Interaction of NS3 with Viral and Host Proteins and RNA During Hepatitis C Virus Replication

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Declaration of Originality

I, Nadia E. M. Sifennasr, hereby declare that the work in this thesis was carried out by myself unless otherwise stated.

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Abstract

Hepatitis C virus (HCV) is a major cause of viral hepatitis. The World Health Organization (WHO) estimates the global prevalence of HCV to be approximately 2.2–3.0% with 130–170 million infected individuals worldwide. HCV has the ability to evade the immune system and to establish persistent infections, which often result in chronic liver disease. HCV encodes a long polyprotein which contain structural and non-structural proteins. The Non-structural protein NS3 is likely to be involved in viral RNA replication by interacting with viral and host components. In this study, a purified recombinant full-length NS3 protein expressed in E. coli was examined for its ability to interact with biotinylated full length HCV JFH-1 genomic RNA and the 3’ terminal of HCV negative strand RNA. The results showed that NS3 could bind to both full length and the 3’ end negative strand RNAs. RNA interference (siRNA) studies were also carried out to investigate the role of host genes RAB40B, RAB27B, TXNIP and Staufen 1, which had previously been shown to be important for HCV replication, on NS3 and NS3/4A proteins expressed by adenovirus vectors using quantitative real-time polymerase chain reaction (qRT-PCR) and FACS analysis. While there were reductions in the levels of the NS3 and NS3/4A transcripts when the expression of these host genes was knocked down by siRNAs, the NS3 and NS3/4A proteins appeared to be more stable when RAB40B, RAB27B, but not TXNIP or Staufen 1, were knocked. One possibility is that although silencing of these host genes may reduce HCV replication, this may also increase in the stability of NS3 or NS3/4A proteins by altering their location within the cell due to the absence of these host proteins. Previously it has been shown that NS3 and NS3/4A protein expression decreased the level of expression of several proteins, which were part of the innate immune response, leading to interferon production. In this study, it was found that the expression of NS3 and NS3/4A proteins, using adenovirus vectors, restored RIG-I, MDA5 and MAVS expression levels in response to dsRNA stimulation, indicating that the NS3 protein may have a role in regulating the expression of these host genes.
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Abbreviations

Aa  Amino acid
apoB  Apolipoprotein B
apoE  Apolipoprotein E
ARFP/F  Alternative reading frame protein / frameshift protein
ANOVA  Analysis of variance
ATP  Adenosine tri phosphate
Bp  Base pair
BSA  Bovine serum albumin
BVDV  Bovine viral diarrhoea
CLDN1  Claudin 1
CpG DNA  Deoxyctydylate-phosphate-deoxyguanylate (CpG)-DNA
CT  Cycle threshold
DAPI  4,6-diamidino-2-phenylindole
DC-SIGN  Dendritic cell-specific intracellular adhesion molecule-3-gRABbing integrin
DEPC  Diethyl pyrocarbonate
DMEM  Dulbecco’s modified Eagle medium
DMSO  Dimethyl sulphoxide
DTT  Dithiothreitol
DNA  Deoxyribonucleic acid
ER  Endoplasmic reticulum
ESCRT  Endosomal sorting complex required for transport
FACS  Fluorescent activated cell sorting
FCS  Fetal calf serum
HIV  Human immunodeficiency virus
HVR  Hypervariable region
GAGs  Glycosaminoglycans
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
G418  Geneticin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl D thiogalactopyranoside</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>JFH-1</td>
<td>Japanese fulminant hepatitis-1</td>
</tr>
<tr>
<td>Huh cells</td>
<td>Human hepatoma cells</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo bases</td>
</tr>
<tr>
<td>LB</td>
<td>Luria bertani</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilo Daltons</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MAVS</td>
<td>Mitochondrial antiviral signaling protein</td>
</tr>
<tr>
<td>MDA5</td>
<td>Melanoma differentiation-associated gene-5</td>
</tr>
<tr>
<td>M-MLV RT</td>
<td>Moloney murine leukaemia virus reverse transcriptase</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propane sulphonic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTTP</td>
<td>Microsomal triglyceride transfer protein</td>
</tr>
<tr>
<td>MW</td>
<td>Membranous web</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Sodiumhydrogenphosphate</td>
</tr>
<tr>
<td>Ng</td>
<td>Nanograms</td>
</tr>
<tr>
<td>NS</td>
<td>Non-structural</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Pfu</td>
<td>Plaque forming units</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Poly (I:C)</td>
<td>Polyriboinosinic:polyribocytidylic acid</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic-acid-inducible gene I</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble NSF attachment protein receptor</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>STAU1</td>
<td>Staufen1</td>
</tr>
<tr>
<td>TEMED</td>
<td>N N N' N' Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TLR3</td>
<td>Toll-like receptor 3</td>
</tr>
<tr>
<td>TXNIP</td>
<td>Thioredoxin interacting protein</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>V/V</td>
<td>Volume to volume</td>
</tr>
<tr>
<td>VAP -A</td>
<td>Vesicle-associated membrane protein-associated protein A</td>
</tr>
<tr>
<td>VAP -B</td>
<td>Vesicle-associated membrane protein-associated protein B</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>WNV</td>
<td>West Nile virus</td>
</tr>
<tr>
<td>YFV</td>
<td>Yellow Fever virus</td>
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INTRODUCTION
1. Introduction

1.1 Background

Hepatitis C virus (HCV) is a small, enveloped positive-sense single-strand RNA virus belonging to the Flaviviridae family. It is a blood-borne pathogen affecting the liver, and causes chronic hepatitis, liver cirrhosis, and HCV hepatocellular carcinoma (HCC) [1, 2]. Presently, the treatment of HCV infection is a combination of peglyated interferon (peg-IFN-α) and the nucleoside ribavirin. This is effective in approximately 40% of patients affected by the HCV genotype 1, as well as 80% of patients with the HCV genotype 2 or 3. Currently, there is no protective vaccine available for HCV [3-5]. The existence of the virus was first indicated in the mid-1970s, where a new agent was implicated in causing post-transfusion hepatitis, which was acknowledged as being different from the Hepatitis A or Hepatitis B virus, or indeed any other viral infection. All attempts to isolate the Non-A, Non-B (NANBH) hepatitis agent or visualise it by electron microscope failed, and the agent remained elusive for over a decade [6]. In 1989, a group of investigators successfully cloned a cDNA sequence encoding an antigenic component of the Non-A, Non-B agent from the RNA molecules of the infectious agent. This cDNA clone was used to identify the rest of the approximate 9,500 nucleotides, of a positive-stranded RNA virus genome, which showed similarities with Togaviridae and Flaviviridae. Following this discovery, the Non-A, Non-B agent was renamed as the Hepatitis C virus and accordingly allocated to the Flavivirus family on the basis of its genome organisation [7]. The origin of HCV is unknown owing to the lack of stored blood samples dating back more than 50 years; however, genotypes 1 and 4 appear to share a common origin. Additionally, a Bayesian analysis suggests that the major genotypes diverged approximately 300–400 years ago from the ancestor virus, whereas the minor genotypes diverged roughly 200 years ago from their major genotypes. However, all of the extant genotypes appear to have evolved from genotype 1 subtype 1b [8, 9].
1.2 Classification and genetic variation

There are three genera classified as being members of the Flaviviridae: Flavivirus, Pestivirus and Hepacivirus. The Flavivirus genus includes yellow fever virus (YFV), dengue fever virus (DFV), and tick borne encephalitis virus; pestiviruses includes bovine viral diarrhoea, classical swine fever, and border disease virus. HCV has been classified as a member of the hepacivirus genus, which also includes the GB virus B (GBV-B) and GB virus C [10, 11]. HCV can be classified into 7 genotypes, each of which is further classified into subtypes (a, b, c, etc.). Furthermore, it has also been shown that HCV circulates in infected individuals as a population of diverse but closely related types known as “quasispecies” [12, 13]. Based on a phylogenetic relationship between the helicase and RNA-dependent RNA polymerase (RdRP) trees of the family Flaviviridae, it was found that the helicase region within NS3 share amino acid sequence identity in six domains, whereas the RdRp protein contains eight conserved motifs. Alignments were performed by hand, based on Koonin et al., alignments [14, 15] that were used as a guide. Helicase sequences of members of the family Flaviviridae fall within the helicase supergroup II, and their RdRp sequences place them into the RdRp supergroup II. Further analysis of the six conserved helicase domains and eight conserved RdRp motifs with their dominant sequences of HCV, GBV-A, GBV-B, GBV-C and GBV-D confirmed that these viruses were related to the Pestivirus and Flavivirus genera within the family Flaviviridae. Therefore, it is proposed to classify GBV-A-like viruses, GBV-C and GBV-D as members of a fourth genus in the family Flaviviridae, named Pegivirus (pe, persistent; g, GB or G); it is also proposed to rename ‘GB’ viruses within the tentative genus Pegivirus to reflect their host origin. (Figure 1.1) [16, 17].

1.3 Epidemiology

The World Health Organization (WHO) estimates that up to 160 million individuals around the world are chronically infected with HCV. In addition, prospective studies have shown that more than 75% of HCV-infected individuals become chronic carriers,
Introduction

Figure 1.1. Phylogenetic tree deduced from the analysis of amino acid sequences of *Flavivirus* genomes. The *Flavivirus* family are more closely related to HCV and include tick-borne encephalitis virus (TBEV); yellow fever virus (YFV); West Nile virus (WNV); dengue virus (DEN), than the pestiviruses: bovine viral diarrhoea virus (BVDV). The *Hepacivirus* include, HCV (genotypes 1a, 1b, 2a, 2b, and 3a) and the most closely related viruses to HCV, GBV-A, GBV-B, GBV-C and HGV [16, 17].

and within 20–30 years, 10–20% of these patients will develop complications, including liver cirrhosis, with 1–2% developing hepatocellular carcinoma (HCC) [18]. The prevalence of HCV around the world varies (Figure 1.2). In Europe the prevalence is approximately 1%, whilst in the United Kingdom at least 200,000 adults carry HCV [19]. The highest prevalence has been recorded on the African continent. In Egypt, it was estimated that 15–20% of population are infected with HCV which has been attributed to the immunisation programme against the parasitic infection Schistosomiasis [20]. Blood products were found to be the main route of transmission until 1992, since then improved screening tests for HCV have greatly reduced this. Risks of transmission also include intravenous drug use, high risk sexual activity, haemodialysis and occupational exposure [19].
1.4 The disease

HCV mainly infects hepatocytes leading to immune-mediated inflammation. The acute phase of hepatitis C is usually asymptomatic, however, patients may experience symptoms such as jaundice, nausea, malaise, dark urine and liver pain [22]. Patients with hepatitis C can be diagnosed 8–12 weeks after initial infection by a rise in alanine aminotransferase (ALT) levels. At that time, HCV specific antibodies and T cells become detectable, and the appearance of HCV-specific T cells in the liver coincides with the first decrease in HCV titre [23, 24]. About 60%–80% of patients will develop chronic hepatitis, which is associated with an increase of membranoproliferative glomerulonephritis (7-fold increase), cryoglobulinemia (11-fold increase), skin disease such as lichen planus (2-fold increase) and porphyria cutanea tarda. Approximately 15–25% of patients will recover spontaneously, whereas the majority will develop to chronic hepatitis. Once the infection has become chronic, patients are less likely to have symptoms, but fatigue, nausea, dark urine can occur [25, 26].
The most common serious complication resulting from a chronic HCV infection is the development of cirrhosis. In cirrhosis, the normal liver tissue is replaced by scar tissue, resulting in liver dysfunction. Approximately 10–15% of patients with chronic hepatitis C will ultimately develop cirrhosis; which may take decades to develop [18]. Further progression of the infection can lead to end-stage liver disease and hepatocellular carcinoma, conditions that require liver transplantation [22]. The outcome of the disease is determined by the efficiency of the host’s antiviral immune response, and the presence of viral escape mutants has been suggested to contribute to the persistence of the virus. Several other features can contribute to severity of the disease e.g. male sex, older age at infection, alcohol consumption, human immunodeficiency virus (HIV) coinfection and hepatitis B coinfection seem to accelerate the progression of the disease [27-29].

1.5 Molecular biology of Hepatitis C virus

Most of the Flavivirus family members share a number of basic characteristics; all possess positive-strand RNA with a nucleotide size ranging from between 9.6 k and 12.3 k nucleotides (nt). Moreover, they all have a lipid bilayer envelope in which the envelope proteins are anchored. A single long Open Reading Frame (ORF) which is flanked at the 5’ and 3’ ends by untranslated regions encodes the viral proteins. The N-terminal part of the ORF encodes the structural protein and the non-structural proteins, are encoded by the remaining C-terminal part of the ORF. HCV shows a number of differences to other Flaviviruses: for example, in contrast to members of the Flavivirus genus where translation is cap-dependent, the 5’ UTR in HCV is cap-independent as is the case of members of the pestivirus genus [30, 31].

HCV genome is a positive-strand RNA, which is 9.6 kb in length, encapsulated by an icosahedral capsid comprised of core protein capsomeres. The genome encodes a long polyprotein precursor of approximately 3000 aa, which is co- and post-translational processed via cellular and viral proteases into 10 structural and non-structural proteins. The ORF that encodes the polyprotein is flanked by 5’ and 3’ untranslated regions (UTR). The structural proteins comprise core and envelope proteins E1 and E2 [32]. The non-structural proteins are NS2, NS3, NS4A, NS4B, NS5A and NS5B. Importantly, the N
terminus of NS3 and NS2 encode a metalloprotease enzyme. The N terminus one-third of NS3 encodes a serine protease, which, along with NS4A, is responsible for cleavages at the NS3-NS4A, NS4A-NS4B, NS4B-NS5A and NS5A-NS5B junctions. The NS3 C-terminal part comprises an NTP/helicase domain (Figure 1.3) [32].

### 1.5.1 Untranslated regions

#### 1.5.1.1 5' UTR

The 5' untranslated region of HCV RNA 341(nt) and is highly conserved amongst HCV subtypes. The 5’ UTR comprises 4 highly ordered domains designated I to IV. The first 40 (nt) consists of domain I, which plays a role in RNA replication. Domains II, III and IV, with the first 12-30 nt of the core, constitute the Internal Ribosome Entry Site (IRES) [33-35]. The IRES plays an important role in HCV replication, directing translation by binding eIF3 and ribosomal S40 ribosomal subunit directly to the mRNA [36]. Furthermore, it can bind 40S ribosomal subunits without the eIF3, eIF4A, eIF4B and eIF4F [37]. Importantly, a genetic interaction between a liver-specific micro RNA, mir-122 and the 5’ UTR region was found to play a role in HCV replication [38-40].

#### 1.5.1.2 3' UTR

The HCV 3’ UTR region, ranging in length from 200–235 nucleotides in the direction 5’ to 3’, contains three distinct regions: a short V variable region of approximately 40 nt, which shows variability between different HCV genotypes; a poly (U) and/or poly (UC) region; followed by a highly conserved sequence X region of approximately 100 nucleotides. The X region forms three stable stem-loop structures designated as SL-1, SL-II and SL-III [41-43]. The 3’ UTR interacts with the NS5B RdRp and with two of
Figure 1.3 The HCV genome structure. The figure shows (A) the location of the 5′ and 3′ UTR regions, the HCV IRES in the 5′ UTR is also indicated. (B) The approximate location of the ATG start codon in the 5′ UTR and termination codon following the NS5B protein coding sequence are indicated by asterisks. The HCV polyprotein diagram colors the structural proteins in purple. White diamond (site of signal peptide peptidase cleavage in the core protein). Black diamonds (signal peptidase cleavage sites in the core, E1, E2, p7, and NS2 proteins). (C) The NS2-3 auto-protease cleavage is designated by the double arrow, indicating cleavage of the NS2-3 bond in cis. The NS3 protease domain, in conjunction with NS4A then cleaves all the remaining polyprotein junctions, as indicated by arrows. The putative functions and amino acid numbering of the mature HCV proteins (numbers on the basis of the Con1 1b isolate of HCV). Circles indicate the location of glycosylation sites in the E1 and E2 proteins [44].

the four stable stem-loop structures located at the 3′ end of the NS5B. The 3′ X region and the 52 upstream nt of the poly(U/C) tract were found to be essential for RNA replication, whereas the remaining sequence of the 3′ UTR appears to enhance viral replication [45-48].
1.5.2 Structural proteins

1.5.2.1 Core protein

The core is a structural protein of 23 kD, located at the N terminus of the polyprotein. The mature form of the core protein contains 191 amino acids [49]. It is divided into three general domains: N-terminal hydrophilic domain I, a hydrophobic domain II, which comprises approximately two-thirds of the C terminal of the protein; and a highly hydrophobic domain III. These domains perform different functions in the HCV lifecycle. Domain I binds RNA and mediate capsid assembly; Domain II mediates the interaction with lipids and membrane proteins; whilst Domain III is believed to form an α-helix at the distal C-terminus of the immature core protein, with the domain cleaved and disappearing from the mature form of the HCV core after translation [50]. The core protein plays an essential role in HCV pathogenesis, interacting with a number of cellular proteins that affect cellular processes, such as lipid metabolism, apoptosis and signalling pathways [50]. Markedly, the HCV core protein possesses both pro and anti-apoptotic functions; it stimulates hepatocyte growth in Huh 7 cells through the transcriptional up regulation of growth-related genes, and it has been implicated in tissue injury and fibrosis progression.

It can also induce the formation of lipid droplets and may play a direct role in the development of steatosis [51-53]. Moreover, it has been found to induce insulin resistance through the impairment of insulin signalling. HCV core activates SOCS-3 by inducing the expression of TNF-α, leading to the impairment of the insulin signalling cascade, resulting in insulin resistance [54]. Furthermore, the core of N-terminal Domain I has a potent RNA chaperone activity, which prevents the misfolding of HCV RNA (Figure 1.3) [55].

1.5.2.2 Envelope proteins

HCV virus has two envelope proteins designated E1 and E2, with molecular weights of 33–35 kDa and 60–72 kDa, respectively, and are localised to the endoplasmic reticulum [56].
Introduction

The envelope proteins E1 and E2 are important constituents of the HCV virion envelope, with each known to play a role in virion entry into host cells through direct binding to the receptors CD81 and scavenger receptor class B type (SR-BI) [57-59]. E1 and E2 are highly glycosylated Type 1 transmembrane proteins, forming two types of stable heterodimeric complexes: a disulfide-linked form of misfolded aggregates, and a non-covalently linked heterodimer, most likely corresponding to the pre-budding complex [60]. The two hypervariable regions, HVR1 and HVR2, respectively and each region can stimulate the production of virus-neutralising antibodies. However, it is recognised that the virus can escape from the neutralising antibodies by inducing random mutations in HVR1 during the course of infection [61-63].

1.5.2.3 Protein p7

The p7 protein is a member of viroporin group of transmembrane proteins. These are membrane ion-channel proteins encoded by different viruses, e.g. HIV-1 and Influenza-A virus, which promote virus release and possibly viral entry. HCV p7 is required for the assembly and release of HCV particles from liver cells [64, 65]. The p7 is a highly hydrophobic polypeptide 63 amino acids in length, which oligomerises to form a heptameric cation channel \textit{in vitro}. The protein is located in the HCV polyprotein between E2 and the carboxy terminus of NS2, although it is not yet classified as either a structural or non-structural protein. Evidence suggests that the ion channel activity can be specifically blocked by several synthetic small molecules, and it could be a possible target for antivirals [66, 67]

1.5.2.4 ARFP/F protein

Alternative reading frame protein of F protein, which is 160 amino acids, is synthesized in the core region by an alternative reading frame. Currently there is little evidence that the F protein plays an important role in the HCV replication cycle [68].
1.5.3 Non-structural proteins

1.5.3.1 NS2

NS2 is a hydrophobic protein with a molecular mass of 23 kDa with cystein protease activity [69, 70]. The NS2 is responsible for the cleavage of the junction between NS2 and the amino terminal domain of NS3 by an autoprotease comprising NS2 and NS3, whilst the cleavage of the N-terminus of NS2 from the C-terminus of p7 is mediated through host signalase enzymes (Figure 1.3) [71-73]. NS2 forms a homodimer. In addition, NS2 has also been shown to interact with all the other HCV non-structural proteins [74]. A study shows that the expression of NS2 in Huh 7 cells resulted in up regulation of transcriptional factor sterol regulatory element-binding protein 1c (SREBP-1c) and fatty acid synthesis, which indicates that NS2 may be involved in steatosis. NS2 has also been suggested as participating in the virus particle assembly [13, 75].

1.5.3.2 NS3

NS3 is a bifunctional, hydrophobic protein of 72 kDa. The mature NS3 comprises five domains: the N-terminal 2 domains form a serine protease which is associated with the NS4A cofactor and the C-terminal three domains, which form an RNA helicase. NS3 protease cleaves at the NS3/NS4A junction and forms a tight, non-covalent complex with NS4A; this is fundamental for the processing of the polyprotein [76].

1.5.3.2.1 The NS3 serine protease domain

NS3/4A serine protease is a non-covalent, heterodimer complex formed by the catalytic subunit located in the N-terminal of NS3 and the activation subunit of the NS4A cofactor. The NS3/4A serine protease cleaves the junctions of the HCV polyprotein precursor at NS3/4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B. The NS3 serine protease domain enhances the activity of the NS3 helicase RNA binding, which is required for RNA unwinding [77, 78]. A conserved sequence is present in all serine proteases comprising the amino acids histidine (His\(^{57}\)), aspartate (Asp\(^{81}\)) and serine (Ser\(^{139}\)), known as the catalytic triad. A substitution of any of these catalytic triad amino acids in HCV NS3 will suppress the cleavage of NS3/4A, NS4/4B, NS4B/NS5A and NS5A/NS5B within the polyprotein [77-81]. Several studies have reported the role of HCV NS3/4A serine protease in break-
ing down the host antiviral response; for example, the HCV NS3/4A blocks the activation of TLR3-dependent and TLR3-independent signal transduction cascades, thus blocking the IFN-induced antiviral response against HCV RNA replication [82]. Importantly, NS4A has been shown to play the role of cofactor with NS3 protease to cleave peptide substrates more efficiently particularly at the NS4B/NS5A junction [80].

1.5.3.2 NS3 helicase domain

The C-terminal of HCV NS3 encompasses the helicase domain. The crystal structure of HCV helicase, viewed as a Y-shaped protein, comprises three domains, the most N-terminal domain (Domain1) and the middle domain (Domain2) are above the C-terminal domain (Domain3) [83-85]. In general, helicases share similarities between each other: for example, they all carry the classical Walker A (phosphate binding loop) and Walker B (Mg+-binding aspartic acid) motifs [86]. The helicases are classified into five superfamilies (SF1-SF5) [87]. Based on the sequence homology, the HCV NS3 helicase belongs to the DEAD-box RNA helicases, and is classified as a member of the DExH family of RNA helicase proteins of superfamily II, which also includes helicases from bovine diarrhoea pestivirus, plum pox potyvirus, and vaccinia virus. Many amino acid sequence motifs can be found conserved amongst RNA helicases of both superfamily I and superfamily II. Furthermore, all helicases possess Rec-A like motor domains, which functions as the core of the helicase molecular motor [87, 88]. However the exact biological role of NS3 helicase remains unclear; it is believed to be involved in several activities such as (i) binding and hydrolyzing nucleoside triphosphates, (ii) interacting with both RNA and DNA, (iii) translocation in a 3′-to-5′ direction, (iv) separating nucleic acid base pairs and (v) displacing nucleic acid binding proteins [86]. The HCV helicase requires a single-stranded region to initiate unwinding with a 3′-end overhang on which to load, and the energy from ATP hydrolysis is believed to fuel both translocation and unwinding.
In contrast to other helicases, HCV helicase cleaves ATP more rapidly in the absence of RNA. This basal ATPase activity is stimulated up to 100-fold by nucleic acids, depending on the nucleic acid sequence and whether or not the protease region is present (Figure 1.4) [89-91]. The most likely role for HCV helicase is assisting the NS5B RNA-dependent RNA polymerase with viral replication by resolving RNA secondary structures and/or double-stranded replication intermediates [92-94]; however, there is the possibility that cellular helicases may perform this function, and recently HCV NS5B has been shown to recruit and interact with cellular RNA helicase p68, which in turn assists in the synthesis of minus-strand HCV RNA [95]. In addition, the NS3 helicase domain has been reported to participate in an early step of viral assembly, suggesting it may have additional or alternative roles [96].
The binding of a polynucleotide by NS3 Helicase in the absence of ATP leaves a large cleft between domains 1 and 2. Binding of ATP occurs with the $\beta$-phosphate binding to residues in motif I (GSGKT) and the $\gamma$-phosphate with Mg$^{2+}$ binding to the conserved acidic residues in motif II (DECH). This results in the closing of the interdomain cleft and the binding of conserved arginines in motif VI (QRRGRTGR) to the ATP phosphates. Val432 and Trp501 disrupt base stacking at either end of the single-stranded region. Closure of the interdomain cleft leads to translocation of the single strand in the 5′ to 3′ direction and forces several bases to slip past Trp501. Hydrolysis of ATP facilitates opening of the cleft and release of ADP. The orientation of Trp501 favors movement of the polynucleotide in only one direction such that opening of the gap results in net movement of the helicase in a 3′ →5′ direction [85].

1.5.3.3 NS4A

NS4 is a small protein of 54 amino acids. It encodes an N-terminal hydrophobic alpha-helix that anchors NS3-4A to cellular membranes, a middle “cofactor” peptide that forms a beta-strand which facilitate the proper folding of the NS3 serine protease domain, and a C-terminal acidic region that forms an alpha-helix at low pH [97-100]. NS4A has been reported to have additional functions, e.g. Interacting with NS5A and regulating NS5A phosphorylation [101, 102]. Moreover, NS4A overexpression in the absence of NS3 inhibits cellular translation process [103, 104], and can lead to mitochondrial apoptosis through activation of caspase-3 [105].
1.5.3.4 NS4B

NS4B is a 27 kDa protein, with both hydrophobic transmembrane (TM) and amphipathic helices [106, 107]. It is an integral membrane protein which localise to the ER-derived membranous compartment.

The expression of NS4B induces membrane proliferation designated as the membranous web, which is an essential structure required for the formation of the viral RNA replication complex [108]. In addition, a cellular protein localized in the early endosome, RAB5, was found to be associated with NS4B in replicon cells and may also play a role in the formation of the replication complex [109].

1.5.3.5 NS5A

NS5A is a phosphoprotein that may exist in two different forms: basal phosphorylated 56 kDa and hyperphosphorylated 58 kDa forms [110]. NS5A is anchored to the membrane of the ER by its N-terminal 30aa, which forms an amphipatic alpha-helix [111]. The role of NS5A remains unclear, although, it has been reported that NS5A plays an important role in virus replication through interaction with other HCV nonstructural proteins as well as various cellular proteins. The interaction with NS5A leads to the assembly of the viral replication complex and may also regulate RNA replication [112-114].

