Investigating Host-Viral Interactions in Liver Lipid Homeostasis and HCV Pathology

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Abstract

Hepatitis C virus (HCV) infects an estimated 170 million people worldwide and is a major cause of chronic hepatitis and hepatocellular carcinoma. As there are limited treatment options, the elucidation of novel host-viral interactions during HCV pathogenesis will be critical for the development of new therapeutics. My thesis work has identified cell death-inducing DFF45-like effector B (CIDEB) as a host factor that is disregulated during HCV infection, and has delineated the relevance of CIDEB's dual roles in apoptosis and lipid metabolism in the context of the HCV lifecycle. Moreover, additional host factors necessary for the HCV lifecycle were investigated using unnatural amino acid (UAA) technology. With this technique, the photo-cross-linking UAA p-azido-phenlyalanine (AZF) and 3'-azibutyl-N-carbamoyl-lysine (Abk) were incorporated into viral proteins by expanding the genetic code of the host organism. This conferred diverse physicochemical and biological properties to these proteins that were exploited to investigate protein structure and function *in vitro* and *in vivo*. In summary, gaining insight into the numerous host-viral interactions that take place during HCV infection will both advance our understanding of HCV pathogenesis and uncover potential therapeutic targets.

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List of Abbreviations

Abk	3’-azibutyl-N-carbamoyl-lysine
ACAT	acyl-CoA cholesterol acyltransferase
ACC	acetyl-CoA carboxylase
Aha	azidohomoalanine
aaRS	aminoacyl–tRNA synthetase
AMPK	AMP-activated protein kinase
ApoB	apolipoprotein B
ApoE	apolipoprotein E
AZF	p-azido-phenlyalanine
CD81	cluster of differentiation 81
CFP	cyan fluorescent protein
CIDEB	cell death-inducing DFF45-like effector B
CPT-1	carnitine palmitoyltransferase type 1
DAA	directed anti-viral agent
DDX3	DEAD box protein
DFF40	DNA fragmentation factor 40
DFF45	DNA fragmentation factor 45

ELISA	enzyme-linked immunosorbent assay
EMCV	Encephalomyocarditis virus
ER	endoplasmic reticulum
FAS	fatty acid synthase
FBS	fetal bovine serum
GAGs	glycosaminoglycans
GFP	green fluorescent protein
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDV	Hepatitis D virus
hnRNP K	heterogeneous nuclear ribonucleoprotein K
HMCR	HMG-CoA reductase
HMCS	HMG-CoA synthase
HNF4A	hepatocyte nuclear factor-4A
HS	human serum
IRES	internal ribosomal entry site
LD	lipid droplet

LDLR	low density lipoprotein receptor
LT-bR	lymphotoxin b receptor
MTP	microsomal triglyceride transfer protein
NS	non-structural
ORF	open reading frame
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1α
PI3K	phosphoinositide III-kinase
PI4KIIIα	phosphatidylinositol-4 kinase III alpha
PKR	protein kinase R
PPARα	peroxisome proliferator activated complex α
SCAP	SREBP cleavage-activation protein
SCD1	steroyl-CoA desaturase 1
SCID	severe combined immunodeficiency
SEM	standard error of the mean
SRB1	scavenger receptor B1
SREBP-1c	steroyl regulatory element binding protein 1c
SREBP-2	steroyl regulatory element binding protein 2
SVR	sustained viral response

TAG	triglyceride
TNFα	tumor necrosis factor α
TNF-R1	tumour necrosis factor receptor 1
TFP	two photon fluorescence
TRAIL	tumor necrosis factor related apoptosis inducing ligand
UAA	unnatural amino acids
VLDL	very low density lipoprotein
WT	wild type

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1.0 General Introduction

1.1 The Hepatitis C Virus

1.1.1 Discovery

The vital role of the liver in maintaining homeostasis has been recognized for thousands of years, which includes its critical functions in nutrient storage, detoxification, and the metabolism of proteins, carbohydrates and lipids (Peltier 2003). Moreover, for nearly as long as the importance of the liver has been acknowledged, the existence of pathogens causing liver-specific maladies has also been speculated. The first appearance of hepatotropic viruses in the literature dates back to the early 1900s when scientists postulated that a viral agent could be responsible for the epidemic of infectious jaundice debilitating soldiers during the First World War (Kuntz 2008). This proposition was later supported by the discovery of Hepatitis B virus (HBV) in 1970 by Dane *et al.* and the identification of Hepatitis A virus (HAV) by Feinstone and Purcell in 1973 (Dane, Cameron et al. 1970; Feinston, Kapikian et al. 1973). Both of these viruses target the liver as their primary disease site, and cause inflammation of the liver, also known as hepatitis, resulting from an infiltration of immune cells in response to hepatic infection (Park S 2014). Due to the shared pathology of these two viruses, they are grouped together as hepatitis viruses, although they are not related taxonomically (Melnick 1994). Soon after their discovery, serological markers for these viruses were identified, which facilitated the diagnosis of infected individuals and drastically reduced the incidence of post-transfusion hepatitis (Alter, Holland et al. 1975). However, it soon became evident that another form of hepatitis, clinically indistinguishable from HAV and HBV infection, was also present, as some patients who did not manifest diagnostic markers for HAV or HBV continued to develop hepatitis after blood transfusions (Feinman, Berris et al. 1980). This new type of hepatitis was referred to as non-A, non-B hepatitis (NANBH), and although it was hypothesized to also be caused by a virus, initial attempts to identify viral antigens and antibodies were not successful (Alter, Holland et al. 1975). However, in 1989, Choo *et al.* were able to identify and sequence the viral genome of the NANBH agent by

screening lambda phage complementary DNA (cDNA) libraries made from nucleic acids isolated from chimpanzees experimentally infected with NANBH (Choo, Kuo et al. 1989). Recombinant peptides were then used to isolate viral antibodies from NANBH patient sera, confirming that these antibodies were present in patients with NANBH all over the world (Kuo, Choo et al. 1989). Thus, the infectious agent of NANBH had been isolated and was called Hepatitis C virus (HCV).

1.1.2 Prevalence

Today, HCV infection is a global epidemic and major health concern persistently infecting an estimated 170 million people worldwide (1999), or approximately 2% of the world population (Shepard, Finelli et al. 2005). The country with the highest seroprevalence is Egypt where between 15 and 25% of residents are persistently infected with HCV (Abdel-Aziz, Habib et al. 2000; Deuffic-Burban, Mohamed et al. 2006). In Canada, HCV infection is also a significant medical and economic burden with a reported prevalence of approximately 0.8-1% (Sherman, Shafran et al. 2007). This number is expected to rise in the future as the number of new infections arising per annum exceeds the estimated number of HCV-related mortalities or cures (Sherman, Shafran et al. 2007).

1.1.3 Genetic Diversity

Using genotyping methods, different HCV isolates have been identified, each having a unique prevalence and global distribution. HCV isolates are currently classified based on nucleic acid sequence into six major genotypes, which share approximately 65% sequence similarity (Simmonds, Holmes et al. 1993). The most predominance genotype in North America and Europe is genotype 1, followed in prevalence by genotypes 2 and 3. Moreover, genotype 4 is most common in North Africa and the Middle East and genotypes 5 and 6 are most common in South Africa and Southeast Asia region respectively (WHO 2002; Bartenschlager 2008). Each HCV genotype comprises a collection of more closely related subtypes which share approximately 80% nucleic acid similarity (Simmonds, Smith et al. 1994). Furthermore, viral isolates within an infected individual

demonstrate extensive genetic variability due to the error- prone nature of the HCV polymerase. The resulting mixture of variants are called quasispecies, and they share 90-99% sequence similarity (Argentini, Genovese et al. 2009). The presence of quasispecies is important for viral persistence as they provide an avenue for viral immune evasion, which complicates development of vaccines and directed antivirals against HCV, as discussed later (Farci and Purcell 2000).

1.1.4 Transmission

HCV is a blood borne pathogen, therefore the most common mode of transmission is through unsafe injection practices such as intravenous drug use, contaminated tattooing or piercing equipment, needle injury in health care professionals, or nosocomial transmission (Wasley and Alter 2000; Alter 2002; Dore, Law et al. 2003; Hauri, Armstrong et al. 2004). Other sources of exposure include vertical transmission from mother to child, sexual transmission, and blood transfusions prior to 1992, before screening for HCV was common practice (Alter 2007).

1.1.5 Diagnosis

When HCV infection is suspected, serological tests can be used to confirm the diagnosis and determine the viral genotype, which will dictate the ideal treatment strategy and aid in predicting prognosis (Pawlotsky 2002). Enzyme-linked immunosorbent assays (ELISAs) can be employed to detect anti-HCV antibodies from patient plasma or serum; however, as the presence of anti-HCV antibodies becomes apparent approximately 50 days post-exposure, up to 30 % of acutely infected patients can test negative for anti-HCV antibodies at the onset of symptoms (Farci, Alter et al. 1991; Glynn, Wright et al. 2005; Chevaliez and Pawlotsky 2007). To date, the most reliable method to identify HCV infection is by specifically detecting HCV RNA using real-time PCR as viral RNA is detectable in the serum within 7-10 days after the initial exposure (Farci, Alter et al. 1991; Scott and Gretch 2007; Chevaliez and Pawlotsky 2008). This technique can also be employed to monitor viral loads during antiviral therapy as a means to evaluate the virologic response to

treatment. Additionally, levels of hepatic enzymes , such as alanine aminotransferase (ALT) or aspartate aminotransferase (AST) can also be used to assess the degree of liver injury that has occurred, where elevated levels of these enzymes can be detected as early as 4-12 weeks after exposure (Santantonio, Sinisi et al. 2003).

1.1.6 Clinical Manifestations, Disease Progression and Risk Factors

One of the most remarkable features of HCV is its ability to induce a persistent chronic infection in the majority of those exposed, making HCV one of the most significant causes of liver disease worldwide. Acute infection is generally asymptomatic, but may be accompanied with mild, non-specific symptoms such as abdominal pain, fatigue, or nausea. When symptoms do present, they are generally not severe enough to warrant consultation of a doctor, making initial exposure to the virus difficult to diagnose. Following exposure to HCV, 20-30% of infected individuals will spontaneously clear the infection, however 70 - 80% will develop chronic infection, which can persist for many years without causing death of the patient (Thomas, Astemborski et al. 2000). Chronic HCV infection is associated with severe, progressive liver damage including steatosis, characterized by the accumulation of lipid within hepatocytes, and fibrosis, where aggregates of extracellular matrix proteins like collagen impair normal hepatic functioning (Tsai and Chung 2010). These pathologies can result in cirrhosis, the final, irreversible stage of liver disease where scar tissue replaces hepatic tissue, ultimately resulting in liver failure (Mengshol, Golden-Mason et al. 2007). Moreover, the extensive damage incurred by fibrosis and cirrhosis puts patients at an increased risk for developing hepatocellular carcinoma, where approximately 1-3% of HCV patients will develop hepatocellular carcinoma after 30 years of chronic infection (Hassan, Frome et al. 2002). Once the liver has incurred irreversible damage, liver transplantation is the only remaining therapeutic avenue. With this being said, rapid re-infection of the newly transplanted graft is a major problem when the underlying cause of HCV has not been addressed (Fafi-Kremer, Fofana et al. 2010). In addition, liver transplantation is limited by a variety of factors, such as long waiting lists and donor shortage.

Overall, experts have predicted that HCV accounts for approximately 70% of cases of chronic hepatitis, 40% of cases of cirrhosis, 60% of cases of hepatocellular carcinoma and 30% of liver transplants in Western countries, which further explains its significant burden on the health care system (Marcellin 1999).

In general, the rate of disease progression is unpredictable in chronic HCV infection. However, several factors are associated with an accelerated progression of liver damage, including an older age at time of exposure, male gender, race, obesity, diabetes, alcohol use and co-infection with HIV or HBV, which allows HCV to act as an opportunistic infection (Soto, Sanchez Quijano et al. 1997; Corrao and Arico 1998; Pontisso, Gerotto et al. 1998).

1.2 Treatment

1.2.1 Vaccine

Currently, no vaccine is available to prevent this disease and conventional therapeutic strategies are insufficient to address the increasing spread of infection (Herker and Ott 2011). The development of an HCV vaccine was hindered for many years due to complications with growing the virus in cell culture and the highly mutable nature of the virus, which results in epitope heterogeneity (Houghton and Abrignani 2005).

1.2.2 Standard Therapy

The FDA-approved standard therapy for HCV consists of pegylated interferon-α (IFN-α) in combination with ribavirin. Although IFN-α does not act on the virus directly, it is capable of initiating antiviral pathways within the cell by binding extracellular receptors, and may also indirectly enhance the function of natural killer cells, cytotoxic T lymphocytes and macrophages (Feld and Hoofnagle 2005; Chevaliez and Pawlotsky 2007). Ribavirin is a nucleoside analogue, and although its mechanism of action is largely unknown, it has been hypothesized to directly interact with and

inhibit the HCV polymerase, thereby decreasing HCV RNA replication (Hofmann, Herrmann et al. 2008). Additionally, despite the fact that treatment with ribavirin alone does not significantly influence viral load, administration of ribavirin substantially augments the antiviral action of IFN-α by modulating IFN-stimulated gene expression (Lin, Noyce et al. 2010).

Overall, the goal of HCV therapy is to achieve a sustained viral response (SVR), defined as the absence of detectable HCV RNA in the serum 24 weeks after treatment has ended (Seeff and Hoofnagle 2002). Unfortunately, treatment success demonstrates considerable variability depending on which HCV genotype a person is infected with, where genotypes 1 and 4 generally demonstrate poor response to standard IFN-based treatments (Hughes and Shafran 2006; Farnik, Mihm et al. 2009). For example, a SVR is achieved with 78 % to 82 % of patients with genotypes 2 and 3, compared to only 42 % to 46 % of genotype 1 infected patients (Flamm 2003). Moreover, there are no approved treatment options for individuals who do not achieve SVR. Other pitfalls of current treatment include the large cost of therapy and the numerous side effects associated with current treatment that prevents patient adherence, and causes 10-20% of patients to prematurely discontinue treatment (1999). Side effects range from fatigue, fever, headache and myalgia, which are experienced by over half of patients being treated with standard therapy (Manns, McHutchison et al. 2001; Fried, Shiffman et al. 2002; Hadziyannis, Sette et al. 2004). Depression is also observed in 20-30% of patients, presumably as a result of the effect of IFN on serotonin activity, while ribavirin leads to a dose-dependent haemolytic anaemia (Fried 2002). Other commonly reported symptoms include flu-like symptoms, neutropenia and thrombocytopenia.

1.2.3 Direct-Acting Antivirals

Recent progress in our understanding of the molecular mechanisms of HCV has led to the development of specific targeted antiviral therapy for hepatitis C (STAT-C), also known as 'direct-acting antivirals' (DAA), which represent a momentous advancement in the treatment of HCV. These

compounds are small molecule inhibitors that specifically target viral enzymes involved in HCV replication, thereby directly impeding the HCV lifecycle. To date, the most promising targets have been the NS3/4A protease and the NS5B RNA dependent RNA polymerase, both of which are required for viral replication. Protease inhibitors such as boceprevir and telaprevir, and polymerase inhibitors such as filibuvir and VCH-222 were shown to be effective in abrogating virion production in clinical trials (Lamarre, Anderson et al. 2003). However, although these DAA are promising, they still need to be used in combination with IFN-α and ribavirin, which is referred to as triple therapy, and as a result, is associated with all the previously described side effects. Additionally, another major problem associated with these targeted antivirals is the development of resistant strains of HCV due to the high replication rates, poor fidelity and lack of proofreading ability of the HCV polymerase. This leads to the acquisition of adaptive mutations upon selective pressure, which explains why DAA monotherapy is ineffective for the treatment of HCV (Hofmann and Zeuzem 2011). Additionally, due to the significant sequence diversity between viral genotypes, it is unlikely that a compound will exhibit equal effectiveness across all HCV isolates (Sarrazin and Zeuzem 2010). However, new clinical trials indicate that the prospect of interferon-free combination DAA therapy for HCV is becoming more of a reality with nearly 90% success rates in the last clinical trials (Stedman 2013).

1.2.4 Treatments Targeting the Host

An alternative therapeutic strategy at the forefront of HCV research aims to target the host cell, rather than the virus itself, to combat viral infection. Proof of this concept has been demonstrated previously with IFN therapy, which stimulates the host's innate immune response to achieve an antiviral affect. Other drugs, such as toll-like receptor agonists, also demonstrate anti-HCV abilities by modulating cellular immune response through cytokine signalling (McHutchison, Bartenschlager et al. 2006). Moreover, the immunosuppressant agent, cyclosporin A has also been shown to suppress HCV replication (Nakagawa, Sakamoto et al. 2005). Furthermore, there are several host-target

strategies currently in development (Salloum and Tai 2012; Baugh, Garcia-Rivera et al. 2013; Zeisel, Lupberger et al. 2013).

Aside from targeting the host's innate defences to restrict HCV infection, altering other cellular pathways that intersect with the viral lifecycle may also be effective at impairing HCV. For example, the inhibition of proviral host factors has been shown to be effective in limiting viral load (Budkowska 2009; Suzuki 2010; Salloum and Tai 2012). For instance, sequestration of the major liver-specific microRNA-122, which plays a crucial role in enhancing HCV replication, can reduce serum HCV RNA and improve liver histology considerably (Heck, Meng et al. 2009; Lanford, Hildebrandt-Eriksen et al. 2010). Overall, a major advantage of targeting the host, rather than the virus, is the reduced risk of developing resistant strains of HCV that result from targeted therapeutics which cause HCV to mutate upon selective pressure. Clearly, a combination of drugs with different but complementary modes of action would be most successful at reducing viral spread and enhancing viral clearance. Furthermore, simultaneously targeting the host, the virus and their interactions will allow for the development of custom therapies that are tailored to genotype and treatment response. In summary, the identification of novel host factors required for the HCV lifecycle will both advance our understanding of HCV pathogenesis and uncover novel therapeutic targets (Li, Brass et al. 2009).

1.3 HCV Experimental Models

1.3.1 Replicon Cells

At first, the study of the HCV lifecycle was hindered as a cell culture system susceptible to HCV infection and replication was difficult to attain. Initial *in vitro* experiments to examine HCV pathogenesis relied on the transient transfection of individual HCV genes into different cell types, which was not the most accurate model to study the effects of an RNA virus. Moreover, attempts to culture the virus by inoculating primary cell lines with infectious cDNA clones derived from patient sera failed to efficiently initiate HCV infection *in vitro*, although these clones were capable of

infecting chimpanzees (Kolykhalov, Agapov et al. 1997; Yanagi, Purcell et al. 1997; Beard, Abell et al. 1999; Lanford, Lee et al. 2001). However, in 1999, the Bartenschlager group reported the first replicon system for HCV in cell culture which revolutionized the study of HCV (Lohmann, Korner et al. 1999). Here, Huh7 hepatoma cells were electroporated with RNA transcribed from a plasmid containing the HCV 5'UTR, a neomycin resistance cassette, the Encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) and the HCV NS3 to 3'UTR region of a modified genotype 1b isolate termed Con1. Cells successfully transfected with this subgenomic replicon were isolated based on their resistance to geneticin, creating a Huh7 cell line that stably harboured autonomously replicating HCV replicons. Furthermore, HCV replication and protein expression was confirmed, indicating that the non-structural proteins are both necessary and sufficient to support HCV replication (Lohmann, Korner et al. 1999).

In addition to subgenomic replicons, this approach was used to develop selectable full-length HCV replicons that can stably persist in Huh7 cells (Blight, McKeating et al. 2002; Ikeda, Yi et al. 2002; Pietschmann, Lohmann et al. 2002; Blight, McKeating et al. 2003). Upon sequencing these genomic replicons, adaptive mutations necessary for efficient replication in cell culture were identified. It is hypothesized that these mutations may explain the previous difficulty in infecting cells lines with WT strains of HCV (Krieger, Lohmann et al. 2001; Lohmann, Korner et al. 2001). Despite the high levels of viral RNA and protein in cells harbouring the genomic replicon, these cells are not capable of producing infectious virions for yet unknown reasons, thereby making some aspects of the HCV lifecycle such as entry and assembly difficult to study with this model (Ikeda, Yi et al. 2002). Overall, these replicon systems are capable of recapitulating the intracellular steps of RNA replication.

1.3.2 Viral Tropism

Interestingly, Huh7 cells are one of the only cell lines capable of permitting high levels of HCV replication, which further supports the notion that host factors play an important role in

determining HCV permissiveness (Zhu, Guo et al. 2003; Ali, Pellerin et al. 2004; Uprichard, Chung et al. 2006). Moreover, within a given group of Huh7 cells, their ability to support HCV replication varies dramatically. This notion led to the creation of highly permissive cell lines designated Huh7.5 and Huh7-Lunet (Blight, McKeating et al. 2002). These optimized lines were obtained by removing the HCV replicons from established clones using an anti-HCV treatment, such as IFN-α or an anti-HCV drug, and then reintroducing the replicon, which often resulted in higher levels of HCV RNA replication compared to the parental cells.

1.3.3 JFH-1 Infectious Cell Culture System

Initial studies on HCV entry relied on the use of HCV pseudoparticles, which were based on retroviruses that contain HCV glycoproteins incorporated into their envelope (Bartosch, Dubuisson et al. 2003; Hsu, Zhang et al. 2003). However, in 2005, a second major breakthrough in the study of HCV pathogenesis occurred, when Wakita's group identified a high-replication molecular clone of HCV, capable of producing authentic HCV particles in cell culture (Kato, Date et al. 2003). This strain was called JFH-1, as it was isolated from a Japanese patient with fulminant hepatitis, and it was identified as genotype 2a (Kato, Date et al. 2003). Remarkably, this viral isolate contained some novel mutations which offered several advantages over other strains previously described for cell culture. When subgenomic replicons of the JFH-1 strain were introduced into cells, a log-order higher replication efficiency was observed compared to Con1 replicons. Additionally, JFH-1 replicons could also replicate in cell lines other than Huh7 and no adaptive mutations were required for this to occur (Date, Kato et al. 2004). Notably, when Huh7 cells were electroporated with full- length JFH-1 replicons, viral particles capable of infecting naïve Huh7 cells, chimpanzees, and chimeric mice with human liver xenografts were produced, and the viruses created by these systems were capable of infecting cultured cells (Bukh, Pietschmann et al. 2002; Lindenbach, Evans et al. 2005; Wakita, Pietschmann et al. 2005; Zhong, Gastaminza et al. 2005; Lindenbach, Meuleman et al. 2006).

