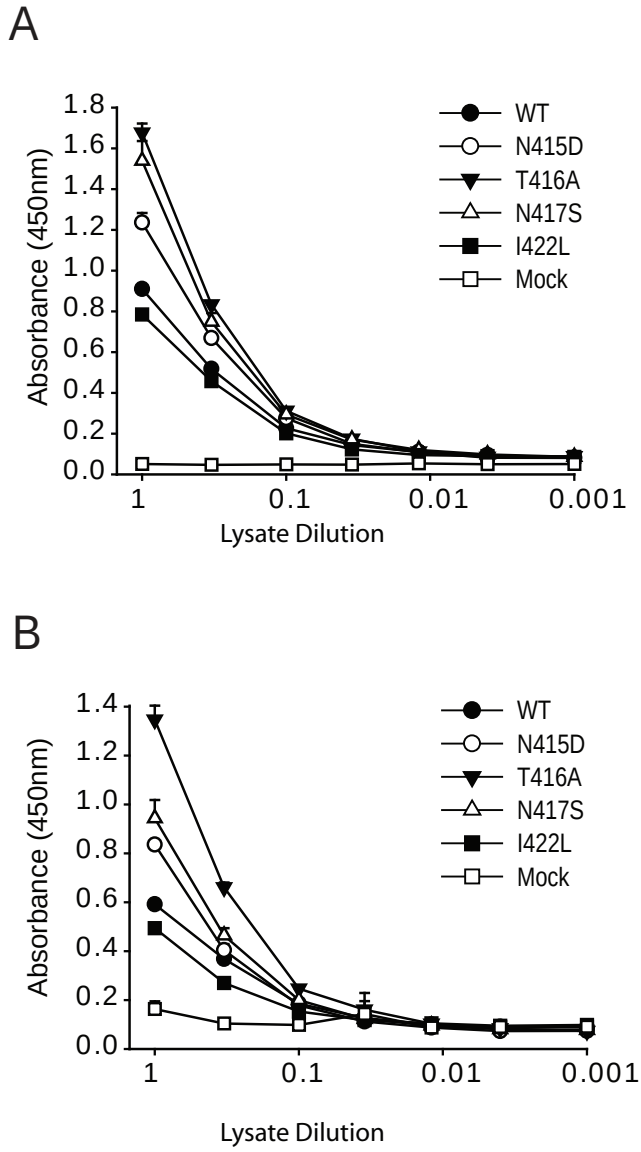
Appendix 2. Reactivity's of WT and Mutants Glycoproteins to Mouse mAb AP33

Cell lysates harvested at 72 h post-electroporation with appropriate viral RNAs were captured onto GNA-coated plates. The bound E2 was detected and the lysate was normalised using CBH-4B. (A and B) Dose curves of lysates normalized for E2 (based on their reactivity to CBH-4B) were then tested in two independent experiments for their ability to bind to mouse mAb AP33 by ELISA. Error bars represent variability of the assays.



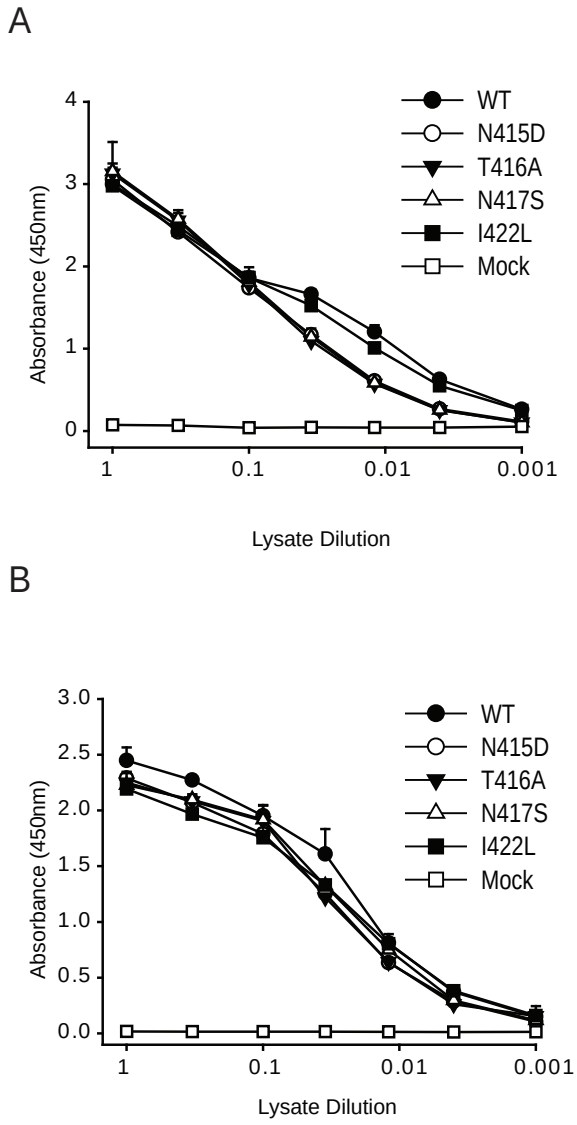
Appendix 3. Reactivity's of WT and Mutants Glycoproteins to Rat mAb 3/11