A study showed that the X-ray crystal structure of domain I has a zinc binding domain, which forms a homodimer with contacts at the N-terminal ends of the molecules; suggesting a location of protein, RNA, and membrane interaction sites at the dimeric interface suggested to be involved in RNA binding during viral replication [115, 116]. Additionally, NS5A has been shown to interact with 3' end of the HCV positive- and negative-strand RNAs suggesting that NS5A may affect the efficiency of RNA synthesis by NS5B. Moreover, NS5A also has been shown to bind with uridylate and guanylate rich RNA, specifically with the large U/G stretches in the 5' IRES, suggesting a role of NS5A in HCV translation and genome multiplication [113, 117, 118].
1.5.3.6 NS5B

NS5B is a 68 kDa tail-anchored protein and is the RNA-dependent RNA polymerase (RdRp) responsible for synthesising the complementary negative strand of the genome as the replication intermediate, and then genomic RNA using the negative strand as the template. Its C terminal 21-aa is responsible for post-translational targeting to the cytoplasmic side of the endoplasmic reticulum [119].

The NS5B organisation is a “right-hand” structure with three sub-domains: thumb, fingers and palm, which is similar to other polymerases such as HIV-1 reverse transcriptase [120, 121]. The NS5B RNA-dependent-RNA polymerase (RdRp) activity plays a principle role in HCV replication, and is the key enzyme required for the synthesis of new RNA genomes. NS5B can initiate RNA synthesis in two way de novo initiation from the 3′-end of RNA template [122, 123] or primer-dependent initiation [124]. An interaction between NS5A and NS5B has been reported [113], suggesting that NS5A might serve as a cofactor for NS5B [112]. Protein interaction between NS5B and cellular proteins that are involved in HCV replication has been reported. PRK2 (protein kinase C-related kinase 2) binds to the fingers domain and may regulate the phosphorylation of NS5B [125]. The glycine/arginine-rich domain of nucleolin mediates its interaction with NS5B, and siRNA (small interfering RNA) directed at nucleolin mRNA suppressed HCV replication in vitro [126]. Furthermore, NS5B also interacts with the NS5A-binding protein hVAP-B, which may be a component of the replication complex [127]. NS5B binds to the retinoblastoma susceptibility protein (pRb) through a site overlapping its polymerase motif, although such an interaction may contribute to the oncogenic property of HCV rather than the replicase function of NS5B [128].

1.6 HCV replication cycle

A number of studies reported that HCV can infect peripheral blood mononuclear cells [129] and replicate in lymphocytes [130, 131]. However, the determinants of HCV-RNA replication process are still unclear. A study found that subgenomic replicon JFH-1 can replicate in non-hepatic cell lines, such as HeLa cells and 293A cells, indicating that the host factors required for HCV replication are not hepatocyte specific [132]. The life cycle of HCV begins once it attaches to the host cell.
It is a multistep process and it can be summarized as: virus attachment and internalization through specific receptors, release of the viral particle into the cytoplasm and uncoating of the genome, RNA genome translation and polyprotein processing, synthesis of a negative strand intermediate from the positive strand genomic RNA to generate new positive strands, and finally virion maturation and release of new HCV viruses (Figure 1.6).

1.6.1 Attachment and entry

The first step in the virus lifecycle is attachment to the host cell. Several cell surface receptors have been identified as being required to mediate the attachment and internalization of the virus into the cell (Figure 1.7). The initial attachment of the virus initiates by a low affinity interaction of E2 with heparan sulfate proteoglycans (HSPGs), on the
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Figure 1.7. HCV attachment and entry. 1. HCV circulate as a lipo-viroparticles (LVPs), particles consisting of lipoproteins (yellow) in complex with HCV particles possessing E1 and E2 glycoproteins (green) on the surface; 2. Interaction with glycosaminoglycans (GAGs) and the low density lipoprotein receptor (LDLR) and specific interactions with SR-B1 and CD81 are mediated by the E1 and E2 glycoproteins; 3. Translocation of viral particles to regions of the membrane possessing tight junction proteins occludin and claudins-1, -6 or -9, forming a co-receptor complex that results in clathrin-mediated endocytosis of the viral particle; 4. the endocytic vesicle containing the virus particle transfers through the cytoplasm where it proceeds to mature into a low pH late endosome resulting in conformational changes, which facilitate the fusion of the viral envelope and endosome membrane, which finally permits viral nucleocapsid to enter the cytoplasm [133].

cell surface and probably the LDL receptor, which may interact with HCV associated lipoproteins (LDL and VLDL) [134]. This is followed by a specific interaction between the Human CD81, scavenger receptor B type I (SR-BI) [58], tight junction proteins Claudin (CLDN)-1, 6, or 9 and Occludin [135-138]. This enables the virus to enter the cell through clathrin-dependent receptor mediated endocytosis. Envelope protein E2 has been observed to bind directly to SR-BI and CD81, thus forming a ternary complex. Furthermore, E2 also binds to CD81 and Claudin, forming a co-receptor complex. Additional interactions may also occur between SR-BI and HCV associated lipoproteins including HDL and LDL [139].
These findings indicate that HCV entry may occur following the formation of a complex of these entry factors. Following attachment, the nucleocapsid is released into the cell cytoplasm through fusion between the viral and cellular membranes. This fusion process is controlled by viral surface glycoproteins, which induce changes for mediating fusion. The Flaviviruses enter target cells by receptor-mediated endocytosis and use Class II fusion proteins [140]. Their resemblance to Flaviviruses, suggests that the HCV envelope protein are Class II fusion proteins [141]; however, in contrast to other Class II fusion proteins, HCV envelope glycoproteins do not appear to require cellular protease cleavage during their transport through the secretory pathway [142].

1.6.2 RNA translation

Once the virus is internalized, the viral nucleocapsid, releases HCV genomic RNA into the viral cytoplasm, which then localises to host ribosomes, at the endoplasmic reticulum, allowing the initiation of the genome translation process. Recent studies have reported that non-coding micro RNAs (miR) are important in controlling gene expression and regulation. Transcription of miR-122 stimulates HCV RNA translation and virus production via the direct interaction of miR-122 with two sites, S1 and S2 in the 5′ UTR, whereas its sequestration in liver cell lines strongly reduced HCV translation [143].

HCV genome translation is under the control of the IRES, which spans domains II to IV of the 5′ UTR, and the first nucleotides of the core-coding region. The IRES mediates the cap-independent internal initiation of HCV polyprotein translation by recruiting both cellular proteins [34, 144], including eukaryotic initiation factors (eIF) 2 and 3 and viral proteins [145-147]. Experiments have shown the generation of three distinct translation initiation complexes (40S, 48S and 80S) [148]. In this regard, the IRES binds directly to a 40S ribosomal subunit, thus forming a stable pre-initiation complex, with such binding potentially occurring even without the canonical translation initiation factors. The 40S subunit aggregates with eIF3, forming a ternary complex, which further assembles with eIF2, GTP, and the initiator tRNA to form a 48S particle, through which tRNA locates in the P site of the 40S subunit and base-pairs to the start codon of the mRNA. After GTP hydrolysis, eIF2 releases the initiator tRNA from the complex, additional GTP hydrolysis step involving initiation factor eIF5B then enables the 60S ribosomal subunit to associate
forming an 80S ribosome, which is responsible for protein synthesis initiation [37, 149, 150]. Several cellular proteins, namely polypyrimidine tract-binding protein (PTB) [151] and La autoantigen [152] were reported to interact with 5’ UTR, and regulate translation. PTB interacts with three distinct pyrimidine-rich sequences within the HCV IRES, and with domain III of the IRES [153]. In addition to the IRES, PTB has also been shown to interact with the 3’ X region to enhance HCV IRES-mediated translation [47, 154]. Moreover, heterogeneous nuclear ribonucleoprotein I (hnRNP I), and several other proteins of the hnRNP family have been shown to interact with the HCV IRES. hnRNP L has also been reported to interact with the 3’ border of the HCV IRES in the core-coding sequence; the binding correlates with the translation efficiency from the IRES [155]. In addition, IRES translational efficiency may also be affected by HCV proteins, including the core [156] and non-structural protein NS4A and NS5B [104].

1.6.3 Post-translation

As a result of the HCV genome translation, a large precursor polyprotein is generated, which is targeted to the ER membrane for the translocation of the E1 ectodomain into the ER lumen, a process mediated by the internal signal sequence located between the core and E1 sequences. The host signal peptidase cleaves the signal sequence, which yields an immature form of the core protein (P23) [157]. The signal peptide is further processed by a host signal peptide peptidase with the aim of yielding the mature form of the core protein (P21) [158]. The host signal peptidase also ensures cleavage at the E1–E2 junction in the ER lumen. An additional signal peptidase cleaves at the C-terminal end of E2 and between p7 and NS2 giving rise to p7. E1 and E2 subsequently undergo several maturation steps. The zinc-dependent NS2-3 auto-protease ensures cis-cleavage of NS3 from NS2. NS3 needs to assemble with its cofactor NS4A to catalyse cis-cleavage at the NS3-NS4A junction and trans-cleavage at all downstream junctions, including NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B [159, 160].

The cleavage sites recognised by the NS3-NS4A protease have the following sequence in common: Asp/GluXXXXCys/Thr-Ser/Ala, with trans cleavages occurring downstream of a cysteine residue and the cis cleavage occurring downstream of a threonine residue.
1.6.4 The HCV replication complex

As has been shown for other positive-strand RNA viruses, HCV replicates in association with intracellular membranes. The HCV NS4B protein induces the formation of a membranous web or membrane-associated foci [108] [161]. It is still unclear whether or not NS4B recruits cellular proteins responsible for vesicle formation or if it induces vesicle formation by itself. However, these are [162] thus required for HCV genome replication [109]. The membranous web contains large amounts of cholesterol and fatty acids and is derived from ER membranes [162, 163]. It is comprised of small vesicles embedded within a membranous matrix, forming a membrane-associated multiprotein complex containing all of the non-structural HCV proteins [108]. HCV replication has been shown to occur in detergent-resistant membranes that co-localise with an essential component of lipid raft domains in the membranous web [164]. In addition, lipid rafts are known to be involved in the formation of the replication complex through protein-protein interactions between hVAP-33 and both NS5A and NS5B HCV proteins [164-166].

1.6.5 HCV RNA replication

The mechanism by which HCV genomic RNA replicates is still unclear. In comparison with other Flaviviruses viruses, HCV replication is thought to be semi-conservative and asymmetric in two steps, with the key control responsible for viral RNA synthesis being NS5B, the catalytic subunit of the replication complex, which has RNA-dependent RNA polymerase (RdRp) activity. Firstly, RNA-positive strands serve as a template for the synthesis of a negative strand. In the second step, negative-strand RNA serves as a template for the production of several strands of positive polarity which are subsequently used for polyprotein translation, the synthesis of new intermediates of replication, or packaging into new virus particles [163]. RNA synthesis initiates at the 3′-end of the plus and minus strands and involves Domain I of the 5′ UTR, which can form a G/C-rich stem-loop, the 3′ UTR and a cis-acting replication element (5BSL3.2), comprising 50 bases located in a large predicted cruciform structure at the 3′ end of the HCV NS5B-coding region [167]. The initiation of RNA-replication is triggered through an interaction between the proteins of the replication complex, the 3′ X region of the 3′ UTR, and 5BSL3.2, which forms a pseudoknot structure with a stem-loop in the 3′ UTR [167-
A phosphorylated form of PTB was found in the replication complex, PTB was shown to interact with two conserved stem-loop structures of the 3′ UTR an interaction thought to modulate RNA replication [170, 171]. The inhibition of PTB expression through means of small interfering RNAs reduces the amount of HCV proteins and RNA in HCV replicon-harbouring Huh7 cells [170].

1.6.6 Virus assembly and release

The early steps of assembly are initiated through an interaction between core protein and lipid droplets (LDs), which are lipid storage organelles that store triglycerides and cholesterol esters [172]. During translation process of the HCV polyprotein, the mature form of core protein is released by signal peptidase and signal peptide peptidase, at the ER membrane allowing transport of the protein to LDs [157, 173, 174]. Core protein possesses two domains (D1 and D2), both of which mediate core LD interaction [175]. The D2 domain interacts with the phospholipid layer surrounding LDs [176]. Several studies have reported that, during infection, replication complexes and newly synthesised virus RNA localises to the lipid droplet surface through the interaction of NS5A with the lipid droplet localised core protein [177-179]. From the above evidence, it is thought that lipid droplets may become embedded in the HCV membranous web structure, allowing the efficient trafficking of the virus genome from replication complexes to virus assembly sites. Another mechanism has been proposed, whereby nucleocapsid formation occurs at the ER membrane, and this requires lipid droplets to function as a core protein transport vehicle, trafficking core from the translation site to where viral assembly takes place [180]. Following virus RNA packaging into the nucleocapsid, the virus undergoes budding to acquire an outer envelope containing envelope glycoproteins E1 and E2, which are embedded in a bilayer lipid. It is unclear where the process of virion-budding occurs; however, NS2 may play a role in this process as it is able to form complexes that are localised adjacent to the core coated lipid droplets, and contains replicase components (NS3 and NS5B), p7, and the envelope proteins (E1 and E2) [181].

The maturation and release of HCV seems to be dependent on the VLDL pathway, based on the observations that the HCV particles isolated from chronically infected serum are often found in low-density forms in association with components of secreted VLDL in-
including triglyceride, Apolipoprotein B100 (ApoB) and Apolipoprotein E (ApoE) [180, 182]. The VLDL are produced in the ER following the fusion of newly translated ApoB protein, with lipid droplet derived triglyceride and cholesterol ester molecules in the lumen of the ER using the microsomal triglyceride transfer protein (MTP) [180, 183]. This process leads to the formation of a pre-VLDL particle, which can further interact with lipoprotein, ApoE which facilitates the fusion of the pre-VLDL particle with luminal lipid droplets, subsequently generating triglyceride-rich VLDL particles [180, 182]. The precise mechanism of pre-VLDL lipidation and localisation is, as yet, unclear, although it probably occurs within the ER or Golgi body. Subsequently, mature VLDL particles are then secreted from the cell using ER-derived COPII-dependent (coat protein complex-dependent) transport vesicles, which are predicted to transfer through the Golgi body to leave the cell [184]. The role of lipid droplets in the VLDL synthesis pathway gives an indication that a specific mechanism, whereby HCV components localised to the surface of lipid droplet are delivered to the VLDL machinery for the process of HCV assembly, maturation, and subsequent secretion. Several studies support this idea: for example, knockdown studies of ApoB and ApoE and inhibition of MTP significantly reduces the titre of HCV [183-186]. Moreover, recent studies have shown that other host proteins involved in VLDL synthesis, such as ACSL3 and ARF1 GTPase (ARF1), are essential in the process of HCV particle secretion. On the other hand, however, other researchers have established that ApoE alone is essential for both HCV assembly and secretion, and interacts directly with NS5A [186, 187]. Other cellular pathways have been implicated in HCV release, for instance; Endosomal cargo sorting is directed by ESCRT (endosomal sorting complex required for transport) protein complexes. Such complexes facilitate the sorting of ubiquitinated endocytosed cargo into intraluminal vesicles, which form within endosomes to create a multivesicular body (MVB). The MVB then fuses with lysosomes, thus allowing the degradation of the intraluminal vesicles and their cargo. ESCRT 0-II controls the process of cargo sorting and the recruitment of ESCRT III [188, 189]. ESCRT III complex is controlled by the ATPase protein, VSP4, which is required for proper functioning of the sorting and degradation process [188, 189]. Recent studies showed that there are several components of the ESCRT pathway may play an essential role in releasing the infectious HCV virions; including the ESCRT complex binding pro-
1.7 The role of cellular host factors in the HCV life cycle

Viruses have relatively few genes compared to their host cells. Even the largest virus genome size encodes only a few hundred genes, while those of host cells encode thousands of genes. Accordingly, during a virus life cycle there are interactions between a relatively few different types of viral components and a plethora of host factors. These host factors confer a suitable environment in which the virus can survive and are necessary resources for gene-poor viruses. Numerous host candidates have been selected and tested by using different methods such as bioinformatics approaches based on siRNA, and others, proteomic approaches, interactome studies and microarray analysis. RAB GTPases and RNA-binding proteins such as Staufen 1 are example of these candidate host proteins.

1.7.1 RAB GTPase proteins: intracellular localisation and function

RAB GTPase proteins are small (20-29 kDa) monomeric Ras-like GTPases [191], they regulate the trafficking of membranes required for the assembly of membrane structures in eukaryotic cells.

In humans there are more than 60 RAB proteins (Figure 1.8), all of which are localised to particular regions of cellular membranes so as to facilitate the creation, trafficking, chaining and fusion of intracellular transport vesicles [192, 193]. Upon activation, the GTP-bound RAB proteins have the capacity to bind to certain effector proteins, namely motor proteins, sorting adaptors and tethering factors. A large number of the RAB proteins recognised thus far have been mapped to a particular cellular location, which is commonly linked with either the trafficking vesicle or a specific intracellular organelle’s membrane; this provides each RAB protein with the potential to control and regulate a certain aspect of the membrane trafficking within the cell. Examples of membrane trafficking events governed by RAB GTPase proteins include the formation of autophagosomes, lipid droplets and melanosomes, protein degradation, protein sorting, and the regulation of endocytosis and exocytosis [193]. To enable these processes, RAB GTPase proteins have been found to behave in a synchronised way through complicated
regulatory networks. For instance, different RAB proteins can be linked to more than one RAB at a time by RAB effect or proteins. Moreover, RAB proteins are also able to crosstalk through either restricting or initiating neighbouring RABs’ GEF and GAP proteins in order to establish a flow of RAB protein activity [193].

1.7.1.1 Regulators of RAB protein

To control the function of RAB GTPase proteins and their adequate control, a number of other host proteins are necessary to facilitate membrane localisation of RAB protein activity. After the translation of a RAB protein, the GDP-bound RAB is delivered to a geranylgeranyl transferase enzyme (RABGGT) by the RAB escort protein (REP), and subsequently transfers a geranylgeranyl motif (Red wavy lines) (Figure 1.9), to either one or two RAB protein C-terminal cysteine residues [194].
Figure 1.8. The intracellular localization of RAB proteins in mammalian cells. (CCV, clathrin-coated vesicle; CCP, clathrin-coated pit; EC, epithelial cells; IC, ER–Golgi intermediate compartment; M, melanosomes; MTOC, microtubule-organizing centre; SG, secretory granules; SV, synaptic vesicles; T, T-cell granules; TGN, trans-Golgi network.), [195]
Following that geranylgeranylated GDP-bound RAB protein is delivered to the target membrane by the REP protein. The activation of the RAB protein is then initiated by a guanine nucleotide exchange factor (GEF) through the exchange of GDP for GTP, with the latter GTP-bound RAB protein then becoming affixed to its particular effector proteins, namely motor proteins, sorting adaptors and tethering factors, so as to carry out its membrane trafficking function. Subsequently, there is interaction between the activated GTP-bound RAB protein and the GTPase-activating protein (GAP), which thus catalyses the GTP hydrolysis to GDP through the RAB GTPase protein (Figure 1.9). This results in the release of the RAB protein from its associated effectors. Thus, there is the removal of the inactive GDP-bound RAB from the membrane, which is achieved through interacting with a RAB GDP disassociation inhibitor protein (GDI), which subsequently becomes attached to GDP-bound RAB proteins, which are then removed from the membrane. The re-insertion of the GDI-bound RAB protein into the target membrane can be achieved by the interaction with a RAB GDI displacement factor (GDF), which recognises and identifies GDI-bound RAB proteins and the subsequent release of the GDI protein from the GDP-bound RAB. Following this there is the reactivation of the membrane localised GDP-bound RAB through the interaction with a GEF protein, at which point the entire process of RAB activation repeats [194].
1.7.2 The RAB27 family

The RAB27 subfamily comprises two isoforms RAB27A formerly designated Ram [196], and RAB27B formerly designated c25KG [197]. RAB27A and RAB27B which form the RAB27 subfamily share 71% amino acid homology [198]. RAB27A is expressed in a wide variety of secretory cell types, including exocrine, endocrine, ovarian, and hematopoietic cells, most of which function specifically in regulated exocytic pathways [199], whereas, RAB27B expression is less abundant and is expressed in platelets, the stomach, large intestine, pancreas, pituitary, and bladder [200], where it exerts its regulatory effect on these organs e.g. the function of pituitary hormone secretion, zymogen secretion from the pancreatic acinar cells, amylase secretion from the parotid acinar cells and controls the process of mast cell degranulation [201]. RAB27B is less well characterized, however, it was found to regulate invasive growth and metastasis in Estrogen Receptor-positive breast cancer cell lines [202]. A recent study showed that RAB27A and RAB27B expression were closely correlated with tumor metastasis and can be prognostic indicators for HCC patients [203]. In general, several effector proteins have been shown to bind to activated RAB27A/B to coordinate vesicle trafficking during exocytosis. Some of the effector proteins are able to bind to the actin cytoskeleton, allowing RAB27 coated vesicles to traffic via actin filaments to the cellular periphery e.g. melanophilin in the trafficking of melanosomes [201, 204]. Other effector proteins mediate the docking of RAB27 bound vesicles at the plasma membrane by binding to the SNARE proteins involved in membrane fusion e.g. granuphilin, which binds to the syntaxin 1a SNARE protein at the plasma membrane during insulin secretion [205].

1.7.3 The RAB40 family

RAB40 family GTP-binding proteins, a member of the Ras oncogen family, comprises RAB40A, RAB40B, and RAB40C, which are highly homologous [206]. The RAB40 family was distinguished from other RABs by possessing the suppressor of cytokine signalling (SOCS) box. It is known that SOCS box-containing proteins interact with an E3 ubiquitin ligase complex (with elongins B and C) that polyubiquitinates target proteins for subsequent degradation [207].
Little is known about the biological function of the RAB 40 family, although, one study has shown that Human RAB40C is regulated directly by a cancer-related miRNA, let-7a, and mediates the biological effects of let-7a in gastric tumorigenesis. Thus, RAB40C proteins have diverse functions in embryogenesis, ubiquitination and cancer [207]. RAB40B was found expressed in the zebra fish brain, embryonic and adult mouse brain, inner ear and heart tissues suggesting a role in neurodevelopment [207]. Although, RAB40A and C are up-regulated in neuronal ganglions and nodes, up-regulation of RAB40B is confined to brain tissue with an expression profile nearly identical to that of RAB3A. RAB40B may define an as yet uncharacterized RAB-regulated hub modulating a linked step in the RAB3A-dependent synaptic vesicle cycle in the brain [208].

1.7.4 Staufen 1

Staufen is a dsRNA-binding protein, which was initially recognised in Drosophila through the trafficking and subsequent translation of cellular mRNAs during the process of embryonic development. In the case of mammalian cells, two different Staufen homologs, namely Staufen 1 and Staufen 2 have been identified, with 51% amino acid homology. These proteins have been found to bind certain motifs on cellular mRNA molecules, to enable the creation of ribonucleoprotein complexes, which are concerned with controlling mRNA trafficking, degradation and translation [209]. Despite the fact that the two mammalian Staufen homologs are believed to perform similar intracellular roles, it is nevertheless known that there are marked differences in terms of their expression patterns, with Staufen 1 expressed universally, whilst the expression of Staufen 2 is neuron specific [209, 210]. Furthermore, a number of different protein isoforms have been recognised for both Staufen homolog genes by alternative splicing, including, for example, the 55KDa and 63KDa isoforms derived from Staufen1, and the 52KDa, 56KDa, 59KDa and 62KDa isoforms derived from Staufen 2. These isoforms are known to differ in terms of their overall ability to bind mRNAs, and many therefore develop distinct ribonucleoprotein complexes in cells [209].

Both Staufen1 and Staufen 2 have been observed to become localised to the rough endoplasmic reticulum in ribonucleoprotein complexes while carrying out their roles of degradation, translation and trafficking of mRNA molecules [209]. Furthermore, it has also
been found that Staufen 1 and Staufen 2 are transported between the cytosol and nucleus to control the localisation of newly synthesised mRNAs [209]. The trafficking role of Staufen proteins is facilitated through its structure which is a tubulin-binding sphere, and thus has the capacity to form complexes encompassing cytoskeletal control proteins (CDC42 and RAC1), cytoskeletal filament proteins (tubulin and actin), and cytoskeletal motor proteins (myosin and kinesin) [211]. Staufen 1 binds to the 3′UTR of cellular mRNAs, which are then targeted for degradation through ‘staufen-mediated decay (SMD)’ [212], this relies on the recruitment of the up-frameshift suppressor 1 protein (UPF1) a cellular host factor commonly concerned with the process of nonsense-mediated decay (NMD), which is known to degrade mRNAs comprising incorrect sequences of translation termination. On the other hand, Staufen 1 binding to the 5′UTR of cellular mRNAs is known to improve overall translation efficiency [213], with Staufen1 also able to further enhance translational activity through its capacity to govern and mediate the balance between polysomes, which implement cellular mRNA translation; and stress granules, which, through translation, repress cellular mRNAs throughout periods of cellular stress [214].

Staufen 1 has been implicated in the replication of a number of viruses with RNA genomes, including Influenza A and the Human Immunodeficiency Virus-1 (HIV-1). In the case of the replication cycle relating to HIV-1, Staufen 1 has been recognised as binding to the HIB Gag precursor protein (Pr55Gag) and the HIV-1 RNA genome, and is present in the HIV-1 virions. Such interactions enable the Gag precursor protein’s multimerisation and facilitate HIV RNA encapsidation during the construction of the nascent HIV-1 particles. Staufen 1 has been observed to relocalise in combination with the HIV-1 RNA and HIV-1 Gag protein to where the assembly of HIV takes place, e.g. the plasma membrane’s lipid raft domains [215]. Moreover, Staufen 1 is also known to adopt a distinct role in the replication cycle of HIV-1 by regulating the rate at which the translation of the HIV-1 Gag protein is carried out [214]. In the replication cycle of Influenza A, Staufen1 is recognised as being part of a viral ribonucleoprotein complex containing viral RNA, viral polymerase and nucleoprotein. Its presence in this complex may enable viral RNA genomic RNA encapsidation into nascent virus particles since reduced expression of Staufen 1 by siRNA decreases the production of the influenza virus particle without im-
pacting the level of virus protein expression or otherwise affecting virus replication. The interaction between the influenza A non-structural protein 1 (NS1) and Staufen 1, remains unclear [216]. In addition to the evidence highlighting the fact that Staufen1 is involved in the replication cycle of both influenza A and HIV-1, Staufen 1 has also been shown to directly bind to the 3’NTR of the HCV genome and the 5’NTR of the negative HCV RNA strand [217, 218]. This clearly suggests that Staufen 1 may be involved in the HCV replication cycle, and that it may also regulate certain processes, including genome replication or encapsidation, throughout the process of nascent virus particles assembly.