Although the JFH-1 cell culture system was capable of producing infectious particles, viral titres were initially low, therefore many groups improved this system using mutagenesis or by creating recombinant intragenotypic and intergenotypic HCV chimeras to enhance viral replication and particle production (Wakita, Pietschmann et al. 2005; Delgrange, Pillez et al. 2007; Gottwein, Scheel et al. 2007; Kato, Matsumura et al. 2007; Scheel, Gottwein et al. 2008; Bungyoku, Shoji et al. 2009). In addition, several reporter systems based on the JFH-1 genome have been characterized where bicistronic or tricistronic luciferase reporter constructs are employed to monitor viral infection (Yanagi, Purcell et al. 1999; Koutsoudakis, Kaul et al. 2006). Overall, the development of an infectious HCV cell culture system continues to be an exceptional tool for investigating various aspects of the HCV lifecycle.

1.3.4 Animal Models

HCV displays a very limited species tropism, with chimpanzees and humans being the only hosts susceptible to HCV infection, yet another demonstration that host factors play an important role in determining HCV permissiveness. Chimpanzees are the most important animal model available for HCV research, as it is the only animal that can be successfully infected with HCV and develop a disease that is comparable to human HCV infection. Accordingly, chimpanzees are useful for testing vaccine and therapeutic candidates, looking at immune responses to HCV, and analyzing viral kinetics and adaptive mutations. Indeed, there are several issues regarding the use of chimpanzees to study HCV, including high costs, limited accessibility and ethical considerations. Therefore, the development of small animal models that support the HCV lifecycle was another important milestone, as rodents are normally non-permissive to HCV growth. These models include transgenic mice overexpressing viral proteins, severe combined immunodeficiency (SCID) mice that have human hepatocytes transplanted into their livers, and most recently, a genetically humanized mouse model that stably expresses human CD81 and OCLN allowing them to complete the entire HCV

lifecycle (Mercer, Schiller et al. 2001; Kremsdorf and Brezillon 2007; Barth, Robinet et al. 2008; Jiao, Wang et al. 2010; Dorner, Horwitz et al. 2013) .

1.4 Structural Biology of HCV

1.4.1 Molecular Organization

HCV is a positive-sense, single-stranded RNA virus in the *Hepacivirus* genus of the *Flaviviridae* family (Pietschmann, Kaul et al. 2006). Its ~9.6 kb genome encodes a single open reading frame (ORF) that is flanked by highly conserved 5' and 3' untranslated (UTR) regions (Moradpour, Penin et al. 2007). The 5' UTR contains an internal ribosomal entry site (IRES) which is bound by host ribosomes, allowing for the translation of the viral genome into a 3000 amino acid polyprotein precursor. After translation, the HCV polyprotein is cleaved by host and viral proteases into 3 N-terminal structural proteins (core, and envelope proteins, E1 and E2) and seven C-terminal non-structural (NS) proteins (p7, NS2–3, NS3, NS4A, NS4B, NS5A, and NS5B) (Lindenbach and Rice 2005) (Figure 1).

1.4.2 Structural Proteins

The structural proteins form the outermost covering of the HCV virion, which have an icosahedral conformation and a diameter of 55-65 nm (Kaito, Watanabe et al. 1994; Shimizu, Feinstone et al. 1996). The viral envelope consists of non-covalent heterodimers of the highly glycosylated E1 and E2 glycoproteins which are embedded in a host derived bi-lipid layer. These glycoproteins mediate viral entry by binding cell-surface factors including cluster of differentiation 81 (CD81), scavenger receptor class B type I (SR-B1) occludin (OCLN) and low density lipoprotein receptor (LDLR) (Pileri, Uematsu et al. 1998; Agnello, Abel et al. 1999; Scarselli, Ansuini et al. 2002; Liu, Yang et al. 2009). Additionally, the E2 protein contains a hypervariable region that can rapidly mutate as a mechanism of immune evasion (Forns, Thimme et al. 2000; Callens, Ciczora et al. 2005).

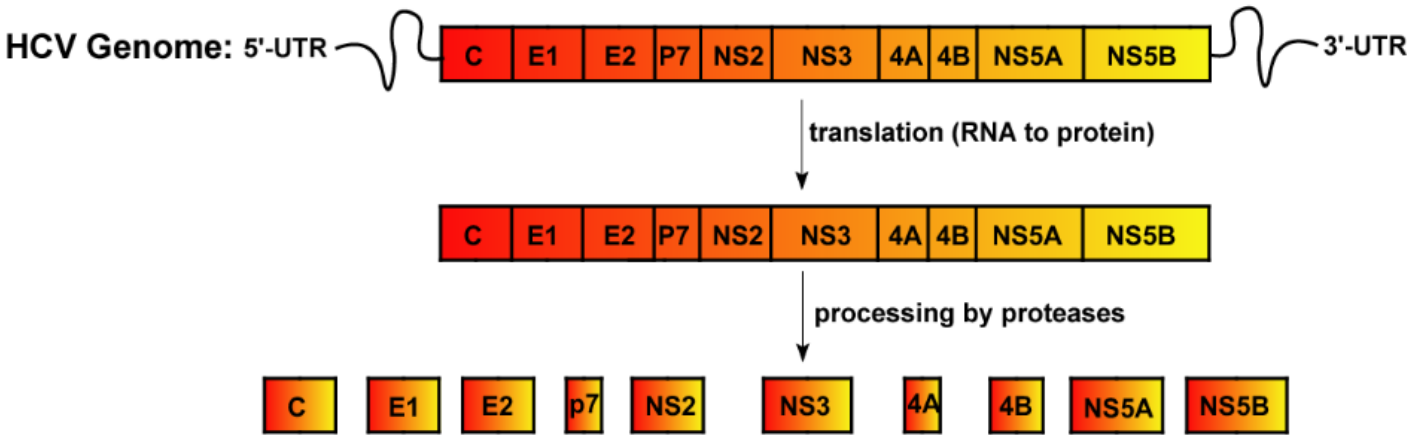

Figure 1. HCV genome organization and polyprotein processing. The 9.6 kb positive sense, single stranded HCV RNA genome, shown in the top panel, is comprised of a coding region flanked by highly conserved 5' and 3' UTRs. Genome translation from the internal ribosome entry site (IRES) produces a 3000 amino acid polyprotein precursor (middle panel) which is cleaved by viral and host proteases into three structural proteins (core, and envelope proteins, E1 and E2) and seven non-structural (NS) proteins (p7, NS2–3, NS3, NS4A, NS4B, NS5A, and NS5B).

Below the viral envelope, the core protein forms the viral capsid that encloses the RNA genome (Ishida, Kaito et al. 2001). The alpha-helical core protein consists of a hydrophilic, N-terminal domain and a hydrophobic, C-terminal domain which mediates lipid droplet (LD) binding, allowing the core protein to localize to the surface of LD (Santolini, Migliaccio et al. 1994; Boulant, Douglas et al. 2008).

p7 is a small hydrophobic protein found between the structural and non-structural regions of the HCV polyprotein (Lin, Lindenbach et al. 1994). Although p7 is not necessary for HCV replication, its role as an ion channel on the ER membrane is required for the formation and release of infectious viral particles, although its mechanism of action has yet to be elucidated (Griffin, Beales et al. 2003; Pavlovic, Neville et al. 2003; Jones, Murray et al. 2007; Steinmann, Penin et al. 2007).

1.4.3 Non-Structural Proteins

All NS viral proteins (NS2–NS5B) associate with the ER membrane after synthesis via transmembrane domains or amphipathic α-helices, except for NS3, whose membrane association is mediated by its cofactor, NS4A.

NS2 is a hydrophobic cysteine protease which autocatalytically cleaves the HCV polyprotein at the NS2-NS3 junction (Grakoui, McCourt et al. 1993; Hijikata, Mizushima et al. 1993). Again, this function is required for the formation of infectious particles, but is not required for HCV RNA replication (Jones, Murray et al. 2007; Jirasko, Montserret et al. 2008). Along with its protease function, NS2 is also involved in host cell apoptosis, proliferation and lipid metabolism (Dumoulin, von dem Bussche et al. 2003; Erdtmann, Franck et al. 2003; Yang, Liu et al. 2006).

NS3 is a primarily cytosolic protein with various roles. In addition to independently demonstrating RNA helicase activity to unwind viral RNA, NS3 aids NS2 in its protease activity and also cleaves the remaining sites in the HCV polyprotein with its serine protease activity, assisted by

its cofactor, NS4A (Kolykhalov, Agapov et al. 1994; Tanji, Hijikata et al. 1994; Han, Hahm et al. 1995; Kim, Gwack et al. 1995).

NS4B is another hydrophobic protein, and its association with the ER membrane induces an alteration in membrane conformation to form the membranous web complex, a compartment that houses the HCV replication complex and is the site of RNA production (Hugle, Fehrmann et al. 2001; Egger, Wolk et al. 2002; Quinkert, Bartenschlager et al. 2005).

NS5A is a hydrophobic phosphoprotein that is phosphorylated by cellular kinases into a basally phosphorylated and a hyperphosphorylated form. Both forms are capable of binding HCV RNA and are necessary for viral replication (Huang, Hwang et al. 2005). Additionally, phosphorylation status can modulate viral RNA replication efficiency, and mutations in NS5A, a common site for cell culture adaptive mutations, are capable of dramatically increasing the rate of viral RNA replication (Kaneko, Tanji et al. 1994; Krieger, Lohmann et al. 2001; Evans, Rice et al. 2004; Neddermann, Quintavalle et al. 2004; Appel, Pietschmann et al. 2005). Moreover, NS5A has also been shown to interact with core and apolipoproteins on the surface of LD and may play a role in hijacking host cell lipid metabolism (Shi, Polyak et al. 2002).

Finally, NS5B protein is the RNA-dependent RNA polymerase of HCV, which replicates the viral RNA genome within the HCV replication complexes (Schmidt-Mende, Bieck et al. 2001; Moradpour, Brass et al. 2004),

1.5 Manipulation of Host Pathways

1.5.1 HCV and Lipid Metabolism

Every step of the HCV lifecycle exhibits an intimate reliance on host cell lipid metabolism and several studies have demonstrated that HCV can be inhibited using drugs that target enzymes in lipid biosynthetic pathways (Su, Pezacki et al. 2002; Lyn, Kennedy et al. 2009; Pezacki, Sagan et al.

2009; Bassendine, Sheridan et al. 2011). Moreover, virtually every stage of viral particle assembly and secretion manipulates some aspect of the very low density lipoprotein (VLDL) secretion pathway (Alvisi, Madan et al. 2011). As successful viral particle formation necessitates a specific lipid environment, several viral proteins associate to the surface of LD and orchestrate their redistribution to the perinuclear region of the cell, towards the HCV replication complexes (Boulant, Douglas et al. 2008). While the later stages of the viral lifecycle, namely assembly, budding, and maturation, remain less explored (Li, Brass et al. 2009), cellular and viral proteins are thought to be packaged together into new virions, a process that is thought to initiate on the surface of LDs (Miyanari, Atsuzawa et al. 2007; Shavinskaya, Boulant et al. 2007). The HCV virions that result are termed lipo-viral particles due to their physical resemblance to VLDL in that they contain apolipoprotein B (ApoB), apolipoprotein E (ApoE) and are rich in triacylglycerols (TAG) and cholesterol esters (Andre, Komurian-Pradel et al. 2002; Nielsen, Bassendine et al. 2006). Finally, newly assembled viral particles are released from infected host cells in a VLDL pathway-dependent manner (Huang, Sun et al. 2007). After circulation in the blood, viral particles will enter hepatocytes by binding lipoprotein receptors such as cluster of differentiation 81 (CD81), scavenger receptor B1 (SRB1), low density lipoprotein receptor (LDLR) and glycosaminoglycans (GAGs) (Herker and Ott 2011). Overall, a considerable similarity between the HCV lifecycle and the VLDL secretion pathway is evident (Figure 2), possibly explaining the tissue tropism.

Given the need for a lipid-rich host cell environment to facilitate HCV proliferation, viral RNA replication strictly depends on fatty acid and cholesterol biosynthesis (Alvisi, Madan et al. 2011).Therefore, HCV hijacks host cell lipid metabolism on three levels to promote viral dissemination (Clement, Pascarella et al. 2009). First, HCV enhances host cell lipogenesis by increasing the activity of lipogenic transcription factors such as the sterol regulatory element binding proteins (SREBP-1c and SREBP-2) which will enhance expression of their downstream target genes resulting in an upregulation of fatty acid and cholesterol synthesis respectively

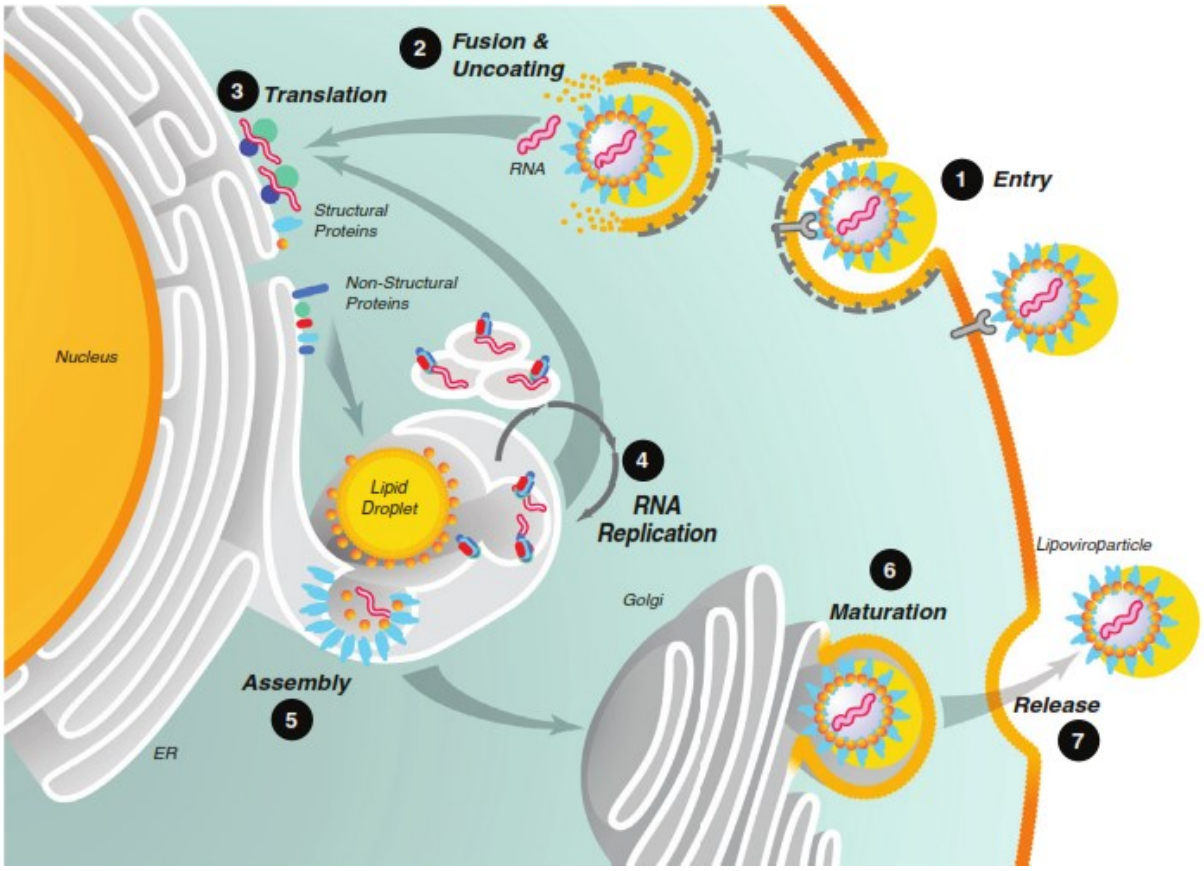

Figure 2.The HCV lifecycle relies on the very low density lipoprotein secretion pathway. HCV lipoviral particles enter target hepatocytes via receptor-mediated endocytosis in part by binding to low density lipoprotein receptor (LDLR) and glycosaminoglycans (GAGs) (step 1). The RNA genomes of newly internalized virions translocate to the endoplasmic reticulum (ER) where they are translated into viral proteins (steps 2 and 3). Association of non-structural viral proteins with the ER alter its conformation by forming membranous web structures which house HCV replication complexes (step 4). Viral particles are assembled on the surface of LDs, and they bud into the ER lumen in order to acquire envelope glycoproteins (step 5). Lipo-viral particles mature in the ER through interactions with lipoproteins (step 6), and exit the cell in a mechanism similar to the VLDL secretion pathway (step 7). Figure adapted from (Herker and Ott 2011).

(Oem, Jackel-Cram et al. 2008; Park, Jun et al. 2009; Li, Lei et al. 2010). Specific targets of SREBP-1c include key enzymes in fatty acid biosynthesis, such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and steroyl-CoA desaturase 1 (SCD1), whereas targets of SREBP-2 are involved in cholesterol biosynthesis (Shimano 2001; Amemiya-Kudo, Shimano et al. 2002). Secondly, HCV impairs host cell lipid degradation by repressing transcription factors involved in activation of fatty acid beta-oxidation such as peroxisome proliferator activated complex α (PPARα) or by inactivation of key regulators of lipid metabolism such as the AMP-activated protein kinase (AMPK) (Mankouri, Tedbury et al. 2010). The HCV core protein also reduces the expression of carnitine palmitoyltransferase type-1 (CPT-1), the rate limiting enzyme of beta-oxidation (Perlemuter, Sabile et al. 2002; Cheng, Dharancy et al. 2005). Finally, HCV reduces host cell lipid export by downregulating VLDL secretion, in part by inhibition of microsomal triglyceride transfer protein (MTP) by core (Perlemuter, Sabile et al. 2002; Hussain, Shi et al. 2003). In summary, the virus seeks to promote a lipid rich environment and modulates the secretion and assembly of VLDL particles to allow for the formation and maturation of viral particles (Figure 3).

1.5.2 Apoptosis

Another host pathway closely involved in viral proliferation is apoptosis. Apoptotic pathways are a principal mechanism by which the host can control viral dissemination, as it gives the host the ability to destroy infected cells, and limit viral spread. However, although hepatic apoptosis is capable of limiting viral spread, it is also detrimental to the host in the sense that it can cause many of the pathologies associated with chronic infection, such as liver damage and liver failure. Furthermore, this situation is complex as it is not well established whether HCV infected or uninfected hepatocytes are undergoing apoptosis during chronic HCV infection (Calabrese, Pontisso et al. 2000; Bantel, Lugering et al. 2001).

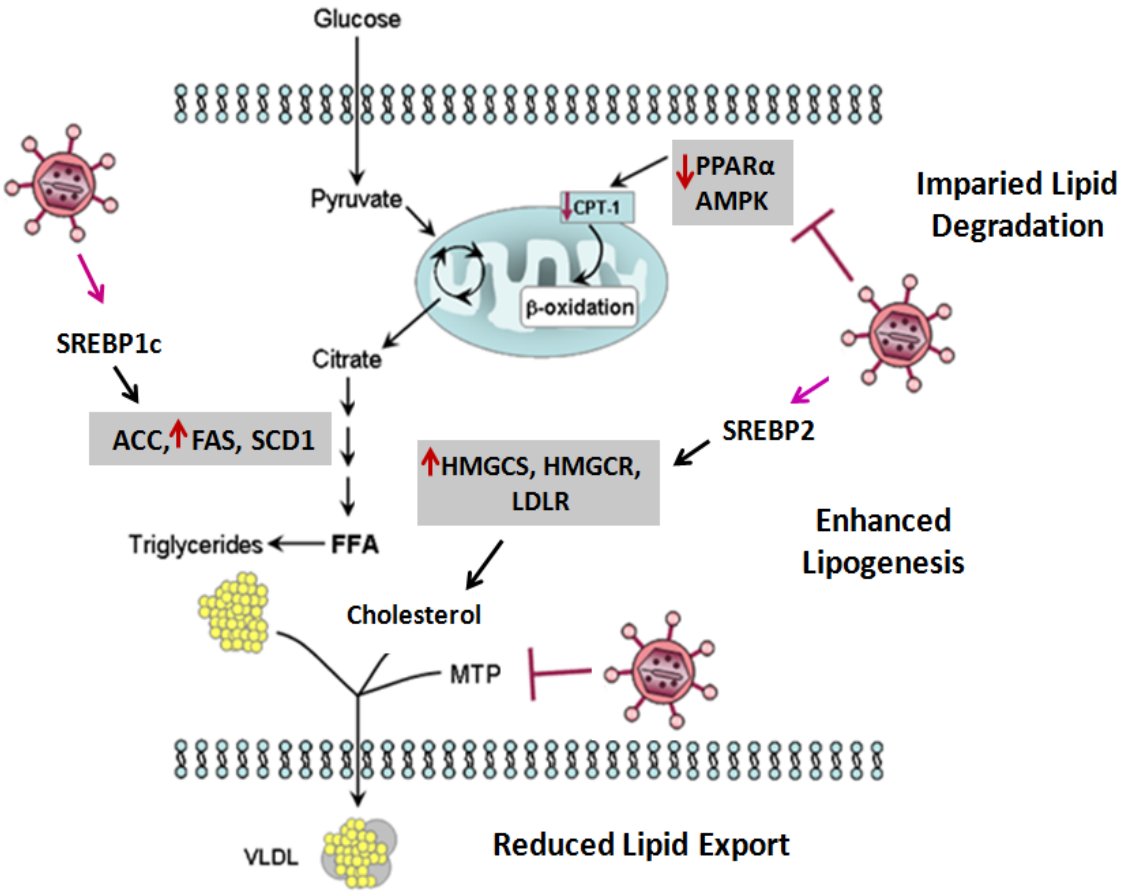

Figure 3. Schematic representation of the effects of HCV on hepatic lipid homeostasis. HCV may interfere with lipid metabolism via at least three distinct mechanisms: (i) Impaired secretion. HCV may interfere with the very-low density lipoprotein (VLDL) assembly and secretion by impairment of microsomal triglyceride transfer protein (MTP) activity by HCV core protein expression. (ii) Increased *de novo* fatty acid and cholesterol synthesis. HCV upregulates the transcription factor sterol regulatory element binding protein 1c (SREBP-1c) which activates lipogenic enzymes such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and steroyl-CoA desaturase 1 (SCD1). HCV also upregulates the transcription factor SREBP-2 which activates enzymes involved in cholesterol biosynthesis such as HMG-CoA synthase (HMCS), HMG-CoA reductase (HMCR) and low density lipoprotein receptor (LDLR). (iii) Impaired fatty acid degradation. HCV expression downregulates factors or the fatty acid beta-oxidation pathway such as peroxisome proliferators-activated receptor α (PPARα), carnitine palmitoyltransferase type 1 (CPT-1) and AMP-activated protein kinase (AMPK). Figure adapted from (Clement, Pascarella et al. 2009).