Cell lysates harvested at 72 h post-electroporation with appropriate viral RNAs were captured onto GNA-coated plates. The bound E2 was detected and the lysate was normalised using CBH-4B. (A and B) Dose curves of lysates normalized for E2 (based on their reactivity to CBH-4B) were then tested in two independent experiments for their ability to bind to rat mAb 3/11 by ELISA. Error bars represent variability of the assays.



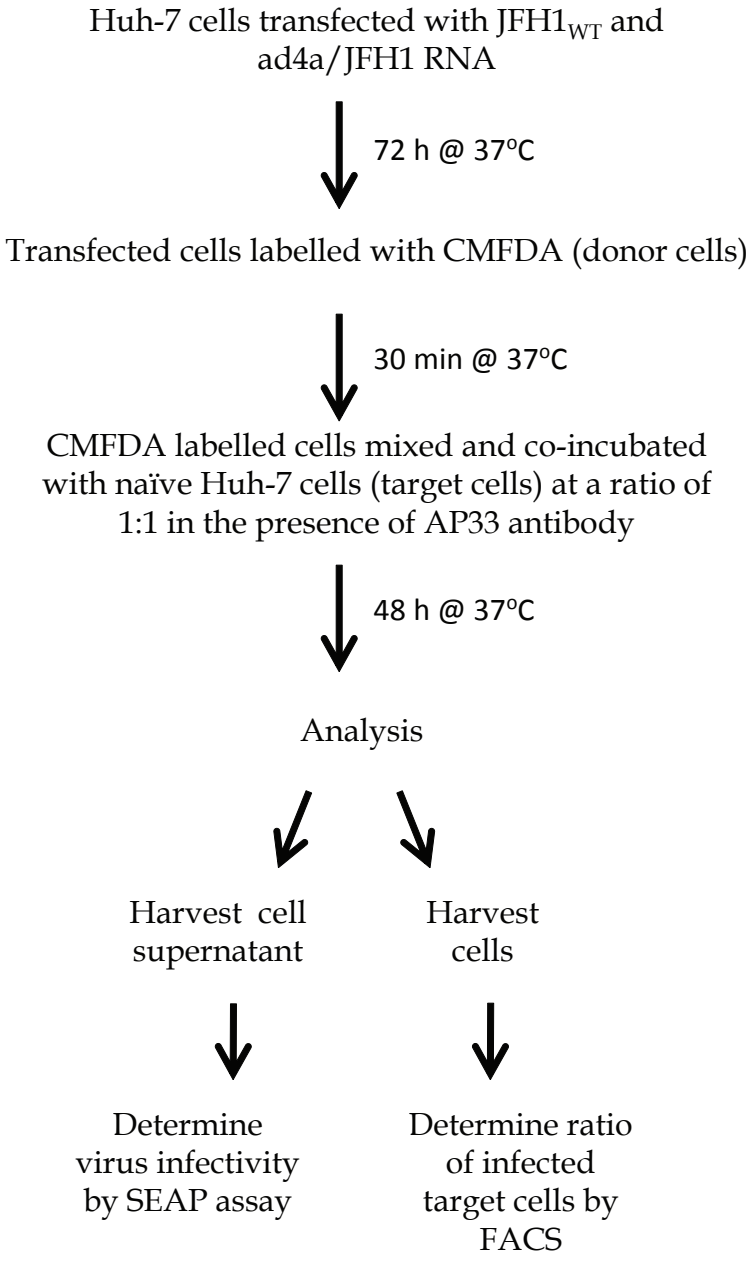
Appendix 4. Reactivity's of WT and Mutants Glycoproteins to hCD81-LEL

Cell lysates harvested at 72 h post-electroporation with appropriate viral RNAs were captured onto GNA-coated plates. The bound E2 was detected and the lysate was normalised using CBH-4B. (A and B) Dose curves of lysates normalized for E2 (based on their reactivity to CBH-4B) were then tested in two independent experiments for their ability to bind to hCD81-LEL by ELISA. Error bars represent variability of the assays.