1.7.5 Thioredoxin-interacting protein (TXNIP)

The Thioredoxin-interacting protein (TXNIP) is a 50kDa protein, it is also known as vitamin D3 upregulated protein (Vdup1) [219], which inhibits the function of thioredoxin (TRX). TXNIP binds to thioredoxin and prevents it from carrying out its function which is to reduce proteins by cystine thio-disulfide exchange [220]. It is believed that the major role of TXNIP is the inhibition of the redox protein thioredoxin; however, other molecular functions of TXNIP such as transcriptional regulation have also been proposed. TXNIP mutations have effects on lipid metabolism, glucose metabolism, and also the NADH/NAD\(^+\) ratios [221]. There is increasing evidence that TXNIP may play a causal role in tumorgenesis. Several studies have shown that TXNIP expression is downregulated in breast and gastric cancers, as well as in various cell lines [222-225].

*In vitro* studies indicate that TXNIP overexpression can inhibit the proliferation of pro-myelocytic leukemia cells and stomach cancer [226, 227]. In a tumor transplant model, TXNIP overexpression suppressed melanoma metastasis [227]. Recently, it was shown that TXNIP interacts with and inhibits Jun activating binding protein (JAB1), a protein that promotes the degradation of p27 (kip1), a cyclin-dependent kinase inhibitor whose expression is reduced in many tumors [228]. Moreover, thioredoxin-interacting protein (TXNIP) has been involved in the development of both type I and II diabetes and its vascular complications [229]. Deficiency of TXNIP leads to improved glucose tolerance and insulin sensitivity in mice fed with a high-fat diet, protects against diabetes, and inhibits glucose-induced pancreatic  β-cell apoptosis [229]. TXNIP also induces caspase-1 inflammasome and innate immune response in pancreatic β-cell and macrophage [229].
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Most significantly the levels of TXNIP expression were shown to increase by about 40 fold during interaction with the human hepatocyte cell line. Huh 7 with HCV using siRNA to target TXNIP reduced HCV replication by 90% indicating that it may play an important role in the replication of the virus [230].

1.8 RNA Binding proteins

RNA binding proteins play an essential role in several biological processes e.g. mRNA splicing, mRNA export, translation, RNA stability and mitochondrial gene expression. RNA binding proteins are mainly classified according to RNA binding domain these include; zinc- finger proteins, dsRBD proteins and RNA helicases. RNA helicases are RNA binding proteins that belong to the DEAD box family, these proteins are involved in several cellular processes, including mRNA splicing, ribosomal assembly, initiation of translation, and RNA transport [231-234]. RNA helicases are thought to be involved in most if not all ATP-dependent processes involving rearrangement of structured RNAs. They are RNA-dependent ATPases with high degree of similarity to DNA helicases [235]. RNA helicase activity has been detected in several viral proteins including the NS3 protein of hepatitis C virus [30], and the NS3 (p80) protein of the pestivirus bovine viral diarrhoea virus BVDV [236].

It is reported that the related NS3 of Kunjin and yellow fever virus as well as pestiviruses such as bovine viral diarrhea virus are involved in formation of infectious viral particles. The mechanism of how NS3 is involved is still unclear, but there is evidence that the helicase domain is important. However, there is genetic evidence for the involvement of two major subdomains of HCV NS3 helicase in the early steps of the assembly process, and this study revealed that the helicase is engaged in particle assembly in a way which is independent of its associated enzymatic activities in a similar way to YFV NS3[237]. The role of HCV NS3 RNA helicase in assembly occurs after interaction of NS5A and NS3 with core protein decorating intracellular lipid droplets and prior to the formation of dense intracellular core protein and non-structural viral protein complexes. It is believed that the role of NS3 in assembly may involve critical interactions of the NTPase and RNA binding domains with one or more other viral or cellular proteins. However, the na-
ture of NS3 interactions and the identity of their binding partners is still under investigation [96]. Host encoded RNA helicases are also involved in HCV replication, for instance, DDX3, a DEAD-box RNA helicase was shown to bind to the HCV core protein [238]. A knockdown study reported the requirement of DDX3 in the HCV replication cycle [239].

1.9 Innate immune responses to HCV infection

Innate immune responses to infected hepatocytes are recognised by cellular proteins known as pattern recognition receptors (PRRs), which contain three major classes of receptors: Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and nucleotide-binding oligomerization domain-like (NOD-like) receptors. Viral interactions with TLRs and RLRs initiate signalling cascades, which leads to the activation of latent transcription factors, including IFN regulatory factors (IRFs) and nuclear factor-κB (NF-κB), and which accordingly culminates in the induction of IRF target genes, Type I IFN, and pro-inflammatory cytokines. TLRs are expressed in endosomes and on the cell surface membrane, and detect viral pathogen associated molecular patterns (PAMPs) in various immune cells, such as macrophages, dendritic cells, B cells, and some types of T cells [240, 241]. Amongst the eleven members of the human TLR family, TLR 2, TLR 3, TLR 4, TLR 7, TLR 8, and TLR 9 are involved in detection of viral PAMPs, e.g. TLR9 recognises unmethylated CpG DNA. TLR3 recognises dsRNA and TLR7/8, recognise ssRNA either on the surface of the cell or most likely in intracellular endocytotic vesicles [240]. The TLRs consists of three family members; retinoic acid inducible gene-I (RIG-I), melanoma differentiation associated gene 5 (MDA5) and LGP2 (Laboratory of Genetics and Physiology 2). RLRs are expressed in the cytoplasm of most cells, including hepatocytes. All RLRs contain 2 N-terminal caspase and recruitment domains (CARD). In addition, all RLRs carry a DExD/H RNA helicase domain and bind to RNA ligands. In this regard, RIG-I has been shown to be important in detection of ssRNA viruses, such as picornaviruses, flaviviruses, orthomyxoviruses, and rhabdoviruses, whilst MDA5 is recognised as essential in the detection of picornavirus, such as encephalomyocarditis virus [242, 243]. The host innate responses to HCV are triggered when conserved PAMPs are
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produced during infection. These are then recognised by TLR3 which recognises viral dsRNA in endosomes, and RIG-I which detects the polyuridine motif of the HCV 3’ UTR in the cytoplasm [244]. Signals from TLR3 stimulate IRF-3 and NF-kB through a MyD88-independent process, which requires the adaptor molecule Toll-IL1 receptor domain, containing adaptor-inducing IFN-β (TRIF). RIG-I, on the other hand, recruits the adaptor molecule IFN-β promoter stimulator protein 1 (IPS-1; also referred to as CARD adaptor interferon, CARDIF, virus-induced signalling adapter [VISA], and mitochondrial antiviral signalling protein [MAVS]). Both pathways result in downstream signalling and nuclear translocation of IFN regulatory factor 3 (IRF3) [244] which activates the expression of the IFN-β gene. The production of IFN-β from the infected cells drives autocrine and paracrine signalling process by binding to the IFN-α/β receptors of the infected cells and cells in local adjacent tissue. This leads to the activation of the JAK/STAT pathway, which results in the activation of interferon stimulated genes (ISGs), such as the OAS1/RNase L system, which degrades viral RNA, and the RNA-specific ADAR1, which converts adenosine residues to inosine residues in dsRNA strands, thus mutating and destabilising viral RNA secondary structures. ISGs also include P56 and PKR, both of which inhibit viral and host RNA translation [244].

1.10 Strategies of HCV to evade immune response

HCV uses several strategies to regulate and escape the host response. The NS3/4A protease blocks IRF-3 activation by inhibiting the host RIG-I network by the protease-dependent disruption of CARD-homology domain signalling, which is induced during RIG-I binding to the HCV RNA PAMP ligand. Moreover, NS3/4A also blocks RIG-I signalling to ablate virus activation of NF-kB. This indicates that NS3/4A inhibits IRF-3 and NF-kB by targeting common factors involved in both IRF-3 and NF-kB activation. The NS3/4A protease also targets TLR3 signalling by cleaving the TRIF adaptor protein. NS3/4A cleavage of TRIF blocks TLR3 signalling, thus inhibiting IRF-3 and NF-kB activation and preventing IFN production [245]. The HCV core protein is also involved in HCV evasion of innate immune responses. Over-expression of the core protein in the cell culture interferes with JAK/STAT signalling and ISG gene activation by
inhibiting STAT 1 activation, through the induction of SOCS3 an inhibitor of JAK/STAT pathway and protein phosphatise 2A (PP2A) and by inducing other inhibitory molecules.

Figure 1.10. Invasion of innate immune response by NS3/4 HCV protein NS3/4A eliminates antiviral signalling by cleavage of MAVS and TRIF [246].

This reduces the transcriptional activity of IRF-3 and inhibits its binding to IFN stimulated response elements. Moreover, HCV NS5A was also found to interfere with ISGs function by the inhibition of 2′-5′-oligoadenylate synthetase (2′-5′ OAS) and the induction of IL8, which subsequently inhibits ISG expression. NS5A also forms heterodimers with protein kinase R (PKR) [245] directly inhibiting its ability to switch of F protein translation. Production of dsRNA by HCV during infection will lead to the synthesis of IFN-β, which in turn leads to production of antiviral proteins known as ISGs. PAMP receptors RIG-I and TLR3 signal through kinases IKKe and TBK1 to activate IRF3 and cause the phosphorylation and degradation of IκB, which allows nuclear translocation of
NF-κB (Figure 1.10). HCV overcomes this response and establishes chronic infection through cleavage of MAVS and TRIF [246].

1.11 Adaptive immunity

Following infection, it is usually 8–20 weeks before HCV antibodies appear, subsequently remaining throughout the remaining course of the chronic infection. On the other hand, HCV-specific T cells, are usually identified between 5 and 9 weeks following infection [245]. HCV neutralising antibodies were first identified in an experimental infection targeting chimpanzees. The antibodies were found against HVR-1 epitope of HCV envelope glycoprotein E2. It has been found that the production of antibodies is at high titre, with cross-reactivity between HCV genotypes upon the progression of the infection to a state of chronicity. Nevertheless, the antibodies’ high titres are not successful in eradicating the virus at this stage with the emergence of virus quasispecies. Furthermore, the frequency of IgG-secreting B cells and the total concentration of IgG are also higher in chronic Hepatitis C, although the majority of the B cells that secrete the IgGs, as well as the IgGs themselves, are not specific to HCV; thus, suggesting that B cells are stimulated by HCV in a B cell-receptor-independent way [245]. This is supported by other findings, which highlight that CD27$^+$ B cells of HCV-infected patients show greater conversion into IgG secreting cells, in addition to a lesser degree of rapid apoptosis and proliferation [245].

The clearance of HCV keenly relies upon HCV-specific T cells [245], with the decline of viral titre beginning with the presence of HCV-specific T cells and the expression of IFN-$\gamma$ in the liver, thereby highlighting the fact that viral clearance is T cell-facilitated. However, it is important to emphasize that it is not yet known whether or not IFN-$\gamma$ levels have a strong link to HCV clearance and ultimately, it may be merely a marker for other T cell functions [245].

During the phases of the production of HCV-specific immune responses, HCV-specific CD4$^+$ T cells are critical. During the process of ALT elevation and clinical presentation, HCV-specific CD4$^+$ T cells, with associated IFN-$\gamma$ and IL-2 production, vigorously pro-
liferate, and this can easily identified in the blood of patients who subsequently recover by eradication of the virus. On the other hand, however, HCV-specific CD4\(^+\) T cell responses are seen to be either weak or entirely lacking amongst those individuals who go on to develop chronic infection. Moreover, there has been a link established between recurrent viremia occurring many months following the perceived control of the virus and the loss of initially strong CD4\(^+\) T cell responses [245]. When comparing CD8\(^+\) T cells with HCV-specific CD4\(^+\) T cells, the former are known to be identifiable in the blood of those patients severely infected, irrespective of the virological outcome. Throughout the process of acute HCV infection, it has been seen that CD8\(^+\) T cells appear paralyzed, show reduced or damaged proliferation and IFN-\(\gamma\) production, and with the presence of cytotoxicity and due to increased levels augmented degrees of programmed death–1 (PD-1) protein [245].

It is not yet known whether the paralysis of the phenotype is a state induced through a viral factor, or whether such a situation derives as a result of a natural progression in terms of the migration and/or maturation of HCV-specific CD8\(^+\) T cells. Importantly, throughout this stage of the infection, antiviral therapy causes a significant and rapid decay of the responses of CD8\(^+\) T cells, thereby suggesting that the majority of HCV-specific CD8\(^+\) T cells are antigen-dependent, short-term cells as opposed to being self-sustaining memory T cells. Nevertheless, HCV-specific CD8\(^+\) T cells’ dysfunction ultimately resolves, with the detection of IL-7 receptor \(\alpha\)-positive (i.e., CD127\(^+\)) memory CD8\(^+\) T cells becoming possible upon the development of HCV-specific CD4\(^+\) T cell responses and the decline of HCV titre [245]. In further support of this finding, the decline of CD4\(^+\) T cells in vivo in the instance of HCV-recovered chimpanzees repeals protective CD8\(^+\) T cell–mediated immunity during the case of re-challenge, thereby suggesting that there is the need of help from CD4\(^+\) T cell concerning the generation and subsequent maintenance of protective CD8\(^+\) T cells [245].

### 1.12 Current HCV therapy

The standard current therapies consist of (peglyated) interferon alpha and the nucleoside analogue ribavirin for a period of 24-48 weeks. However, many HCV infected patients do
not achieves sustained virological responses with this therapy which is also associated with serious side effect. Moreover, the effect of this treatment is virus genotype specific [247, 248]. Interferon alpha induces the host to initiate innate immune responses. Ribavirin is a guanosine analogue that acts against several RNA and DNA viruses. Several mechanisms have been proposed for the mechanism of action ribavirin, including inhibition of inosine monophosphate dehydrogenase (IMPDH) by ribavirin 5’–monophosphate, inhibition of the HCV polymerase activity by ribavirin 5’-triphosphate or induction of a so called error catastrophe as the result of the accumulation of a lethal number of mutations in viruses that have been cultured with ribavirin. [249]. In yellow fever virus (YFV), a flavivirus with similarity to HCV, ribavirin may inhibit inosine monophosphate dehydrogenase leading to depletion of intracellular GTP pool. However, it is still unclear whether in HCV ribavirin acts only by inhibiting IMPDH, as there is some evidence that ribavirin may modulate the Th1 response [249].

The regimen recommended by NICE (National institute for Health and Clinical Excellence) in the UK for patients with genotype 1 or 4, was 180µg of peglyated interferon alpha 2a (Roferon, Roche) per week plus 1000-1200 mg ribavirin per day for 48 weeks. Patients infected with genotype 2 or 3 were advised to be treated with 180µg of peglyated interferon alpha 2a (Roferon, Roche) per week plus 800 mg ribavirin per day for 24 weeks.

However, adverse events have been reported with this treatment such as flu-like symptoms, depression and nausea, often leading patients to decline or stop treatment. With this combination of treatment a sustained virological response (SVR) will be achieved in 40-50% of those with genotype 1 or 4 and in 80% of those with genotype 2 or 3 [250]. The NS3 protein is one of the most intensively studied targets for new anti-HCV treatment. The HCV NS3 protease domain together with the cofactor NS4A forms a heterodimeric protease, which cleaves the viral polyprotein into single functional proteins. Although the NS3/4A protease is analogous to trypsin, a serine protease, the substrate specificity is different from that of host cell proteases. The substrate binding region of NS3 protein is considered to be shallow or flat, which makes it difficult to design selective inhibitors. HCV persistence depends on the inhibition of host immune response. It has been shown that HCV inhibits phosphorylation and effector action of interferon regulatory factor -
3(IRF-3), a key cellular antiviral signaling molecule. Interference of NS3/4A function by mutation or peptidomimetic inhibitor relieved this blockade and restored IRF-3 phosphorylation after cellular challenge with unrelated virus. Moreover, dominant-negative or constitutively active IRF-3 mutants, respectively, enhanced or suppressed HCV RNA replication in hepatoma cells, therefore, NS3/4A protease can be targeted in two ways, the inhibition of which may both block viral replication and restore IRF-3 control of HCV infection [251]. The macrocyclic peptidomimetic inhibitor BILN 2061 (also known as Culoprevir Boehringer Ingelheim), is a non covalent reversible complex forming serine protease inhibitor. The protease inhibitor activity of this compound has been demonstrated in a HCV subgenomic replicon cell model, and it was found that the polyprotein processing was inhibited at all NS3-dependent cleavage sites. Based on these results, BILN 2061 was the first HCV protease inhibitor to enter clinical trials. After oral administration of this compound the patients showed decline in HCV titers within hours. Also, the efficacy of BILN 2061 is higher against genotype 1 than genotype 2 or 3. On the other hand, it was found that replicons containing single mutations in the NS3 protease gene (A156V or D168V), showed high resistance levels to BILN 2061.

However, BILN 2061 was shown to be cardiotoxic, therefore the development of this compound was halted [249]. VX-950 (Telaprevir) is another peptidomimetic inhibitor of the NS3 viral protease; it forms a covalent reversible complex with HCV NS3/4A by a slow binding and slow dissociation mechanism with a half life of about 1hr, which may confer a sustained inhibitory antiviral effect. Moreover, VX-950 shows good activity against HCV genotype 2.

Several other related HCV protease inhibitors are under study in clinical trials including VX-500 (phase I), VX-813 (phase I), MK-7009 (phase II) [249]. Polymerase enzymes are also considered another target for anti-viral drugs. Several nucleoside inhibitors have been used as inhibitors for HCV (NS5B) RNA-dependent RNA polymerase enzyme for instance; NM-283 (Valopicitabine), a nucleoside analogue. It is a prodrug of the nucleoside analog 2’-C methylcytidine; the compound undergoes intracellular phosphorylation of the 5’-triphosphate which results in the formation of the active inhibitor of the viral polymerase. Oral administration of Valopicitabine 800mg daily for 2 weeks results in a dose dependent decline in HCV RNA in patients infected with HCV genotype-1.
nation of Valopicitabine with interferon resulted in a further decline of detectable viral load, and absence of viral RNA [249]. Other selective inhibitors are also being considered for instance; Non-nucleoside inhibitors such as benzothiadiazines, benzimidazoles/diamides. However, a number of treatments are also in development targeting the host factors required for virus replication these include cyclophilin inhibitors SCY-635(Scynexis) and DEBIO-25 (Debio) which are still under clinical trials [249].

1.13 Vaccination

Several approaches have been used to produce vaccines against HCV. Among the earliest attempts HCV peptide-based vaccines with different adjuvants have been used to induce Th1 and cytotoxic T cell response in chronic HCV patients. One of these is IC41, which consists of 5 conservative peptides from HCV core, NS3 and NS4 proteins, which are conserved within genotypes 1 and 2, and includes 4 known HLA-A2 epitopes and 3 promiscuous CD4\(^+\) epitopes. However, the clinical trials showed that the response was not sufficient to clear the viral load [252]. Another vaccine candidate which was delivered by attenuated virus vectors induced effective CD4\(^+\) and CD8\(^+\) T cell responses. Modified virus of Ankara is one of the vectors, a highly attenuated poxvirus strain; it has been used in several vaccine designs, such as HIV, tuberculosis, colorectal cancer, and melanoma. Vaccines based on MVA vector expressing HCV antigens including NS3, NS4, and NS5B have been shown to induce IFN-\(\gamma\)-secreting CD4\(^+\) T cells and specific CD8\(^+\) T cells capable of secreting IFN-\(\gamma\) and killing in vitro and in vivo when tested in transgenic mice [252]. A recent study using adenovirus based HCV protein expression showed T-cell responses against CD4\(^+\) and CD8\(^+\) cell subsets, which recognized HCV genotypes 1a and 3a. Moreover, the T cell response was found to be sustained for at least 12 months. These findings indicate that adenovirus vector strategy can induce a sustained T cell response of a magnitude and quality associated with protective immunity and may open the way for studies of prophylactic and therapeutic vaccines for HCV [253].
1.14 HCV cell culture systems

Several attempts have been made to propagate HCV virus in cell culture. Replication was detected in hepatoma, B and T cell lines, primary cultures of human and chimpanzee hepatocyte. However, the replication titers were very low and could not be detected by stringent RT PCR and thus were not amenable for further HCV replication studies [254]. The first subgenomic replicons described were based on genotype 1b HCV (an isolate called Con1) [255]. Subsequently new replicons were developed using a genotype 1a HCV consensus clone called H77 [256]. The subgenomic replicon consist of HCV 5’ UTR (containing the IRES) to initiate RNA translation of neomycin resistance gene (*neo*) gene for antibiotic selection. The replicon also contains the coding sequence of the HCV non-structural proteins, from NS3 to NS5B under the translational control of an EMCV (Encephalomyocarditis virus) IRES. The 3’ UTR is also included in the replicon. During the development of HCV replicons, it was found that mutations are required for sufficient levels of replication. Adaptive mutations were found in NS3 and NS5A. The function of these mutations was to enhance HCV polyprotein cleavage, and HCV interaction with cellular components of the replicase complex or by inhibiting the cellular anti-viral defence [256]. In 2003 a genotype 2a clone isolated from a patient with fulminant hepatitis designated as JFH-1, was found to replicate without the requirement of adaptive mutations and transfecting JFH-1 clone into Huh-7 cells was found to produce virus (designated as HCVcc for cell derived HCV) that is infectious for Huh-7 cells and allowing the study of the full replication cycle of HCV [257, 258].

1.15 Methods of detecting RNA-protein interaction in vitro

Proteins interact with RNA through electrostatic interactions, hydrogen bonding and hydrophobic interactions. Protein-RNA interactions are dependent on the secondary and tertiary structure of the RNA molecule, which means both RNA and protein, must be folded correctly to permit precise binding. Several approaches can be used to detect protein RNA interactions which all have advantages and disadvantages in their application.
1.15.1 RNA Electrophoretic mobility shift assay

Electro mobility shift assay (EMSA), or simple gel shift assay, is a sensitive technique used to detect Protein-RNA interactions. In EMSA, a $^{32}$P–labeled or biotinylated nucleic acid fragment containing a specific DNA or RNA sequence is incubated with a target DNA or RNA binding protein. The protein–nucleic acid complexes are then separated from unbound nucleic acid by electrophoresis through non-denaturing polyacrylamide gel. The protein- nucleic acid complexes migrate more slowly than a free DNA/RNA probe through the acrylamide gel. EMSA has a number of advantages. The technique is simple to perform; it can be used either with radioisotope or biotin labeled nucleic acid, with a wide range of nucleic acid size and structures. On the other hand, there are limitations for this assay such as Protein-DNA/ RNA complexes that are not at chemical equilibrium during the electrophoresis step, so that some complexes may dissociate rapidly during electrophoresis which prevents detection of the complex. Moreover, EMSA does not provide detailed information about the location of the nucleic acid sequences that are bound by proteins [259].

1.15.2 RNA Pull-down assay

RNA pull down assays selectively extract RNA-protein complexes from a cellular extract. The protein is expressed as a fusion protein. An RNA probe can be biotinylated, then the complex extracted from a cell lysate and than purified using magnetic beads or agarose, then evaluated by Northern blotting or through RT-PCR , whereas the protein can be detected by Western blotting or mass spectrometry [260].

1.15.3 Fluorescent in situ hybridization co-localization

Fluorescent in situ Hybridization (FISH/ISH) co-localization techniques can be used to detect both an RNA transcript and a protein of interest using RNA probes and antibodies. FISH/ISH detects the position and abundance of a RNA and protein in a cell or tissue sample. The reaction can be visualized by microscopy and a co-localized signal for both the RNA and protein of interest to indicate possible complex formations. A labeled RNA probe must be generated for detection of a particular sequence of RNA and the protein
may be detected using antibody staining or by using fluorescent tagged proteins [261] [262].

1.16 Hypothesis and Aims

NS3 HCV has protease RNA helicase and NTPase activities that are important for HCV replication. RNA helicases in other viruses have been found to interact with RNA and also have RNA chaperone activities and play a role in virus assembly. This project will investigate the hypothesis that HCV NS3 helicase also possesses these activities, by exploring the interaction of NS3 with viral RNA, and viral and host proteins. In addition, the role of the NS3 protein in regulating the expression of innate immune response genes.

- To investigate whether there is an interaction between the recombinant HCV NS3-1a with the full length HCV JFH-1 RNA and the 5’ end of JFH-1.

- To investigate the effect of the gene silencing of four host proteins RAB27B, RAB40B, TXNIP and Staufen 1 on HCV NS3 expressed using adenovirus vectors, and whether they have a direct role in gene expression.

- To investigate the effect of the HCV NS3 protease inhibitor BILN 6021 on NS3 from different HCV genotypes expressed in adenovirus vector and the effect on RIG-I, MDA5 and MAVS.
MATERIALS & METHODS
2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>BDH</td>
</tr>
<tr>
<td>Agarose</td>
<td>Bioline</td>
</tr>
<tr>
<td>Ammonium persulphate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Sigma</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Boric acid</td>
<td>BDH</td>
</tr>
<tr>
<td>Buffered phenol</td>
<td>Sigma</td>
</tr>
<tr>
<td>CHAPS</td>
<td>A.G.Scientific,INC</td>
</tr>
<tr>
<td>Chloroform</td>
<td>BDH</td>
</tr>
<tr>
<td>DABCO (1,4-diazabicyclo-[2,2,2]-octane)</td>
<td>Sigma</td>
</tr>
<tr>
<td>DAPI (4’-6-Diamidino-2-phenylindole)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Diethylpyrocarbonate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Dimethyl sulphoxide</td>
<td>Sigma</td>
</tr>
<tr>
<td>DTT</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>EDTA</td>
<td>BDH</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Analar, Normapur</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>Analar, Normapur</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Analar, Normapur</td>
</tr>
</tbody>
</table>
Materials and Methods

- Glacial acetic acid: BDH
- Hydrochloric acid: BDH
- IPTG: CHALBIOCH
- Imidazole: Sigma
- Isopropanol: BDH
- Magnesium chloride: BDH
- Methanol: BDH
- MOPS: Sigma
- Mowiol 4-88: Sigma
- Neutral red: Sigma
- RNA Zap: Ambion
- Sodium dodecyl sulphate (SDS): BDH
- Sodium di hydrophosphate: BDH
- Sodium hydroxide: BDH
- Sodium chloride: BDH
- N,N,N”,N”-tetramethyl-ethylenediamine: Sigma
- Tris base: BDH
- Triton-X: Invitrogen
- Tween 20: Sigma
- Urea: Sigma
2.1.2 Tissue culture materials and reagents

Dulbeco’s modified Eagle’s medium (Invitrogen)  
DMEM growth medium supplemented with 2 mM L-glutamate,  
10% fetal calf serum (FCS), 100  
IU/ml penicillin and 100µg/ml streptomycin.

Fetal calf serum (FCS) (Invitrogen)  
Inactivation of complement in FCS,  
by incubation 56ºC for 60 minutes.