Regardless, many viruses have developed mechanisms to inhibit the induction of host cell apoptosis to allow for viral proliferation. Virtually all HCV proteins have been demonstrated to modulate apoptotic pathways through a variety of mechanisms. although it is not known which interactions are physiologically relevant (Bantel and Schulze-Osthoff 2003; Kountouras, Zavos et al. 2003; Fischer, Baumert et al. 2007). Generally, HCV has been demonstrated to upregulate the major hepatic death ligands, which include the CD95 ligand, tumor necrosis factor α (TNFα) and tumor necrosis factor related apoptosis inducing ligand (TRAIL) (Bantel and Schulze-Osthoff 2003). However, this topic is complicated as studies supporting both pro and anti-viral affects for individual HCV proteins have been published (Dumoulin, von dem Bussche et al. 1998; Ray, Meyer et al. 1998; Zhu, Khoshnan et al. 1998; Marusawa, Hijikata et al. 1999; Benali-Furet, Chami et al. 2005; Saito, Meyer et al. 2006). Although the differences observed are thought to be attributed to the model used, viral strain etc., it is still not clear how HCV is influencing apoptosis *in vivo*. This suggests that HCV proteins play a significant, yet complex role in altering host cell survival and apoptosis that is not yet entirely understood, and is likely influenced by metabolism and liver homeostasis. Additionally, hepatic apoptosis also plays an important role during liver remodeling and regeneration, where several pro-apoptotic cytokines and transcription factors are required to initiate these pathways (Schlossberg, Zhang et al. 1996; Fausto 2000; Huh, Factor et al. 2004). Moreover, the influence of HCV's anti-apoptotic role on the development of hepatocellular carcinoma, a common complication of HCV infection, is also largely unknown (Balsano and Alisi, 2007). Overall, insight into HCV's manipulation of host cell apoptosis will be crucial in order to gain a complete understanding of HCV pathogenesis.

1.5.3 Targeting Host Factors to Impair HCV

As the viral lifecycle demonstrates an intimate reliance on host pathways, host factors with novel regulatory roles in either pathway represent potential therapeutic targets. With this being said, a finer comprehension of the molecular mechanisms governing the complex array of host-viral

interactions crucial for viral proliferation is required in order to develop novel therapies against HCV. One host factor of particular interest is the cell death-inducing DFF45-like effector B (CIDEB) protein. CIDEB's established interactions with viral proteins, in addition to its role a master regulator of apoptosis and hepatic lipid metabolism, make it an exceptional candidate for involvement in the HCV lifecycle. My thesis work aims to identify the role of CIDEB in the context of HCV pathogenesis.

2.0 Chapter 1:

Investigating the Role of Cell Death-Inducing DFF45-like Effector B in HCV Replication

2.1 Abstract

Cell death-inducing DFF45-like effector B (CIDEB) is an apoptotic protein previously found to interact with the HCV non-structural protein 2 (NS2). Recently, CIDEB has been implicated in lipid homeostasis and VLDL lipidation, which suggests additional roles for CIDEB in the HCV lifecycle, due to HCV's fundamental reliance on host cell lipid metabolism for successful proliferation. Herein, I have delineated the relevance of CIDEB's dual roles in apoptosis and lipid metabolism in the context of the HCV lifecycle. I have demonstrated that host cells upregulate CIDEB expression during HCV infection, and accordingly CIDEB overexpression inhibits HCV replication. Furthermore, I have characterized a CIDEB mutant, KRRA, which is deficient in lipid droplet fusion and demonstrated that CIDEB-mediated inhibition of HCV is independent of the protein's role in hepatic lipid homeostasis. My results suggest that higher levels of CIDEB expression, which favour an apoptotic role for the host factor, may inhibit HCV replication. Collectively, my data demonstrates that CIDEB is a host factor that is disregulated during HCV infection that contributes to altered triglyceride homeostasis.

2.2 Introduction

2.2.1 CIDEB Mechanism and Regulation

CIDEB is one isoform of the CIDE family of proteins which are found in various tissues throughout the body (Yonezawa et al, 2011). Expression of the CIDEB isoform is highly restricted to the liver and small intestine as a result of direct binding of transcription factor hepatocyte nuclear factor-4A (HNF4A) to its internal promoter (Da, Li et al. 2006). CIDEB mRNA expression is additionally controlled by peroxisome proliferator-activated receptor-γ coactivator (PGC-1α), Sp1, and Sp3 (Da, Li et al. 2006; Chen, Norris et al. 2010). CIDE family proteins are highly conserved at their N-terminal domain that also shares homology to the DNA fragmentation factor 45 (DFF45), which enables this group of proteins to activate cellular apoptosis (Wu, Zhang et al. 2008) (Figure 4). CIDEB-induced apoptosis requires homodimerization via its C-terminal domain, as well as mitochondrial localization which ultimately induces the release of cytochrome *c* from the mitochondria, allowing for the delivery of caspase activating molecules during apoptosis (Chen, Guo et al. 2000).

2.2.2 CIDEB and HCV

Consistent with this notion of viruses inhibiting the induction of host cell apoptosis to promote their proliferation, CIDEB was found to interact with the HCV non-structural protein 2 (NS2) and co-expression of CIDEB and NS2 caused a decreased percentage of apoptotic cells compared to overexpression of CIDEB alone (Erdtmann, Franck et al. 2003). Furthermore, during adenoviral infection in transgenic mice expressing the HCV polyprotein, the level of CIDEB protein content was decreased to 1% of the level observed in WT mice (Erdtmann, Franck et al. 2003). It remains to be seen whether HCV infection induces downregulation of CIDEB expression; however this previous study suggests that either the virus-induced downregulation of CIDEB or the binding observed between CIDEB to NS2 may inhibit CIDEB's dimerization or mitochondrial localization.

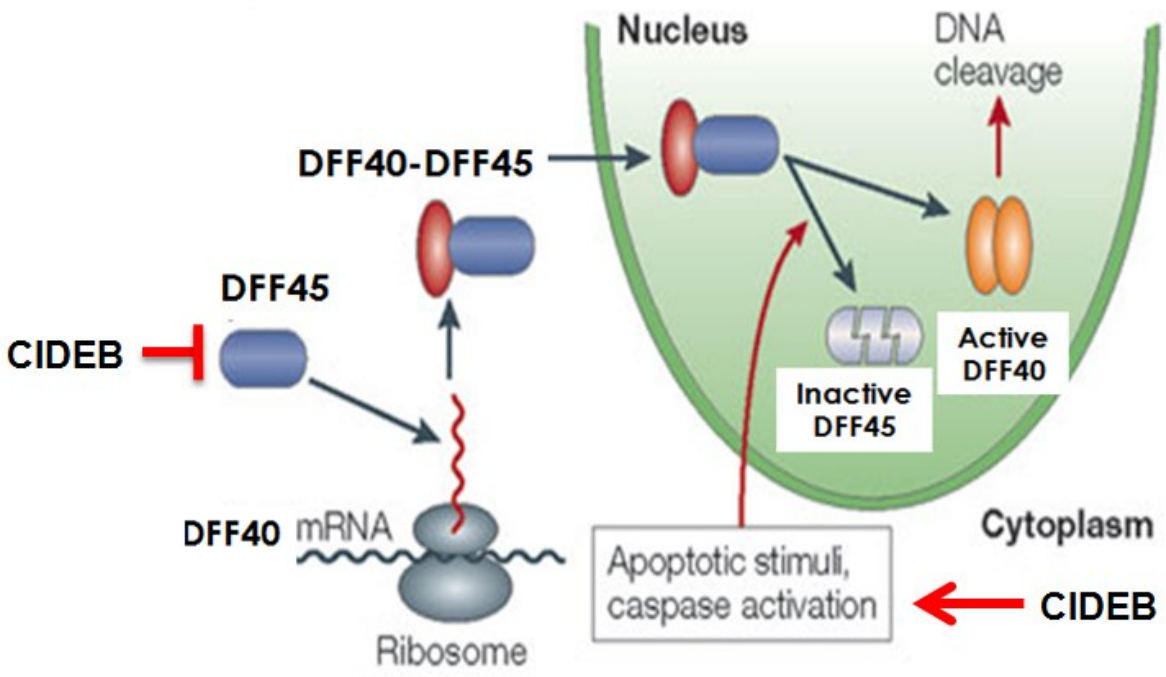

Figure 4. Role of CIDEB in cellular apoptosis. DNA fragmentation factor 40 (DFF40) is a caspase activated DNase which is bound by its inhibitor, DNA fragmentation factor 45 (DFF45), upon translation under normal circumstances. When cells undergo apoptosis, caspases cleave DFF45, liberating DFF40, allowing it to perform its nuclease activity after dimerization and nuclear localization. Cell death-inducing DFF45 like effector B (CIDEB) shares homology with DFF45 at its N-terminal domain, allowing it to bind this inhibitor upon apoptotic stimulation which frees DFF40 to cleave DNA. Figure adapted from (Samejima and Earnshaw 2005).

which are necessary for induction of apoptosis. Overall, this suggests that the suppression of CIDEB may be a mechanism for HCV to overcome the apoptotic pathway.

2.2.3 CIDEB and Lipid Metabolism

Recently, an additional role of CIDEB in lipid metabolism has been elucidated which may be of relevance to the HCV lifecycle. CIDEB-null mice are resistant to obesity and liver steatosis, have increased whole body metabolism and demonstrate increased insulin sensitivity (Li, Ye et al. 2007). This phenotype may be explained due to CIDEB's role as a master regulator of hepatic fatty acid and cholesterol homeostasis (Li, Lei et al. 2010). Li *et al.* argued that CIDEB exerts its control over hepatic lipid homeostasis by regulating the expression of SREBP cleavage-activation protein (SCAP) (Li, Ye et al. 2007). Under normal conditions, CIDEB expression upregulates SCAP, increasing its ability to activate its target genes SREBP-1c and SREBP-2 and resulting in increased *de novo* fatty acid and cholesterol biosynthesis (Li, Lei et al. 2010). When considering CIDEB's role in fatty acid biosynthesis, CIDEB-null mice showed decreased lipogenesis and reduced expression levels of the transcription factor SREBP-1c as well as its downstream targets ACC, FAS and SCD1 (Li, Ye et al. 2007). Moreover, CIDEB expression may result in downregulation of LDL receptor (LDLR) and acyl-CoA cholesterol acyltransferase (ACAT), thereby decreasing hepatic cholesterol uptake and storage (Li, Lei et al. 2010). Additionally, CIDEB was shown to play a role in the homeostatic regulation of energy metabolism and insulin sensitivity as CIDEB deficiency results in increased glucose disposal rate and improved insulin sensitivity (Gong, Sun et al. 2009) (Figure 5).

Furthermore, CIDEB plays diverse roles in controlling lipid secretion and storage in hepatocytes. CIDEB is a target gene of the transcriptional coactivator PGC-1α which provides a mechanism whereby PGC-1α can partition triglycerides (TAGs) toward the secretory pathway in hepatocytes (Chen et al. 2010). Additionally, CIDEB directly interacts with ApoB and localizes to the surface of LDs and the ER membrane (Ye, Li et al. 2009). Likewise, CIDEB-null mice have impaired

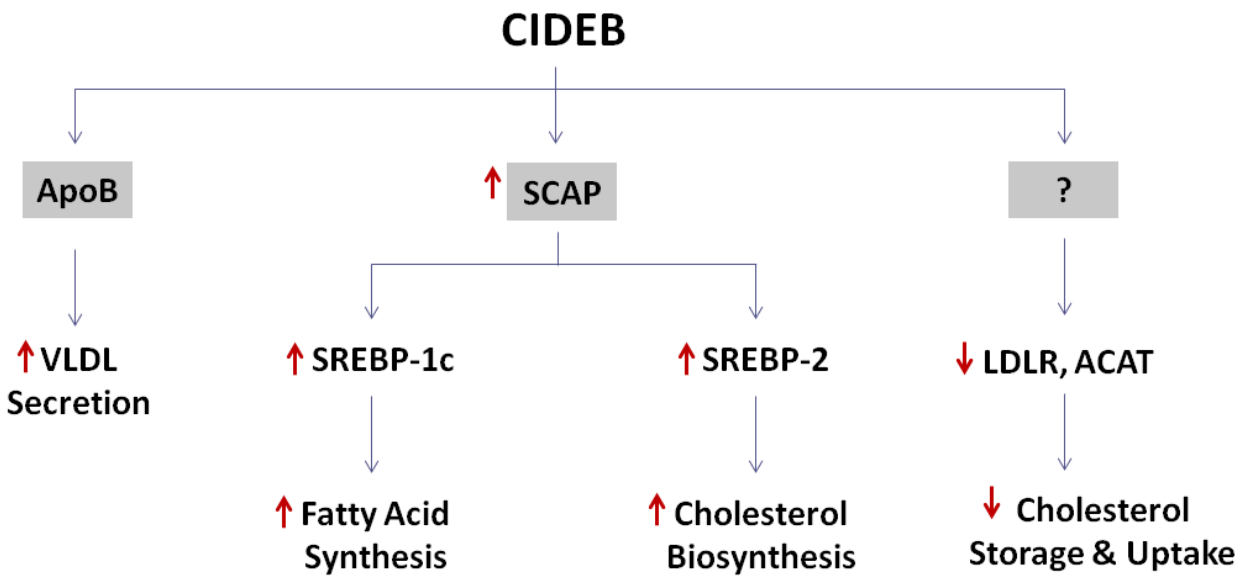

Figure 5. CIDEB is a master regulator of hepatic lipid metabolism. Cell death-inducing DFF45 like effector B (CIDEB) interacts with apolipoprotein B (ApoB) to upregulate the lipidation and maturation of very low density lipoproteins (VLDL). CIDEB expression results in upregulated levels of SREBP cleavage-activation protein (SCAP), increased cleavage and activation of sterol regulatory element binding proteins (SREBP-1c and SREBP-2) and upregulated *de novo* fatty acid and cholesterol biosynthesis in the liver. CIDEB also decreases the expression of low density lipoprotein receptor (LDLR) and acyl-CoA cholesterol acyltransferase (ACAT), thereby downregulating hepatic cholesterol uptake and storage. Overall, CIDEB appears to play a role as a master regulator of fatty acid and cholesterol biosynthesis as well as lipid import and secretion. Figure adapted from (Li, Lei et al. 2010).

VLDL lipidation as they contain significantly lower levels of TAG making these VLDL much smaller and denser (Ye, Li et al. 2009). The role of CIDEB in VLDL secretion may also explain reduced hepatic cholesterol export in CIDEB-null mice (Li, Lei et al. 2010). Based on these findings, a model for CIDEB's role in lipid metabolism was developed. Initially, CIDEB is found on the exterior of LDs and on the ER and it interacts with ApoB on immature VLDL precursors to allow for their lipidation and maturation. Once mature, VLDL will enter the ER lumen and CIDEB will be dissociated (Ye, Li et al. 2009) (Figure 6). Overall, there is strong evidence that CIDEB is also involved in hepatic lipid homeostasis and the VLDL pathway.

2.3 Hypothesis and Objectives

CIDEB is a host factor that is disregulated during HCV infection that modulates HCV's lifecycle through its effects on lipid metabolism and apoptosis. In order to investigate the hypothesis, the role of CIDEB in human hepatic lipid homeostasis was characterized, the effect of HCV on CIDEB expression was elucidated and the effect of CIDEB overexpression in the context of HCV was investigated. Additionally, my thesis work aimed to delineate the relative contribution of CIDEB's dual roles in apoptosis and lipid metabolism to viral pathogenesis.

2.4 Material and Methods

2.4.1 DNA Plasmid Subcloning

a. CIDEB constructs

The human CIDEB gene (GenBank Accession No. AAH35970.1) was PCR-amplified from cDNA obtained from the HepG2 hepatoma cell line using the primers that are listed in Table 1 (Appendix) which contain restriction sites for subcloning. The resulting PCR products were purified.

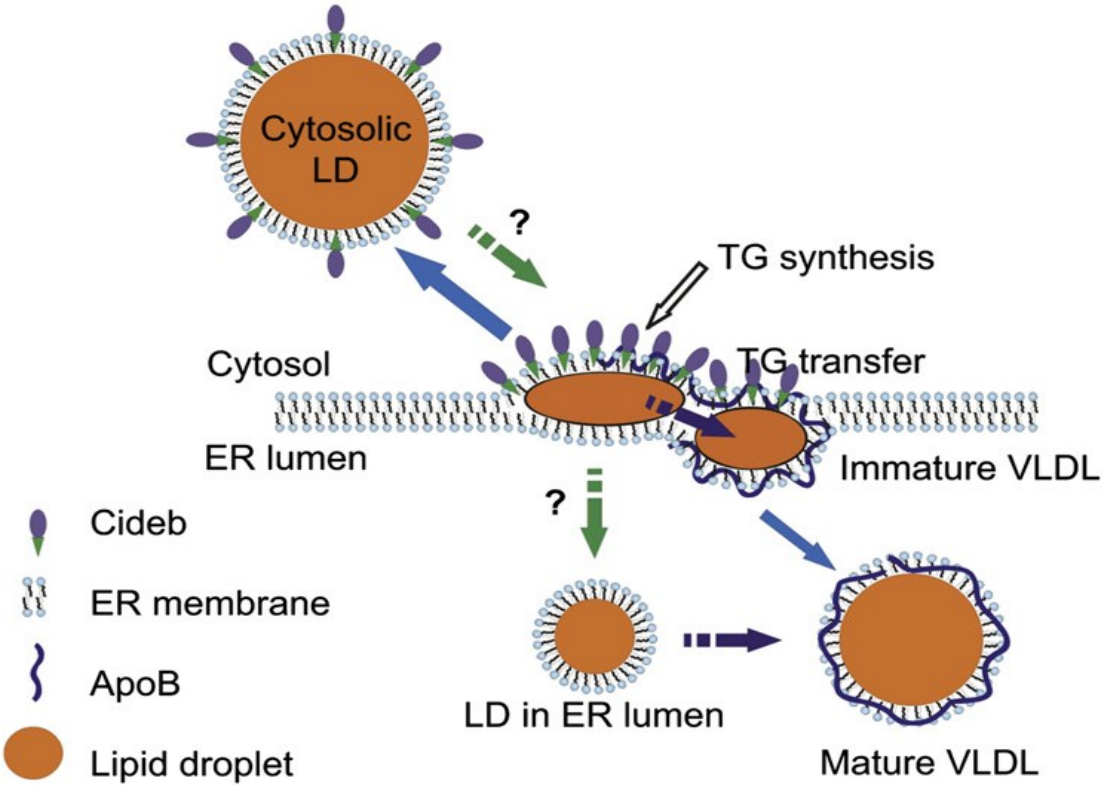

Figure 6. CIDEB is involved in VLDL lipidation and maturation. Cell death-inducing DFF45 like effector B (CIDEB) is localized to the exterior of lipid droplets and on the endoplasmic reticulum (ER) where it interacts with apolipoprotein B (ApoB) on immature very low density lipoprotein (VLDL) precursors to allow for their lipidation and maturation. Once mature, VLDL will enter the ER lumen and CIDEB will be dissociated, allowing for secretion of mature VLDL. Figure adapted from (Ye, Li et al. 2009).

with the QIAquick PCR Purification Kit (Qiagen) and digested with the corresponding restriction enzymes (New England Biolabs). Restriction products were isolated and purified following manufacturer's specifications using the QIAquick Gel Extraction Kit (Qiagen). Purified restriction fragments were ligated into digested pCMVβ or pIRES2-EGFP (bicistronic construct expressing EGFP) mammalian expression vectors (Marker Gene Technologies Inc., Lab Life Inc.), transformed into One Shot(R) TOP10 Chemically Competent *E. coli* (Invitrogen) and plated onto 0.1% ampicillin agar plates. The plasmid DNA of positive colonies were extracted using the QIAquick Spin Miniprep Kit (Qiagen) and sequenced using the primers listed in Table 1. Two single conserved point mutations were found, therefore site-directed mutagenesis was performed as per the QuickChange(R) Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using the primers listed in Table 1 (Appendix). Again, the plasmid DNA of positive colonies were extracted using the QIAquick Spin Miniprep Kit (Qiagen) and sequenced using the primers listed in Table 1 (Appendix). Plasmid DNA containing the desired sequence was purified large scale using a Maxiprep Kit (Qiagen). The QuikChange site-directed mutagenesis kit was used to create the KRRA and Y160F mutants in both the pCMVβ and pIRES2-EGFP constructs using the primers in Table 1. The control pTriEx4-CFP plasmid was derived from a previously described pOpsin-CFP construct using primers listed in Table 1 (Koukiekolo, Jakubek et al. 2009).

b. pCMVβ- NS2-FLAG construct

The region corresponding to the HCV NS2 protein was PCR-amplified from the Con1 isolate of HCV genotype 1b (Genbank Accession No. AJ238799.1) using the primers listed in Table 1 (Appendix) which contain the NotI restriction site for subcloning. Again, resulting PCR products were digested, purified and ligated into digested pCMVβ as per the manufacturer's protocol. The plasmid DNA of positive colonies were sequenced using the primers listed in Table 1 (Appendix) and plasmid DNA containing the desired sequence was purified large scale.