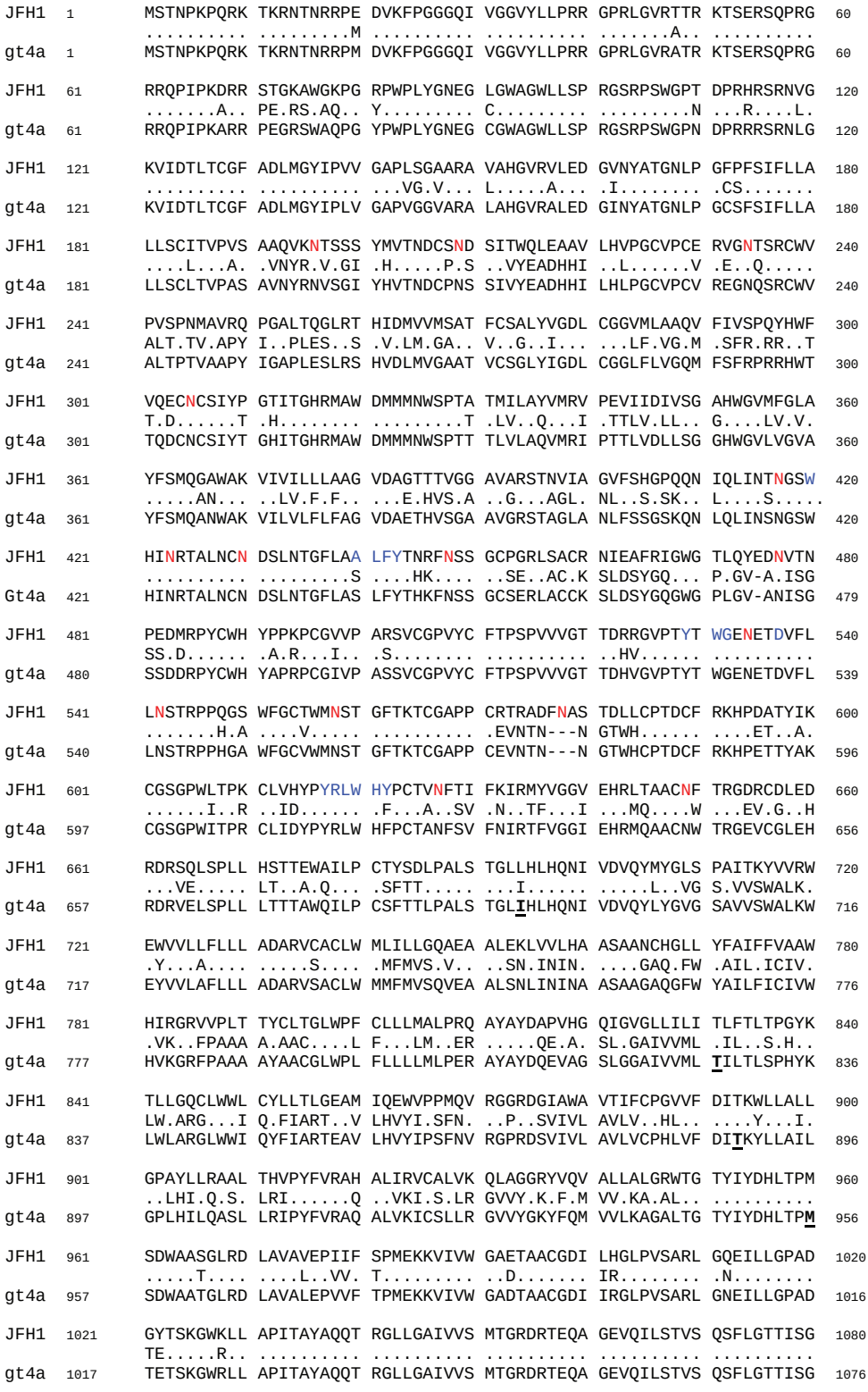
Appendix 5. HCVpp Lysate Reactivity in ELISA

The intracellular glycoproteins within cell lysates harvested from HEK-293T at 48 h post-transfection were captured onto GNA-coated plates and the bound E2 was detected using either (A) CBH-4B or (B) D3.7. Error bars represent variability of the assays.



Appendix 6. The Infectious Co-Culture Assay.

Flow chart outlining the method adapted from Brimacombe *et al*, (2010).



Appendix 7. Amino Acid Alignment Comparing the Core to NS2 Regions of genotype 4a ED43 sequence to JFH1

The core to NS2 region of genotype (gt) 4a (bottom row) was aligned to the JFH1 sequence (top row). Dots represent amino acids that are identical in both sequences (middle row). Blue highlights the critical CD81 binding residues in E2 (Owsianka *et al.*, 2006; Drummer *et al.*, 2003; Yagnik *et al.*, 2000). Red highlights the glycosylation sites in E1E2 (Goffard and Dubuisson, 2003). The cell culture adaptive mutations identified in this study are indicated in bold and underlined.

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