Trypsin – EDTA (TE) (Invitrogen)  
1X (TE-EDTA)

Penicillin-streptomycin (Gibco, Invitrogen)  
10,000 units penicillin, 1,000µg  
streptomycin.

2.1.3 Reagents used for siRNA, RNA extraction and miscellaneous as-
says

Lipofectamine 2000  
Invitrogen

Opti-MEM  
Invitrogen

Geneticin (50mg/ml)  
Invitrogen

Proteinase K solution  
Invitrogen

poly (I:C) (polyinosinic-polycytidylic acid dsRNA)  
Sigma  
10µg/ml

Trizol  
Invitrogen
Ni-NTA agarose  
Qiagen

Bradford Reagent  
Sigma

Nuclease- free water  
Ambion

Mung bean nuclease (10u/µl)  
New England Biolab

2.1.4 Buffers and solutions

All solutions were prepared with Milli Q water unless otherwise stated. For pH calibration, a pH meter 3320 (Jenway) was used.

Agarose 4%  
4g of agarose was dissolved in milli Q water to give a final volume of 100 ml. The solution was sterilized by autoclaving at 121°C for 20 minutes. Before use the solution was prewarmed at 65°C in a water bath.

DEPC water  
Milli Q water was treated with 0.1% v/v diethylpyrocarbonate (DEPC) (Sigma) for 24 hours at 37°C and then autoclaved (at least 15 min) to inactivate traces of DEPC.

Buffer A  
50 mM NaH$_2$PO$_4$, [pH8.0] 300 mM NaCl, 8 M Urea.

Buffer B  
50 mM NaH$_2$PO$_4$, [pH8.0] 300 mM NaCl, 8 M Urea, 20 mM imidazole.

Buffer C  
50 mM NaH$_2$PO$_4$, [pH8.0] 300 mM NaCl, 8M
Materials and Methods

Buffer D

Urea, 100 mM imidazole.

50 mM NaH$_2$PO$_4$, [pH 8.0] 300 mM NaCl, 8 M Urea, 250 mM imidazole.

10X TBE

890 mM Tris base

890 mM Boric acid

20 mM EDTA [pH 8.0]

1X TE

10 mM Tris-HCl

1 mM EDTA [pH 8.0]

Mounting medium with DAPI

2.4 g of Mowiol 4-88 was added to 6 g of glycerol and mixed well with a magnetic stirrer. 6 ml of milli Q water was added to the mixture and left at room temperature until dissolved. 12 ml of 0.2 M Tris-Cl [pH 8.5] was added to the mixture and heated to 50°C for 10 min. After the Mowiol had dissolved, it was then clarified by centrifugation at 3000 g for 15 minutes. 0.72 g DABCO 1,4-diazabicyclo[2.2.2] octane and 0.1% 4, 6-diamidino-2-phenylindole, dilactate was added to the medium. Aliquoted and stored at −20°C.

10% Neutral red stock

1 mg neutral red dye in 10 ml PBS.

Protein refolding buffer

25 mM Tris-Cl [pH 7.2], 10% (v/v) glycerol, 50 mM NaCl, 1 mM DTT, 0.1% CHAPS.

Resuspending buffer

8 M urea in PBS, 1 mM DTT.

20 x SSC buffer

175.3 g of NaCl, 88.2 g of sodium citrate, adjusted to pH [7.0], sterilized by autoclaving.
2.1.5 Bacterial growth media and reagents

Agar
Bacto-agar added to 2% (w/v) to required medium.

Luria broth (LB)
1% (w/v) tryptone, 0.5 (w/v) yeast extract, 1% (w/v) NaCl.

SOC medium
2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$, 20 mM glucose.

Ampicillin stock 1000x
1 g of ampicillin was dissolved into 10 ml of milli Q water, then sterilized by using a 0.22 µm filter (Sterillin). Then aliquot and store at -20°C.

IPTG stock
1M IPTG dissolved in water, sterilized using 0.22 µ filter. Stored 1ml aliquots at -20°C.

LB freezing solution
LB broth with 50% glycerol (sterilized).

Lysozyme stock solution
50 mM Tris-HCL, [pH 8.0], 10mg/ml lysozyme.

2.1.6 SDS-PAGE

Coomassie blue statin
0.1% Coomassie R 250, 10% acetic acid (v/v), 40% (v/v) methanol.

Destaining buffer
12.5% (v/v) Isopropanol and 10% (v/v) glacial acetic acid in Milli Q water.

Protein lysis buffer
10 mM Tris-HCl
150 mM NaCl
0.5% Nonidet-P40
Materials and Methods

Halt Protease Inhibitor Cocktail 1mM, 800nm Aprotinin, 50 µM Bestatin, 15µM E64, 20µM Leupeptin, 10µM Pepstatin A).

SDS-PAGE separation gel 30% (w/v) acrylamide, 0.8 % (w/v) bis-acrylamide (National Diagnostics) 1.5M Tris-HCl, [pH8.8], 0.1% (w/v) Sodium dodecyl sulphate (SDS) 0.1% (w/v) ammonium persulphate (APS) 0.5% (v/v), 0.5% (v/v)N,N,N”,N”-tetramethyl-ethylenediamine (TEMED).

SDS-PAGE stacking gel 30% (w/v) acrylamide, 0.8 % (w/v) bis-acrylamide (National Diagnostics), 1M Tris-HCl, [pH 6.5] 0.1% (w/v) SDS 0.1% (w/v) ammonium persulphate (APS) 0.5% (v/v)N,N,N”,N”-tetramethyl-ethylenediamine (TEMED).

SDS-PAGE running buffer (10x) 25 mM Tris, 1.92 M Glycine, 3.5 mM SDS (National Diagnostics).

2.1.7 Western blot reagent

Ammonium persulphate (APS) 10% (w/v) 0.5 g of APS dissolved in 5ml of water, aliquoted and stored at -20°C

10x Tris–EDTA reducing buffer Prepared by adding: 10% (v/v) Beta mercaptoethanol, 0.5 M Tris HCl, 8% (w/v) SDS. Aliquots and stored -20°C.
Materials and Methods

Bromophenol- blue/glycerol
0.02% Bromophenol blue in EDTA = 5ml
5 ml glycerol + 5 ml bromophenol blue.

Transfer Buffer
25 mM Tris, 192 mM Glycine, 20% (v/v) Methanol

PBS/Tween buffer solution
1x PBS/0.1% (v/v) Tween

PBS/Tween/skimmed milk
1x PBS/0.1% (v/v) Tween/2% (w/v), 4% (w/v) skimmed milk powder.

PBS/Tween/BSA
1x PBS/0.1% (v/v) Tween/ 2% (v/v) 1% (w/v) BSA.

2.1.8 Molecular biology kits

Qiaprep spin miniprep  Qiagen

T7 & SP6 MEGAscript kit  Ambion

ECL western blotting detection reagents and analysis system  Thermo

Quantitect SYBER Green PCR  Qiagen

Quantitect SYBER Green RT-PCR  Qiagen

5’ EndTag Nucleic Acid Labeling System  Vectorlab

3’ EndTag Nucleic Acid Labeling System  Vectorlab

UltraSNAP Detection Kit for Nucleic Acid Blots  Vectorlab

Wizard SV Gel and PCR clean up system  Promega

RNAeasy mini kit  Qiagen
2.1.9 Mammalian cell lines

<table>
<thead>
<tr>
<th>Cells</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Huh 7 cell line</td>
<td>Differentiated human hepatoma cell line established from a hepatocellular carcinoma. Huh7 cells were a gift from John Monjardino.</td>
</tr>
<tr>
<td>293 A cell lines (Invitrogen)</td>
<td>293A Cell line is a sub-clone of the cell line 293 which established from primary embryonal human kidney transformed with sheared human adenovirus type 5 DNA. The genes encoded by the E1 region of adenovirus (E1a and E1b) are expressed in these cells and participate in the some viral promoters, allowing these cells to produce very high levels of protein. E1 also complements the E1-deletion in recombinant adenoviral vectors, allowing viral replication.</td>
</tr>
</tbody>
</table>

Table 2.1. Mammalian cell lines

2.1.10 Flow cytometry reagents

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FACS staining buffer</td>
<td>PBS, pH 7.4-7.6, 2% (v/v) heat inactivated FCS, 0.2% (w/v) sodium azide.</td>
</tr>
<tr>
<td>Formaldehyde buffer (Fixation buffer)</td>
<td>14 ml of 37.5% formaldehyde, 57.2 ml PBS [pH 7.4].</td>
</tr>
<tr>
<td>Permeabilization (PERM) Buffer</td>
<td>FACS Staining + 0.5% saponin</td>
</tr>
<tr>
<td>SUPERPERM buffer</td>
<td>75% (v/v) PERM buffer+ 25% (v/v) filtered).</td>
</tr>
</tbody>
</table>
2.1.11 Oligonucleotides

All oligonucleotides were synthesised by Invitrogen. The stock primers were dissolved in nuclease free water at a concentration of 100 μM and stored at -20ºC.

2.1.11.1 Primers used for adenovirus quantification

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Position</th>
<th>bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS3-2a qRT-PCR sense</td>
<td>GGCGCCATAGTGGTGAGTAT</td>
<td>3473-3492</td>
<td></td>
</tr>
<tr>
<td>NS3-2a qRT-PCR antisense</td>
<td>TCCGTGGTAAACAGTCCACA</td>
<td>3585-3604</td>
<td></td>
</tr>
<tr>
<td>NS3-1a qRT-PCR sense</td>
<td>AGGGCTACAAGGTTGTTGTG</td>
<td>4081-4100</td>
<td></td>
</tr>
<tr>
<td>NS3-1aqRT-PCR antisense</td>
<td>GGTGCCAGTGGGTAATTGTT</td>
<td>4199-4209</td>
<td></td>
</tr>
<tr>
<td>NS3-1b qRT-PCR sense</td>
<td>ATGGCGCCTATTACGGCCTA</td>
<td>3424-3437</td>
<td></td>
</tr>
<tr>
<td>NS3-1bqRT-PCR antisense</td>
<td>ACACACGCCATTGAGCAG</td>
<td>3557-3576</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2. Primers for adenovirus quantification

2.1.11.2 DNA primers for qRT-PCR

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH sense</td>
<td>5’TCTCTGCTCCTCTCCGTGAAC’</td>
</tr>
<tr>
<td>GAPDH antisense</td>
<td>5’CGGATTTGGTCGTATTGGG3’</td>
</tr>
<tr>
<td>JFH-1 sense</td>
<td>5’TCTGCAGAACCAGGTGAGTA3’</td>
</tr>
<tr>
<td>JFH-1 antisense</td>
<td>5’TCAGGCACTACCACAAGGC3’</td>
</tr>
<tr>
<td>RIG-I sense</td>
<td>5’GGAAGAGGTGCAUAUAUU3’</td>
</tr>
<tr>
<td>RIG-I antisense</td>
<td>5’AAUAUAUCUGCACUCUCUCC3’</td>
</tr>
<tr>
<td>MDA5 sense</td>
<td>5’GGUGAAGAGCGAUAUCAG3’</td>
</tr>
<tr>
<td>MDA5 antisense</td>
<td>5’CUGAAUCUGCUCCCUCC3’</td>
</tr>
</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAVS</td>
<td>5’UAGUUGAUCUCGCAGACGA3’</td>
</tr>
<tr>
<td>MAVSantisense</td>
<td>5’UCGUCCGCGAGAUCAACUA3’</td>
</tr>
<tr>
<td>RAB 40B sense</td>
<td>5’CATCTCAGATCCCCGTTCC3’</td>
</tr>
<tr>
<td>RAB 40B antisense</td>
<td>5’CAAGAGCTTCATGCACATCC3’</td>
</tr>
<tr>
<td>RAB 27B sense</td>
<td>5’AATCTGACACCTGCTCTCCC3’</td>
</tr>
<tr>
<td>RAB27B antisense</td>
<td>5’AGAAATGTGTCTTCCCCACC3’</td>
</tr>
<tr>
<td>TXNIP</td>
<td>5’TAAAAGCCCCTTTGAAACCC3’</td>
</tr>
<tr>
<td>TXNIP antisense</td>
<td>5’GCTCAGTGCTCTGACACAGG3’</td>
</tr>
<tr>
<td>Staufen 1 sense</td>
<td>5’GCTCACGCTAGACACATGGG3’</td>
</tr>
<tr>
<td>Staufen 1 antisense</td>
<td>5’GGTCACGCTGAGTAGGAAGC3’</td>
</tr>
</tbody>
</table>

Table 2.3. DNA primers for qRT-PCR
2.1.12 siRNA molecule sequences

All siRNAs were purchased from Ambion, except for the HCV, Non-Targeting 1 and was purchased from Sigma.

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sense strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAB27 B sense</td>
<td>5’GACUUAUAUCAUGAAGCGAAAdTdT3’</td>
</tr>
<tr>
<td>RAB40 B sense</td>
<td>5’CGCUGGGUCUUUGACGCAAdTdT3’</td>
</tr>
<tr>
<td>Stufen 1 sense</td>
<td>5’GAACGAAUUUGUAUCUCUAdTdT3’</td>
</tr>
<tr>
<td>TXNIP sense</td>
<td>5’CAUCCUUCAAAGGAAAAAUAdTdT3’</td>
</tr>
<tr>
<td>Non-Targeting 1</td>
<td>5’ GAUCAUACGUGCGAUCAGAdTdT3’</td>
</tr>
</tbody>
</table>

Table 2.4. siRNA molecule sequences

2.1.13 Restriction digestion enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>Incubation conditions</th>
<th>Conc./dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td>Promega</td>
<td>Buffer H 37°C (10x)</td>
<td>1µl (10u/µl)</td>
</tr>
<tr>
<td>ApaI</td>
<td>New England Biolab</td>
<td>Buffer 3 37°C (10x)</td>
<td>1µl (10u/µl)</td>
</tr>
<tr>
<td>BamHI</td>
<td>Promega</td>
<td>Buffer E 37°C (10x)</td>
<td>1µl (10u/µl)</td>
</tr>
<tr>
<td>NdeI</td>
<td>New England Biolab</td>
<td>buffer 4 37°C (10x)</td>
<td>1µl (10u/µl)</td>
</tr>
<tr>
<td>XbaI (HC)</td>
<td>Promega</td>
<td>Buffer D 37°C (10x)</td>
<td>1µl (100u/µl)</td>
</tr>
</tbody>
</table>

Table 2.5. Restriction digestion enzymes
2.1.14 Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Supplier</th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-V5</td>
<td>Mouse IgG</td>
<td>Invitrogen</td>
<td>IF: 1:300, WB: 1:1000, FC: 1:300</td>
</tr>
<tr>
<td>Anti-NS3</td>
<td>Mouse IgG</td>
<td>Virogen</td>
<td>IF: 1:100, WB 1:500</td>
</tr>
<tr>
<td>Alexa Flour 488 donkey anti-mouse IgG</td>
<td>Mouse IgG</td>
<td>Invitrogen</td>
<td>IF, FC: 1:1800</td>
</tr>
<tr>
<td>Alexa Flour 594 donkey anti-mouse IgG</td>
<td>Mouse IgG</td>
<td>Invitrogen</td>
<td>IF, FC: 1:1800</td>
</tr>
<tr>
<td>Anti IgG2a Isotype control</td>
<td>Mouse IgG</td>
<td>Immunokontact</td>
<td>FC: 1:300</td>
</tr>
</tbody>
</table>


Table 2.6. Antibodies

2.1.15 Molecular weight markers and loading dyes.

<table>
<thead>
<tr>
<th>Ladder/Marker</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bench Mark protein ladder (Invitrogen) (Appendix 6.2.II)</td>
<td>Ladder for SDS-polyacrylamide gel electrophoresis, the ladder consists of 15 engineered proteins ranging in molecular weight from 10 to 220 kDa.</td>
</tr>
<tr>
<td>Pre-stained protein ladder (Invitrogen)(Appendix 6.2.1)</td>
<td>Ladder for SDS-PAGE, consists of 10 pre-stained protein bands in the range of 10-200 kDa.</td>
</tr>
<tr>
<td>Millenium RNA marker (Ambion) (Appendix 6.1)</td>
<td>Contains 10 discrete RNA transcripts of 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, and 9 kilobases.</td>
</tr>
<tr>
<td>Blue/Orange Loading Dye, 6X (Promega)</td>
<td>0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10mM Tris-HCl [pH 7.5] and 50 mM EDTA [pH 8.0].</td>
</tr>
</tbody>
</table>

Table 2.7. Molecular weight markers and loading dyes
2.1.16 Bacterial *E. coli* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM 109(DE3)</td>
<td><em>endA1, recA1, gyrA96, thi, hsdR17 (rK⁻, mK⁺), relA1, supE44, λ⁻, Δ(lac-proAB), [F’, traD36, proAB, lacI³ZΔM15], IDE3</em></td>
</tr>
<tr>
<td>DH5α</td>
<td><em>F⁻ Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK⁻, mK⁺) phoA supE44 λ⁻ thi-1 gyrA96 relA1</em></td>
</tr>
</tbody>
</table>

Table 2.8. Bacterial *E. coli* strains

2.1.17 Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJFH-1</td>
<td>Provided by Dr. Takaji Wakita Department of Virology, National Institute of Infectious Disease, Tokyo, Japan.</td>
</tr>
<tr>
<td>pPAW3</td>
<td>Constructed in Dr. Michael McGarvey laboratory, Department of Hepatology, Imperial College London (Wardell, 1999).</td>
</tr>
<tr>
<td>pJFH_1 IRES</td>
<td>Provided by Dr. Peter Karayiannis, Department of Hepatology, Imperial College London.</td>
</tr>
</tbody>
</table>

Table 2.9. Plasmids

2.1.18 X-ray films

Hyperfilm Blue film (GE healthcare) was used for Western blot and Electromobility shift assay.
2.2 Construction of non structural NS3 protein expression plasmid

The full length HCV NS3 [1027- 1657] protein was constructed by Wardell, 1999 [263].

2.2.1 Preparation of chemically competent cells

A starter culture was prepared by taking a loopful of glycerol stock of *E. coli* JM 109 (DE3) strain and streaked onto Luria agar (Invitrogen) plate. The next day, a single colony was inoculated into 5 ml of Luria Broth (Invitrogen) and incubated at 37°C for 16 hours, with shaking at 250 rpm. A 1ml aliquot of this culture was inoculated into 100 ml of LB and incubated at 37°C in orbital shaker, the cell density of the culture was measured using spectrophotometer CO 8000 Biowave, Biochrom. The spectrophotometer was blanked using LB broth. Every 30 minutes, a 1mL aliquot was sampled from the batch culture and transferred to a 10 mm cuvette, and the optical density was measured at 600 nm, until the OD$_{600}$ reached 0.4. The culture was cooled on ice for 10 minutes and centrifuged using a bench top centrifuge (Thermo scientific) for 10 minutes at 2,700 g at 4°C. The pellet was resuspended with 1.6 ml of ice cold 100 mM CaCl$_2$ and incubated on ice for 30 minutes followed by centrifugation at 2,700 g for 10 minutes at 4°C. The cell pellet was the resuspended with ice cold 100 Mm CaCl$_2$ containing 15% glycerol and incubated on ice for 20 minutes where then frozen at -80°C.

2.2.2 Transformation of chemically competent cells

Frozen competent cells were left on ice until just thawed and aliquots of 100 µl were transferred to fresh tubes. 56 ng of pAW3 DNA plasmid encoding NS3 protein in a volume of 6 µl, were added to the cells and placed on ice for 10 minutes. The cells were heat shocked at 42°C for 45 seconds in a water bath, thereafter; the tube was placed on ice for 2 minutes. 900 µl of SOC medium was added to the transformed cells and incubated for 60 minutes at 37°C with shaking at 225rpm. The transformation reaction was then diluted 1:10 and 1:100 and 100 µl of each dilution were plated on Luria agar plates containing 100 mg/ml ampicillin and incubated at 37°C for 16 hours.
2.2.3 pAW3 DNA purification by QIAprep spin miniprep Kit

pAW3 DNA was extracted with QIAprep miniprep Kit (Qiagen) from cultured cells according to the manufacturer protocol. One colony of *E. coli* containing a plasmid was inoculated in 5ml of Luria broth medium containing 100 mg/ml of ampicillin overnight at 37°C shaking at 225 rpm. The cells were then pelleted by centrifugation at 6,800g for 3 minutes at room temperature. The pellet was resuspended in 250 µl of resuspension buffer P1 and lysed with 250 µl P2 lysis buffer for 3 minutes. 350 µl neutralizing buffer P3 was added and centrifuged for 10 minutes at 13,000 g. The supernatant was applied to the DNA binding column and centrifuged for 30 seconds, then washed by adding 0.5 ml Buffer PB and centrifuged for 30 seconds. The column was then washed by adding 0.75 ml of buffer PE and centrifuged for 1 minute. An additional 1 minute centrifugation was used to remove residual wash buffer. Finally, the column was placed in a clean microcentrifuge tube and the pAW3 DNA was eluted by adding 50 µl of water.

2.2.4 Restriction enzyme mapping

All enzymes used for pAW3 DNA restriction digestion were used according to the manufacturers recommendations. Generally, in a total volume of 20 µl, up to 1 µg DNA was digested with 0.5-1µl of restriction enzyme for 2 hours at 37ºC with the appropriate buffer. Double digests were performed by using a one step reaction containing both enzymes. Enzymes are inactivated at a temperature recommended by the manufacturer. For large scale digestions the enzyme concentrations and volumes were increased.

2.2.5 Agarose gel electrophoresis

Agarose gels were prepared using a Horizontal gel electrophoresis apparatus. 1.0 % - 0.75% (w/v) agarose in 1X TBE-buffer was melted in a microwave oven. After cooling to approximetly to 65°C ethidium bromide was added to a final concentration of 0.5µg/ml and poured into the preassembled apparatus. Once the gel had set 1x TBE buffer was poured on the surface of the gel and the combs were removed to reveal the wells. The DNA sample was mixed with of 6x Orange/blue loading dye (Promega) in a volume of 10 µl , then the DNA samples were loaded in the wells along with 1kb DNA (Promega) (Appendix 6.1). The DNA was then resolved by gel electrophoresis according to their
size at 10 Volt per centimeter of gel. The DNA fragments were visualised on a UV transilluminator (GelDoc-It Image, UVP).

2.2.6 Testing for plasmid stability

Before induction cells were tested for plasmid stability. LB agar plates containing different additions were used in plating: (i) with 100mg/ml ampicillin, (ii) with 1mM IPTG, (iii) with both ampicillin and IPTG, or (iv) with neither added to the plate. Cells were grown in LB broth until they reached OD$_{600}$ 0.5. 10-fold serial dilution of cell suspension was made including a 10$^5$ and 10$^6$ dilution. Cells were plated at a dilution of 10$^5$ on IPTG containing plates and on ampicillin and IPTG containing plates. Whereas, cells at 10$^6$ dilution were plated on LB plates and on the ampicillin plates.

2.3 Protein methods

2.3.1 Recombinant protein overexpression

Recombinant proteins were over expressed using the JM109 (DE3) E. coli strain bearing the pET16b expression vector carrying the gene HCV NS3 pAW3 [1027-1657] fused with a histidine tag (Appendix 6.3). An overnight culture was used to inoculate 4 liter of LB containing 100 mg/ml ampicillin and grown at 37°C in orbital shaker incubator until reached OD$_{600}$ of 0.5. The culture was then cooled to 30°C. 500µl of the non induced sample was collected, pelleted and resuspended in sample buffer. NS3 protein expression was then induced by adding 0.5mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The culture then transferred to refrigerated orbital shaker incubator (Thermo scientific) and left for three hours at 25°C, then 500µl of induced culture was collected, pelleted and re-suspended in sample buffer.

2.3.2 Purification of NS3 protein expressed in E. coli

The expression of NS3 protein from pAW3 DNA expressed in E. coli, mainly resulted in insoluble protein called inclusion bodies. Inclusion bodies have high density and can be pelleted by centrifugation and this procedure enabled the separation of NS3 protein which formed inclusion bodies from the soluble fraction. Contaminating soluble proteins were
removed by a series of washes and sonication in a wash buffer, followed by centrifugation and to separate the inclusion body pellet from the supernatant which contaminated the soluble fraction. The final protein pellet which contained only inclusion bodies was solubilised with 8M urea containing 1mM DTT. High concentrations of chaotropic agents such as urea or guanidinium hydrochloride are needed to disrupt the non-specific interactions between the protein molecules in inclusion bodies. After protein expression, *E. coli* cells were harvested by centrifugation (Thermo scientific) at 3000 g for 15 min at 4°C and resuspended in 10 ml resuspension buffer containing (0.2 M NaCl, 50 mM Tris-HCl [pH 7.4], 1% Triton X-100 and 1mg/ml lysozyme) per 100 ml culture. The resuspended cells were then transferred to 30 ml Oakiridge tubes in 20 ml aliquots and kept on ice.

The bacterial suspension was then disrupted with a (Sonifier 450, Ultrasonicson, Branson) on ice (settings at 5 x 5 sec, 60% duty cycle, output 5. The cell lysate was then centrifuged at 10,000g using an XL. 90 Ultracentrifuge (Beckman) for 15 minutes at 4°C. This sonication/resuspension washing was repeated 7 times, and the final pellet of inclusion bodies was resuspended in cold 8M urea in PBS, 1mM DTT and sonicated as before and centrifuged for 40 minutes at 4°C. The supernatant that contain solubilised NS3 inclusion bodies was then collected.

### 2.3.3. Protein quantification by Bradford assay

The concentration of the protein was quantified by using the Bradford assay. The protein concentration was estimated by using a standard curve of bovine serum albumin (BSA) samples with a concentration range of 0.25-1.25 mg/ml. Briefly, 5µl of protein sample was mixed with 250µl Bradford reagent (Sigma) in a 96-well plate (Nunc) along with the BSA, and mixed gently on a shaker for 5 minutes. The absorbance was measured at A595 nm by using VersaMax microplate reader.

### 2.3.4. Purification of small scale protein under denaturing conditions

The expression of foreign genes in *E.coli* often results in the production of insoluble recombinant protein which forms inclusion bodies [264]. Inclusion bodies are aggregates of protein, but it is still not understood precisely how they are formed, although some studies indicate that the formation of these inclusions is due to specific intermolecular inter-
action between proteins [265, 266] or can otherwise be attributed to the mis-folding of the expressed polypeptide, often involving the formation of unwanted disulphide bond bridges[267]. A borosilicate liquid chromatography column (Sigma) 1.0 cm internal diameter × 10 cm in length, bed volume 8 ml, with a bottom cap was assembled according to the manufacturer’s instructions. A volume of 3 ml of 50% Ni-NTA agarose (Qiagen) was washed twice with milli Q water, and centrifuged using bench top centrifuge (Thermo scientific ) at 1,000 g for 50 seconds to pellet the resin. It was then equilibrated with denaturing buffer A (50 mM NaH$_2$PO$_4$, [pH8.0] 300 mM NaCl, 8 M Urea).