2.4.2 Cell Culture

Cell monolayers of the human hepatoma cell lineages Huh7 and Huh7.5, as well as Huh7 based lineages expressing HCV full genomic replicon (Huh7.5-FGR), HCV subgenomic replicon (Huh7-SGR) or Huh7-SGR-Luc cells were grown in an incubator at 37°C with 5% CO_2, in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Burlington, ON), supplemented with 100 nM nonessential amino acids (NEAA), 50 U/mL penicillin, 50 μg/mL streptomycin and 10% fetal bovine serum (FBS; PAA Laboratories, Etobicoke, ON) or alternatively 2% human serum (HS; Invitrogen, 34005-100, pooled human AB serum, lot numbers 1215151). G418 Geneticin was added at a concentration of 250 μg/mL in cell lineages expressing HCV replicons (FGR or SGR) to maintain the stable expression of viral proteins. Where described, cells were supplemented with 300 µM BSA-conjugated oleic acid (Sigma-Aldrich, St. Louis, MO).

Plasmids that contain HCV subgenomic replicons, namely pFK-I_{389}neo/NS3-3'/5.1 and pFK-I_{389}neo/NS3-3'/Δ5B (Lohmann, Korner et al. 1999) were obtained from Ralf Bartenschlager (Institute of Hygiene, University of Heidelberg, Heidelberg, Germany). The replicons contain the neomycin phosphotransferase gene at the 5' end which acts as a selectable marker and express HCV non-structural proteins (NS3 to NS5B) from the encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES). The pFK-I_{389}neo/NS3-3'/Δ5B plasmid carries an in-frame 10-amino acid deletion (Δ) at the active site of the NS5B RNA polymerase which makes the replicon replication defective (Lohmann, Korner et al. 1999; Pezacki, Sagan et al. 2009). The Huh7.5 cell line stably expressing the full-length HCV genotype 1b replicon with a S2204I adaptive mutation in NS5A (Huh7.5-FGR) was a kind gift from Dr. Charles M. Rice (Rockefeller University, New York, USA) and Apath LLC (St. Louis, MO, USA) (Blight, Kolykhalov et al. 2000). The Huh7-SGR-Luc cells were previously described (Pezacki, Sagan et al. 2009).

2.4.3 Transient Expression of CIDEB

For transient expression of CIDEB, Huh7 cells were seeded at 7.0×10^5 cells per 60 mm dish culture media. Cells were transfected 24 hours post-seeding once they had reached approximately 70% confluency. Transfection complexes were prepared using CIDEB DNA plasmid and Lipofectamine 2000 (Invitrogen) as per manufacturer's specifications. Prior to adding the transfection complex mixture, cells were washed twice with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and then with transfection media, while transfection complexes were incubating. Each transfection complex mixture was then added to the cells and incubated for 4 hours at 37 °C. After 4 hours, one equivalent volume of recovery media, i.e., antibiotic-free DMEM containing 20% (v/v) FBS and 200 nM minimal nonessential amino acids, was added. Transfected cells were then incubated at 37 °C and 5% CO_2 for 48 hours.

2.4.4 Determination of Relative Cellular Levels of RNA by qRT-PCR

3.5 x 10^5 cells were seeded per well of a 6-well plate, transfected 24 hours post-seeding once they had reached approximately 70% confluency (as above) and incubated for 48 hours post transfection. Cells were washed twice with 0.5 mL of PBS then RNA isolation was performed using TriZol (Invitrogen) as per the manufacturer's protocol. RNA concentration was determined on a Nanodrop ND-1000 spectrophotometer and RNA integrity was verified via a native 0.8% agarose gel in 1X TBE (Ambion, Austin, TX), stained with 1X SYBR® Safe dye (Invitrogen). Single stranded cDNA was synthesized using SuperScript™ II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. The reaction for the real-time quantitative PCR was set up in a total volume of 20 µL and included 50 nM of both forward and reverse primers, 10 µL of iQ™ SYBR(R) Green Supermix (Bio Rad) and 1 uL of cDNA. Real-time quantitative RT-PCR was performed using an iQ5 Multicolour Real-time PCR Detection System (Bio-Rad). The reaction mixture was first incubated for 2 min at 50 °C and then for 10 min at 95 °C and finally cycled 40 times between 95 °C for 15 s and 60 °C for 1 min. C_t values for the replicon and large ribosomal protein cDNAs were calculated

for each sample using the instrument software. To calculate the relative cellular abundance of the HCV replicon, the following method was used (N-fold difference = 2_t^- (ΔC_t treated – ΔC_t control), where ΔC_t is the difference between the replicon and large ribosomal protein cDNA C values) as previously described (Nasheri, Singaravelu et al. 2011). Primers are described in Table 1.

2.4.5 Triglyceride Measurements

TAGs were extracted from Huh7 or Huh7.5-FGR cells transiently overexpressing CIDEB for 48 hours and quantified using a triglyceride assay kit (BioVision, Mountain View, CA) as per manufacturer`s specifications. The absorbance was detected at 570 nm with a SpectraMax M2 Microplate Reader (Molecular Devices, Sunnyvale, CA). Total protein levels in lysates were quantified with the DC protein assay (Bio-Rad, Hercules, CA). TAG levels were normalized by protein content.

2.4.6 Coherent Anti-Stokes Raman Scattering (CARS) Microscopy

8.0×10^4 cells were seeded per Lab-Tek Chambers (Thermo scientific), transfected with the bicistronic CIDEB construct 24 hours post-seeding once they had reached approximately 70% confluency (as above) and incubated for 48 hours post transfection. Cells were fixed by incubating them with 0.5 mL fixing solution (PBS, 4% formaldehyde, 4% sucrose) at 4 ^oC for 15 minutes. Cells were washed with PBS 3 times, each for 5 minutes, prior to imaging. The CARS microscopy system was setup as previously described (Pegoraro, Ridsdale et al. 2009). The imaging and subsequent quantitative voxel analysis of TAG content was performed as previously described (Lyn, Kennedy et al. 2009). LD counting and sizing was performed using the ImageJ plugin for Particle Counting and Analysis.

2.4.7 Western Blot Analysis

Cells were washed twice with PBS and lysed with a lysis buffer heated to 95 °C consisting of 50 mM Tris-HCl (pH 6.8), 2% SDS, and 10% glycerol 48 hours post-transfection. A protease inhibitor cocktail mix (Roche Diagnostics, Penzberg, Germany) was added to each extract. The protein concentration of the whole cell lysate was quantified by using the Bio-Rad DC Protein Assay according to the manufacturer's protocol. Prior to loading, a final concentration of 100 mM DTT and 0.1% v/v bromophenol blue was added to each sample. A total of 30-60 μg protein/well was loaded onto a SDS-PAGE gel (15% resolving, 4% stacking gel). The resolved proteins were transferred to a Hybond-P (Amersham Biosciences, Piscataway, NJ) poly(vinylidene difluoride) membrane. The membrane was blocked for one hour at room temperature in 5% skim milk in TBS-T (180 mM NaCl, 10 mM Tris, 0.05% Tween 20). Membranes were probed using a mouse anti-NS5A (1:3000 dilution, Virogen, Boston, MA), mouse anti-NS3 (1:3000 dilution, Virostat, Portland, ME), mouse anti-CIDEB (1:200 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA), JL-8 (anti-CFP) (Clontech Laboratories, Inc., Mountain View, CA), mouse anti-core (1:1000 dilution, Abcam, Boston, MA), goat anti-sE2 (1:500 dilution, Abcam, Boston, MA), or mouse anti-PTP1D (1:10000 dilution; Sigma, Saint Louis, MO) primary antibodies overnight followed by a secondary (HRP)-conjugated goat anti-mouse and donkey anti-goat IgG antibody (1:10,000 dilution, Jackson ImmunoResearch Laboratories, Inc., Westgrove, PA) for one hour. Membranes were washed with TBS-T five times for 5 minutes and the blot was developed using ECL Plus Western Blotting Detection Reagents (GE Healthcare, Baie d'urfe, QC) according to the manufacturer's protocol. The band intensities were measured by densitometry using ImageJ software (National Institutes of Health).

2.4.8 Luciferase Assays

Luciferase assays were performed as previously described (Nasheri, Singaravelu et al. 2011).

2.4.9 Caspase assay and inhibitors

The activity of caspase-3 was measured 48 hours post-transfection using the caspase-Glo® 3/7 assay kits (Promega, Madison, USA), as per the manufacturer's protocol. Caspase inhibitors Z-VADK-FMK and Z-DEVD-FMK (Figure 7, R&D Systems, Minnesota, USA), treatments were performed simultaneously with transfections.

2.4.10 Cytotoxicity Assays

WT Huh7 or Huh7.5-FGR cells were seeded into 12-well plates at a density of 80×10^5 cells per well. Cells were transfected 24 hours post-seeding once they had reached approximately 70% confluency. Transfection complexes were prepared using CIDEB DNA plasmid and Lipofectamine 2000 (Invitrogen) as per manufacturer's specifications. Half of the cells had recovery media containing 300 µM oleic acid (Sigma-Aldrich, Saint Louis, MO). Transfected cells were then incubated at 37 °C and 5% CO_2 for 48 hours. 3 h before each of the desired time points, 100 µl of formazen (Sigma-Aldrich, Saint Louis, MO) was added into each well and cells were incubated for the remaining time at 37°C. The medium was removed and 200 µl of DMSO was added into each well. The plate was gently rotated on an orbital shaker for 10 min to completely dissolve the precipitation. The absorbance was detected at 570 nm with a Microplate Reader (Molecular Devices, Sunnyvale, CA). This assay has been previously described (Kennedy, McKay et al. 2011).

2.4.11 Statistical analysis

Student's unpaired t-test was used to analyze data, and *P*-values less than 0.05 were deemed significant.

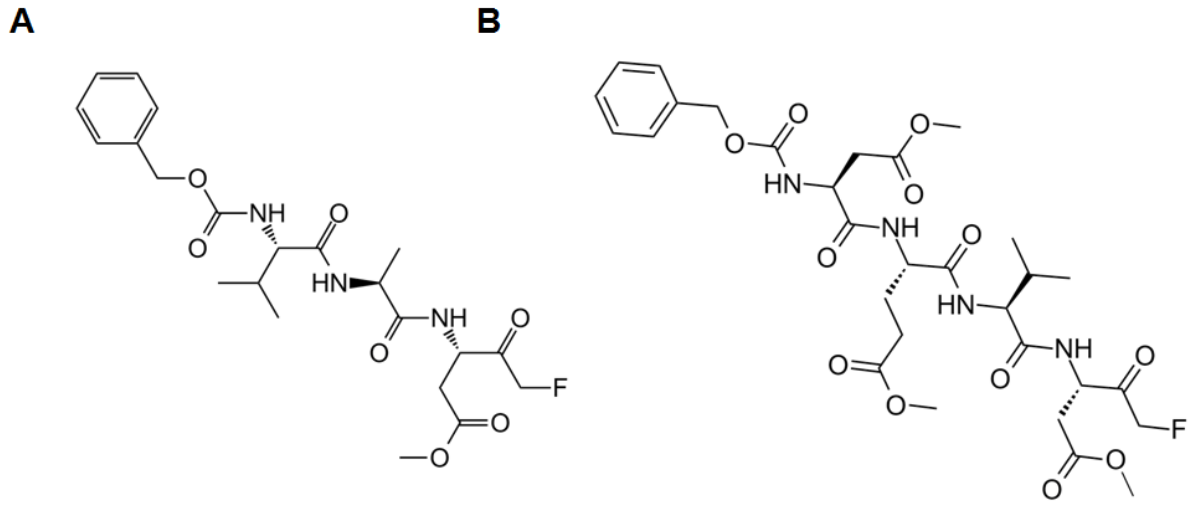

Figure 7. Structure of caspase inhibitors. Structures of (A) the pan-caspase inhibitor Z-VAD-FMK (chemical name benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone) and (B) caspase-3 inhibitor Z-DEVD-FMK (chemical name methyl(4S)-5-[[(2S)-1-[[(3S)-5-fluoro-1-methoxy-1,4-dioxopentan-3-yl]amino]-3-methyl-1-oxobutan-2-yl]amino]-4-[[(2S)-4-methoxy-4-oxo-2 (phenylmethoxycarbonylamino) butanoyl] amino]-5-oxopentanoate).

2.5 Results

2.5.1 CIDEB Pathway Activation

Due to the low levels of endogenous CIDEB in several HCV models, two approaches were used to activate this pathway in order to evaluate the role of CIDEB in human lipid homeostasis and HCV pathogenesis. First, the coding region of the CIDEB protein was cloned into a mammalian expression vector as a means to overexpress CIDEB in various cell lines. The cDNA used for cloning was obtained from HepG2 human hepatoma cells, which demonstrate a higher level of CIDEB expression than Huh7 based cell lines, yet are less permissive to the HCV lifecycle (Sainz, Barretto et al. 2012) (Figure 8). CIDEB cloning also required a round of site-directed mutagenesis to correct a conserved substitution mutation that changed the third last codon from a histidine to a tyrosine. When overexpression was performed in Huh7 cells, increasing levels of CIDEB could be detected via Western blot (Figure 9). In addition to CIDEB overexpression, the CIDEB pathway was also activated by supplementing cell culture media with human serum (HS) in place of fetal bovine serum (FBS), as HS treatment has been previously shown to upregulate CIDEB levels by upregulation of its positive transcriptional regulator PGC1-α (Singaravelu, Lyn et al. 2013). HS treatment also causes cells to be more differentiated and therefore this is more physiologically relevant. Overall, the effect of an activated CIDEB pathway on human lipid metabolism and HCV was investigated using these models.

2.5.2 CIDEB Upregulates Lipogenesis in Human Hepatoma Cells

Initially, the role of CIDEB in human lipid homeostasis was investigated, as previous studies made use of other animal models (Li, Ye et al. 2007; Ye, Li et al. 2009; Li, Lei et al. 2010; Li, Ye et al. 2012; Tiwari, Siddiqi et al. 2013). The relative transcript expression of lipogenic genes fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD) and acetyl-CoA carboxylase (ACC) was quantified

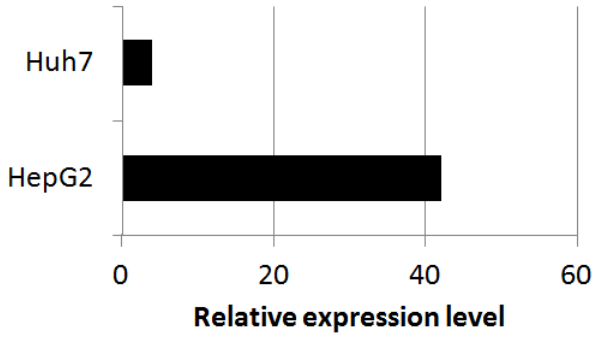

Figure 8. CIDEB expression in human hepatoma cells. Relative CIDEB expression in two hepatoma cell lines, HepG2 and Huh7s is depicted. HepG2s are non-permissive to HCV replication while Huh7s are known to be permissive to the entire HCV life cycle. Figure was adapted from BioGPS online gene annotation portal (http://biogps.org). Median expression level was ~3.47 over all NC160 cell lines sampled in microarray.

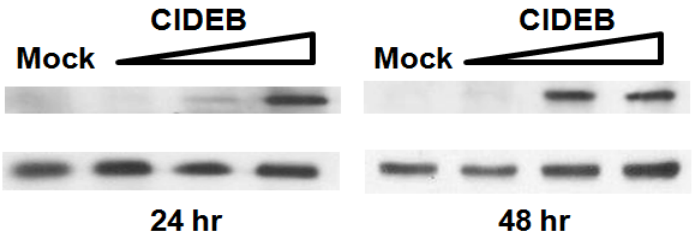

Figure 9. Optimization of CIDEB overexpression in human hepatoma cells. Western blot demonstrating CIDEB overexpression at increasing concentrations for 24 or 48 hours in Huh7 cells. Proteins were detected via immunoblotting using an anti-CIDEB antibody. Expression levels of the housekeeping gene PTP1D serve as a loading control.

in human hepatoma cells overexpressing CIDEB via qRT-PCR. All three genes were upregulated during CIDEB overexpression, which is in agreement with previous studies in mice and rats that demonstrate that CIDEB enhances cellular lipogenesis (Figure 10A).

Furthermore, the level of hepatic TAG accumulation in human hepatoma cells overexpressing CIDEB was investigated. Levels of hepatic TAG were compared to mock cells that were treated with transfection reagent alone. Moderate levels of CIDEB overexpression resulted in an increase in cellular TAG (Figure 10B), however the highest level of CIDEB overexpression resulted in a diminished accumulation of hepatic TAG (Figure 23B).

Moreover, the hepatic lipid content of human hepatoma cells overexpressing CIDEB was visualized using Coherent Anti-Stokes Raman Scattering (CARS) Microscopy. The CARS microscope allows for label-free imaging of cell lipids by tuning in to the inherent C-H bond vibrational resonance. This technique may also be combined with simultaneous two-photon fluorescence (TPF) imaging, therefore a bicistronic construct expressing GFP and CIDEB was cloned. Here, GFP expression served as a marker for cells which overexpress CIDEB after transfection. The cells were fixed 48 hours post-transfection and were subsequently imaged. This experiment was performed in the presence of oleic acid, as previous studies have shown that treating cells with oleic acid decreases the cytotoxicity of CIDE family proteins' expression by promoting their localization to LDs (Liu, Zhou et al. 2009). Cells in the mock treatment, which were treated with transfection reagent alone, display a homogenous distribution of lipids with no distinct changes in LD morphology between cells (Figure 11A). Comparatively, cells overexpressing CIDEB display significantly larger LDs and a decrease in average LD number per cell, which is in agreement with previous reports (Li, Ye et al. 2007; Ye, Li et al. 2009; Li, Lei et al. 2010) (Figure 11B). Moreover, CIDEB-expressing cells had a larger proportion of LDs with diameters greater than 1 μm compared to mock transfected Huh7 cells (Figure 24C).

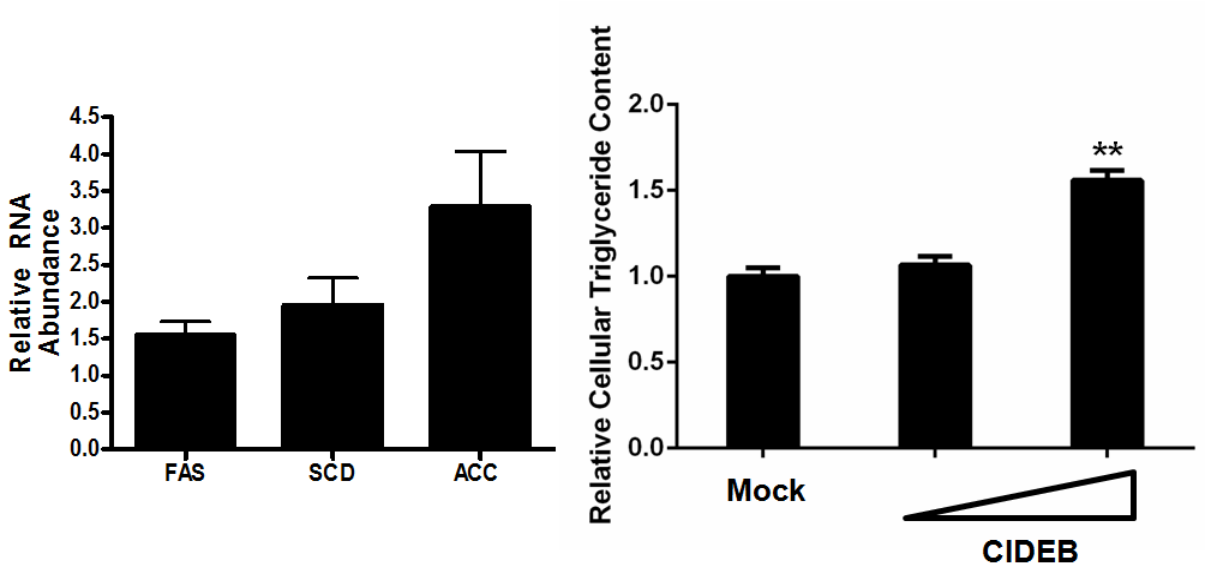

Figure 10. CIDEB in human lipid homeostasis. (A) Relative transcript expression of genes fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD) and acetyl-CoA carboxylase (ACC) were quantified via qRT-PCR in Huh7 cells transiently transfected with CIDEB for 48 hours. Each bar represents the mean ± SEM values (n=3). (B) TAG content was isolated and quantified from Huh7 cells transiently overexpressing CIDEB at increasing concentrations for 48 hours. Relative TAG levels were normalized to mock. Each bar represents the mean ± SEM values (n=3). **p<0.01.