About 10 ml of the solubilised NS3 protein was mixed with 3 ml of Ni-NTA agarose and mixed gently by shaking on a rotary shaker at 10 rpm for 1 hour in a cold room. The supernatant-Ni-NTA mixture was then loaded into the column with bottom cap still attached. Once the resin had settled, the bottom cap removed and flow-through collected. The column was washed with 2 times the column volume of denaturing buffer B (50 mM NaH$_2$PO$_4$, [pH8.0] 300 mM NaCl, 8M Urea, 20mM imidazole), and the wash was collected. The protein was then eluted with 4 ml of denaturing buffer C (50mM NaH$_2$PO$_4$, [pH8.0] 300mM NaCl, 8M Urea, 100 mM imidazole) in four separate tubes, and a final elution with 4ml of denaturing buffer D (50 mM NaH$_2$PO$_4$, [pH8.0] 300 mM NaCl, 8M Urea, 250 mM imidazole). SDS-PAGE analysis using 12.5 µl aliquots of the flow-through, wash fractions and the elution fractions was performed. The elution fractions that contained significant quantities of a band at ~70 kDa were pooled and then subsequently used to refold the protein.

2.3.5. Dialysis tubing

A dialysis tube with an MW cut-off 12,000 (Invitrogen) and 30 mm width was boiled prior to use in 2% sodium bicarbonate (w/v) and 1mM EDTA for 10 minutes; it was then rinsed in Milli Q water and boiled again for 10 minutes in mM EDTA, then rinsed with milli Q water before use.
2.3.6. Protein refolding

To produce soluble and enzymatically active NS3 from denatured protein, a refolding procedure was used. Urea is a strong denaturing chaotropic agent, it contains two amino acid groups which forms hydrogen bonds that interfere with intramolecular hydrogen bonds of proteins, thus denaturing and solubilising the insoluble protein. To refold proteins solubilised in urea, it is necessary to remove the urea which then allows the proteins to reform intramolecular bonds and reforms their tertiary structure. Step-wise dialysis was carried out to refold the protein to its native state. The buffers used in refolding were those previously used by Wardell, 1999 et.al [268], to enhance protein refolding to the native confirmation, these buffers include; sodium chloride which prevent non-specific protein-protein interactions that may cause mis-folding and aggregation of proteins. DTT prevents the formation of disulphide bonds between cysteine amino residues. The pH was also adjusted to a physiological pH to mimic that of the mammalian cell cytoplasm. CHAPS detergent was added for optimal NS3 protease activity [269, 270] Stepwise dialysis was performed to remove the urea from NS3 protein preparations and all the purification process was performed at 4ºC when possible. 6 ml of NS3 protein solution was placed in the dialysis tube, the ends were sealed with plastic clips and the urea was removed by stepwise dialysis using refolding buffer (25 mM Tris-HCl [pH 7.2], f (v/v) glycerol, 50 mM NaCl, 1M DTT, 0.1% CHAPS) containing 4M, 2M, and 1M urea, followed by three buffer changes without urea. The dialysis changes continued for 2 hours for each change of buffer. The dialysed protein was clarified by refrigerated centrifuge XL. 90 Ultracentrifuge (Beckman) at 16,000 g for 1 hour at 4ºC and dialysed against refolding buffer containing 50% glycerol and stored at -20ºC.

2.3.7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

2.3.7.1 Resolving gel

A Biorad (mini-gel) vertical apparatus was used to prepare SDS-PAGE gels. Two glass plates separated by 0.75 mm spacers were assembled after cleaning with 70% alcohol. 10% resolving gel was made by the addition of 3.3 ml protogel (30% acrylamide, 0.8%
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w/v bis-acrylamide, National Diagnostics), 2.5 ml resolving buffer (1.5M Tris, pH 8.8, 0.1 ml of 10% SDS, National Diagnostics), 100 μl of 10% ammonium persulphate, 10μl TEMED (Sigma) and made up to 10 ml with milli Q water. The ingredients were mixed thoroughly and poured into the pre-assembled glass plates, and covered with 1ml isopropanol and left to polymerise for 30 minutes. Once the gel was set the top of the gel was rinsed with milli Q water.

2.3.7.2 Stacking gel

A stacking gel mixture was prepared by adding 1.5 ml of protogel (30% acrylamide, 0.8 % w/v bis-acrylamide), 2.5ml stacking gel buffer (containing 0.5M Tris, 0.1 ml 10% SDS, National Diagnostics), 100 μl of 10% ammonium persulphate, 10μl TEMED and 5.7ml of milli Q water. Again, all the ingredients were mixed, and then 1-2 ml applied on the top of the polymerized resolving gel, and a suitable well-forming comb was inserted into the stacking gel. Following polymerisation, the gel was transferred to the running gel apparatus (Bio-Rad) and 1x running buffer (1.92 M glycine, 0.25 M Tris and 3.5mM SDS) was poured into the upper and lower compartments of the tank and the combs removed carefully from the stacking gels.

2.3.8. Sample preparation

A volume of 12.5μl of protein was added to sample buffer containing (2.5μl 10x Tris–EDTA-reducing buffer and 10μl bromophenol blue/glycerol), and then loaded into the gel in a total volume of 25μl. The samples were boiled in a heat block at 90ºC for 5 minutes. 15μl of SDS-PAGE prestained molecular weight marker (Invitrogen) (Appendix 6.2.I) was also loaded onto the gel. The gel was run at a constant voltage of 100V for 1 hour, or until the dye front was ~ 0.5cm to the end of the bottom. The power was switched off and the gels were prepared for either Coomassie staining or Western blot.

2.3.9. Comassie blue staining

Coomassie blue staining was performed by using (Coomassie blue stain 0.1%, Coomassie R250 (w/v), 10% acetic acid (v/v), 40% methanol (v/v)). This acid-methanol mixture acts as a denaturant to fix the protein in the gel, which prevents the protein from diffusing within the gel or being washed out while it is being stained. The gel was left to
stain for several hours with gentle agitation, at room temperature. Following the staining, the commassie stain was removed and the gel rinsed twice with H₂O and destained by using a destain solution (12.5% isopropanol and 10% glacial acetic acid) for at least 3 hours, or until the background stain has been removed from the gel. Once sufficiently destained, the gel was placed on a transparent plastic sheet for scanning.

2.3.10. Western blot and immunodetection

Following electrophoretic protein separation, the gel was transferred to nitrocellulose Hybond ECL membrane (GE healthcare) which had been previously soaked in protein transfer buffer (1.92 M glycine, 0.25 M Tris and 3.5 mM SDS, 20% methanol). The transfer was performed by using a mini trans plot-cell (Biorad) according to the manufacturer’s protocol, then the transfer were carried out at 120V for 1 hour at 4°C. The nitrocellulose membrane was then blocked in a buffer containing 4% skim milk for 1 hour at room temperature to prevent non-specific antibody binding. The membrane was then incubated with 3µg/ml mouse anti-HCV NS3 antibody (Virogen) diluted in PBS/0.1% Tween-20/BSA for overnight at 4°C. Unbound antibodies were removed by washing the membrane 3 times with 10 ml PBS/0.1% Tween-20 for 10 minutes. The secondary antibody horseradish peroxidase (HRP) labelled anti-mouse (Invitrogen) was added to the membrane at a dilution of 1:1000 in blocking buffer and incubated for 1 hour at room temperature before washing three times with PBST. Blots were visualized by using SuperSignal West Pico Chemiluminescent Substrate (Thermo scientific) and developed on autoradiography film (GE healthcare).

2.3.11 ATPase activity assay

ATPase activity was detected by using an ATPase colorimetric assay kit (Innova Bioscience) the reactions was performed according to the manufacturer’s protocol. A substrate /buffer (SB) was prepared by mixing 20 µl of 0.5M Tris [pH 7.5], 5µl of 0.1M MgCl₂, 10 µl of 10 mM ATP and the reaction buffer made up to 100 µl. The reaction performed by mixing 100 µl of 1 nM protein plus 100 µl substrate. 50 µl of Gold mix reagent containing (Pi ColorLock Gold and accelerator) was added to stop the reaction. Two minutes later, 20 µl of stabilizer provided with the kit was added. After 30 minutes, the absorbance was measured at a wavelength 650 nm on a microtiter-plate reader Veramax. Along-
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side, a standard curve was prepared using 0.1mM Pi standard stock as described by the manufacturer. To calculate enzyme activity a standard curve was performed by preparing a set of Pi standards using the 0.1mM Pi stock (included in the kit). A duplicate set of wells containing 200 µl of each standard and 50 ml of Gold mix was prepared. Two minutes later 20 µl of Stabiliser was added. The plate was read after 30 minutes, using plate reader.

2.3.12 RNA methods

2.3.12.1 RNA transcription and purification (modified)

18 µg of pJFH-1 DNA was linearized with (100U) of restriction enzyme Xba I (Promega) in a reaction mixture contains 5µl of 10x buffer D (Promega) and total volume of 50µl. The mixture was incubated for 2 hours in a water bath at 37ºC. After digestion samples were cleaned using a Wizard SV gel and PCR clean up system (Promega) according to the manufacturer’s recommendations. In a total reaction volume of 50 µl containing 43µl of digested DNA, 2µl (20U) of Mung bean nuclease (10 U µl), and 5 µl Mung bean nuclease buffer (10X) were added and the mixture was incubated for 30 minutes in a heat block at 30ºC. Then the DNA sample was treated with proteinase K by combining the 50µl of Mung bean nuclease–treated solution, with 10% (wt/vol) SDS, 2µl Proteinase K solution (20μg µl) and nuclease free water to a total volume of 100µl, this was then incubated for 1 hour in a heat block at 50 C°. Then the DNA sample was purified by using a Wizard SV gel and PCR clean up system and the pellet resuspended in 20 µl TE buffer. RNA transcripts of the JFH-1 plasmid and 3’ end JFH-1 negative RNA were synthetised using a T7 MEGA script and SP6 MEGA script RNA transcription kit (Ambion), respectively as described by the manufacturer. In brief, 2- 4 µl (0.5-1 µg) of linear template DNA were mixed with the following reagents 2µl of ATP (75mM), 2µl CTP (75mM), 2µl GTP (75mM), 2µl UTP (75mM), 2µl reaction buffer(10X), 2µl enzyme mix and the total volume was adjusted to 20µl by adding 4-6µl of nuclease free water. The sample was incubated in a water bath at 37ºC for 4 hours. 1µl of DNase was added and incubated for 15 minutes in an incubator at 37ºC. The RNA was cleaned up by using QIAeasy RNA extraction kit (Qiagen) following the manufacturer instructions.
2.3.12.2 Measurement of RNA concentration and integrity

RNA concentration was measured by nano drop as described in section 2.15.3 and the integrity of the linearized RNA was assessed by formaldehyde agarose gel.

2.3.12.3 Formaldehyde agarose gel for RNA

RNA retains much of its secondary structure during electrophoresis unless it is first denatured. The addition of formaldehyde to the agarose gel maintains the RNA in a linear (denatured) form, by reducing secondary structure formation. Electrophoresis equipment was cleaned with RNA Zap (Ambion) and rinsed with DEPC treated water then rinsed again with Milli Q water. A 1.25% agarose gel was prepared by dissolving 1.2 g agarose in 81 ml autoclaved Milli Q water. The gel was cooled down to 65°C, 0.5 μg/ml ethidium bromide, 10 ml 10x MOPS buffer and 5 ml formaldehyde were added and the gel was immediately poured into preassembled electrophoresis apparatus (Flowgen ioscience) and equilibrated in running buffer (1x MOPS buffer, 12.3M formaldehyde) for 30 minutes. Approximately 3μl of the RNA sample (~ 2-10 μg) was mixed with equal volume of gel loading buffer II (Ambion). The sample was incubated at 65°C for 10 min and transferred on ice, and then loaded into the gel, Ambion Mellinium RNA ladder (Ambion) (Appendix 6.1), were included for RNA size estimation. The gel was run at 75 volt and 100 mA.

2.3.12.4 5’ Labelling end of RNA

Full-length RNA was prepared for labelling at the 5’ end by using a 5’ end tag Nucleic acid labelling system according to the manufacturer instructions. Using the 5’ EndTag Nucleic Acid Labelling System (Vectorlab) allows the covalent attachment of a variety of fluorescent dyes, haptens, or affinity tags to the 5’ end of unmodified oligonucleotides or 5’-OH modified RNA. 5’ end labeling is achieved by transferring a thiol group from ATPγS to the 5’ hydroxyl group of the RNA by T4 polynucleotide kinase. After incorporating the thiol functional group, a thiol-reactive label is chemically coupled to the 5’ end of the RNA. To label the RNA, 1μl of universal reaction buffer was combined with 0.6 nmols of 5’end in 8μl and 1μl alkaline phosphatise in a microcenrifuge tube. The total reaction was adjusted to 10μl with deionised water. After a 30-minute incubation period at 37°C, the mix was combined with 2μl of universal reaction buffer, 1 μl of ATPγS, and 2 μl of T4 nucleotide kinase. Then the total reaction was brought to 20μl with deionised...
water and incubated for 30 minutes at 65°C. 70 µl of water and 100µl of buffered phenol were added and it was then vortexed briefly.

The supernatant was removed to a clean centrifuge. 5µl of precipitant and 263 µl of 95% ethanol were added to the aqueous fraction and mixed. The precipitated nucleic acid was centrifuged at 13,000g in an eppendorf tube for 30 minutes. The pellet was dried and re-suspended in TE buffer (10mM Tris, 1mM EDTA, [pH 8].

2.3.12.5 Detecting by dot Plot

Labelling was confirmed by comparing dot blots of the reaction to those of the labelled DNA control supplied with the kit. 1µl spots of 82 ng of biotinylated RNA was two-fold in 6X SSC buffer were applied to a nitrocellulose membrane. After UV crosslinking using a transilluminator for 8 minutes and wavelength 250 nm. A Dulox chemiluminescent kit was used to detect the biotinylated RNA.

2.3.12.6 3’ end labeling

The 3’end of the JFH-1 negative strand was labeled using a 3’End Tag DNA labelling system (Vectorlab) according to the manufacturer instructions. The kit contains terminal transferase (TdT) which catalyzes nucleotide incorporation at the 3’ ends of single-stranded or double-stranded DNA. Labeling is achieved in two steps: (I) TdT incorporates SH-GTP at the 3’ end (SH-GTP is a modified guanosine triphosphate that contains a chemically active thiol group). (ii) After introducing the thiol functional group, a thiol-reactive label is chemically coupled to the 3’ end of the nucleic acid. The following components were combined, 0.5 nmols of 3’ ends, 2 µl 10x TdT reaction buffer, 2 µl SH-GTP, 2 µl TdT in a total volume reaction of 20 µl. The mix was incubated for 30 minutes at 37°C. To the reaction, 10µl of thiol-reactive label was added, mixed and incubated for 30 minutes at 65°C in a heat block. 70µl of water and 100 µl of buffered phenol was added, and vortexed. The mix was centrifuged for 5 minutes at 4,000g. The aqueous phase was transferred to clean tube, then 5µl of precipitant and 300 µl of 95% ethanol was added to the aqueous phase. The precipitated RNA was then centrifuged for 15 minutes at 13,000g. The pellet was left to dry and reusupended in TE buffer pH 8.0. The concentration of 5’ or 3’ RNA was calculated as follows:
\[ \frac{A \times V}{B \times C} = \text{nmols of 5' or 3' ends/μl} \]

A = nucleic acid concentration (μg/μl).
B = average molecular weight of nucleotide.
C = total number of bases or the number of base pairs.
V = volume of nucleic acid.

2.3.12.7 Electromobility shift assay

2.3.12.7.1 Native TBE polyacrylamide gel electrophoresis

TBE polyacrylamide gels were prepared and used with the Bio-Rad mini Protean II electrophoresis vertical apparatus. Two glass plates, which were separated by a spacer cleaned with 70% ethanol and clamped together and locked into the clamp assembly, then transferred to the casting stand. The composition of the polymerizing mixture was prepared as given in Table 2.1, mixed well and poured gently into the glass plate assembly. A suitable well-forming comb was immediately inserted and the gel left to polymerise at least for 2 hours. After gel polymerisation the comb was removed and the well rinsed with 0.5x TBE. Before loading the samples, the gel was run for 1 hour. The samples were adjusted to 1x agarose gel sample buffer in a total volume of 20μl and loaded into the wells, then run at 10 mA for 1.30 hrs at 4°C to prevent disruption of Protein-RNA interactions. Progression of electrophoresis was monitored by the migration of xylene cyanol and bromophenol blue bands.
### Materials and Methods

<table>
<thead>
<tr>
<th></th>
<th>Volume (ml for each gel)</th>
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</thead>
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<td></td>
<td>10% polyacrylamide gel</td>
</tr>
<tr>
<td>40% Acrylamide bis (37.5:1)</td>
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</tr>
<tr>
<td>5X TBE</td>
<td>1</td>
</tr>
<tr>
<td>Distilled water</td>
<td>6.39</td>
</tr>
<tr>
<td>10% Ammonium persulphate</td>
<td>0.1</td>
</tr>
<tr>
<td>TEMED, N,N,N,N tetramethylene diamine</td>
<td>0.01</td>
</tr>
<tr>
<td>Total</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

Table 2.10. Solutions and volumes used for the preparation of a 10 and 7% polyacrylamide gel

#### 2.3.12.7.2 Binding reaction

Sample binding reactions were prepared by mixing different NS3 protein concentrations ranging from 100 nM, to 600 nM with 5’ and 3’ end biotinylated RNA, in 20 µl of 1X binding buffer (100 mM Tris-HCl [pH 7.5], 5 mM MgCl₂, 2 mM DTT, 0.10mg/ml BSA), 0.01µg Poly (I:C) as a non-specific competitor and finally 55 pmol of 5’ or 3’ end biotinylated RNA, then incubated for 30 minutes at 4°C. 5µl of 50% (v/v) in PBS was added to each reaction mixture before loading onto a native polyacrylamide acrylamide bisacrylamide gel (37.5:1). Electrophoresis was carried out in 0.5x Tris-borate-EDTA (TBE) buffer at 80 Volt for ~ 2 hours.

#### 2.3.12.8 Transfer to nylon membrane

Gels were blotted onto Hybond positively charged nylon membrane (GE healthcare) using a Mini Trans-Blot cell apparatus. In a gel holder cassette (10 x 7.5 cm), a sandwich of sheets which contained 1x sponge pad, 2x Whatman paper, the gel, a Nylon membrane, 2x Whatman paper and 1x sponge pad, was assembled. Bubbles between the sheets were
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removed by adding 0.5x buffer and carefully rolling the sheets with a pipette. After placing the sandwich in the transfer tank, a cooling unit was added and the tank was filled with 0.5x TBE buffer, then run at 120 Volt for 1 hour at 4°C.

2.3.12.9 UV Crosslinking

The nylon membrane was placed face down on a transilluminator and exposed to UV light for 8 minutes at the 302 nm setting.

2.3.12.10 Detection of biotinylated RNA

An Ultrasnap detection kit (Vectorlab) was used to detect biotinylated RNA. The kit detects biotinylated RNA with streptavidin coupled to alkaline phosphatase. The blot was blocked with 20 ml of 1x Polyblock reagent for 30 minutes at room temperature with gentle shaking, then the blot was incubated with 20µl of AP-streptavidin diluted in 20 ml 1x Polyblock for 30 minutes at room temperature with gently shaking. Then the blot was washed with 30 ml of 1x wash A for 10 minutes at room temperature 3 times with gentle shaking, then the blot was rinsed in 30 ml of 1x Wash B, and excess reagent was removed by touching the edges to filter paper. In a dark room, the blot was placed on a level surface and covered with 5ml of Dulox substrate (Vectorlab) and left for 5 minute. The blot was then washed in 30 ml of 1x Wash buffer for 1 minute at room temperature, then the blot was touched with filter paper to remove excess liquid, and wrapped in plastic wrap and exposed to X-ray film.

2.3.13 Adenovirus methods

2.3.13.1 Growth and amplifying adenovirus

Stocks of replication defective recombinant adenoviruses containing HCV NS3, or NS3/LacZ expression cassette containing recombinant virus were used. All work with adenovirus was performed under Biosafety Level 2 (BL-2). A crude viral stock was obtained from (Al-hababi, 2008) used to generate a new high titre viral stock.

A 25cm² flasks of 293A cells which had previously been growing was trypsinized and 3x10⁶ cells in DMEM containing 10% FCS and 10% penicillin/streptomycin were transferred to a new 25cm² flask. The next day, a 300 µl inoculum of crude adenoviral stock
was added to the cells, which then incubated in humidified incubator at 37°C and 5 % CO₂. The infection was continued until 80-90% of the cells were rounded and detached from the flask, which indicates the cells contain adenoviral particles. The medium containing detached cells was transferred to 15 ml falcon tube. This was kept at -80°C for 30 minutes, and then placed in a 37°C water bath for 15 minutes to thaw. This step was repeated twice to lyses the cells. Finally, the cell lysate was centrifuged at 3,000 g for 15 minutes at room temperature. The supernatant which contains the viral particles was aliquoted into cryogenic tubes, and stored at -80°C.

2.3.13.2 Tittering adenovirus stock by plaque assay

In a 12-well plate, 293A cells were seeded such that they would be 80-90% confluent at the time of infection. On the day of infection, vials of each of adenovirus stocks were thawed for 10 min in a 37°C water bath. 10-fold serial dilutions ranging from 10⁴ to 10¹³ were prepared by dilution in 10% FCS DMEM with a final volume of 1ml. Addition of virus dilution to the wells in the plate was followed by incubation overnight at 37°C in a humidified CO₂ incubator. The following day, medium that contained the virus inoculums was removed. An overlay solution was prepared by mixing 12 ml of prewarmed 2% FCS DMEM at 37°C and 1.2ml of pre-warmed at 65°C 4% agarose in PBS. 1.5 ml of the pre-warmed agarose at 37°C overlay, was then added to each well. The 12-well plate was then left in a tissue-culture hood at room temperature until the agarose overlay solidifies, and then incubated at 37°C in a humidified CO₂ incubator. After two days, an additional 0.75 ml of 4% agarose overlay solution was added to the wells, and left until solidified before returning the plate to the incubator. The plate was monitored until plaques were visible. For each well 300 µl of 1% neutral red in H₂O was added to each well and incubated for 3 hours at 37°C in a humidified CO₂ incubator. Finally, the plaques were counted and the titer of adenoviral stocks was calculated and expressed as plaque forming units (pfu) per ml. The titer was calculated as follow: the number of plaques which appears as lytic areas were counted, then the following formula was used to determine the titer (pfu/ml) of each viral stock.
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Number of plaques per well

The Viral titer = ______________________ = pfu/ml

Dilution factor X volume (ml) of diluted virus added to the well

2.3.13.3 Determining the transduction efficiency (multiplicity of infection)

A day before transduction, $1 \times 10^5$ of Huh7 cells were seeded so that their confluence at the time of transduction would be 50-70% in each well of a 24 plate prior to transduction and incubated at 37°C in an atmosphere of 5% CO$_2$ for 48 hours. From each adenovirus stock, different doses of MOI 1 to 100, were added to the wells, and then the plate was incubated at 37°C overnight. Next day, the medium was replaced and left for a further 24 hours incubation. The volume of virus stock needed to achieve for a particular MOI was determined as follows:

I. Number of cells to be infected x desired MOI = total PFU needed.

II. ($\text{total PFU needed}$) / (PFU/ml) = total volume (ml) of virus needed to reach the desired MOI.

2.3.14 Cell culture methods

2.3.14.1 Cell culture growth

Human hepatoma cells (Huh7) were grown and maintained in Dulbecco’s modified Eagle (DMEM) medium (Invitrogen) supplemented with 1x non essential amino acids, 10% heat inactivated fetal calf serum, 100 units/ml penicillin and 100µg/ml streptomycin at 37°C in a humidified incubator with 5% CO$_2$. The cells were split at approximately 80% confluence and washed with phosphate buffered saline (PBS) (Invitrogen) and trypsinised with 0.05% Trypsin, 0.02% EDTA. Cells were then incubated for 2-5 minutes at 37°C in the CO$_2$ and then resuspended in complete medium. The cell suspension was centrifuged at 1,000 g for 5 minutes. The supernatant was removed and the cell pellet resuspended in complete medium. Aliquots of this suspension was used to seed new tissue culture flasks. Additionally, G418 (Geneticin) (GIBCO, Invitrogen) at a final concentration of 50mg/ml was added to Huh7 cells harboring subgenomic HCV replicons. The cells were then in-
cubated at 37 °C in 5% CO₂. Cells were passaged 2 to 3 times per week or before the cells became confluent.

2.3.14.2 Cell storage

All of the cell lines were grown in T75cm² tissue culture flasks until confluent and trypsinised as for passaging of cells and then counted using a haemocytometer. Cells were centrifuged using at 1000 g for 5 min. The supernatant was decanted and the cells resuspended in freezing medium (DMEM containing 20% FCS and 10% DMSO) to an approximate density of 5.0 x10⁶. Aliquots of 1 ml were transferred to cryovial tubes (Nunc) and frozen at -80°C overnight, then transferred to cryogenic storage tubes for long-term storage in liquid nitrogen.

2.3.14.3 Adenovirus transduction and siRNA knockdown of host genes

A day before transduction with recombinant adenovirus, Huh7 cells were washed with phosphate-buffered saline (PBS), trypsinized and resuspended in complete medium. Cells were then seeded in 24-well plates at a density of 0.5 x10⁵ cells/ml, in 10% FCS DMEM medium. The next day, the cells were transduced with adenovirus at an MOI of 50 or 100 pfu/cell. The cells were incubated with adenovirus for 36 hours at 37°C in a humidified incubator with 5% CO₂. After 36 hours the medium was removed and the cells were washed with 1ml PBS and then 500µl of OPTI-MEM (Invitrogen) was added to the well. The optimal concentration of siRNA was determined [271]. Each siRNA used was initially diluted from the original stock of 20µM to obtain the desirable concentration of 25nM. For each transfection sample, 1.5 µl siRNA was mixed with 48.5 µl OPTI-MEM medium. In a separate eppendorf tube, 1µl Lipofectamine 2000 (Invitrogen) was diluted in 49µl OPTI-MEM I reduced medium, mixed gently and incubated for 5 minutes at room temperature. This solution was then combined with the previously diluted siRNA solution and incubated for 20 minutes at room temperature to allow the formation of siRNA Lipofectamine 2000 complexes. After 20 minutes, the 100 µl of the transfection mixes were added to wells 500 µl of OPTI-MEM medium and then the cells were incubated at 37 °C in 5% CO₂ for 48 hours. Finally, after eight hours the OPTI-MEM medium was replaced with 500 µl of DMEM medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100µg/ml streptomycin and the plate was incubated.
for a further 48 hours. Alongside, controls containing GAPDH and non-targeting siRNAs were used to determine the efficiency of transfection.

2.3.15 Sample preparation for RT-PCR

2.3.15.1 RNA extraction

Intracellular RNA was extracted using TRIzol reagent (Invitrogen) according to manufacturer instructions. Growth medium was removed from the 24 well plates, and 0.35 ml TRIzol reagent was added and left for 5 minutes to allow for complete dissolution of the cells. TRIzol-cell lysates were then homogenised by pipetting up-and-down and then transferred into 1.5ml eppendorf tubes. 250µl of chloroform was added to each tube and the homogenate was shaken vigorously for 15 seconds and incubated at room temperature for 5 minutes. The homogenate was then centrifuged at 12,000g for 15 minutes at 4ºC, which will separate the homogenate into different phases, the aqueous (upper) phase was carefully transferred to a 1.5ml clean tube. An equal volume of isopropyl alcohol was then added to the aqueous phase to precipitate the RNA. The mixture was incubated at room temperature for 15 minutes and then centrifuged at 12,000 g for 30 minutes at 4ºC. The RNA was pelleted, washed with 70% ethanol and resuspended in 30µl of DEPC-treated water and stored at –80ºC until further use.

2.3.15.2 DNase treatment

DNase treatment was performed using a DNA-free DNase Kit (Ambion), to remove genomic DNA. 3 µl 10x volume of DNase I Buffer and 1µl of DNase I (2units) were added to 0.5-2 µg of RNA, and mixed gently by pipetting up and down and then incubated at 37ºC in water bath for 20-30 minutes. 0.1 volume of DNase Inactivation Reagent was added to the mixture, the mixture was then vortexed, and incubated for 2 minutes at room temperature and then centrifuged at 10,000 g for 1 minute to separate the DNase Inactivation Reagent from the RNA. The RNA was then transferred to a new tube and stored at -80ºC.

2.3.15.3 RNA concentration and quality

The concentration and the purity of RNA samples were determined using a nanoDrop ND-1000 full spectrum (220-750nm) UV spectrophotometer (Thermo Scientific), which
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calculates the concentration of a sample based on the absorbance detected using Beer’s law, and measures high concentrations of RNA without dilution. Before measuring the RNA concentration, RNase Free water was used to perform a blank reading. From each sample 2 µl of RNA was pipetted onto the nano Drop pedestal. A second pedestal was brought into contact with the liquid sample to allow the spectrometer to analyze the light that passes through the sample. The nanoDrop ND-1000 software calculate the quantity of RNA in the sample using an OD$_{260}$ absorbance reading. It also simultaneously measured the OD$_{280}$ of the samples allowing the purity of the sample to be estimated. The ratio of A$_{260}$/A$_{280}$ gives an estimation of the purity of the sample.

2.3.15.4 qRT-PCR

Quantitative Real–Time PCR analysis was performed using a QuantiTech SYBR RT-PCR (Qiagen) kit. The principle of this kit is based on the fluorescent dye SYBR Green that binds to all double-stranded DNA molecules during the PCR reaction, emitting a fluorescent signal on binding. The fluorescent signal increases as the number of dsDNA molecules also increases. All reactions were performed in a final volume of 10µl. For each reaction, a master PCR mix was combined as follows: 5µl of SYBR Green, 0.1µl of RT enzyme, 0.5µl of (3.2µM) of each reverse and forward primers. The master mix was then distributed into 1.5ml eppendorf tubes, mixed and transferred into PCR capillaries (Roch). 150ng of each RNA sample was added to the individual capillaries and then centrifuged for about 5 seconds and then loaded into a lightcycler machine (Roche).

The conditions of the PCR were ; initial activation step at 95°C for 15 minutes, denaturating at 94°C for 15 seconds, annealing at between 55-60°C depending on the primer used for 30 seconds, and extension at 72°C for 30 seconds. PCR capillaries were placed in the LightCycler machine (Roche) and the cycling program was started. Finally, the results were plotted on graphs using PRISM software.

2.3.15.5 Relative quantification

Relative quantification measures the changes in mRNA levels of a gene across multiple samples and expresses it relative to the levels of an internal control RNA. Housekeeping genes are usually used as a reference gene and can be co-amplified in the same tube in a multiplex assay or can be amplified sperately. Accordingly, relative quantification does
not require standards with known concentrations and the reference can be any transcript, as long as its sequence is known. To calculate fold change in expression, the cycle threshold (CT) values obtained following qRT-PCR analysis were used in the ΔΔCT equation [207].

**The ΔΔCT equation:**

\[
\text{CT target gene} - \text{CT reference gene} = \Delta \text{CT} \\
\Delta \text{CT adenovirus transduced sample} - \Delta \text{CT negative infected sample} = \Delta \Delta \text{CT} \\
\text{Fold change in gene expression} = 2^{-\Delta \Delta \text{CT}}
\]

To normalise the gene expression data, GAPDH was used as the reference gene for all experiments.

### 2.3.15.6 Primer design

Primers for qRT-PCR were designed using online resources. The gene sequences were obtained from the National center for biotechnology information website (http://www.ncbi.nlm.nih.gov/). Primers for qRT-PCR were designed using Primer 3 software to amplify a product between 100bp and 150bp in size. The annealing temperature used for all primer pairs was 58°C. Primers were synthesized by Invitrogen.

### 2.3.16 Indirect immunofluorescence staining

Indirect immunofluorescence staining was performed to detect protein expressed in either or Huh7 transduced with adenovirus containing V5 tag.

#### 2.3.16.1 Evaluation of Anti V5 by indirect immunofluorescence staining

Glass cover slips (22 x 22 mm) (VWR) were sterilized by autoclaving and placed in the wells of 24 plate. Huh7 cells were seeded at 2 x 10^5 per well with complete DMEM medium. Once the cells grown to 70% confluence they were transduced with the following adenovirus constructs; AdNS3-1a, 1b, 2a, AdNS3/4A-1a, 1b, 2a and Ad LacZ recombinant adenoviruses at MOI 100. After 48-72 hours post infection the cells were washed with 500µl PBS followed by 2ml of 4% paraformadehyde in PBS fixation solution and incubated for 25 minutes at room temperature. The fixing solution was then discarded from the wells and the cells were carefully washed with PBS. The cells were permeabilised by adding 1ml of 0.1% Triton X-100 in PBS for 15 minutes and then washed with
1ml of PBS. The cover slips were transferred from the wells and placed, cell coated face down, onto or 30µl of mouse anti tag V5 antibody (anti-V5) diluted 1:300 in 1% BSA in PBS on a strip of parafilm for one hour at room temperature. Unbound antibody was washed off three successive times in 1ml PBS and the coverslips were then placed face down onto 50µl of or AlexaFluor 594-conjugated anti-mouse IgG (Invitrogen), diluted 1:800 in sterile PBS on a clean parafilm and incubated for one hour at room temperature. The cover slip was washed again three times in 1ml PBS, and placed face down on 15µl of Mowiol mounting medium with DAPI nuclear stain on a clean microscope slide. The cells were examined using oil lens 60x magnification using Fluorescence microscope, Nikon Eclipse TE2000-S.

2.3.17 FACS analysis of HCV core intracellular staining

One day prior to the infection, Huh7 cells were seeded in 24 well plates at a density of 2x10^5, and were then subsequently infected with HCV JFH-1 at different MOI, as detailed in (section 2.9.1). For FACS staining, all of the steps prior to fixation were carried out on ice. Subsequently, the cells were harvested through trypsinization for 2–3 minutes at 37°C, with the addition of 2 ml of PBS and cell suspensions were then transferred to Falcon 12x75 mm polypropylene tubes, and 1,500 g centrifuged at 4ºC for 5 minutes. Subsequently, the supernatant was discarded, and 1 ml of PBS was then added to the cell pellet, mixed gently, and again centrifuged at 1,200 g at 4ºC for 5 minutes. Again the supernatant was discarded, and 300µl of fixative (4% paraformaldehyde and formaldehyde in PBS) was added to each cell pellet, mixed gently, and incubated on ice for at least 30 minutes. Following the period of incubation, each tube received 1 ml of PBS, which was mixed gently, and again centrifuged at 1500 g at 4ºC for 5 minutes. Subsequently, the supernatant was discarded and the cell pellet was then re-suspended in 200µl PBS and transferred to a V-shape 96 well plate (Sarstedt). For intracellular staining, the same procedures as described above were carried out with the use of saponin containing buffers. Initially, 200µl PERM buffer (FACS staining buffer with 0.5% (w/v) saponin) was added to each of the wells, with thorough mixing and subsequent centrifuging at 1200 g for 5 minutes with the use of a plate centrifuge (Sigma). This washing procedure was again repeated with a 200µl SUPERPERM buffer. The supernatants were then discarded, then
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80μl (1:300 in PBS) of the mouse monoclonal anti V5 antibody (Invitrogen) added to the wells with the exception of the negative control. This was then left at room temperature for 1 hour. Following this stage, there was the washing of the cells with 200μl PERM buffer per sample, with centrifugation carried out as described before. Following the removal of the supernatant, the cells pellets were resuspended in 100μl of the Alexa 594 donkey anti-mouse IgG- secondary antibody (Invitrogen), diluted 1:800 in PBS, and left at room temperature for of 1 hour. As previously, the supernatant was removed by centrifugation. The cell pellets were then washed with 200μl of PERM buffer, spun, and the supernatants discarded. Following the final stage, PBS was once again used to wash the cells, which were then re-suspended in 200μl of staining buffer (2% FCS in PBS), gently mixed and then transferred to FACS analysis tubes (BD). A FACS C6 Flow Cytometer (Accuri) was used to determine cell fluorescence signals. Sample analysis was carried out with the use Accuri software.
RESULTS
3. Results

3.1 The interaction of NS3 with HCV RNA

HCV NS3 protein has been shown to have a range of activities, including protease, ATPase, helicase enzyme activities as well as RNA binding and protein-protein interaction activities. To investigate the interaction of NS3 with RNA, NS3 protein and HCV RNA were produced and used in cell free interaction studies. NS3 protein was characterised for enzyme activity and synthetic RNAs corresponding to full-length HCV genome and the 3'UTR were synthesised.

3.1.1 Expression and purification of NS3 protein

To determine the presence of the full length HCV NS3 pAW3 [102-1657], the plasmid DNA was digested using BamHI restriction enzymes. A 1% agarose electrophoresis gel was used to confirm that the correct plasmid DNA was present. As shown in (Figure 3.1) lane 1; contains the 1Kb DNA ladder, lane 2 contains the undigested plasmid, whereas lane 3 contains two fragments resulting from the double digestion, the parental (pET16b) is 5,711 bp, whereas the NS3 pAW3 plasmid insert is approximately 1,800 bp.

3.1.1.1 Determination of plasmid stability

To determine which fraction of the cells contain the functional NS3 pAW3 plasmid. Four plates containing different additives (i) 100mg/ml ampicillin, (ii) 1mM IPTG, (iii) both 100mg/ml ampicillin and 1mM IPTG, or with neither (iv) were used to grow *E. coli* containing pAW3. 10-fold serial dilutions were made from 10^1 to 10^6. Then cells at a dilution of 10^5 were plated on 1mM IPTG or 1mM IPTG and ampicillin. Whereas cells at a dilution of 10^6 were plated on LB or 100mg/ml ampicillin. As seen in (Figure 3.2), bacterial colonies grow on both LB medium and LB containing antibiotic, which indicates that these cells are still carrying the target plasmid. Whereas, no growth was detected on the plates that contains both 1mM IPTG and IPTG with antibiotic.

The absence of growth may be explained when the IPTG is present, because cells carrying a protein production plasmid dedicated all their resources to the production of the recombinant protein instead of cell maintenance, only mutants that retain the plasmid but
have lost the ability to express the target gene can grow in LB medium contain IPTG and antibiotic.

Figure 3.1. 1% agarose gel electrophoresis shows restriction digestion of pAW3 plasmid. Lane 1; 1Kb DNA ladder, lane 2; undigested pAW3, lane 3; pAW3 digested with Ndel and Bam H1 enzymes.
Figure 3.2. Plasmid stability test. (i) LB plate contains viable cells, (ii) LB plates with antibiotics which contains cells still carry the target plasmid, (iii) LB plate with IPTG with no growth, (iv) LB plate with antibiotic and 1mM IPTG with no growth.

3.1.1.2 Protein purification from inclusion bodies

Inclusion bodies are formed from the expression of high levels of recombinant proteins in *E. coli* which leads to protein aggregation. Proteins contained in inclusion bodies are biologically inactive and need to be solubilised, refolded and purified before they can be used. Solubilising protein aggregates is usually achieved by using high concentrations of a chaotropic agent e.g. Urea along with a reducing agent e.g DTT and then refolded by the gradual removal of the denaturant.

Inclusion bodies were isolated by repeated sonication and washes of samples corresponding to total protein extracts from cells induced with IPTG and uninduced cells. Soluble protein, from washes 3-6 and insoluble protein were examined by SDS-PAGE
(Figure 3.3). No protein bands were detected in the supernatant of the final wash prior to solubilisation of the inclusion bodies with a buffer containing 8 M urea and DTT. (lane 3-9), demonstrating the removal contaminating soluble protein. The final lane (10) shows the purified inclusion bodies containing undiluted NS3 protein prior to purification.

### 3.1.1.3 Purification of NS3 protein under denaturing conditions by liquid chromatography column

Protein purification under denaturing conditions was performed using Ni-NTA agarose. A His tagged protein is an amino acid motif that consists of at least 6 histidine (His) residues either at the C-terminal or N-terminal of the tagged protein. The His tag attaches to the Ni²⁺ ions on the column through the imidazole ring in the histidine residue of the 6xHis tag. Applying imidazole which itself contains the histidine ring, displaces the His-tag from nickel co-ordination, hence freeing the His-tagged proteins (Figure 3.4).
Results

Figure 3.3. Purification of inclusion bodies containing NS3 protein. Samples were resolved by SDS-PAGE on a 10% gel and stained with coomassie blue. Lane M: molecular weight ladder, lane 1: uninduced cells, lane 2: induced cells, lane 3-9: wash, lane 10: inclusion bodies solubilized with 8M urea.
Results

Figure 3.4. 10% SDS-PAGE of purification of 6xHis tagged NS3 protein under denaturing conditions using Ni-NTA nickel-nitrilotriacetic agarose column. Lane M; molecular weight marker, lane 1; flow-through, lane 2; wash 1, lane 3; wash 2, lane 4-6 eluted with buffer C, 8-9 eluted with buffer D.

3.1.2 Protein refolding

The purified NS3 protein was refolded to its bioactive form by stepwise dialysis of the urea solubilised protein to remove the urea and allow protein refolding. The protein sample was dialysed stepwise using refolding buffer (25 mM Tris-HCl [pH 7.2], 10% (v/v) glycerol, 50 mM NaCl, 1M DTT, 0.1% CHAPS) containing 4M, 2M, and 1M urea, followed by three buffer changes without urea. The protein was then dialysed against buffer A containing 50% glycerol for 24 hours and stored at 20 °C. All dialysis steps were carried out at 4°C. During the dialysis there was a large amount of precipitated protein. However, this solution was clarified by high speed centrifugation at 16,000g for 30 minutes at 4°C to remove the precipitate. The yield of soluble protein was ~ 0.7mg/ml. Figure 3.5.A and B demonstrate the presence and purity of the NS3 protein after refolding, using a 10% SDS-PAGE and western blot.
Figure 3.5. A. 10% SDS-PAGE shows the differences between the uninduced, 1mM IPTG induced cells and the refolded NS3 protein. Lane 1; un induced cells, 2; induced cells, lane 3; shows the refolded NS3 protein. B. Western blot analysis shows these extracts with the anti NS3 antibody. Samples were resolved by SDS-PAGE on a 10% gel before western transfer. B. Western blotting was performed with anti NS3 (3µg/ml) and HRP conjugated antibody. Lane M; molecular weight marker, lane 1; induced cells, 2; refolded NS3 detected with anti NS3 antibody.
3.1.2.1 Determination of NS3 protein ATPase activity

ATPase is an enzyme that catalyzes the decomposition of adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and free phosphate. ATPase has a wide range of functions e.g. Transmembrane ATPases import many of the metabolites necessary for cell metabolism and export toxins, wastes, and solutes that can hinder cellular processes. Plasmid pAW3 that encodes a full length HCV NS3 protein and was previously characterised for helicase, ATPase activity, the effects of Mg\(^{2+}\), NaCl ions and pH on these enzyme activities. The ATPase activity of NS3 can therefore be used to assess the activity of NS3 protein after refolding. To do this, a colorimetric assay was used to measure the production of inorganic phosphate from ATP by ATPase activity. A standard curve was created to calculate enzyme activity, then the OD\(_{260}\) of inorganic phosphate (Pi) was measured every 10, 20, 30, 50 and 60 minutes. Figure 3.6 shows the generation of Pi by NS3 protein ATPase versus time which shows a linear relationship. This indicates that the NS3 protein is enzymatically active.

![Figure 3.6. Time course of HCV NS3 ATPase activity shows a linear relationship between ATPase activity of NS3 versus time. The experiments were carried out in the presence of 1mM NS3 (●), and the control contains reaction buffer without NS3 (▲). A standard curve was created to calculate enzyme activity, then the OD260 of inorganic phosphate Pi (mM) was measured every 10, 20, 30, 50 and 60 minutes.](image-url)
3.1.3 Synthesis and analysis of full length JFH-1 RNA

The discovery of the HCV genotype 2a isolate called JFH-1 has facilitated the study of the HCV life cycle from host-cell attachment and entry, through replication, to the assembly and release of progeny virions. To investigate the binding activity of NS3 to HCV RNA, synthetic RNA corresponding to the full length HCV genome was produced. The preparation of in vitro transcribed RNA from plasmid DNA was performed as described by Wakita et al, 2006 [272]. Plasmid JFH-1 was grown in E. coli DH5α to generate pJFH-1 DNA. After extraction of pJFH-1, the plasmid were then linearized using Xba I enzyme to produce linear DNA (Figure 3.7). An agarose gel was used to confirm the size of both the plasmid and the linearized JFH-1 (Figure 3.8). The linearized pJFH-1 was used as a template to synthesize the full length JFH-1 RNA in vitro using T7 RNA polymerase. The plasmid was first purified by column-based clean up. The pJFH-1 DNA was then enzymatically treated with mung bean nuclease and proteinase K and purified. RNA was synthetized by in vitro transcription and further purified. The presence of the RNA transcripts of the desired size was confirmed by formaldehyde agarose gel electrophoresis (Figure 3.9).

Figure 3.7. JFH-1 DNA plasmid map. pJFH-1 (Red arrow), Ampicillin resistance gene (Green arrow).
Figure 3.8. Linearization of pJFH-1. 1% agarose gel electrophoresis shows a restriction digestion of pJFH-1. Lane M; 1Kb DNA ladder, lane 1; undigested pJFH-1, lane 2; pJFH-1 linearized with Xba I.
Figure 3.9. Analysis of transcribed pJFH-1 RNA. Electrophoresis of 1% agarose-formaldehyde gel stained with ethidium bromide. M; molecular weight ladder (1µg), 1, transcribed full length JFH-1 RNA (1µg).
3.1.4 Synthesis and analysis of the 3’ end JFH-1 negative RNA

The 5’ end of the JFH-1 HCV genome was transformed in *E. coli* JM109 (DE3) cells (Figure 3.10). The DNA was then linearized with *Apa I* enzyme. Double digestion with *Nde I* and *Ecor I* was used to show the presence of the 5’ end. The size of the plasmid was estimated by agarose gel electrophoresis (Figure 3.7). The linearized DNA was then used as a template for *in vitro* SP6 RNA transcription, to produce the 3’ end negative strand RNA. The transcript size was confirmed by formaldehyde gel electrophoresis (Figure 3.12).

![Figure 3.10. DNA plasmid of HCV IRES. IRES (Red).](image-url)
Figure 3.8. Restriction digestion of 5’end. 1% agarose gel electrophoresis shows restriction digest of JFH-1 5’ end. Lane M; 1Kb DNA ladder, lane 1; undigested pJFH-1 5’ end, lane 2; double digest with EcoR I showing cleaved pJFH-1 5’ end and the insert (500bp); lane 3, linearised pJFH-1 5’ end (3808 bp).

Figure 3.9. Analysis of transcribed 3’end JFH-1 RNA. 1 µg was loaded onto a 1% agarose-formaldehyde gel and stained with ethidium bromide. M; RNA Millennium molecular weight ladder (2µg), 1, transcribed 3’ end JFH-1 HCV negative strand.
3.1.5 Detection of HCV RNA-NS3 protein interaction by electro mobility shift assay

The electrophoretic mobility shift assay (EMSA) is a powerful and qualitative tool used to detect protein-RNA interaction. It is based on the observation that the electrophoretic mobility of a protein-nucleic acid complexes slower than that of the free nucleic acid. The sensitivity of the assay allows them to be performed with low protein and nucleic acid concentrations ranging from 0.1 nM or less. A wide spectrum of nucleic acid sizes from short oligonucleotides to several Kbp. It can be performed with different nucleic acid structures; single-stranded, duplex, triplex and quadruplex nucleic acids and small circular nucleic acid. The electro mobility shift assay can be performed with proteins ranging in size from small oligo peptides to protein complexes with $M_r \geq 10^6$. On the other hand, it can be used with both highly-purified proteins and crude cell extracts. Moreover, the nucleic acid can be labeled with radio isotopes, covalent or non covalent fluorophores or biotin. Biotin has several advantages comparing with radioactive isotopes, it is safe and the shelf life is long. In this study biotin (Long Arm) maleimide was used to label HCV RNA. The presence of the 6-aminohexanoate spacer arm between the maleimide group and biotin reduces the possibility of steric hinderance. Biotinylated probes were detected using streptavidin coupled to alkaline phosphatase.

3.1.5.1 Screening of biotinylated RNA

Figure 3.13 A shows the titration of the biotinylated RNA by dot blot. Labeled RNA was detected using dot blot assay as described in section 2.3.12.10. 2-fold dilution of 1µl of 25nM of in vitro transcribed biotinylated RNA was used. Visually the result showed strong signals at concentrations of 25 nM, 12 nm, 6 nM and 3 nM which gradually decrease in intensity from the concentration 1.5 nM. The densitometry of the dots was also analyzed using scanning integrated optical density software (Image J), and different concentrations are presented as relative percentages (Figure 3.13.B).
3.1.5.2 Detection of the interaction between 3’end biotinylated HCV RNA and NS3 protein

The transcription process is the first step of gene expression and it begins with the formation of the pre-initiation complex. Once this complex has formed RNA protein interactions takes place to splice, protect, translate or degrade the message. After transcription initiation, the complementary promoter sequence in the mRNA is cleaved and the capping machinery incorporates a “GpppN” cap at the 5’ end of the mRNA. Elongation of
the transcript then occurs through the recruitment of elongation factors to the message. Elongation is followed by 3’ end processing and splicing, resulting in a mature RNA transcript that is exported to the cytoplasm for translation. All of these processes require significant protein:RNA interactions and are highly regulated and complex. Many of the regulatory elements for this process lie within the 3’ and 5’ untranslated regions (UTRs) of the mRNA. Coding sequence for regulatory microRNAs (miRNAs) have been located in coding regions of exons, the introns and exons of non-coding genes and also in repetitive elements. An increased emphasis has been placed on the significance of the non-coding RNAs and their roles in cellular regulation and disease states. However, tools for the study of critical protein:RNA interactions have been limited. The UTR regions of mRNA contain sequence elements that recruit RNA-binding proteins for post-transcriptional regulation and protein translation.

In addition, these elements promote transcript stability or degradation and can direct subcellular localization of the RNA. These RNA regulatory elements vary in length, but rely on both primary and secondary structure for RNA-protein binding interactions. The 5’ and 3’ ends of nucleic acid are common sites for labeling with biotin, since labeling at these sites minimizes the steric hindrance. In order to examine the interaction of full length HCV NS3-1a protein with the full length HCV JFH-1 RNA and the 3’ end of negative strand HCV RNA, electromobility shift assays were performed using biotinylated RNA probes corresponding to the full length JFH-1 (2a) with the full length NS3-1a protein.

For the full length JFH-1 RNA experiment, 7% native acrylamide gel was used and 10% native acrylamide gel was used for the 3’ end. Equal mole fractions of bound and free RNA which was 55 pmol, were combined with different concentrations of NS3-1a protein ranging from 100 to 600 nm. The RNA was mixed in binding buffer with NS3 protein, Poly (I:C) was added as a competitor and the mixture was equilibrated for 30 minutes at 4ºC. As demonstrated in (Figure 3.14.A) lane 1, contains free RNA in binding buffer, it shows high signal intensity, lane 2; contains RNA and 100 nM of NS3-1a protein, the intensity of the biotin began to fade with increased protein concentration, which indicates binding of RNA and protein. However, the reaction showed slight shift. Binding of the RNA to the protein was assessed by measuring the decrease of chemoluminescence.
Results

(CL) signal intensity of the RNA bands using Image J. Software (Figure 3.14 C). Figure 3.14 C shows a histogram presenting relative intensity for each reaction band. Figure 3.12.A shows the interaction of full length HCV NS3-1a protein with the full length JFH-1. The intensity of the biotin began to fade with increased protein concentration, which indicates binding of RNA and protein. Similar results were seen in (Figure 3.15.A) for the interaction of 3’ end of the negative RNA strand and HCV NS3-1a protein.

<table>
<thead>
<tr>
<th>RNA/Protein mixture</th>
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</thead>
<tbody>
<tr>
<td>Lane 1 Free RNA</td>
<td>100.00</td>
</tr>
<tr>
<td>Lane 2 55pmol RNA with 100 nM NS3</td>
<td>49.001550</td>
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<td>Lane 3 55pmol RNA with 200 nM NS3</td>
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<tr>
<td>Lane 5 55pmol RNA with 600 nM NS3</td>
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Figure 3.11. Electro mobility shift assay of the interaction of JFH-1 RNA and NS3 protein. A. RNA-protein interaction was detected by adding 55pmol fraction of biotinylated of RNA to different NS3 protein concentrations ranging from 100-600 nM, in binding buffer at 4°C for 30 min and loaded into 7% acrylamide gel, lane 1; free RNA, lane 2-5 different NS3 protein concentrations. B. Representation of quantification of each band of RNA/Protein was calculated using the Image-J program. C. Histogram presents relative intensity for each band.
### Results

<table>
<thead>
<tr>
<th>Lane</th>
<th>RNA/Protein mixture</th>
<th>Intensity reading</th>
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<tbody>
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</tr>
<tr>
<td>Lane 2</td>
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</tr>
<tr>
<td>Lane 3</td>
<td>55pmol RNA with 200 nM NS3</td>
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</tr>
<tr>
<td>Lane 4</td>
<td>55pmol RNA with 400 nM NS3</td>
<td>61.74514</td>
</tr>
<tr>
<td>Lane 5</td>
<td>55pmol RNA with 600 nM NS3</td>
<td>19.26387</td>
</tr>
</tbody>
</table>

Figure 3.12. Electromobility shift assay of the interaction of 3’ end of the negative RNA strand and NS3 protein. A. RNA-protein interaction was detected by adding 55pmol fraction of biotinylated RNA to different NS3 protein concentrations ranging from 100-600 nM in binding buffer at 4°C for 30 min and loaded into 10% acrylamide gel, lane 1; free RNA, lane 2-5 different NS3 protein concentrations. B. Representation of quantification of each band of RNA/Protein was calculated using the Image-J program. Relative Intensity was calculated by dividing the absolute intensity of each sample band (on the table) of each band by the absolute intensity of control. C. Histogram presents relative intensity for each band.
3.2 The interaction of NS3 protein with Host proteins

NS3 has been shown to be present in HCV RNA complexes and to interact with several HCV non-structural proteins; NS4A, NS5A and NS5B [273-275]. Host proteins are also likely to play a role in these RNA replication complexes and to interact with the viral proteins [276]. Recombinant adenoviruses that express NS3 and NS3/4A proteins from different HCV genotypes were used to investigate the interaction of NS3 with host proteins that may be important for HCV RNA replication and their stabilizing effects on NS3.