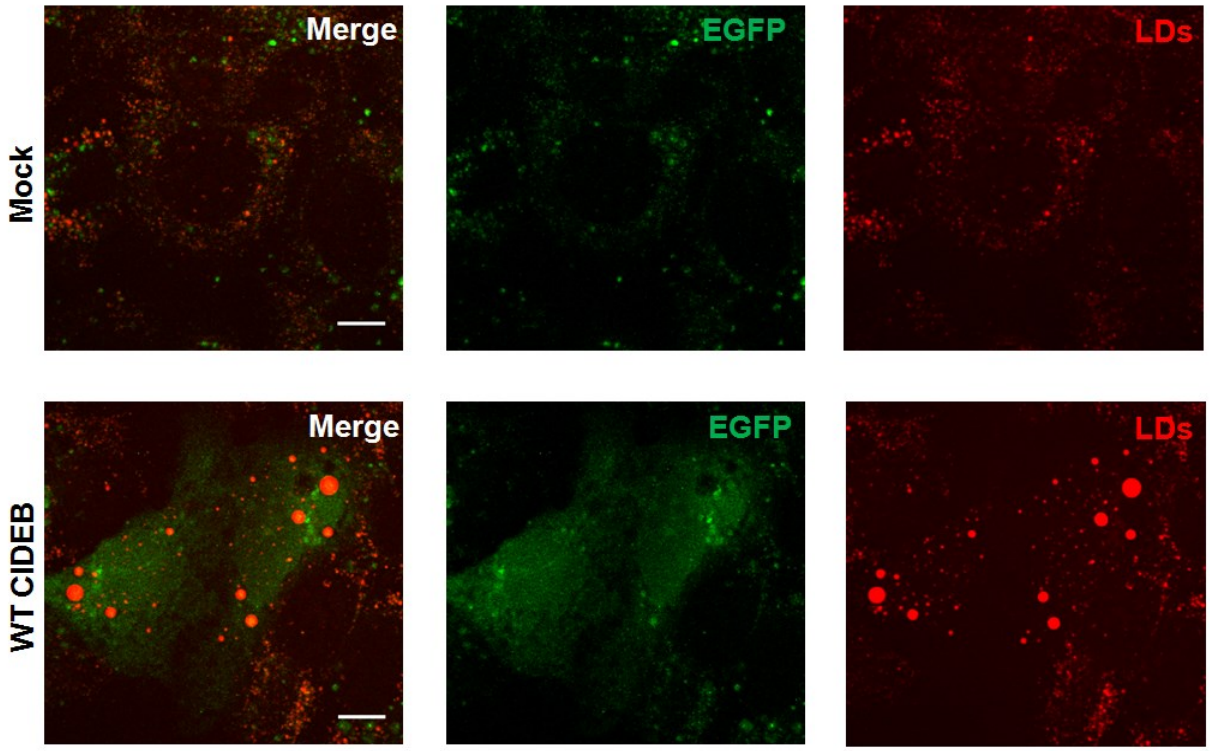

Figure 11. Lipid droplet phenotype in human hepatoma cells overexpressing CIDEB. The hepatic lipid content of Huh7 cells incubated with 300 µM oleic acid and either (A) mock treated or (B) overexpressing a bicistronic GFP and CIDEB construct were fixed 16 hours post-transfection and imaged with CARS microscopy and two-photon fluorescence (TPF). Representative CARS, TPF, and merged images are shown of twelve independent trials. Scale bar represents 22 µm.

As other CIDE family proteins have been shown to cause larger LD by inducing lipid fusion, live cell imaging experiments were carried out in Huh7 cells treated with oleic acid and transfected with CIDEB for 16 hours. Cells were subsequently imaged every 30 minutes using CARS microscopy. Here, the formation of larger LD can be visualized directly, as over time, smaller LD colocalize to form the larger LD that can be observed after longer incubation periods (Figure 12). Overall, the results demonstrate that CIDEB induces lipid fusion, just as other CIDE family proteins (Gong, Sun et al. 2011).

2.5.3 CIDEB and Apoptosis

Previous studies with other CIDE family proteins have demonstrated that higher expression levels favour an apoptotic, rather than a lipogenic role for these proteins in cell culture (Keller, Petrie et al. 2008). To confirm this effect in our own cell culture system, cell proliferation assays were performed on Huh7 cells transfected with a gradient of CIDEB overexpression, treated with or without oleic acid (OA). Previous studies indicate that oleic acid may serve as a signal shifting the activity of CIDE family proteins towards a lipogenic vs. an apoptotic role (Gong, Sun et al. 2011). It was hypothesized that the lower level of CIDEB overexpression would demonstrate limited apoptosis and here CIDEB's role in lipid metabolism would predominate, whereas at the higher levels of CIDEB overexpression, its apoptotic role would be prevalent. As expected, a drastic decrease in cell viability was observed at the highest level of CIDEB overexpression compared to mock, which is consistent with previous reports (Liu, Zhou et al. 2009). Interestingly, similar levels of cytotoxicity were observed regardless of the presence or absence of oleic acid, with only a modest decrease in apoptosis observed in OA treated cells (Figure 13).

2.5.4 CIDEB is a Host Factor that is Disregulated during HCV Infection

Now that the role of WT CIDEB had been clarified, the role of CIDEB in the context of the HCV lifecycle was investigated. First, the level of endogenous CIDEB was compared in naïve Huh7

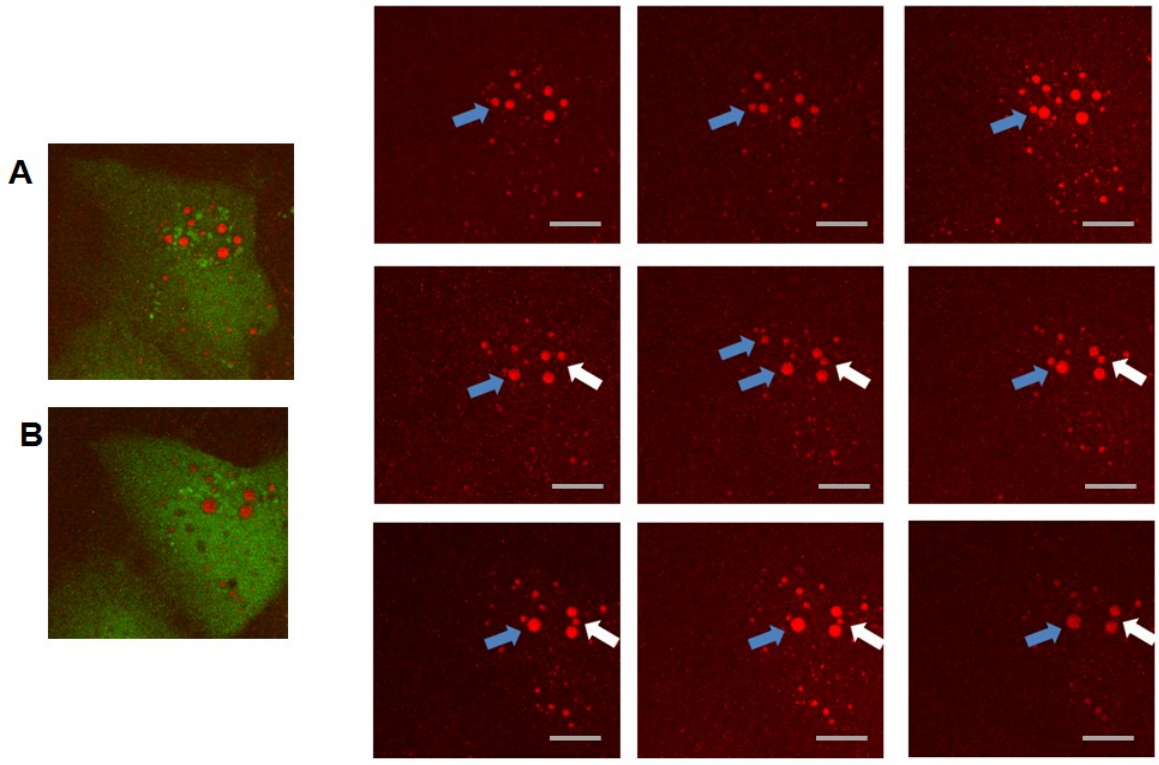

Figure 12. CIDEB overexpression induces lipid droplet fusion in human hepatoma cells. Huh7 cells were incubated with 300 µM oleic acid, transfected with a bicistronic GFP and CIDEB construct and imaged every 30 minutes 16 hours post-transfection using CARS microscopy with simultaneous fluorescence imaging. Time is increasing as the images go from left to right. Arrows represent areas of LD fusion, where the blue arrow and white arrow both represent unique LD fusion events. Green cells represent (A) initial and (B) final cell images using two photon fluorescence demonstrating persistent CIDEB expression. Scale bar represents 22 µm.

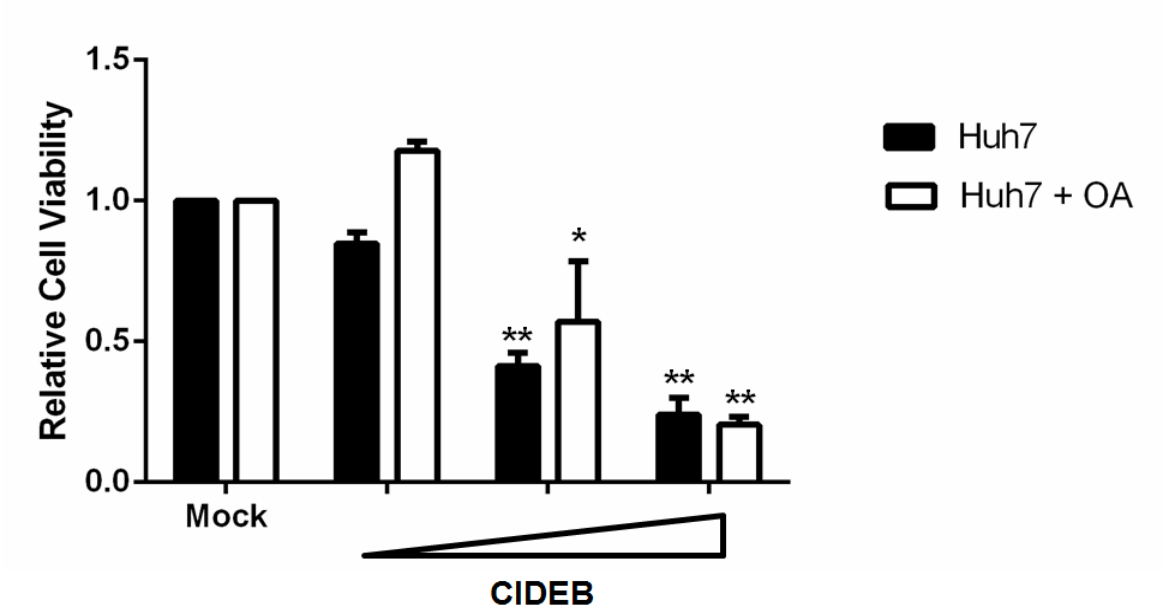

Figure 13. Cell viability during CIDEB overexpression in human hepatoma cells. CIDEB was overexpressed for 48 hours at increasing concentrations in Huh7 cells in the presence or absence of oleic acid. An MTT assay was performed to determine cell viability. Relative cell viability normalized to mock is shown. Each bar represents the mean ± SEM values (n=5). **p<0.01.

cells, Huh7.5 cells stably expressing the full-length HCV genomic replicon from the Con1 isolate (genotype 1b; Huh7.5-FGR; Figure 14A) and Huh7.5 cells stably expressing the subgenomic HCV replicon from the Con1 isolate (genotype 1b; Huh7-SGR; Figure 14B). Interestingly, there was no statistical difference in the levels of CIDEB in these three cell lines when cells were cultured in normal FBS, although detection of CIDEB in these models is difficult due to the low level of endogenous CIDEB in the Huh7 line and its derivatives (Figure 14C). Moreover, when CIDEB levels were compared in FBS treated Huh7.5 cells either infected with a cell-culture adapted strain of HCV high titer JFH-1, JFH-1_T (genotype 2a) , or a mock infection, CIDEB levels were not seen to vary as a result of infection (Russell, Meunier et al. 2008) (Figure 14D). Collectively, these results demonstrate that at the low level of endogenous CIDEB found in Huh7 cells cultured in FBS, HCV does not alter CIDEB expression, regardless of HCV genotype.

Next, the level of endogenous CIDEB was compared in cells treated with HS for 8 days, as a means to activate the CIDEB pathway and obtain more physiologically relevant levels of CIDEB. CIDEB levels in Huh7-SGR cells were compared to naïve Huh7 cells (Figure 15A) or cured Huh7-SGR cells which no longer expressed viral proteins after being treated with two weeks of NS5B siRNA and two months of IFNγ treatment (200 units/ml) (Blais, Lyn et al. 2010) (Figure 15B). Interestingly, CIDEB was upregulated at the protein level in hepatoma cells expressing SGR, but had lower levels in the naïve and cured Huh7 cells. This indicates that CIDEB levels may be disregulated during HCV infection as a means to abrogate the viral lifecycle, consistent with its pro-apoptotic role.

2.5.5 CIDEB Overexpression Inhibits HCV Replication

To further confirm CIDEB's role as a host factor disregulated during HCV infection, the effect of CIDEB overexpression in the context of full length or subgenomic HCV genome was investigated. Using the previously established low and high levels of CIDEB overexpression, luciferase assays were performed to investigate how CIDEB influences HCV replication. This assay

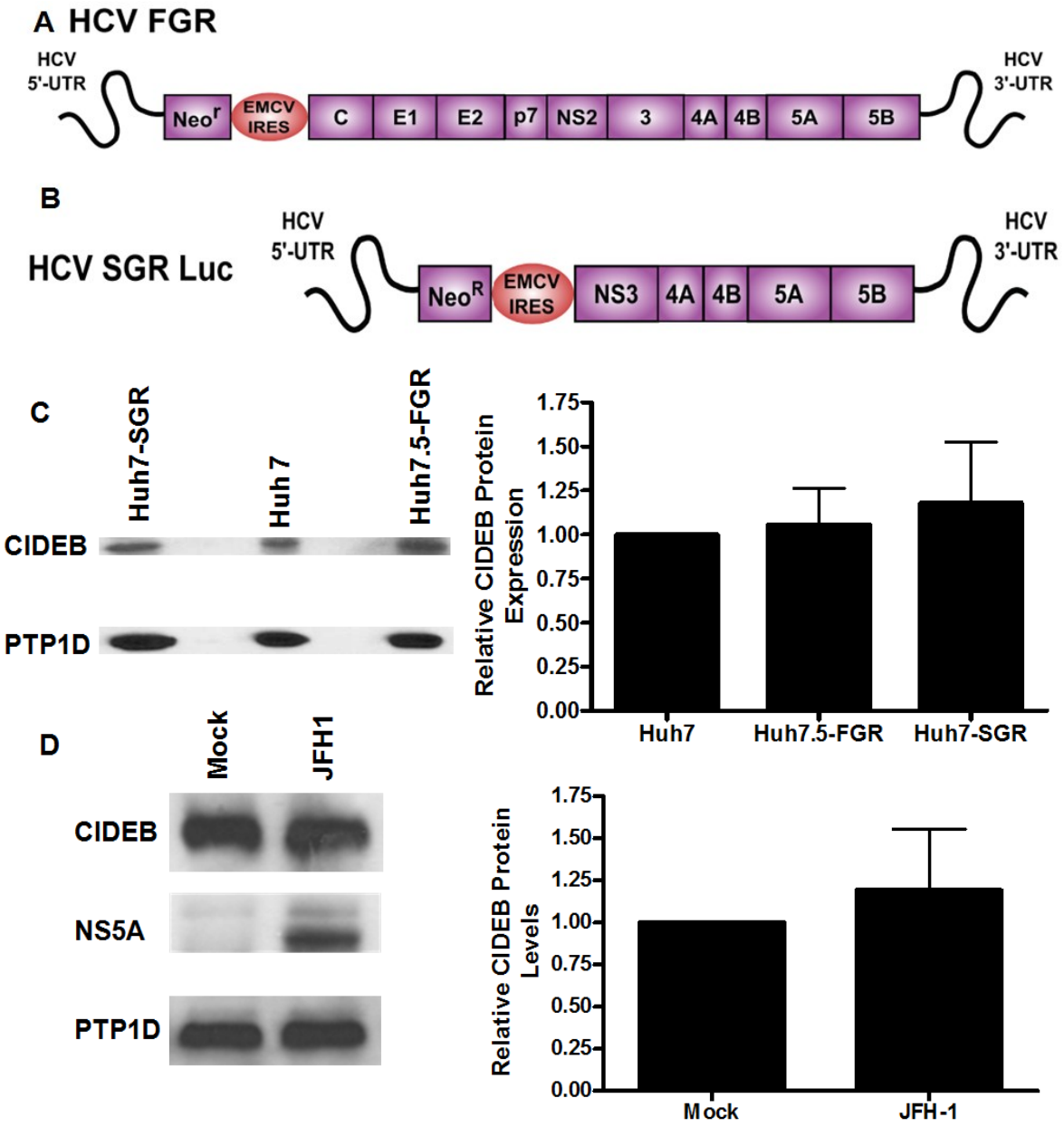

Figure 14. Relative CIDEB expression in human hepatoma cells expressing HCV. (A) Diagram depicting HCV full-genomic replicon (HCV FGR) stably expressed in the Huh7.5 cells (Huh7.5-FGR) used in this study. The bicistronic construct contained a neomycin resistance (Neo^r). The FGR consists of three HCV structural proteins (core, E1, and E2) and seven HCV non-structural proteins (p7 and NS2-NS5B). (B) Diagram depicting HCV sub-genomic replicon (HCV SGR) stably expressed in the Huh7 cells (Huh7-SGR) used in this study. The bicistronic construct contained a neomycin resistance (Neo^r) and expressed HCV non-structural proteins necessary and sufficient for viral replication (NS3-NS5B). (C) Relative CIDEB protein levels were quantified via Western blot densitometry in (A) naïve Huh7 cells, Huh7.5-FGR or Huh7-SGR (n=3) or (B) mock and JFH1-infected Huh7.5 cells (n = 3). Each bar represents the mean ± SEM values. PTP1D was used as a loading control.

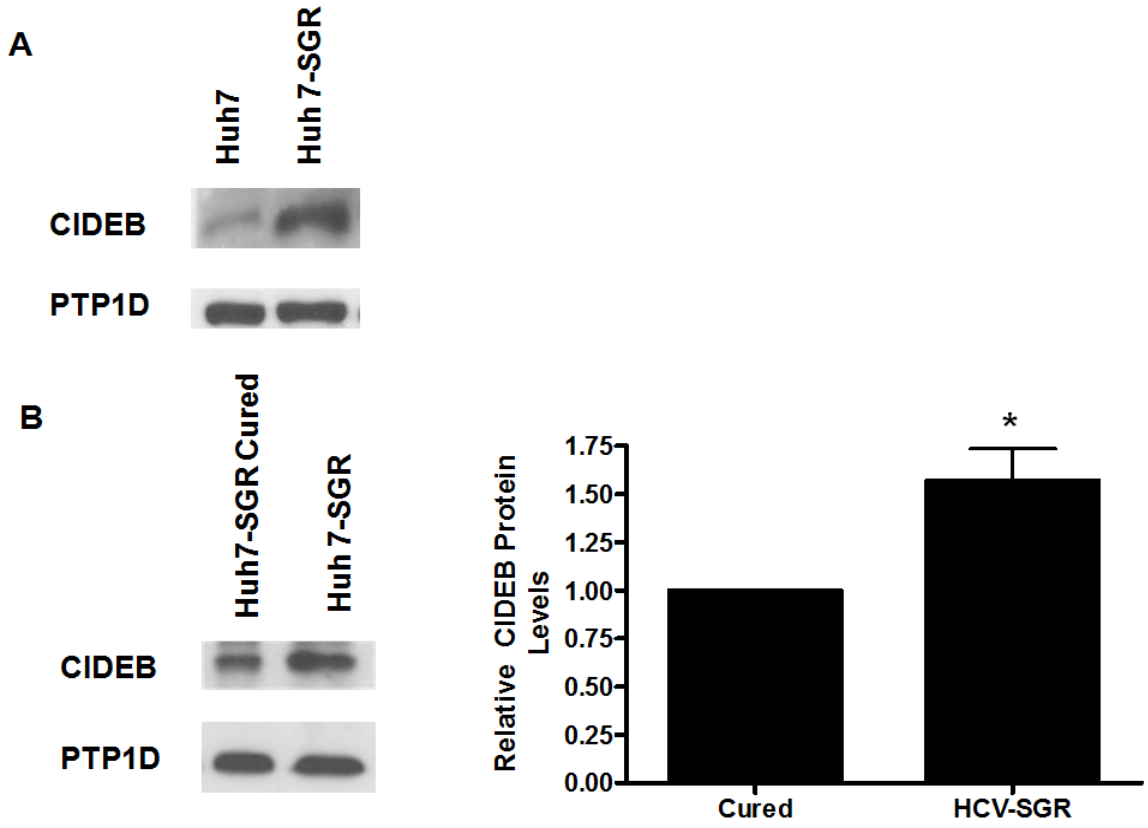

Figure 15. Relative CIDEB expression in HS treated human hepatoma cells expressing HCV. (A) Comparison of CIDEB levels in naïveHuh7 cells or Huh7-SGR (Huh7-SGR) (n=3) or (B) cured Huh7-SGR and Huh7-SGR cells. PTP1D was used as a loading control. Relative CIDEB protein levels were quantified via Western blot densitometry in (B). Each bar represents the mean ± SEM values (n = 3). **p<0.05.

makes use of Huh7 cells stably expressing a tricistronic replicon harbouring an HCV internal ribosomal entry site (IRES) driving the expression of a luciferase gene and an encephalomyocarditis virus (EMCV) IRES driving constitutive expression of the HCV subgenomic replicon (NS3-NS5B) (HCV-SGR-Luc; Figure 16A). These cells harbour all of the HCV proteins necessary and sufficient to form a functional HCV replication complex, and therefore decreased luciferase signal here correlates to decreased viral replication (Lohmann, Korner et al. 1999). Using this model, both levels of CIDEB overexpression effectively inhibited HCV replication (Figure 16B).

The effect of CIDEB on HCV replication was further investigated at the protein level using Western blot analysis. When CIDEB was overexpressed in HCV7.5-FGR cells at the two established expression levels, only higher levels of CIDEB expression yielded decreased levels of HCV viral proteins NS5A and NS3 (Figure 16D,E). Moreover, a decrease in HCV RNA levels could also be observed at the highest level of CIDEB overexpression in Huh7.5-FGR cells using qRT-PCR (Figure 16C). Moreover, MTT assays confirmed that CIDEB overexpression continued to exert its apoptotic effect in the Huh7.5-FGR model (Figure 17).

The difference in antiviral activity seen in HCV-SGR-Luc vs. Huh7.5-FGR cells at the lower level of CIDEB overexpression was initially thought to be attributable to the difference in transfection efficiencies between these two cells lines. However, after comparing the transfection efficiencies of these two cells types using the control protein cyan fluorescent protein (CFP), it was observed that HCV-SGR-Luc cells were not as easily transfected as Huh7.5-FGR cells, which would have explained the antiviral affect observed at the lower level of CIDEB overexpression in this line (Figure 18). Therefore, the difference in HCV inhibition observed at lower levels of CIDEB overexpression is thought to be attributable to the presence of HCV structural proteins and NS2, which are not present in the HCV-SGR-Luc cells.