3.2.1 Expression of target protein in recombinant adenoviral vector

Adenoviral vectors (AdV) have been used to express a wide variety of viral and cellular genes in mammalian cells. Proteins produced with the adenovirus expression system have been used for biochemical characterization of proteins as well as in gene therapy experiments. Generally, the adenoviral vectors used are based on human adenovirus serotypes 2 (Ad2) and 5 (Ad5) of subgroup C. The most widely used adenoviral vectors for gene delivery are the called “first-generation” replication-deficient vectors, in which the E1 region of the genome is deleted. Deletion of the E1 region, while retaining the ITR and packaging signal, is designed to prevent expression of the E2 genes and thus block viral DNA replication and the synthesis of late structural proteins.

E1-deleted Ad vectors are therefore propagated in complementing human cell lines that provide the E1 proteins in trans. In order to provide additional cloning space in the vector, the E3 region, which is not necessary for viral replication in culture, is also commonly deleted. Because adenoviruses can encapsidate DNA ranging from 75 to 105% of the length of the wild-type viral genome, these modifications allow up to 7.5 kb of foreign DNA to be accommodated. Adenoviral vectors allow for transmission of their genes to the host nucleus but do not insert them into the host chromosome. The 293A cell line is used for viral production. This is a human kidney cell line which has been stably transfected with the E1A region of the adenoviral genome. This allows the vector to be made and matured within the 293A cell, although, vectors prepared from this cell line will lack the E1A region and they are replication-deficient. Adenoviral vectors enables generation of high titer adenoviral stocks and are capable of containing DNA inserts up to 7.5 kilobases, and can infect both replicating and differentiated cells.
3.2.2 Adenovirus stock amplification

In this study, adenoviruses containing individual NS3 and NS3/NS4A inserts from genotypes 1a, 1b and 2a containing V5 were constructed, Al-Hababi, 2008 [277], using the Vira Power Adenovirus Expression System (Invitrogen), and stored at - 80°C. All adenovirus stocks containing HCV NS3 and NS3/NS4A inserts from genotypes 1a, 1b and 2a were used to generate new high titre viral stocks. 293A cells were infected with the Adenovirus vectors which bind to the Coxsackie/Adenovirus Receptor (CAR) [278]. Adenoviruses are then internalized via integrin-mediated endocytosis [279], followed by active transport to the nucleus. Within the nucleus the early events of gene expression are initiated i.e. transcription and translation of the adenovirus early proteins, followed by expression of the adenoviral late genes and viral replication. These events depend on E1 that is produced by the 293A cells.

After 24 hours post infection, morphological changes appear in 293A cells. These showing discrete large rounded cells together with some normal cells. After approximately 48 hours 80-90% of the cells were rounded up and became detached from tissue culture flask. By 96 hours post infection, all the cells were floating which indicates that cells are filled with adenoviral particles. The cells were then harvested and the titers were determined by plaque assay (Figure 3.13).
3.2.3 Determination of adenovirus titer by plaque assay

In order to determine the optimum quantity of virus needed to produce proteins in the expression experiments, plaque assay was used. The assay was carried out using a 293A cells monolayer. These cells were infected with a 10-fold serial dilutions ranging from $10^4$ to $10^{13}$ of adenovirus stock, and the cells were then covered with a pre-warmed 4% agarose overlay, and incubated for 8-10 days until the plaques were visible. In this experiment early cytopathic effects (CPE) started to appear by day 3. Early cytopathic signs...
Results

appear as patches of rounding dying cells. As the infection proceeds, cells containing viral particles lyse and infect neighbouring cells. By day 10 plaques are clearly visible (Figure 3.17).

Once the plaques were visible and distributed all over the wells, they were stained with 1% neutral red and incubated for 3 hours at 37°C in a humidified CO$_2$ incubator. The virus plaques appeared as clear areas with clear plaques surrounded by a pink monolayer of infected cells, since only live cells take up the dye. The plaques were counted and the titer of adenoviral stocks concentration was determined between $10^7$-$10^8$ plaque forming unit (pfu) as shown in (Figure 3.18).
Figure 3.17. Plaque formation in 293A cells infected with AdNS3-1a. A. Day 3 post-infection, cells producing adenovirus first appear as patches of rounding, dying cells. B. Day 6-8 post-transfection, cells containing viral particles lyse and infect neighboring cells and plaques begins to form. C. Day 8-10 post-transfection, infected neighboring cells lyse, forming a plaque that is clearly visible.
## Results

<table>
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<th>Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS3/1a</td>
<td>$9.8 \times 10^7$ pfu/ml</td>
</tr>
<tr>
<td>NS3/4A/1a</td>
<td>$3.9 \times 10^8$ pfu/ml</td>
</tr>
<tr>
<td>NS3/1b</td>
<td>$1.2 \times 10^8$ pfu/ml</td>
</tr>
<tr>
<td>NS3/4A/1b</td>
<td>$3.5 \times 10^8$ pfu/ml</td>
</tr>
<tr>
<td>NS3/2a</td>
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</tr>
<tr>
<td>NS3/4A/2a</td>
<td>$7.0 \times 10^7$ pfu/ml</td>
</tr>
<tr>
<td><em>LAC Z</em></td>
<td>$4.1 \times 10^8$ pfu/ml</td>
</tr>
</tbody>
</table>

Figure 3.18. Titres of recombinant adenoviruses. The Ad NS3, NS3/4A and Ad *LacZ* adenovirus vectors were titrated by plaque assay.
3.2.4 Determine transduction efficiency (multiplicity of infection) by flow cytometry

Multiplicity of infection was determined using flow cytometry. Different M.O.I doses ranging from 10-200 of six AdNS3, AdNS3/4A adenoviruses carrying the V5 epitope were added to Huh 7 cells. The amount of virus needed to add for a certain MOI was calculated as described in section 2.3.12.2. After 48 hours the cells were trypsinsed and washed and all the subsequent staining was performed. Firstly, the cells were fixed by 4% paraformadylehyde to preserve the general structure of cellular organelles, and then permeabilized with 0.1% Triton X-100 to allow the primary antibody to penetrate the cells. The primary and secondary antibodies were titered before staining, to find the optimal antibody concentration. Anti-V5 mouse monoclonal IgG2a antibody of a dilution of 1:300 in PBS, was found to be the optimal concentration. The secondary antibody gave good results at 1: 800. Before performing the experiment, the C6 Flow Cytometer (Accuri, Cytometers) was validated using validation beads containing a mixture of fluorophores that enable their excitation with the blue laser (488 nm) to validate the channels of flow cytometers. The controls used in this experiment were, 1) Isotype antibody control IgG2a, to confirm that the primary antibody binding is specific and not the non-specific Fc receptor binding or other protein interactions. 2) Cells only as a negative and background autofluorescence control and for gating the cell population. 3) Treated secondary antibody for non-specific binding of secondary antibody on treated cells. However, in this experiment Isotype was also used as the control in flow cytometry experiments to distinguish between the positive and negative cells (Figure 3.19).

3.2.5 Detection of recombinant NS3 protein expression by Immunofluorescent staining

Recombinant adenovirus vectors containing coding sequences for HCV NS3 and NS3/4A from genotypes 1a, 1b and 2a and LacZ recombinant proteins were used to transduce Huh7 cells at 100 MOI for 48 hours. To determine the percentage of Huh7 cells expressing HCV NS3 or NS3/4A, indirect immunofluorescent staining was performed. Mouse monoclonal anti-V5 antibody followed by Alexafluor 945 donkey anti mouse antibody was used to detect the recombinant proteins. The staining indicated that
most of the cells were transduced with the recombinant adenovirus vectors to express HCV NS3 or NS3/4A protein (Figure 3.20). Control Huh 7 cells without NS3 protein, did not show fluorescence with tag V5 antibodies.
Figure 3.19. Determination of transduction efficiency (multiplicity of infection) by flow cytometry. Upper panel (M2) Marker M2 is used to identify the positive stained cells. 1; Untransduced Huh 7 cells (Black), 2; Huh 7 cells transduced with Ad NS3-1a stained for isotype IgG (Red), 3; Huh 7 cells transduced with Ad NS3-1a stained with anti V5 tag antibody and secondary antibody (Blue), 4; Transduced Huh 7 cells with Lac Z stained (Yellow). FACS panels shows the genotype and MOI titre. Lower panel Shows the mean fluorescence intensity of adenovirus stock.
Figure 3.15. Immunofluorescence staining of Huh-7 cells transduced with adenovirus expressing NS3 and NS3/4A protein tagged with V5 epitope. Staining of V5 tagged peptide (red) with anti-V5 antibody to localize HCV-antigen expression. Each slide represents expressed NS3 or NS3/4A from different genotype as indicated. Huh7 cells transduced with V5 tagged LacZ (A-C), Huh 7 negative control (D-F), Huh 7 transduced with adenovirus NS3-1a (G-I). Huh 7 transduced with NS3/4A-1a (J-L), Huh 7 transduced with NS3-2a (M-O), Huh 7 transduced with NS3/4A-2a (P-R), Huh 7 transduced with NS3-1b (S-U) Huh 7 transduced with adenovirus vector expressing NS3/4A-1b (V-X), first column, DAPI nucleus staining, second column V5 tag antibody staining, third column merged images.
3.2.6 Silencing of host cell gene expression by siRNA

Previously a study using siRNA silencing found a number of cellular genes which are involved in virus replication and secretion, these include TXNIP, RAB27A/B, RAB40 and Staufen 1. However, the precise role of these genes in HCV replication cycle is not known. Also, it is not known if their inhibition would have direct effects on NS3 protein expression or stability. siRNA knockdown of RAB27B, RAB 40B TXNIP and Staufen 1 was carried out to determine the effects on NS3 expression [230].

The efficiency of gene silencing in cells transduced with adenovirus expressing NS3 was tested, using targeting siRNA and non-targeting siRNAs as a negative control (“scrambled RNA sequence” termed SiCon). Cells were transfected with 25nM of the HCV siRNA or 25nM of the non-targeting siRNAs for 48h hours using Lipofectamine 2000 reagent (Invitrogen). Additionally, the non-targeting siRNAs were used to compare the effect on cells transfected with non-targeting siRNA or cells transfected with siRNA knockdown and to ensure non-specific changes in cellular gene expression did not occur either due to the transfection process or the toxic effect of siRNA used in the culture medium. After transfection, qRT-PCR was used to quantify intracellular target gene RNA
levels. Some difficulties were encountered due to variation in cell density at the time of transfection. Low cell density resulted in poor recovery of RNA from the transfected cells while a high cell density resulted in the inhibition of cell growth. To obtain an optimal cell density of 90–95% confluency after 48 hours, the cell density at the time of transfection was optimised at around 50% confluency.

Changes in gene expression due to the knockdown of host genes with specific siRNAs were compared to GAPDH which was used as a reference gene and its expression was considered as a value of 1. Following transfection with the siRNA the effect of the knockdown and changes in gene expression were measured against this reference point using the ∆∆CT equation. Changes in gene expression below 1 show a successful knockdown for the target whilst the changes above 1 indicate the knockdown was unsuccessful.

Figure 3.21 shows the levels of gene silencing using siRNA specific for RAB 27B. It showed decrease of 98% in both NS3-1a and NS3/4A-1a. Also, RAB 27B silencing showed a reduction in the levels of RAB 27B by 86% in genotype NS3-2a and by 94% in genotype NS3/4A-2a. RAB 27B levels showed a decrease in NS3-1b and NS3/4A-1b genotypes of 92% and 88%, respectively, compared with the negative control.

Figure 3.22 RAB 40B RNA reduced gene expression of 78% in cells carrying genotypes NS3/1A and by 96% in cells that express genotype NS3/4A-1a. RAB 40B silencing showed a reduction in the level of RAB 40B of 84% in genotype NS3-2a and by 95% in genotype NS3/4A-2a. RAB 40B levels showed a decrease in NS3-1b and NS3/4A-1b genotypes of 97% and 85%, respectively, compared with the control non-targeting siRNA (siCon) control. Figure 3.23 the Staufen 1 reduced gene expression of 88% in genotype NS3/1A and by 98% in genotype NS3/4A-1a. Also, Staufen 1 silencing showed a reduction of 82% in genotype NS3-2a and by 83.6% in genotype NS3/4A-2a. Staufen 1 levels showed a reduction in cells with NS3/1A and NS3/4A-1b of 77% and 92%, respectively, compared with the control.
Figure 3.21. Quantification of RAB 27B siRNAs inhibition levels. Huh7 cells transduced with adenovirus expressing NS3 were transfected with specific siRNA in triplicate and 48 hours post transfection, the target genes were quantified by qRT-PCR. GAPDH siRNA was used to measure the efficiency of transfection. Control non-targeting duplex siRNA (siCon), with a scrambled sequence, was used as negative control.
Figure 3.22. Quantification of RAB 40B siRNAs inhibition levels. Huh7 cells transduced with adenovirus expressing NS3 were transfected with specific siRNA in triplicate and 48 hours post transfection, the target genes were quantified by qRT-PCR. GAPDH siRNA was used to measure the efficiency of transfection. Control non-targeting duplex siRNA (siCon), with a scrambled sequence, was used as negative control.
Results

Figure 3.23. Quantification of Staufen 1 siRNAs inhibition levels. Huh7 cells transduced with adenovirus expressing NS3 were transfected with specific siRNA in triplicate and 48 hours post transfection, the target genes were quantified by qRT-PCR. GAPDH siRNA was used to measure the efficiency of transfection. Control non-targeting duplex siRNA (siCon), with a scrambled sequence, was used as negative control.
Figure 3.24. Quantification of TXNIP siRNAs inhibition levels. Huh7 cells transduced with adenovirus expressing NS3 were transfected with specific siRNA in triplicate and 48 hours post transfection, the target genes were quantified by qRT-PCR. GAPDH siRNA was used to measure the efficiency of transfection. Control non-targeting duplex siRNA (siCon), with a scrambled sequence, was used as negative control.
Figure 3.24 shows TXNIP gene was with a decrease of 85% and 95% respectively, in both genotypes NS3-1a and NS3/4A-1a. Also, TXNIP silencing shows reduction in the levels of TXNIP by 86% in genotype NS3-1a and by 94% in genotype NS3/4A-2a. On the other hand, TXNIP levels showed decrease in cells expressing both NS3/1b and NS3/4A-1b genotypes by 82% and 88%, respectively, compared with the control.

3.2.6.1 Effect of RAB 27B gene silencing on NS3 protein expression

The RAB family of proteins is a member of the Ras superfamily of monomeric G proteins. RAB GTPases regulate many steps of membrane trafficking, including vesicle formation, vesicle movement along actin and tubulin networks, and membrane fusion. It is expressed in platelets, the stomach, pituitary, and bladder [200].

To determine whether or not RAB27B gene knockdown has effects on HCV NS3 expression, Huh7 cells were transfected with 25nm RAB27B siRNAs for 24 hours followed by transduction with adenovirus expressing HCV NS3 protein at MOI of 100 for 48 hours. The levels of NS3 and NS3/4A gene expression were determined by qRT-PCR. Figure 3.25 shows the effect of RAB 27B knockdown on NS3 and NS3/4A protein expression. The NS3-1a and NS3/4A-1a showed decrease in expression by 25% and 33%, respectively, while the effect of silencing RAB 27B was 26% on genotype NS3-2a and 36% on genotype NS3/4A-2a compared to the negative controls transfected with scrambled siRNA (siCon). The levels of genotypes NS3-1b and NS3/4A-1b were also determined and they showed reduction in their levels by 40% and 37%, respectively.

3.2.6.2 Effect of RAB 40B gene silencing on NS3 expression

RAB40B was found expressed in E14.5 embryonic and adult mouse brain, inner ear and heart tissues, the role of this protein in HCV replication is still unknown, also it is unknown whether this protein has direct interaction with HCV protein [207]. As shown in (Figure 3.26), there was a decrease of 36% in NS3-1a expression, while the effect of RAB 40B silencing was 28% on NS3/4A-1a expression. The genotype NS3-2a and NS3/4A-2a showed decreases in their expression by 27% and 15%, respectively. The genotype NS3-1b also showed a considerable decrease of 40%, while NS3/4A-1b showed a reduction of 37%. 
Figure 3.25. The effect of RAB 27B siRNA on adenovirus expressing NS3 and NS3/4A genotypes 1a,1b and 2a. Huh7 cells were transduced with adenoviruses expressing NS3 and NS3/4A genotypes 1a,1b and 2a. Total cellular RNA was extracted and qRT-PCR was used to quantify the changes in gene expression. Fold change in NS3 expression was normalized using GAPDH.
Figure 3.16. The effect of RAB 40B siRNA on adenovirus expressing NS3 and NS3/4A genotypes 1a, 1b and 2a. Huh7 cells were transduced with adenoviruses expressing NS3 and NS3/4A genotypes 1a, 1b and 2a. Total cellular RNA was extracted and qRT-PCR was used to quantify changes in gene expression. Fold change in NS3 expression was normalized using GAPDH.
3.2.6.3 Effect of Staufen 1 gene silencing on NS3 expression

Staufen 1 is a dsRNA-binding protein, localised to the rough endoplasmic reticulum in ribonucleoprotein complexes, it play a role in the degradation, translation and trafficking of mRNA molecules. As shown in (Figure 3.27), there is decrease in NS3-1a level by 16%, while the effect of staufen 1 gene silencing was 23% on NS3/4A-1a expression. NS3-2a and NS3/4A-2a show reductions in their expression by 40% and 39%, respectively. On the other hand, NS3-1b was also showed decrease in its level by 37%, whereas, NS3/4A-1b showed less reduction levels by 17%.

3.2.6.4 Effect of TXNIP Gene Silencing on NS3 expression

Thioredoxin-interacting protein (TXNIP) is encoded by the TXNIP gene. TXNIP has multiple functions. Silencing of TXNIP gene (Figure 3.28) showed reduction of 18% on NS3-1a and 20% for NS3/4A-1a following the knockdown. Reduction of NS3-2a was achieved by 30%, whereas, the NS3/4A-2a levels lowered by 25%, which was almost the same level in reduction of full length NS3 or with full length with NS3/4A. Silencing of TXNIP showed a reduction of 20% in NS3-1b and 25% in NS3/4A-1b.
Figure 3.27. The effect of Staufen 1 siRNA on adenovirus expressing NS3 and NS3/4A genotypes 1a, 1b and 2a. Huh7 cells were transduced with adenoviruses expressing NS3 and NS3/4A genotypes 1a, 1b and 2a. Total cellular RNA was extracted and qRT-PCR was used to quantify fold change in gene expression. Fold change in NS3 expression was normalized using GAPDH.
Figure 3.28. The effect of TXNIP siRNA on adenovirus expressing NS3 and NS3/4A genotypes 1a, 1b and 2a. Huh7 cells were transduced with adenoviruses expressing NS3 and NS3/4A genotypes 1a, 1b and 2a. Total cellular RNA was extracted and qRT-PCR was used to quantify fold change in gene expression. Fold change in NS3 expression was normalized using GAPDH.
3.2.7 Flow cytometry analysis of V5 tag NS3 protein expression

Flow cytometry analysis was carried out on Huh7 cells transduced by NS3 or NS3/4A adenovirus vector. To determine the effect of host proteins RAB 27B, RAB 40B, TXNIP and Staufen 1 on V5 tagged NS3 protein expression. Huh7 cells previously transduced with the adenovirus vectors were transfected with target siRNA and then stained using an anti-V5 tag antibody for tagged NS3 protein using the protocol described in section 2.3.13.2. An isotype antibody which has no specificity for target protein, was run alongside each knockdown sample as a negative control. Figure 3.29 shows a FACS plot generated for gene silencing. For each cell line unstained Huh 7 cells were used to gate the population of live cells (Black). Transduced Huh 7 cells with Ad-NS3-1a were used as a positive reference and run with each set of siRNA samples also with Isotype antibody as a negative control. FACS analysis was performed in order to detect V5 tagged NS3 in Huh 7 cells. Cells were stained with monoclonal anti-V5 antibody and secondary anti-mouse Alexa-Flour 594 to detect V5 tagged NS3. The positive cells were identified by a shift towards the right of the plot (M2) (Blue), when compared to stained cells with the secondary antibody (Red) or with the isotype control (Yellow). The results were presented as the mean fluorescence intensity (MFI) measured by Accuri flow cytometry software and reported in arbitrary units.

Figure 3.29. FACS plot of positive staining for NS3 protein.

Marker M2 is used to identify the positive stained cells. 1; Untransduced Huh 7 cells (Black), 2; Huh 7 cells transduced with Ad NS3-1a stained for Alexa-Fluor 594 conjugated IgG (Red), 3; Huh 7 cells transduced with Ad NS3-1a stained with anti V5 tag antibody and secondary antibody (Blue), 4p; Transduced Huh 7 cells with Ad NS3-1a protein stained for Isotype IgG (Yellow).
3.2.7.1 Silencing effect of host genes RAB 27B and RAB 40B on V5 tag NS3 protein

Fig 3.30 A shows the effect of silencing of RAB27B on Huh 7 cells transduced with different adenovirus genotypes. Transduced cells were stained intracellularly with mouse anti-V5 tag antibody and a secondary anti-mouse Alexa-Flour 594 antibody. Panels (i-vi) shows the flow cytometry analysis of RAB 27B gene silencing, each panel defines a specific genotype, all genotypes show a shift to the right compared with the NS3 positive untreated control, which indicates that silencing of RAB27B was up regulating NS3 protein expression. To detect whether this shift is significant or not, the results were analyzed as the mean florescence intensity (MFI) of each genotype, also the level of expression was compared to its specific positive untreated control, the results were analysed using analysis of variance (ANOVA), all of the genotypes showed significant difference compared with the control indicating (Fig 3.30. B). Additionally, silencing of RAB 40B was also tested for its effect on NS3 protein in different genotypes. (Figure 3.31.A) panels (i-vi) represent the result of the flow cytometry which shows detectable positive shift on all genotypes but the shift was higher above the control positive in Huh 7 cells transduced with genotypes NS3-1a, NS3/4A-1a, NS3-2a and NS3/4A-1b. The MFI of these genotypes was analyzed and showed statistical significantly change ($P > 0.05$) (Figure 3.31.B).

3.2.7.2 Silencing Effect of Host Genes TXNIP and Staufen 1 on V5 tag NS3 protein

Fig 3.32 A shows the effect of TXNIP on NS3 V5 tagged protein. Flow cytometry panels (i-vi) shows analysis of TXNIP host gene silencing, each panel defines specific genotype, all genotypes did not shows positive shift to the right which indicate that silencing of TXNIP has no effect on NS3 and NS3/4A protein. The MFI of the samples was measured; the level of expression was compared to the positive control, which showed no significant change in NS3 protein all of the genotypes (Figure 3.32 B). Staufen 1 gene knockdown was also tested for its effect on all transduced cells with all NS3 protein genotypes. Figure 3.33. A shows the effect of Staufen 1 on NS3 protein, which did not show changes in NS3 protein expression above the normal levels of NS3 protein control.
Figure 3.30. Flow cytometry analysis of the effect of RAB 27B silencing on NS3 V5 tagged protein. (A) Panels (i-vi) show the effect of RAB 27B gene silencing on NS3 V5 tagged protein stained with anti-V5 antibody and anti-mouse Alexa-Flour 594, each panel represents a specific NS3 or NS3/4A genotype. Changes in expression of NS3 protein (showed as shift to the right in the flow cytometry plots) compared to the control. (B) The (MFI) of NS3 and NS3/4A protein genotypes showed significant changes in their levels compared to the control ($P > 0.05$). The mean florescence intensity (MFI) represented in arbitrary units, and the error bars represent the standard deviation from two independent experiments.
Figure 3.31. Flow cytometry analysis of the effect of RAB 40B silencing on NS3 V5 tagged protein. (A) Panels (i-vi) shows the effect of RAB 40B gene silencing on NS3 V5 tagged protein, each panel represents a specific NS3 or NS3/4A genotype. Changes in expression of NS3 protein (showed as shift to the right in the flow cytometry plots) compared to the control. (B) The MFI shows significant change in genotypes NS3-1a, NS3/4A-1a, NS3-2a and NS3/4A-1b ($P > 0.05$). The mean florescence intensity (MFI) represented in arbitrary units, and the error bars represent the standard deviation from two independent experiments.
Results

Figure 3.32. Flow cytometry analysis of the effect of TXNIP on V5 tag NS3 protein stained with anti-V5 antibody and anti-mouse Alexa-Flour 594. (A) Panels (i-vi) shows flow cytometry analysis of TXNIP gene silencing on NS3 V5 tagged protein, each panel represents a specific NS3 or NS3/4A genotype. (B) Shows the (MFI) of NS3 and NS3/4A protein genotypes did not show significant changes in their levels compared with the control. The mean florescence intensity (MFI) represented in arbitrary units, and the error bars represent the standard deviation from two independent experiments.
Figure 3.33. Flow cytometry analysis of the effect of Staufen 1 on V5 tag NS3 protein stained with anti-V5 antibody and anti-mouse Alexa-Flour 594. (A) Panels (i-vi) show flow cytometry analysis of Staufen 1 gene silencing on NS3 V5 tagged protein, each panel represents a specific NS3 or NS3/4A genotype. B. Shows the (MFI) of NS3 and NS3/4A protein genotypes did not show significant changes in their levels compared with the control. The mean florescence intensity (MFI) represented in arbitrary units, and the error bars represent the standard deviation from two independent experiments.
3.3 Effects of inhibition of NS3 protease activity on interferon pathway gene expression

NS3 protease activity has been shown to inhibit the interferon response pathway by proteolysis of the signalling intermediates, TRIF and MAVS. Evidence that NS3 can also inhibit the expression of interferon response pathway genes has recently been obtained Al-Hababi, 2011 [277]. To investigate whether this inhibition is due to the NS3 protease, the effect of the NS3 specific protease inhibitor BILN 2061 on host gene expression was examined.