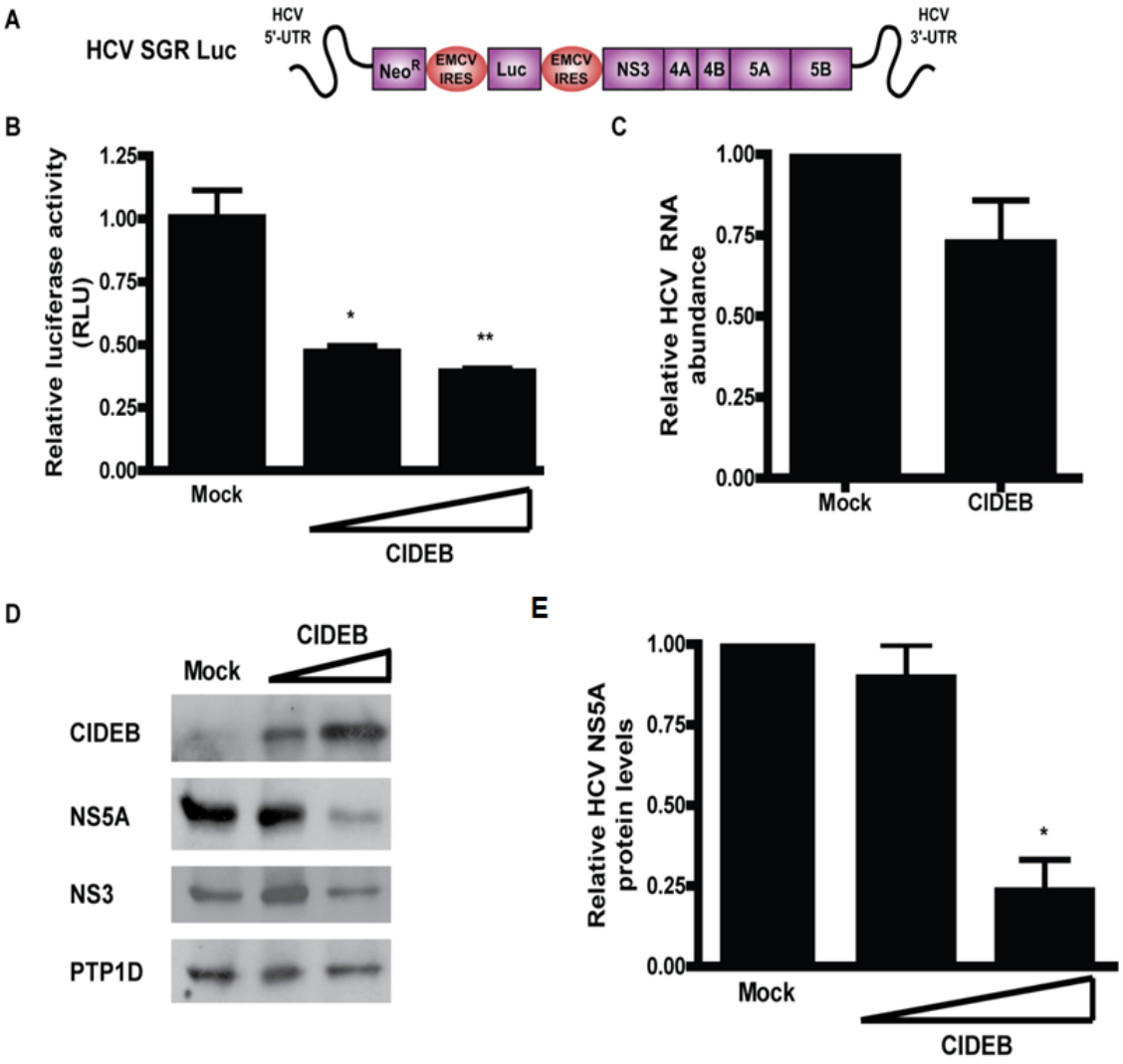

Figure 16. CIDEB overexpression inhibits HCV replication. (A) Diagram depicting tricistronic HCV subgenomic replicon construct expressed in Huh7-SGR-Luc (HCV-SGR-Luc). Construct harbours HCV IRES-driven neomycin resistance (Neo^r), and luciferase gene (Luc) and subgenomic replicon (SGR) expression (NS3-NS5B) driven by separate EMCV IRES motifs. (B) Huh7-SGR-Luc cells were transfected with wild type CIDEB. Cells were lysed 48 hours post-transfection and luciferase activity was measured. Relative luciferase activity relative to mock is shown. Luciferase signals were also normalized by protein content. C-E. Huh7.5-FGR and Huh7 cells were transfected as in B. (C) Total RNA samples were isolated from Huh7.5-FGR cells 48 hours post-transfection, and qRT-PCR was performed for HCV RNA levels. Relative HCV RNA levels are shown after normalization by 18S rRNA levels. (D) (E) Protein samples were isolated from Huh7.5-FGR cells 48 hours post-transfection, and Western blotting was performed for NS5A, NS3, and CIDEB protein levels. PTP1D was used as a loading control. A representative blot is shown (n=3). (E) Densitometry of NS5A levels from the three independent Western blot trials. PTP1D levels were used for normalization. Each bar represents the mean ± SEM values ($n \geq 3$). *$p<0.05$; **$p<0.01$.

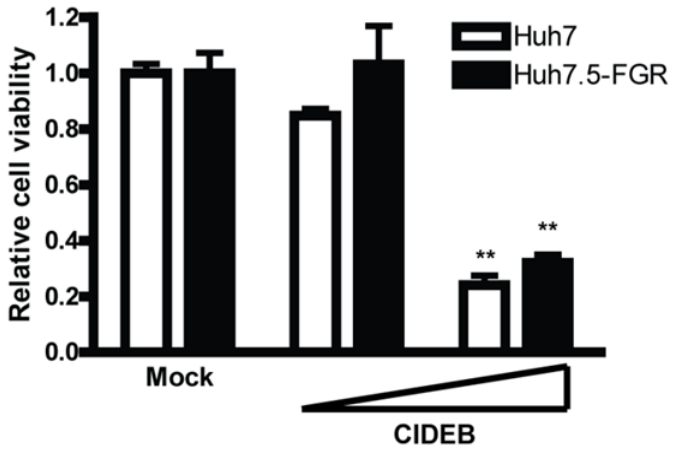

Figure 17. Cell viability during CIDEB overexpression in human hepatoma cells. CIDEB was overexpressed for 48 hours at Huh7 or Huh7.5-FGR cells. An MTT assay was performed to determine cell viability. Relative cell viability normalized to mock is shown. Each bar represents the mean ± SEM values (n=5). **p<0.01.

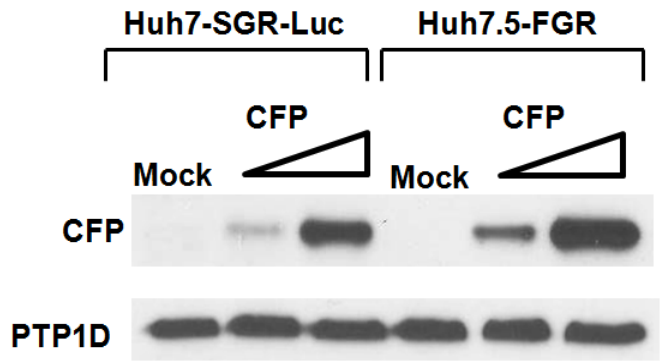

Figure 18. Transfection efficiency of HCV cell lines. CFP was overexpressed at increasing levels for 48 hours at Huh7-SGR-Luc or Huh7.5-FGR cells. Western blot analysis allowed for the comparison of transfection efficiency between cell lines. PTP1D was used as a loading control.

Moreover, to confirm that the antiviral effect of CIDEB overexpression was specific to CIDEB and not a result that can be observed when any protein is overexpressed to similar levels, Huh7.5-FGR cells were transfected with the same amount of CFP expressing plasmid. Western blot analyses revealed no change in HCV protein levels during high levels of CFP overexpression (Figure 19A). This supports the claim that HCV inhibition caused by CIDEB overexpression is specific to CIDEB.

Furthermore, to confirm that the levels of CIDEB overexpression used in this study were physiologically relevant, this level of CIDEB protein expression was compared to the endogenous levels of CIDEB in primary human hepatocytes. Western blot analyses revealed an approximate 5 fold increase in CIDEB levels in the transfected Huh7 cells compared to basal levels in the hepatocytes, calculated from the average of five trials using densitometry (representative Figure 19B). As clinical studies have demonstrated that CIDEB expression is modulated in diseased states (Yu, Wang et al. 2013), and fatty acid treatment has been shown to induce similar magnitude changes in CIDEB expression in pancreatic beta cells (Li, Song et al. 2012), this higher level of CIDEB overexpression, in specific metabolic states, was deemed as physiologically relevant.

Interestingly, when the levels of viral protein NS5A were investigated in Huh7.5-FGR cells that were transfected with CIDEB after 6 days of HS treatment, the antiviral effect of CIDEB could not be observed (Figure 20). This suggests that the lipid rich environment that results from HS treatment partially offsets CIDEB's apoptotic role by promoting CIDEB's localization to LDs, thereby interfering with CIDEB's ability to impair HCV replication, as there would be more LDs and therefore a larger proportion of CIDEB bound to LDs rather than localized to the mitochondria. Overall, it can be hypothesized that CIDEB's apoptotic role may be of particular importance for its ability to modulate the HCV lifecycle.

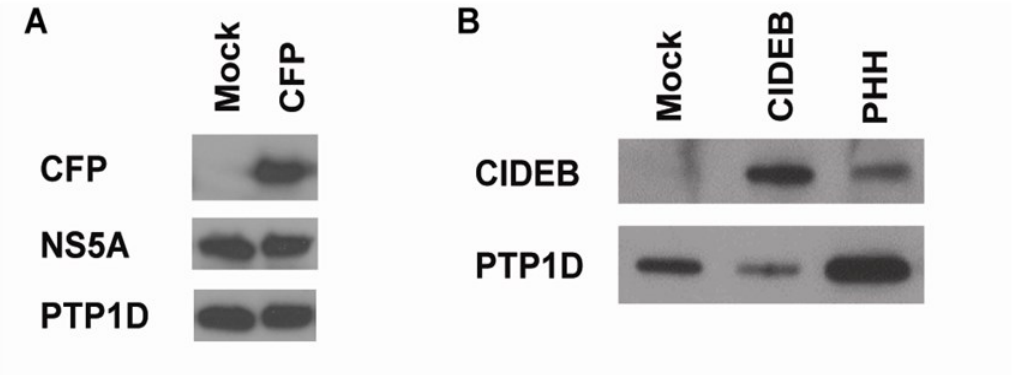

Figure 19. CIDEB's impairment of HCV replication is specific and physiologically relevant. (A) CFP was overexpressed for 48 hours in Huh7.5-FGR cells. (B) The level of CIDEB overexpression in Huh7.5-FGR cells was compared to primary human hepatocytes via Western blot analysis and densitometry. Representative blot of five trials is depicted. PTP1D was used as a loading control.

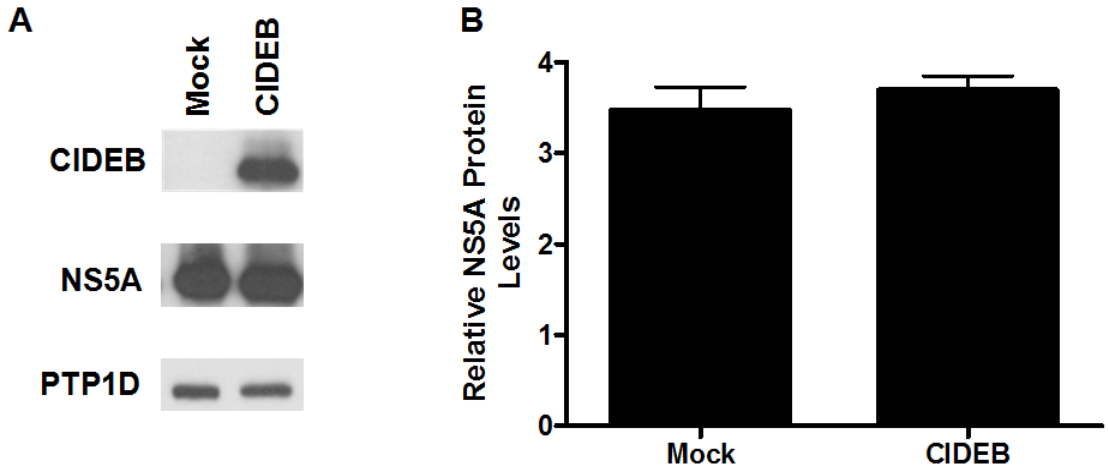

Figure 20. CIDEB overexpression fails to inhibit HCV replication after HS treatment. (A) Protein samples were isolated from Huh7.5-FGR cells treated with HS for 8 days and transfected with CIDEB for 48 hours, and Western blotting was performed for NS5A and CIDEB protein levels. PTP1D was used as a loading control. A representative blot is shown (n=3). (B) Densitometry of NS5A levels from the three independent Western blot trials. PTP1D levels were used for normalization. Each bar represents the mean ± SEM values (n=3).

2.5.6 The Effect of CIDEB and HS on Viral Secretion

In addition to activating CIDEB expression, HS treatment has also been demonstrated to activate the HCV viral lifecycle by increasing the production of very-low density lipoproteins and increasing the secretion of viral particles by 1000 fold (Singaravelu, Lyn et al. 2013; Steenbergen, Joyce et al. 2013). This finding was supported with our own models when Huh7.5-FGR cells treated for 6 days with HS were observed to secrete considerably more viral proteins than FBS treated cells (Figure 21). As HS has been shown to activate both HCV and CIDEB, and since we previously showed that HS treated cells stably expressing HCV proteins have upregulated CIDEB levels due to upregulation of its positive transcriptional regulator PGC1-α (Singaravelu, Lyn et al. 2013), I sought to investigate the effect of knocking down CIDEB in HS treated cells. When CIDEB was knocked down with siRNA in Huh7.5-FGR cells treated with HS, a pronounced increase in intracellular and secreted viral proteins could be observed (Figure 22AB). Moreover, CIDEB could not be detected in the serum, which is in agreement with previous studies that demonstrate that CIDEB dissociates form nascent VLDL when they enter the ER for secretion (Figure 22B). Again, this signifies that CIDEB is playing an antiviral role, as its absence causes an increase in viral proliferation. Furthermore, when viral protein secretion was investigated in cells overexpressing CIDEB, higher levels of CIDEB overexpression was found to decrease viral protein secretion as expected (Figure 22C).

2.5.7 CIDEB's Dual Roles in its Capacity to Modulate the HCV Lifecycle

In order to delineate the relative contribution of CIDEB's dual roles in cellular lipid metabolism and apoptosis in its capacity as a host factor during HCV infection, two mutants were created. The first mutant was based on a CIDEC mutant previously described by Gong *et al.* where three basic residues in the protein's C terminal domain were mutated to alanine which rendered CIDEC deficient in LD clustering and fusion (Gong, Sun et al. 2011). These three residues are highly conserved within the CIDE family proteins and are found within an amphipathic α-helix, in a K/RK/RR motif,

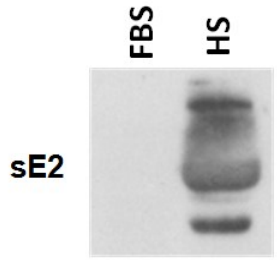

Figure 21. HS treatment increases viral protein secretion. (A) Protein samples were isolated from the serum of Huh7.5-FGR cells treated with FBS or HS for 8 days, and Western blotting was performed for sE2 protein levels. 150 ug of serum protein were acetone precipitated and loaded per well. A representative blot is shown (n=3).

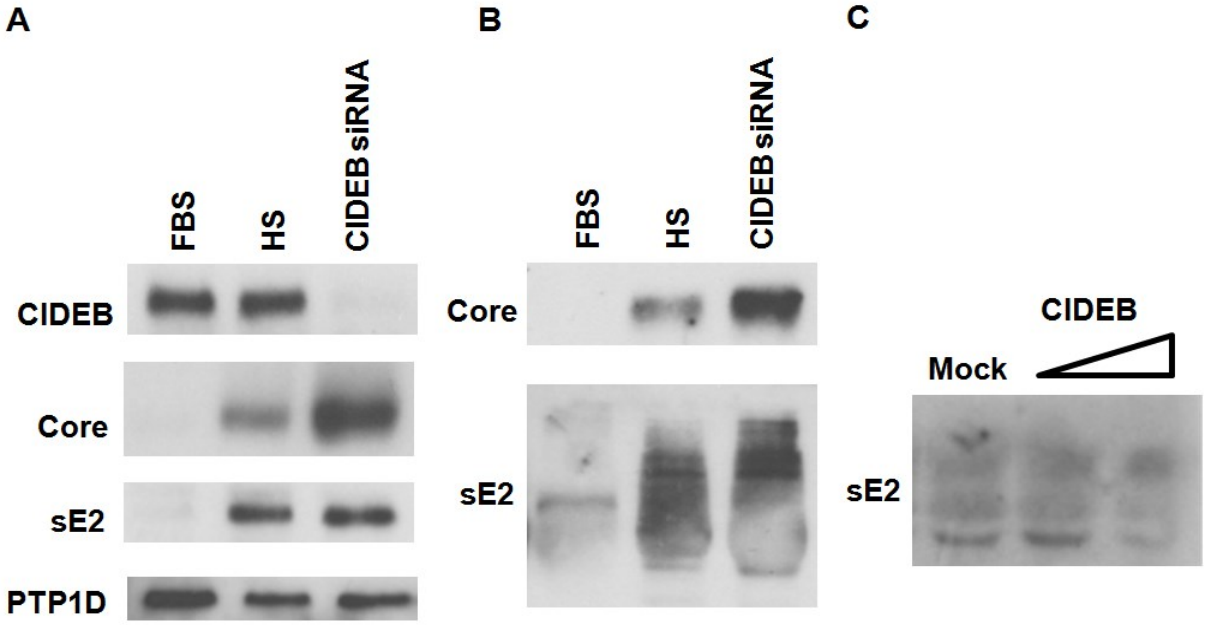

Figure 22. CIDEB siRNA increases intracellular and secreted viral proteins. A-B Huh7.5-FGR cells treated with FBS or HS and a non-sepcific siRNA 8 days, or HS and and CIDEB siRNA for 8 days. (A) Protein samples were isolated from the cell lysates of cells and Western blotting was performed for CIDEB, core, sE2 protein levels. PTP1D was used as a loading control. (B) 150 ug of serum proteins were acetone precipitated and loaded per well. Western blotting was performed for CIDEB and sE2 protein levels (CIDEB could not be detected). (C) Huh7.5-FGR cells were treated with HS for 8 days and transfected with increasing levels of CIDEB for 48 hours. 150 ug of serum proteins were acetone precipitated and loaded per well. Western blotting was performed for sE2 protein levels. A representative blot is shown (n=3).

comprised of K/RyxxK/RyxxR with x representing a hydrophobic residue and y representing a hydrophilic residue (Figure 23A). Accordingly, this CIDEB mutant was termed KRRA, and this mutant was hypothesized to demonstrate reduced LD fusion activity. Moreover, a second mutation in CIDEB converting the tyrosine at residue 160 to a phenylalanine, was created. This mutant was thus termed Y160F, and this mutation was previously shown to disrupt the proposed interaction between HCV NS2 and CIDEB, which renders NS2 incapable of abrogating CIDEB's apoptotic activity (Erdtmann, Franck et al. 2003).

Subsequently, the influence of these mutants on TAG accumulation and LD fusion relative to wild type (WT) CIDEB was characterized. Huh7 cells were transfected with either WT CIDEB or a mutant construct, and TAG levels were analyzed 48-hours post-transfection (Figure 23B). A similar reduction in cellular TAG was observed for both WT CIDEB and the Y160F mutant, suggesting that this mutation had no impact on CIDEB's role in hepatic lipid homeostasis. By contrast, the reduction in cellular TAG content in KRRA overexpressing cells was less pronounced, and this decrease wasn't statistically significant (based on a $p<0.05$ threshold; $p=0.058$). This suggests that the KRR → AAA mutation partially disrupts CIDEB's lipogenic role. Moreover, the effect of the mutants on LD clustering and fusion was investigated as before using CARS microscopy. Cells overexpressing the Y160F mutation displayed similar LD morphology to WT CIDEB, with no statistical difference in the number of LD per cell or their size distribution (Figure 23C). Accordingly, the Y160F mutant was concluded to have no influence on CIDEB's ability to cluster and fuse LDs. In contrast, the KRRA mutant did display a difference in LD morphology as expected. KRRA overexpressing cells displayed a smaller average LD size compared to WT CIDEB ($P<0.005$) (Figure 23C), with very few LDs with diameter above 1.5 μm (Figure 24C) and no statistically significant change in the average number of LDs per cell compared to mock ($P>0.05$) (Figure 24B) unlike WT CIDEB expressing cells. With this being said, the LD fusion activity of the KRRA mutant was not completely abrogated as this mutant was still able to induce a larger average LD diameter than mock (Figure 24A).

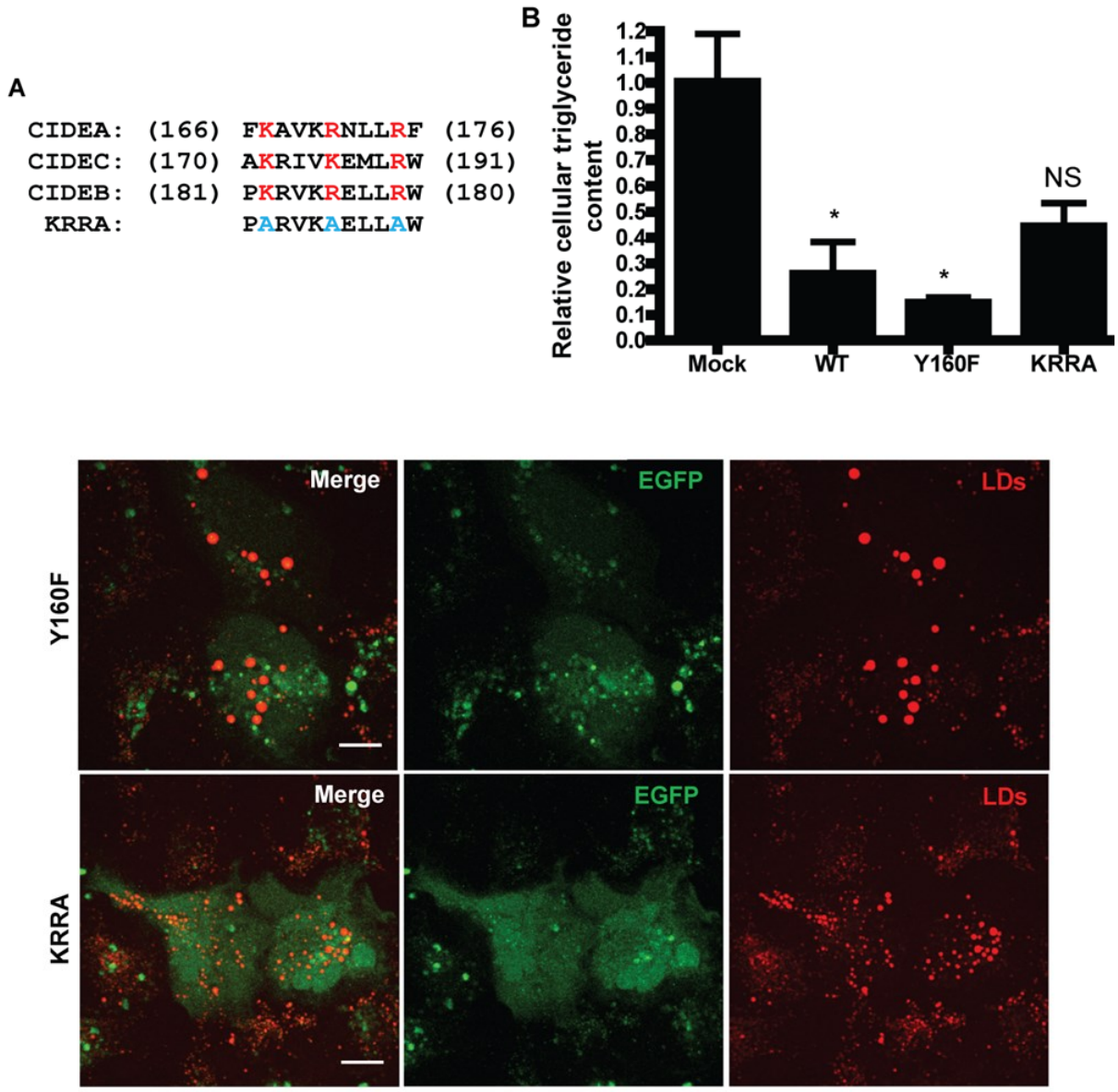

Figure 23. Characterization of CIDEB mutant deficient in lipid droplet fusion. (A) Alignment of KRR motif sequences conserved in CIDE family of proteins. KRR residues are highlighted in red and were mutated to alanine residues (blue) in CIDEB KRRA mutant construct (adapted from Gong *et al*.). (B) Huh7 cells were transfected with the highest expression level of CIDEB wild type (WT) and mutant (Y160F and KRRA) constructs. Triglyceride assays were performed 48 hours post-transfection, with values normalized by protein content. Cellular triglyceride content is shown relative to mock transfected cells. Each bar represents the mean ± SEM values (n=3). *$p<0.05$; NS – not significant. (C) Huh7 cells were transfected with bicistronic constructs expressing GFP and either CIDEB wild type or mutant constructs and then incubated with 300 µM oleic acid. 16 hours post-incubation, cells were fixed and imaged with CARS microscopy and two-photon fluorescence (TPF). Representative CARS, TPF, and merged images are shown for mock, CIDEB WT, Y160F, and KRRA mutants. Scale bar represents 22 µm.

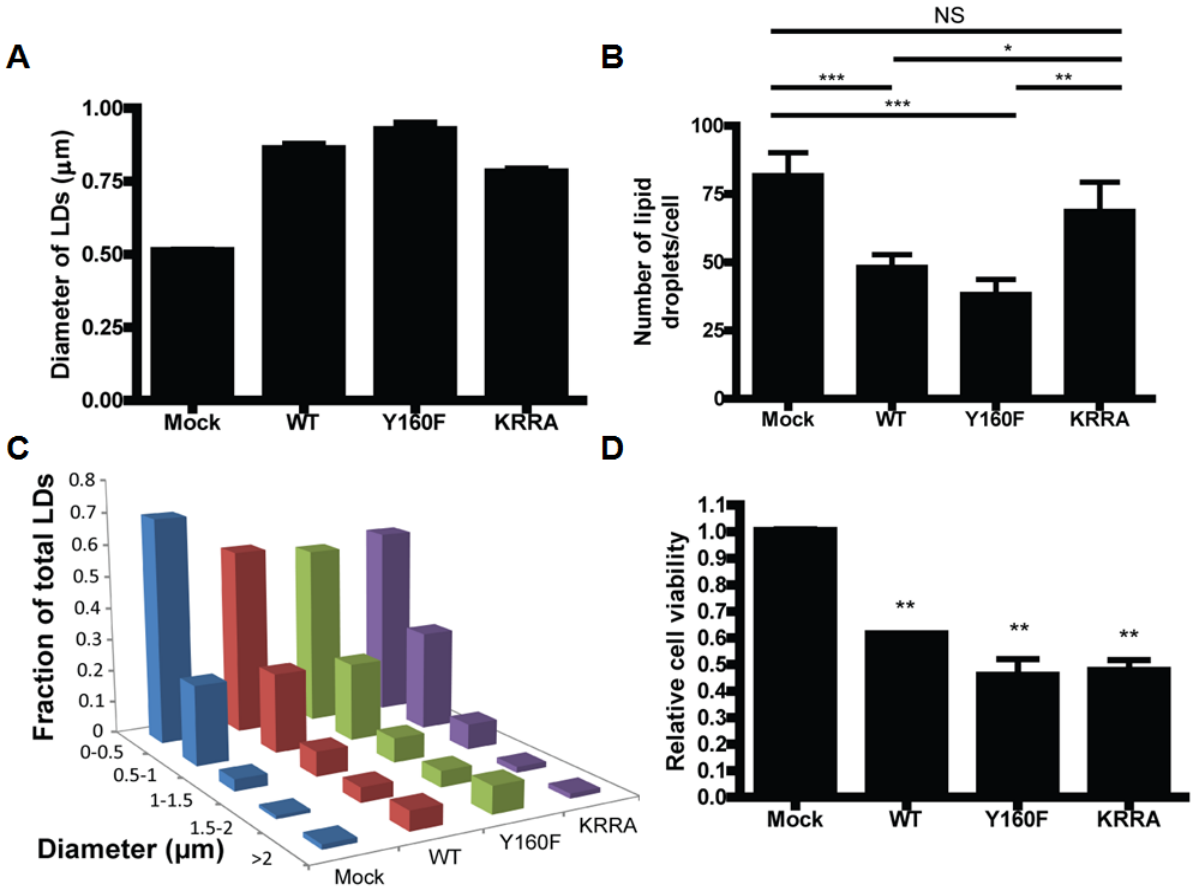

Figure 24. Analysis of CARS Images. CARS images from figures 9 and 21 were analyzed. (A) Average diameter of lipid droplets (n≥813 LDs). (B) Lipid droplet counts were performed on the CARS images for each cell and averages for each construct are shown (n≥18 for each sample). Each bar represents the mean ± SEM values. (C) Size distribution of lipid droplets (n≥813 LDs). (D) MTT assays were also performed in Huh7 cells 48 hours after transfections to assess cell viability. Relative cell viability normalized to mock is shown. Each bar represents the mean ± SEM values (n=3). *p<0.05; **p<0.01; ***p<0.0005; NS – not significant.

Collectively, these results demonstrate the KRRA mutation impairs CIDEB's ability to induce LD fusion.

2.5.8 CIDEB's Lipogenic Role is Maintained in the Presence of Viral Proteins

The ability of WT and mutant CIDEB to maintain this lipid phenotype in the presence of viral proteins was also confirmed. TAG assays and CARS analysis of LD phenotype was also carried out in Huh7.5-FGR cells for all mutants (Figure 25 and 26). The presence of viral proteins did not alter CIDEB's ability to induce lipogenesis and LD fusion, although the basal amount of lipids found in the Huh7.5-FGR cell line is higher than naïve Huh7 cells due to HCV's general upregulation of host cell lipid metabolism.

2.5.9 CIDEB Mutants and Apoptosis

In order to characterize CIDEB's apoptotic and lipogenic roles during the HCV lifecycle, the ability of the mutants to induce apoptosis was examined, via MTT assay (Figure 24D). Interestingly, both the KRRA and Y160F mutants maintained WT CIDEB's ability to induce apoptosis, which indicates that the Y160F mutation has no effect on either of CIDEB's roles in apoptosis or metabolism. Conversely, the KRRA mutation was found to render CIDEB deficient in LD fusion while maintaining its ability to induce apoptosis.

2.5.10 CIDEB Inhibits HCV Replication Independent of its Ability to Induce LD Fusion

Finally, I investigated the ability of the CIDEB mutants to influence the HCV lifecycle. Both mutants maintained their ability to inhibit HCV replication at the highest level of CIDEB overexpression, resulting in decreased luciferase activity in the Huh7-SGR-Luc cells (Figure 27A) and a decrease in NS5A protein levels in Huh7.5-FGR cells (Figure 27BC). Moreover, qRT-PCR analyses supported these findings at the RNA level (Figure 27D). In all models, the HCV inhibition resulting from CIDEB mutant overexpression was similar in magnitude to that observed for WT

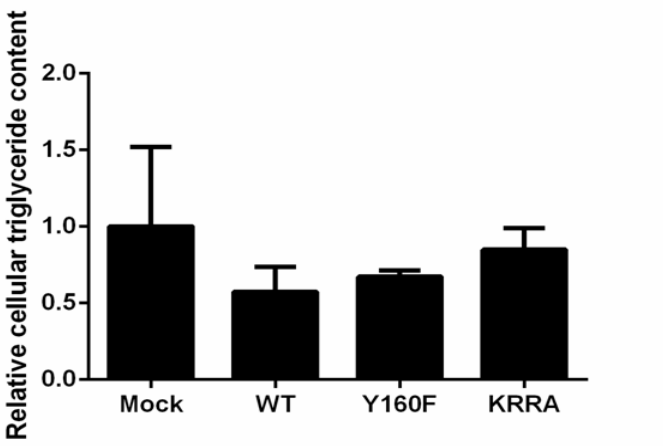

Figure 25. Lipid droplet phenotype in Huh7.5-FGR cells. Huh7.5-FGR cells were transfected with the highest expression level of CIDEB wild type (WT) and mutant (Y160F and KRRA) constructs. Triglyceride assays were performed 48 hours post-transfection, with values normalized by protein content. Cellular triglyceride content is shown relative to mock transfected cells. Each bar represents the mean ± SEM values (n=3).

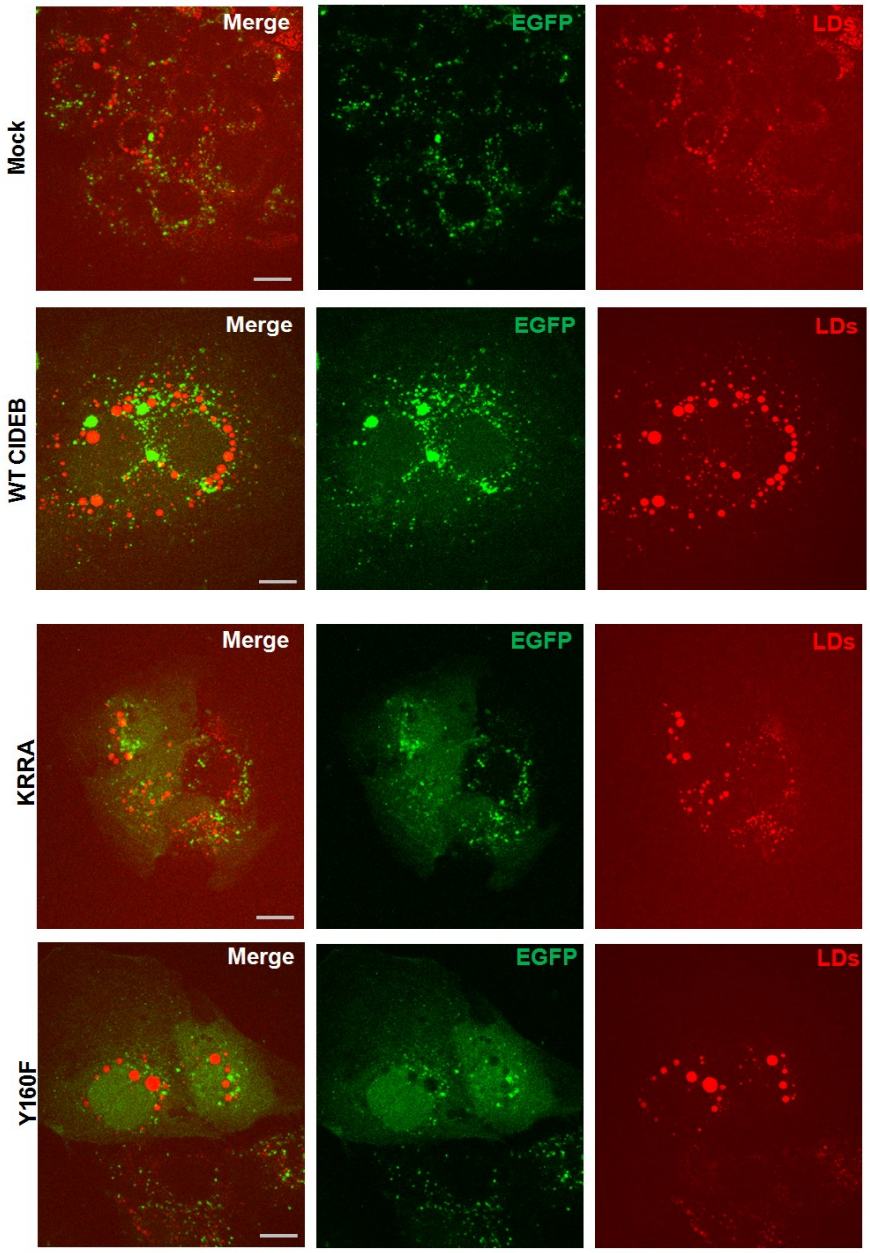

Figure 26. Lipid phenotype of WT CIDEB and mutants in HCV expressing cell lines. Huh7.5-FGR cells were transfected with bicistronic constructs expressing GFP and either CIDEB wild type (WT) or mutant constructs and then incubated with 300 µM oleic acid. 16 hours post-incubation, cells were fixed and imaged with CARS microscopy and two-photon fluorescence (TPF). Representative CARS, TPF, and merged images are shown for mock, CIDEB WT, Y160F, and KRRA mutants. Scale bar represents 22 µm.

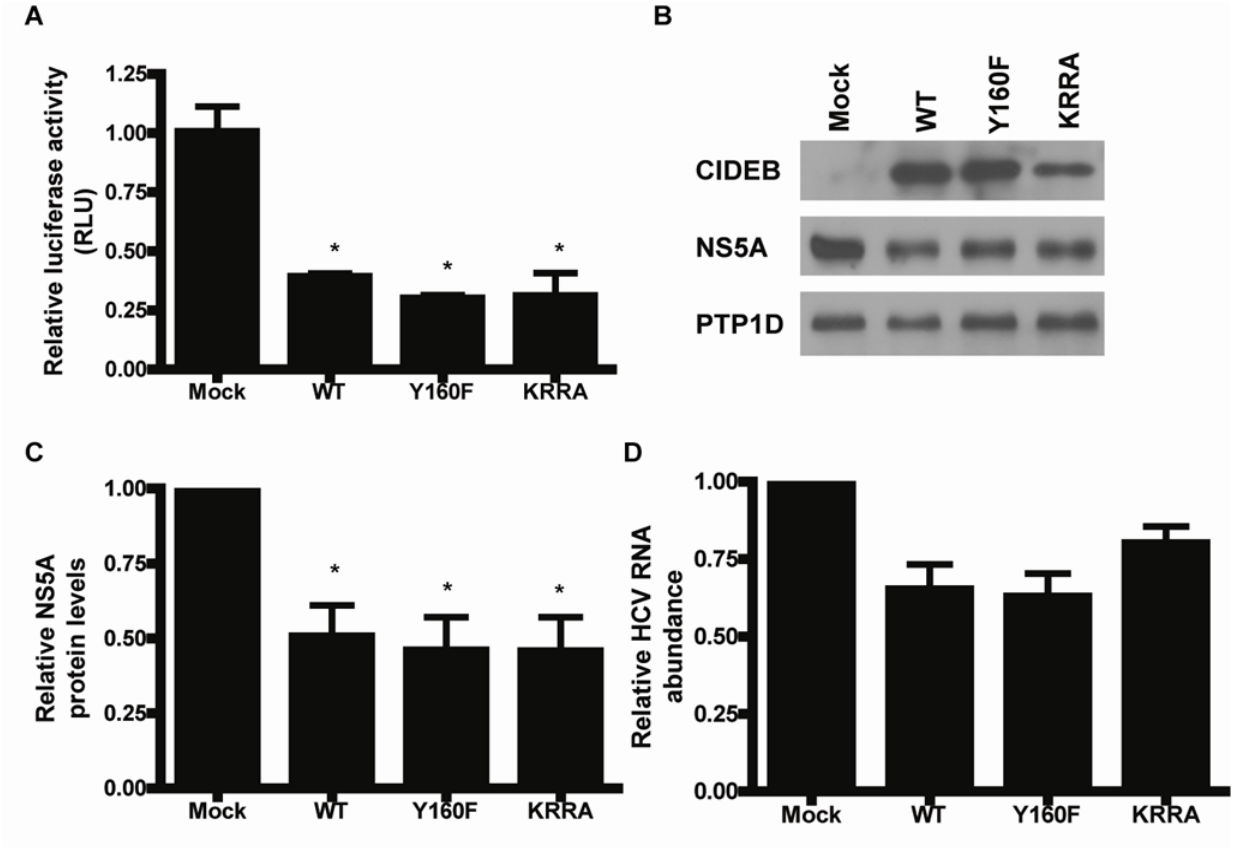

Figure 27. CIDEB inhibits HCV replication independent of its LD fusion activity. (A) Huh7-SGR-Luc cells were transfected with CIDEB wild type (WT) and mutant (Y160F and KRRA) constructs. Luciferase assays were performed 48 hours post-transfection, with values normalized by protein content. Normalized luciferase activity is shown relative to mock transfected cells. B-D Huh7.5-FGR cells were transfected as in (A). (B) Protein samples were isolated 48 hours post-transfection, and Western blotting was performed for NS5A and CIDEB protein levels. PTP1D is shown as a loading control. A representative blot is shown (n=3). (C) Densitometry of NS5A levels from the three independent Western blot trials. PTP1D levels were used for normalization. (D) Total RNA samples were isolated from Huh7.5-FGR cells 48 hours post-transfection, and qRT-PCR was performed for HCV RNA levels. Relative HCV RNA levels are shown after normalization by 18S rRNA levels. Results shown are the average of two independent experiments done in triplicate. Each bar represents the mean ± SEM values. *$p<0.05$.

CIDEB overexpression in the same model (Figure 27). These results suggest that neither disruption of the proposed NS2-CIDEB interaction nor CIDEB's role in lipid fusion affects CIDEB's role as a modulator of HCV. Additionally, when considering the relative expression levels of WT CIDEB and the CIDEB mutants, it appears as though the KKRA mutant is expressed at a lower level compared to WT or Y160F CIDEB (Figure 27B). Although this trend was not observed consistently in all trials, it was proposed that this lower expression could result from a reduced solubility of the KKRA mutant. Although the KKRA mutation may partially alter the structure of CIDEB resulting in reduced solubility of the protein, this mutant still demonstrated the same potency in antiviral effect as WT CIDEB or the Y160F mutant, therefore this mutation does not appear to abrogate the antiviral activity of CIDEB and if anything, this mutation is thought to demonstrate an enhanced ability to impair HCV replication. Collectively, these results demonstrate that CIDEB's impairment of HCV replication is independent of its role in hepatic lipid storage, although, as shown earlier, CIDEB does affect levels of secretion.