3.3.1 Effect of protease inhibitor BILN 6021 on NS3 protein expression and IFN pathway

To investigate the effect of antiviral protease inhibitor BILN 6021 on the IFN pathway cytoplasmic receptors, MAVS, RIG-I and MDA5, in cells expressing NS3 and NS3/4A. BILN 2061 (10µM) were added to the culture medium of Huh7 cells transduced with adenovirus expressing NS3 and NS3/4A for 24 hours. The antiviral activity of BILN 2061 compound alone and with addition of poly (I:C) for each genotype to stimulate the toll-like receptor (TLR) 3 was confirmed by measuring mRNA levels by qRT-PCR. Data were analysed using one-way analysis of variance (ANOVA), and Bonferroni post-tests. Figure 3.34.A showed slight reduction in expression of NS3-1a when treated with BILN 2061. Additionally, BILN 2061 reduced the expression of MAVS, RIG-I and MDA5 genes significantly compared to the control levels. Moreover, the effect of BILN 2061 and poly (I:C) showed slight changes in MAVS, RIG-I and MDA5 expression levels. Treatment of Huh7 expressing NS3/4A-1a with BILN 2061 showed significant decrease in NS3/4A-1a expression (P <0.05) in comparison to the control, whereas, RIG-I, MDA5 and MAVS host genes showed significant reduction in comparison with the control levels. The effect of BILN 2061 and poly (I:C) reversed MAVS, RIG-I and MDA5 genes to control levels (Figure 3.34.B).
Figure 3.34. Effects of BILN 2061 on HCV NS3-1a (A) and NS3/4A-1a (B) on MAVS, RIG-I and MDA5 and expression. Huh7 cells were grown in 24 well plates followed by transduction with AdNS3, AdNS3-4a for 48 hour. Cells were stimulated with poly (I:C) (5ng/μl) for 8 hours. Total RNA was extracted, and the mRNA expression was determined by real-time PCR and normalized using GAPDH.
Figure 3.35. Effects of BILN 2061 on HCV NS3-2a (A) and NS3/4A-2a (B) on MAVS, RIG-I and MDA5 expression. Huh7 cells were grown in 24 well plates followed by transduction with AdNS3, AdNS3/4A for 48 hour. Cells were stimulated with poly (I: C) (5ng/μl) for 8 hours. Total RNA was extracted, and the mRNA expression was determined by real-time PCR and normalized using GAPDH.
Figure 3.35.A shows slight reduction in NS3-2a levels treated with protease inhibitor BILN 2061. MAVS, RIG-I and MDA5 genes did not show significant changes in their expression levels when treated with BILN 2061 or with a combination of BILN 2061 and poly (I:C). NS3/4A-2a treated with BILN 2061 showed significant decrease in NS3/4A-2a expression (P <0.05) compared to the control. MAVS, RIG-I and MDA5 gene expression levels were reduced when treated with BILN2061. Additionally, BILN 2061 and poly (I:C) combination reversed MAVS, RIG-I and MDA5 genes to the control levels in response to poly (I:C) stimulation (Figure 3.35.B).

Figure 3.36.A shows slight reduction in NS3-1b levels when treated with BILN 2061. In addition, BILN 2061 did not show significant changes on MAVS, RIG-I and MDA5 genes, whereas, BILN 2061 and poly (I:C) reversed slightly MAVS, RIG-I and MDA5 genes. NS3/4A-1b treated with BILN 2061 showed significant reduction in NS3/4A-1b expression (P <0.05) in comparison to the control levels. Additionally, MAVS, RIG-I and MDA5 showed significant reduction when treated with protease inhibitor BILN2961, whereas, BILN 2061 and poly (I:C) reversed MAVS, RIG-I and MDA5 genes in response to poly(I:C) stimulation. (Figure 3.36. B).
Figure 3.36. Effects of BILN 2061 on HCV NS3-1b (A), and NS3/4A-1b (B) on MAVS, RIG-I and MDA5 expression. Huh7 cells were grown in 24 well plates followed by transduction with AdNS3, AdNS3/4A for 48 hour. Cells were stimulated with poly (I: C) (5ng/μl) for 8 hours. Total RNA was extracted, and the mRNA expression was determined by real-time PCR and normalized using GAPDH.
3.4 Summary of the interaction of NS3-1a protein with JFH-1 RNA

Full-length NS3 [1027-1657], has been expressed as a histidine-tagged fusion protein in *E. coli* and isolated from inclusion bodies and purified under denaturing conditions using chaotropic agents urea and imidazole. Expression of NS3 protein was confirmed as a band of approximately 70 kDa observed by SDS-PAGE analysis and Western blotting (Figure 3.5). The NS3 protein was then refolded by sequential dialysis to remove the urea and imidazole. A colorimetric assay for NS3 protein ATPase activity showed a linear relationship between, the time of the reaction between enzyme substrate, and the level of ATPase activity demonstrating that the NS3 protein was enzymatically active. To determine the interaction of full length HCV NS3-1a protein with the full length HCV JFH-1 RNA, an electro mobility shift assay (EMSA) was used, at different concentrations of HCV NS3-1a protein, ranging from 100nM to 600nM, and 55pmol of full length biotinylated JFH-1 RNA. The results showed a decrease in the chemoluminescence signal intensity of the biotinylated RNA and NS3 protein complex with an increase in NS3 protein concentration, which indicated binding between RNA and NS3-1a protein. The RNA protein complex showed a slight migration compared with the control; however this was not significant (Figure 3.14). Similar results were seen for the interaction of the biotinylated 3’end HCV the negative strand RNA and HCV NS3-1a protein (Figure 3.15).

3.5 Summary of silencing effect of host genes RAB 27B, RAB 40B, Staufen 1 and TXNIP on NS3 and NS3/4A protein expression

Adenovirus stocks containing individual NS3 and NS3/NS4A inserts from genotypes 1a, 1b and 2a containing a V5 tag were used to generate high titre viral stocks and titered by plaque assay. An MOI (multiplicity of infection) of 100 was determined to be appropriate for use in subsequent experiments. These stocks of adenovirus vectors that express HCV NS3 and NS3/NA4A proteins were used to test the effect of the silencing of host protein genes, RAB 27B, RAB 40B, TXNIP and Staufen 1 on NS3 stability. Firstly, the efficiency of RAB 27B, RAB 40B, TXNIP and Staufen 1 gene silencing by siRNAs was
Results

quantified using qRT-PCR and all of the host genes showed a significant decrease in expression.

The effect of host gene RAB27 knockdown on NS3 gene expression was determined and it showed a decrease in transcript levels in all NS3 genotypes. The NS3-1a and NS3/4A-1a genotypes showed a decrease in expression by 25% and 33%, respectively, while the decrease in expression was 26% for NS3-2a and 36% for NS3/4A-2a compared to the negative control. The NS3-1b and NS3/4A-1b gene expression showed a greater decrease in their levels, by 40% and 37%, respectively (Figure 3.24). RAB 40B gene silencing showed decrease of 36% in NS3-1a expression and 28% in NS3/4A-1a gene expression. NS3-2a and NS3/4A-2a showed decreases in their expression of 27% and 15%, respectively. Additionally, NS3-1b showed a considerable decrease in NS3 expression by 40%, while NS3/4A-1b showed a reduction in expression by 37% (Figure 3.25). RAB 27B and RAB 40B host gene knockdown both lowered the expression levels of all NS3 genotypes, which indicate that these proteins may be required by functional HCV NS3 protein. Silencing of the Staufen 1 gene gave a 16% decrease in NS3-1a a 23% decrease in NS3/4A-1a expression. NS3-2a and NS3/4A-2a showed reductions in their expression by 40% and 39%, respectively. NS3-1b expression was reduced by 37%, whereas, NS3/4A-1b only showed a reduction of 17% (Figure 3.26). Moreover, silencing of host gene TXNIP showed a reduction of 18% on NS3-1a and 20% for NS3/4A-1a following the knockdown, whereas, NS3-2a showed a reduction of 30%. The NS3/4A-2a levels were lowered by 25%, which was similar to the level of reduction by full length NS3. Silencing of TXNIP showed a reduction of 20% in NS3-1b and 25% in NS3/4A-1b (Figure 3.27), these results showed that both Staufen 1 and TXNIP reduced the levels of gene expression in all NS3 genotypes. The effect of host gene proteins RAB 27B, RAB 40B, TXNIP and Staufen 1 knockdown was also tested for all V5 tagged NS3 at the protein level by flow cytometry. Knockdown of RAB 27B and RAB 40B showed a detectable increase in the levels of NS3 and NS3/4A proteins in all genotypes, whereas, silencing of Staufen 1 and TXNIP had no detectable effect on HCV NS3 protein levels in all genotypes.
3.6 Summary of the effect of protease inhibitor BILN 2061 on NS3 and NS3/4A protein expression and IFN pathway

Huh 7 cells expressing NS3-1a, NS3-2a and NS3-1b protein treated with the antiviral protease inhibitor BILN 2061 showed slight reduction in expression levels of these genes, whereas, Huh 7 cells expressing NS3/A-1a, NS3/A-2a and NS3/A-1b protein showed significant reduction in NS3/A-1a, NS3/A-2a and NS3/A-1b expression levels. The high reduction in NS3/4A-1a, 2a and 1b genotypes expression levels could be due to the presence of the co-factor NS4A. The effect of the antiviral protease inhibitor BILN 2061 on cytoplasmic receptor components of the IFN pathway, namely MAVS, RIG-I and MDA5, were tested in Huh 7 cells expressing NS3 and NS3/4A. Cells expressing NS3-1a, NS3-2a and NS3-1b genotypes treated with BILN 2061, showed little reduction in expression of MAVS, RIG-I and MDA5 levels compared to control levels, whereas BILN2061 and poly (I:C) reversed MAVS, RIG-I and MDA5. Treatment of Huh7 cells expressing NS3/4A-1a, NS3/4A-1b protein with BILN 2061 showed significant changes in expression levels of MAVS, RIG-I and MDA5 genes, and slight reduction in NS3/4A-2a, whereas, BILN 2061 and poly (I:C) reversed RIG-I, MDA5 and MAVS levels to in response to poly(I:C). (Figures 3.34, 3.35, 3.36).
DISCUSSION


4. Discussion

4.1 RNA-protein interactions

RNA–protein interactions are important in many biological processes, such as all the steps in gene expression and its regulation. Generally, the genomes of positive-stranded RNA viruses are involved in at least three major processes: They act as mRNAs to direct the synthesis of viral proteins; they serve as templates for genome replication; and they are packaged along with structural proteins during viral assembly [74]. Studies on HCV replication have been expanded after the discovery of HCV strain JFH-1 (genotype 2a) that can be grown in cell culture. The HCV genome contains important elements which are critical for virus viability, for instance; the NS3 helicase, which is responsible for unwinding of the viral RNA during replication, the NS5B polymerase which is required for viral RNA replication and 5’ and 3’ UTRs which do not encode any viral proteins but act as functional elements essential for regulating translation and initiating replication [280]. NS3 protein contains a serine protease activity at its N-terminal end, which is responsible for the downstream cleavage in the nonstructural region, thus playing a fundamental role in viral RNA replication [31, 50]. Additionally, The X-ray crystallography structure of NS3 and structure-based mutagenesis of NS3 has identified important amino acid residues required for helicase and ATPase activities [9, 29, 37, and 59]. Generally, Helicases are involved in many RNA metabolic processes, such as transcription, RNA splicing, RNA export, translation, and RNA degradation [275].

A recent study showed that DDX3 which is a cellular helicase is involved in HCV replication, and thus might also be involved in HCV assembly. Moreover, it may be incorporated into the HCV virion by interacting with the core to act as a chaperone [239]. However, viral helicases are also found to play a role in viral replication. A study on NS3 of Yellow Fever Virus (YFV) reported that a mutation of conserved tryptophan 349 in the helicase domain of NS3 impeded the production of virus particles, thus indicating that NS3 might play a role in the virus assembly, in addition to its known enzymatic properties [281].
In this study we demonstrated that the HCV NS3 protein from amino acid [1027-1657] was expressed as a histidine-tagged fusion protein in E. coli. It was then isolated and purified from inclusion bodies [264]. The purification of NS3 protein was achieved using a Ni-NTA column under denaturing conditions and imidazole, and the expression of NS3 was confirmed by SDS-PAGE analysis and Western blot. However, despite all the adjustments that were made to produce pure NS3 protein e.g. varying the imidazole concentrations in the elution buffer, and purification at 4°C, it was difficult to produce high levels of pure NS3 protein. This could be due to several reasons e.g. the position of the 6X His tag may influence the ability of NS3 to bind to the Ni-NTA column or the interaction of NS3 with other proteins when expressed at high levels which might even influence it solubility. However, detection of NS3-1a protein unbound to nickel in flow-through was detected previously [268]. It was observed that large amounts of NS3-1a precipitated during the refolding process, this aggregation of protein during refolding may be due to the absence of cellular factors such as chaperones and cell organelles e.g. ER which would be present during viral RNA translation, or viral proteins which would normally be present during viral RNA translation. Additionally, other viral proteins which would normally be present during HCV replication such as NS4A which is known to associate strongly with NS3 protein [282] [283], may also be required for the correct NS3 folding. SDS PAGE analysis demonstrated that the refolded protein was partially purified i.e. with some cellular protein contaminants, however, several studies have previously used partially purified protein to successfully study RNA–protein interactions [284].

Generally, RNA helicases of DEAD-box protein family possess an ATPase activity that is dependent on, or stimulated by RNA. However, ATPase activity does not require a specific RNA substrate [88]. Several studies revealed cooperativity between RNA binding and ATP binding with the binding of RNA being stronger in the presence of ATP [88]. The characterization of the full-length NS3-1a protein was studied previously by Wardell et al. 1999 [268], NS3 was able to hydrolyse a variety of NTPs and dNTPs and showed a preference for ATP. The biochemical characteristics of HCV helicase and NTPase activity were similar to those of other viral and non-viral RNA helicases, for example the yeast protein PRP22 [285], and the bluetongue virus VP6 protein [286], which both exhibited polynucleotide stimulated ATPase activity. In this study the ATPase activ-
The activity of NS3 protein was tested and a linear relationship was demonstrated between the time of the reaction between enzyme and substrate and the level of ATPase activity. This showed that the protein possesses ATPase activity indicating that the refolding process was successful in producing functionally active NS3 protein.

Electrophoretic mobility shift assays (EMSA) are used to detect a complex of proteins bound to a labelled RNA probe. The RNA–protein complex is separated by agarose gel electrophoresis and transferred to a positively charged nylon membrane. Although, the sensitivity of the biotin-labelled EMSA is not as high as that with radioisotope labelling, there are practical advantages with this technique. Biotin-labelled RNA probes avoid the hazard and inconvenience of RNA probes with radioisotope labelling [287] [288]. In this study we found that there is binding activity between the full length JFH-1 RNA and NS3-1a protein which was detected by a decrease in the chemiluminescence signals of the labelled JFH-1 RNA. The interaction between the full length NS3-1a and the 3’ end of the negative strand was also detected by observing a reduction in the biotin intensity also; the band shifts were more obvious compared to the full length JFH-1 RNA.

Shifting of JFH-1 RNA-NS3-1a protein complex bands was not great which may be attributed to the differences in the RNA-protein ratio [287]. Moreover, the observed binding between JFH-1 RNA and NS3-1a and the 3’ end of the negative strand with NS3-1a protein indicates that the interaction between NS3 protein and JFH-1 RNA (genotype 2a) is not genotype specific. Studies using $^{32}$P-labelled RNA have reported that NS3 protein specifically binds to the 3’ ends of both the positive- and negative-strand RNAs of HCV [289]. However, in the present study the observed interactions between the JFH-1 RNA and genotype 1a NS3 protein may indicate that NS3-RNA interactions are not dependent on highly conserved nucleic acid sequences.

There are many possible reasons why NS3-1a protein may be flexible in the sequences that it can bind. One possibility is that although that 3’ and 5’ terminal sequences are well conserved, in quasispecies that are generated during HCV replication they have sequence mismatches compared to the canonical genotype sequence. However, flexibly in sequence recognition by the HCV RNA replication machinery would help to ensure that they can also be replicated. It is also possible that cell-free protein nucleic acid binding
experiments lack other host or virus components, or the physiological conditions needed to replicate in the intracellular environment. The binding of NS3 to viral genome sequences directly implicates NS3 protein in the multiple processes within the viral life cycle involving the genome (translation, RNA replication, and assembly). In this regard, the interaction of NS3 with the 3′ end negative strand and the full-length JFH-1 could be a valid target for antiviral intervention.

4.2 Host genes involved in Protein–Protein interaction

Many biological functions involve the formation of protein-protein complexes. Protein-protein interactions play important roles at several levels of cell function e.g. in the structure of sub-cellular organelles, the transport machinery across the various biological membranes, packaging of chromatin, the network of sub-membrane filaments and signal transduction and regulation of gene expression [290]. The regulation of cell function brought about by the interactions of these proteins is balanced by the relative affinities of the various protein partners and the modulation of these affinities by the binding of ligands, other proteins, nucleic acids, ions such as Ca2+, and covalent modification, such as specific phosphorlyation or acetylation [290]. Microarray analysis study published by Dr McGarvey’s group [271], found that several host genes play a role in the replication of HCV. It was shown that HCV infection altered the expression level of about 2000 host genes; subsequent siRNA knock down analysis showed that several of these appeared to be important for HCV replication. One possibility is that some of these host proteins may play a role in the formation of HCV RNA replication complexes and that knocking out the expression of these genes could destabilize these replication complexes and increase the turnover of HCV proteins involved in RNA replication. NS3 or NS3/4A protein is thought to be important in the replication complexes. Therefore, this study looked at the alterations in NS3 levels when host genes TXNIP, Staufen 1, RAB278 or RAB40B expression were knockdown. Thioredoxin-interacting protein (TXNIP) is a multifunctional protein which regulates critical biological processes, such as inflammation, stress, and apoptosis. It also has a tumor-suppressive function is under-expressed in some human cancers. Moreover, it has been found that TXNIP down regulation may play a role in
liver carcinogenesis, because TXNIP deficiency is able to induce HCC in experimental mice [291]. In addition, TXNIP is involved in the regulation of lipid metabolism and may therefore impact on the HCV [271]. Moreover, TXNIP was identified through microarray analyses, validated by qRT-PCR, and recognized as highly up regulated during HCV-infection. The knockdown of TXNIP by RNA interference inhibits HCV replication by up to 90%, this implies that TXNIP is required for HCV replication [230]. In this study, the effect of TXNIP silencing on NS3 and NS3/4A gene expression from different genotypes in siRNA transfected cells was measured. A reduction in NS3 and NS3/4A levels was found in genotypes 1a, 2a and 1b. Despite the variation in the level of reduction, this was not significant between each genotype. It is not known whether TXNIP knockout suppresses NS3 expression directly or indirectly. Directly, in which TXNIP itself suppresses IL-3 receptor and cyclin A2 promoter activity, or the silencing of TXNIP may downregulate directly the NS3 expression or indirectly by suppressing other cellular genes which have a regulatory effect on NS3[292].

The RAB proteins are involved in membrane transport regulation. They are membrane secretory vesicle proteins and are thought to be involved in releasing mature virus particles from within an infected cell [271]. Silencing of both host genes RAB 27B and RAB 40B showed a reduction in NS3 levels. The genotypes NS3-1b and NS3/4A-1b showed considerable decreases of 40% and 37% respectively. Also, NS3-1a levels reduced to 36%. However, silencing RAB27B and RAB40B also had a considerable effect on the intracellular JFH-1 RNA levels, indicating that these factors may play multiple roles in the HCV replication cycle [271].

Staufen1 is an RNA-binding protein that controls the translation, trafficking and degradation of cellular mRNA molecules [209]. Staufen 1 has been shown to facilitate virus particle assembly in the replication cycles of HIV and Influenza A [216, 293] and to stimulate translation of the HIV genome. Previous investigations to identify RNA binding proteins that interact with the HCV genome have also demonstrated that Staufen1 binds directly to the HCV genome, at the 3'UTR of the positive RNA strand and 5'UTR of the negative RNA strand [294]. The effect of Staufen 1 silencing on Huh 7 cells transduced with NS3 and NS3/4A protein demonstrated that Staufen 1 knockdown reduces the levels of NS3 proteins in all genotypes. The largest reduction was found with NS3-2a and
NS3/4A-2a genotypes showing reductions in their expression by 40% and 39%, respectively. Staufen 1 knockdown also reduced NS3 levels in JFH-1 infected Huh 7 cells, which replicates the complete cycle of HCV [230].

The levels of RAB27B and RAB40B gene expression were investigated for their effects on NS3 protein expression and stability. Huh7 cells transduced with adenovirus 1a, 2a, and 1b were transfected with the RAB27B and RAB40B siRNA’s. Following transfection the cells were stained and analyzed using flow cytometry analysis. FACS analysis revealed that the RAB27B knockdown and RAB40B median fluorescence intensity (MFI) staining signals showed that high levels of NS3 protein were expressed in both RAB27B and RAB40B knockdown experiments.

RAB27B and RAB40B have important roles in regulating the trafficking of secretory vesicles. Additionally, the expression of RAB27B was found to increase significantly during JFH-1 infection, which indicates that RAB27B play role in the trafficking of secretory vesicles to the plasma membrane, which may be important for virus replication [230]. Moreover, reduced levels of RAB27B and RAB40B may prevent NS3 from being transported to intracellular locations that required for RNA replication or even virus particle assembly. However, NS3 may still be expressed and remain at sites which increase its stability.

The expression of TXNIP was also found to be elevated significantly during JFH-1 infection [271]. Efficient silencing of TXNIP protein expression was observed following transfection of Huh7 cells with TXNIP siRNA. The effect of TXNIP silencing on NS3 protein expression was determined, the MFI signals did not show significant differences in NS3 expression compared to the positive control. This suggests TXNIP may reduce overall HCV replication but may not have direct effect on NS3 stability. The effect of Staufen 1 silencing on NS3 protein expression was investigated and the intensity of the MFI did not show statistical differences in NS3 expression compared to the positive control. This could be because the localization of Staufen 1 has a discrete position rather than adjacent to the target protein NS3, which minimize its effect on NS3. Moreover, a study conducted on Staufen 1 of Drosophila embryogenesis found that the localization of Staufen 1 and Prospero protein which are cellular proteins in drosophila co-localized.
It seems that Staufen 1 may not associate directly with NS3 or other cellular or viral proteins which may play a role to co-localize Staufen 1 to NS3 protein.

4.3 Effect of NS3 on IFN pathway gene expression

Pegylated interferon and ribavirin is the standard combination treatment for HCV. The Efficacy of treatment is 80% in patients infected by HCV genotype 2 or 3, whereas in patients infected by HCV genotype 1 the response to treatment is less than 50% [296-298]. BILN 2061 is a potent antiviral developed to inhibit HCV NS3/4A serine protease. BILN 2061 was used to study its effect on NS3 and NS3/4A protein expression and IFN pathway. In this study, Huh 7 expressing NS3-1a, NS3-2a and NS3-1b treated with the antiviral protease inhibitor BILN 2061 showed slight reduction in their expression levels, whereas Huh 7 cells expressing NS3/A-1a, NS3/A-2a and NS3/A-1b showed significant reduction in expression levels. These differences could be due to the presence of co-factor NS4A. It was reported that all inhibitors have higher affinity for NS3 in the presence of NS4A [299]. The result demonstrated that BILN 2061 has direct effect on NS3 protein especially NS3/4A-1a, 2a and 1b genotypes. Additionally, the results did not show genotypic specificity in inhibition with BILN 2016. HCV NS3/4A complex can inhibit host cell adaptors proteins of the innate immune response TRIF and MAVS [300]. In this study, treatment of Huh 7 cells expressing NS3/A-1a, NS3/A-2a and NS3/A-1b protein with BILN 2061 reduced the expression levels of RIG-I, MAVS and MDA5 more efficiently than in Huh 7 cells expressing NS3-1a, NS3-2a and NS3-1b protein, and reversed the NS3/4A-mediated inhibition of RIG-I, MDA5 and MAVS signaling in response to poly (I:C) stimulation. These results may indicate the role of NS3 protein in the inhibition of IFN induction mediated by dsRNA stimulation. Although, the role of HCV NS3/4A protein has been detected in IFN inhibition by direct targeting both TRIF and MAVS adaptors proteolysis [300-302]. However, the mechanism of how NS3/4A inhibit IFN components at transcription level is still unclear. It has been found in a previous study using subgenomic HCV RNA replicons that the expression of NS3 and NS3/4A proteins, using adenovirus vectors, restored RIG-I, MDA5 and MAVS expression levels in response to dsRNA stimulation,
indicating that the NS3 protein may have a role in regulating the expression of these host genes [277]. Additionally, BILN 2061 has been shown to have some inhibitory effect on RIG-I, MAVS and MDA5, this could be due to dose toxicity of this compound or, the NS3 protein may have more complex role in the regulation of expression of these genes.

In conclusion, this study aimed to investigate whether there is an interaction between recombinant HCV NS3-1a and full length HCV JFH-1 RNA and 3’ end of JFH-1 negative strand RNA. It can be concluded that although there was little altered migration of the RNA-protein complexes, the binding between NS3-1 protein and the target HCV RNAs JFH-1 and the 3’ negative RNA strand was clearly shown. It was also demonstrated that gene silencing of RAB27B and RAB40B had an effect on HCV NS3 and NS3/4A expressed by adenovirus vectors, but that silencing of Staufen 1 and TXNIP did not. The NS3 protease inhibitor BILN 2061 was shown inhibit NS3 and NS3/4A suppression of RIG-I, MDA5 and MAVS gene expression. This was contrary to the expected outcome which indicates that NS3 protein may have more complex role in the regulation of expression of these genes than was originally thought. Additionally, It was found that the expression of NS3 and NS3/4A proteins, using adenovirus vectors, restored RIG-I, MDA5 and MAVS expression levels in response to dsRNA stimulation, indicating that the NS3 protein may have a role in regulating the expression of these host genes.
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6. Appendix

6.1 RNA and DNA molecular weight ladders

Ambion Millennium RNA ladder (Ambion)                      1KB DNA ladder (Promega)

Figure 6.1. RNA and DNA molecular weight ladders
6.2 Protein molecular weight ladders

7. SDS-PAGE prestained molecular weight marker (Invitrogen)

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</table>

Figure 7.1. SDS-PAGE prestained molecular weight marker (Invitrogen)
8. SDS-PAGE molecular weight marker (Invitrogen)

Figure 8.1. SDS-PAGE molecular weight marker (Invitrogen)
6.3 DNA plasmid map

pET16b expression vector containing His-tagged fragment of pAW3 [1027-1657] used to produce full length HCV NS3 protein.

Figure 8.2. DNA plasmid map