2.5.11 CIDEB Inhibits HCV Replication in a Caspase-Independent Manner

As the previous results with the KRRA mutant suggest that CIDEB's ability to impair HCV replication was independent of its regulation of lipid metabolism (Figure 27), it was hypothesized that CIDEB's ability to modulate the HCV lifecycle was dependent on its ability to induce apoptosis. This hypothesis was also supported as inhibition of HCV replication could be observed at the higher concentration of CIDEB overexpression in both Huh7.5-FGR and HCV-SGR-Luc cells (Figure 16), which was demonstrated to be where CIDEB's apoptotic role was favoured (Figure 13). Although one previous study investigating the apoptotic mechanism of transiently expressed CIDEB in HeLa cells demonstrated that CIDEB induces cytochrome C release from the mitochondria which subsequently results in caspase-3 activation (Erdtmann, Franck et al. 2003), it still remains unclear whether CIDEB-induced cell death occurs in a caspase-dependent manner (Erdtmann, Franck et al. 2003). When the apoptotic mechanisms of the other CIDE family proteins were compared, CIDEC

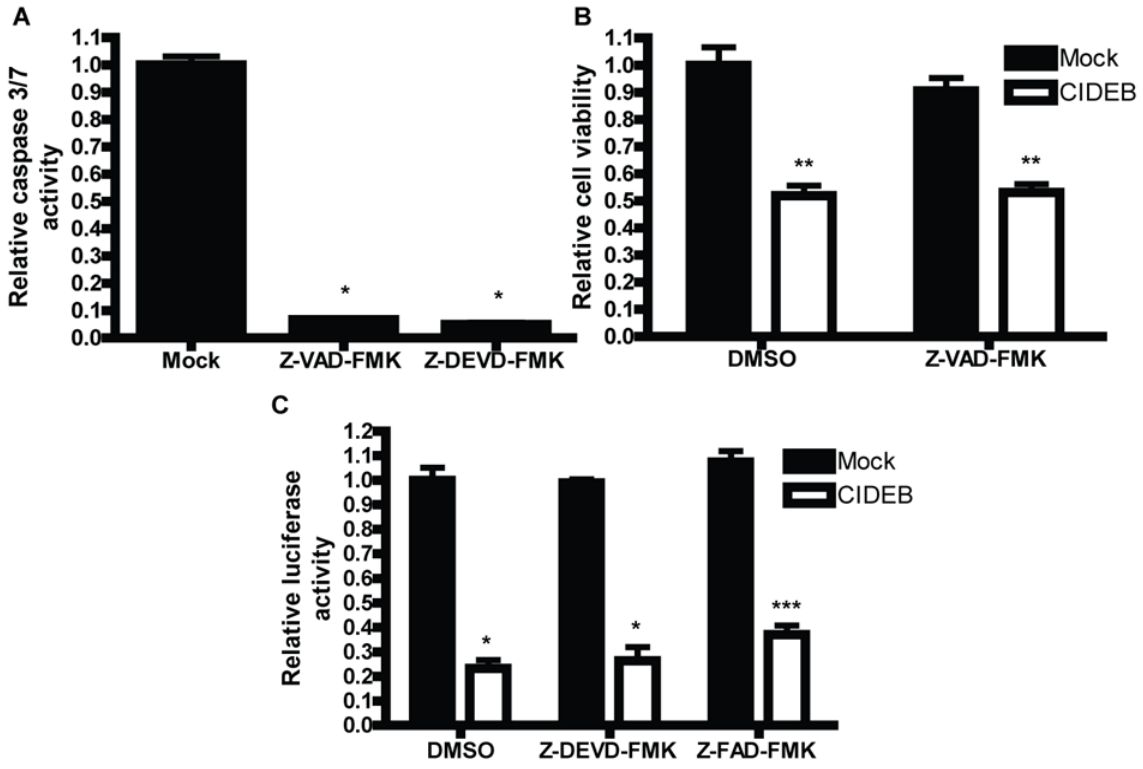

Figure 28. CIDEB inhibits HCV replication in a caspase-independent manner. (A) Huh7.5-FGR cells were transfected with wild type CIDEB and simultaneously treated with either DMSO (mock), pan-caspase inhibitor (Z-VAD-FMK), or caspase-3 inhibitor (Z-DEVD-FMK). 48 hours post-transfection, caspase 3/7 proteolytic activity was measured via a standard caspase assay. Caspase activity is normalized relative to CIDEB-transfected cells. (B)-(C) Huh7-SGR-Luc cells were treated as in A. (B) Cell viability was measured 48 hours post-transfection via MTT assay. (C) Luciferase activity was assayed 48 hours post-transfection. In B and C, values were normalized relative to mock transfected and DMSO treated cells. Each bar represents the mean ± SEM values (n=3). *p<0.05;**p<0.01;***p<0.0001.

appears to induce apoptosis in a caspase-dependent manner (Liu, Zhou et al. 2009; Tang, Xing et al. 2011), while CIDEA-induced cell death appears to occur in a caspase-independent manner (Inohara, Koseki et al. 1998). Therefore, in order to examine whether CIDEB induces apoptosis and inhibits HCV replication in a caspase pathway dependent manner, CIDEB transfected Huh7-SGR-Luc cells were supplemented with either the pan-caspase inhibitor (Z-VAD-FMK) or caspase-3 inhibitor (Z-DEVD-FMK). Initially, the activity of the caspase inhibitors was confirmed using an assay that assesses caspase-3/7 proteolytic activity (Figure 28A). Subsequently, MTT assays were performed on cells supplemented with caspase-inhibitor, and this treatment was unable to rescue CIDEB-induced cell death (Figure 28B), which suggests a caspase-independent mechanism of cell death and is consistent with the results of an analogous experiment performed for CIDEA (Inohara, Koseki et al. 1998; Tait and Green 2008). Furthermore, caspase inhibitor treatment could not rescue CIDEB mediated inhibition of HCV replication (Figure 28C). Overall, these results demonstrate that CIDEB-induced cell death and HCV inhibition occur in a caspase-independent manner.

2.6 Discussion

Every stage of the HCV lifecycle depends on a series of host-viral interactions to allow for the assembly, maturation and secretion of viral particles. Successful viral proliferation has a profound reliance on host cell lipid metabolism as specific lipid environments are necessary for viral particle formation. Although the early stages of the viral lifecycle are largely elucidated, later stages, namely assembly, budding, and maturation, remain less explored (Li, Brass et al. 2009). With this being said, further insight into the role of the novel host factor CIDEB during HCV pathogenesis may advance our understanding of the molecular mechanisms of the HCV lifecycle. CIDEB's recently elucidated role in hepatic lipid metabolism is of particular interest as a greater understanding here may uncover additional mechanisms of how HCV manipulates host cell lipid metabolism to allow for viral propagation. Delineation of the relative contribution of CIDEB's dual roles in apoptosis and lipid metabolism to viral pathogenesis is also necessary to fully comprehend the role of this protein in the

viral lifecycle. Herein, the role of CIDEB in human hepatic lipid homeostasis as well as its role a host factor in viral pathogenesis has been investigated.

Initially, the CIDEB construct was cloned due to the low levels of endogenous CIDEB detected in Huh7 cells via western blotting and immunofluorescence. The cDNA used for cloning was obtained from HepG2 human hepatoma cells which express CIDEB to a high degree in order to be overexpressed in Huh7 cells which serve as one of the main cell culture models for HCV (Figure 8). This may be explained as Huh7 cells and its derivatives express several proviral and entry factors required for viral entry and replication, such as miRNA-122, which are not found in HepG2 cells (Podevin, Carpentier et al. 2010). Furthermore, HepG2 cells act more like normal liver cells in that they are more polarized and differentiated, which is inhibitory towards the virus (Mee, Harris et al. 2009). Several studies suggest that CIDEB expression may be downregulated in hepatoma cells in general due to their de-differentiated state, as the transcription factor HNF-4α, which regulates CIDEB expression, is also involved in promoting cellular differentiation and is therefore downregulated in these models (Bukong, Lo et al. 2012). This is further supported by Ye *et al.* when CIDEB expression levels in the kidney was found to be downregulated during renal carcinoma (Yu, Wang et al. 2013).

When considering the phenotype of Huh7 cells, their impaired VLDL secretion may also aid HCV. The impaired VLDL pathway may be partly explained by their low levels of CIDEB, which was recently shown to play a key role in VLDL lipidation and secretion (Meex, Andreo et al. 2011). Furthermore, the decreased expression levels of CIDEB found in Huh7 cells may attribute to making this cell line such an excellent model for HCV, given our evidence, alone with previous work (Erdtmann, Franck et al. 2003), suggesting that it plays an antiviral role in the HCV lifecycle. As my results demonstrate that upregulation of CIDEB by the host is a mechanism to impair HCV replication, the low levels of CIDEB present in Huh7 cells may procure a suitable host environment to allow for the stable propagation of viral proteins in culture. This should be tested in the future by

the creation of a Huh7 cell line stably expressing CIDEB, which may reduce the ability of these cells to serve as a model for HCV.

Initially, the role of CIDEB in the context of human lipid homeostasis was investigated as previous studies have largely been conducted in mouse and rat models (Li, Ye et al. 2007; Ye, Li et al. 2009; Li, Lei et al. 2010; Li, Ye et al. 2012; Tiwari, Siddiqi et al. 2013). When quantifying the relative transcript expression of lipogenic genes in CIDEB overexpressing cells, all three genes (FAS, ACC and SCD1) were upregulated during CIDEB overexpression (Figure 10A). This is consistent with previous work done by Li *et al.* using a mouse model where CIDEB-null mice had lower levels of these and other lipogenic genes, suggesting that CIDEB levels correlate with the expression of lipogenic genes (Li et al 2007). Therefore, this result suggests a role for CIDEB in human lipid homeostasis.

As a likely result of the increased cellular lipogenesis in CIDEB overexpressing cells, an increase in hepatic TAG was also observed at low levels of CIDEB overexpression (Figure 10B). Again, this is in accordance with previous mouse studies demonstrating that CIDEB-null mice have decreased hepatic steatosis, as well as decreased adiposity index (Li et al 2007). However, when higher levels of CIDEB was overexpressed, the increase in TAG accumulation was no longer observed (Figure 23B), which may be explained in part that at high concentrations, CIDEB's apoptotic role becomes more prominent and its role in lipid metabolism declines. This is supported by our cell viability assays where the higher levels of CIDEB overexpression resulted in significant cell death, whereas the lower level of expression had minimal cell death in naïve and HCV harbouring Huh7 cells (Figure 13 and 17). This result is similar to what was observed for CIDEC, another member of the CIDE family of proteins (Keller, Petrie et al. 2008).

Moreover, the LD phenotype of CIDEB overexpressing cells was also observed via CARS microscopy (Figure 11). Overexpressing CIDEB in Huh7 cells decreased the number of LD per cell,

and increased the number of larger LDs in comparison to mock transfected cells (Figure 24). Interestingly, the hepatic lipid content of human hepatoma cells stably expressing FGR displayed the same lipid phenotype (Figure 26). These larger LD are hypothesized to result from CIDEB's upregulation of hepatic lipogenesis, or due to a compensatory mechanism where CIDEB overexpression stimulates the formation of TAGs due to activation of the VLDL pathway. However, as Huh7 have an impaired VLDL secretion pathway, cells cannot secrete these excess lipids resulting in large hepatic LDs (Meex, Andreo et al. 2011). It was shown previously that another member of the CIDE family, CIDEC (Fsp27), promotes lipid droplet growth by lipid exchange and transfer at lipid droplet contact sites, thereby contributing to the formation of larger LDs (Keller, Petrie et al. 2008; Gong, Sun et al. 2011). CIDEB was confirmed to function via a similar mechanism as live cell imaging studies visualized the movement of smaller LD closer together to form larger ones (Figure 12). Future work should examine whether CIDEB also has a similar role in LD fusion by cloning a CIDEB-GFP fusion construct that will allow for the visualization of CIDEB localization.

Overall, the results obtained when investigating CIDEB's role in human lipid homeostasis were in accordance with previous results obtained with other CIDE family proteins, and in other models. Due to CIDEB's established role as an enhancer of hepatic lipogenesis, the hypothesis that the host upregulates CIDEB during HCV infection may seem counter-intuitive, as this could be regarded as pro-viral rather than antiviral. However, one must keep in mind CIDEB's alternate role in cellular apoptosis, and how several apoptotic proteins are also upregulated during HCV infection, as a means to regulate viral propagation (Bantel and Schulze-Osthoff 2003). Moreover, the HCV lifecycle also relies on manipulation of the VLDL secretion pathway, in addition to host cell lipogenic pathways, as a means to mediate viral particle secretion and decrease lipid export. The virus is known to inhibit parts of the VLDL secretion pathway in order to mediate viral particle assembly and hepatic lipid accumulation, for example, HCV-core is known to inhibit MTP as a means to enable proper maturation and secretion of viral particles (Perlemuter, Sabile et al. 2002). Therefore,

upregulating CIDEB and its role as a regulator of lipid secretion may impede with HCV's manipulation of the VLDL pathway, thereby impairing viral secretion and serving an antiviral role. This was supported by the observation of an increase in intracellular and secreted viral proteins observed in Huh7.5-FGR cells treated with CIDEB siRNA (Figure 22AB), and a corresponding decrease in viral particle secretion was detected in Huh7.5-FGR cells overexpressing CIDEB (Figure 22C).

Considering the dual role of CIDEB in lipogenesis and apoptosis, the effect of overexpressing CIDEB on hepatoma cell death was investigated. Cell proliferation assays were performed in WT human hepatoma cells either in the presence of absence of oleic acid (Figure 13). Oleic acid was used as previous studies with other CIDE family proteins indicated that oleic acid may serve as a signal shifting their activity more towards a lipogenic vs. an apoptotic role (Gong, Sun et al. 2011). However, only a modest decrease in cytotoxicity was observed in the presence of oleic acid, which indicates that when CIDEB levels are very high, even a lipogenic environment cannot overcome its apoptotic role (Figure 13).

As the role of CIDEB in human lipid homeostasis has been delineated, the role of CIDEB as a host factor that is disregulated during HCV infection was explored next. Interestingly, a significant difference in CIDEB levels could not be observed in naïve Huh7 cells compared to Huh7 cell stably harbouring full-length or sub-genomic HCV replicons (Figure 14C), or JFH1 infected Huh7.5 cells, (Figure 14D), which can be attributed to the low endogenous levels of CIDEB in these cell lines. In order to overcome this problem, cells were treated with HS, as this has previously been shown to activate the CIDEB pathway through upregulation of its transcriptional upregulator, PGC-1α (Singaravelu, Lyn et al. 2013). After 8 days of HS treatment, the level of endogenous CIDEB was upregulated at the protein level in human hepatoma cells expressing stably expressing the HCV SGR, compared to naïve Huh7 cells or cured Huh7-SGR cells (Figure 15) (Blais, Lyn et al. 2010). As HS cells harbour more physiologically relevant levels of CIDEB, this model is hypothesized to be

favourable over cells cultured in normal FBS. It is important to note that this upregulation of CIDEB is not in agreement with work by Erdtman *et al.* who observed downregulated CIDEB in HCV transgenic mice (Erdtmann, Franck et al. 2003), but this is probably due to the discrepancies between models used. They proposed that the virus inhibits CIDEB in order to allow for viral propagation by inhibiting apoptosis in infected cells, however the authors failed to consider how the host's manipulation of CIDEB could be an antiviral defence mechanism, as demonstrated in Figure 16 when increased levels of CIDEB impaired HCV replication.

The effect of CIDEB overexpression in the context of SGR or FGR HCV genome was investigated next. When CIDEB was overexpressed in Huh7-Luc-SGR cells for 48 hours, a decreased luciferase signal, indicative of impaired viral replication, was observed at both low and high level of CIDEB overexpression, where the lipogenic and apoptotic roles of CIDEB would predominate, respectively (Figure 16B). Contrastingly, when these two levels of CIDEB were overexpressed in Huh7.5-FGR cells, only the higher level of CIDEB overexpression caused a substantial downregulation of HCV viral protein and RNA levels (Figure 16C-E). This CIDEB-mediated inhibition of HCV was found to be both specific and physiologically relevant (Figure 19). To explain why the low level of CIDEB overexpression was only able to inhibit HCV replication in cells harbouring the SGR and not the FGR, the transfection efficiency of both of these stable cells lines was investigated. Huh7.5-FGR were actually easier to transfect (Figure 18), signifying that the presence of NS2 or structural proteins may partially impair CIDEB's inhibitory role. It is hypothesized that as these viral proteins are not found in the subgenomic replicon cells, more pronounced HCV impairment is observed in this cell line. Future work aims to elucidate if specific viral proteins are modulating CIDEB activity and determine the nature of this association (ie. whether the virus is modifying CIDEB activity by localization, degradation, physical inhibition, etc.).

Interestingly, the CIDEB – mediated inhibition of HCV was not maintained in Huh7.5-FGR cells treated with HS for 6 days prior to CIDEB overexpression (Figure 20). This suggests that the

lipid rich environment that results from HS treatment partially offsets CIDEB's apoptotic role, thereby interfering with its ability to impair HCV replication. This can be understood by the fact that more CIDEB will locate to LDs in the HS treated cells as these cells are more LD rich. An alternate explanation is that as HS treatment causes Huh7.5 cells to be more differentiated, they could be harder to transfect than naïve Huh7.5 cells, and therefore the levels of CIDEB overexpression here is actually less than in non-human serum treated cells (Steenbergen, Joyce et al. 2013).

Now that the role of WT CIDEB in HCV pathogenesis had been characterized, I sought to delineate which of CIDEB's cellular roles had the greatest impact on HCV. To investigate CIDEB's role in lipid homeostasis, the KRRA mutant was cloned, based on previously described mutations in CIDEC that impaired its ability to induce LD fusion (Gong, Sun et al. 2011) (Figure 23A). As expected, cells transfected with the KRRA mutant displayed a larger number of LD that were significantly smaller compared to cells overexpressing WT CIDEB, which suggests that CIDEB's LD clustering and fusion ability has been successfully abrogated (Figure 23C). Moreover, cells overexpressing the KRRA mutant displayed increased TAG levels compared to the CIDEB WT (Figure 23B). This suggests that the KRRA mutation also impairs CIDEB's role in VLDL assembly; resulting in reduced lipid export and hence intracellular lipid accumulation (Figure 23B). Overall, the KRRA mutant demonstrates imbalanced hepatic lipid homeostasis due to its impaired ability to regulate the VLDL secretion pathway. In order to determine if CIDEB's role as a regulator of hepatic lipid homeostasis was necessary for its functioning as a host factor involved in HCV pathogenesis, the effect of the KRRA mutant on HCV replication was examined. The KRRA mutant was able to inhibit HCV in cells stably harbouring both the SGR and FGR HCV genomes, indicating that CIDEB's inhibition of HCV replication is independent of its ability to induce LD fusion and VLDL assembly (Figure 27).

As work with WT CIDEB demonstrated that a higher level of CIDEB overexpression, one where the cytotoxic role of the protein predominates, was most effective in impairing HCV replication, it

was hypothesized that CIDEB's apoptotic role is required for the modulation of the HCV lifecycle, rather than its lipogenic role. In order to investigate if this, CIDEB's mechanism of action was explored using both a caspase-3 inhibitor and a general caspase inhibitor drugs. Interestingly, caspase inhibitor treatments were unable to rescue CIDEB's induction of cell death and repression of HCV replication (Figure 28). Although CIDEC is known to have a caspase dependent mechanism of action, CIDEA has been shown to have a caspase-independent mechanisms of cell death, therefore this result suggests that CIDEB is more similar to CIDEA with this regard (Tait and Green 2008). This caspase independent induction of cell death supports an alternative mechanism, the one that gave the CIDE family proteins their name, where CIDEB's N-terminal domain binds and sequesters DFF45, which frees binding partner DFF40, allowing it to enter the nucleus and trigger DNA fragmentation, ultimately, resulting in apoptosis (Lugovskoy, Zhou et al. 1999). In summary, my data suggests that CIDEB's inhibition of HCV replication is independent of its role in LD fusion and caspase activation.

As previous work by Erdtmann *et al.* supported a role for the viral protein NS2 in altering CIDEB-mediated apoptosis, and slight differences in HCV inhibition in cells expressing sub-genomic vs. full-genomic HCV replicons was observed, the importance of this proposed interaction in CIDEB mediated inhibition of HCV was investigated by creating the Y160F mutant, which is deficient in NS2 binding (Erdtmann, Franck et al. 2003). Using our models, the Y160F mutant behaved almost exactly like WT CIDEB, in that it continued to induce the formation of larger LDs, induce cell death and impair HCV replication to similar levels (Figure 23-27). This suggests that CIDEB's inhibition of HCV is independent of the proposed NS2 interaction. However, the validity of this proposed interaction is questionable as the authors' experiments were performed in cell lines generally considered non-permissive to HCV (Erdtmann, Franck et al. 2003). Moreover, a following study could not detect this interaction in a Huh7 derived cell line, which these authors speculated to be a result of the transiency of the interaction or differences in experimental systems (Stapleford and Lindenbach 2011). Furthermore, as the Y160F mutant results in the removal of an OH group from

the 160th amino acid of CIDEB, this mutation is minimally perturbative and therefore may have a subtle effect on CIDEB's function, which explains why we cannot see a difference between WT CIDEB and the Y160F mutant. This could potentially be resolved by using a lower level of Y160F overexpression, which may influence the protein's equilibrium, as equilibriums are concentration dependent.

Future studies aim to further investigate the reported interaction between CIDEB and NS2, to determine if this is responsible for the discrepancy observed between HCV models. A NS2-FLAG construct has been cloned for this purpose as specific antibodies for this protein is not commercially available. Studies should aim to investigate the colocalization of CIDEB and NS2 using immunofluorescence, to investigate if any other proteins are interacting with this CIDEB-NS2 complex via immunoprecipitation and to demonstrate the effect of NS2 overexpression in the context of CIDEB. It is hypothesized that the NS2-CIDEB interaction acts like a signal resulting in the translocation of CIDEB away from the mitochondria where it exerts its role in apoptosis.

2.6.1 Future Directions

The role of CIDEB in human hepatic lipid metabolism should be further investigated by looking at lipoprotein secretion profiles of CIDEB overexpressing cells, along with the potential roles of CIDEB in lipolysis or in LD fusion, which results in larger LDs. It is hypothesized that CIDEB overexpressing cells will secrete lipoproteins of lower density due to an increased TAG content as a result of increased VLDL lipidation. In a recent study, HS treatment was shown to induce differentiation in Huh7.5 cells, allowing them to secrete TAG-rich lipoproteins which is not normally observed with Huh7.5 cells cultured in traditional FBS (Steenbergen, Joyce et al. 2013). Moreover, as HS treatment also promotes CIDEB expression, I hypothesize that HS-induced differentiation of Huh7.5 cells may rescue lipoprotein secretion by activating CIDEB expression (Singaravelu, Lyn et al. 2013). To test this hypothesis, lipoprotein secretion studies should be completed in HS treated

cells overexpressing CIDEB or with CIDEB knocked down to see if the level of CIDEB expression correlates with lipoprotein secretion.

Additionally, the contribution of CIDEB's role in cholesterol homeostasis to its antiviral role should be investigated. Previous studies have demonstrated that CIDEB is a key regulator of cholesterol uptake, storage and biosynthesis (Li, Lei et al. 2010). Moreover, inhibition of cholesterol synthesis has been shown to inhibit HCV by affecting geranylgeranylation of the protein FBL2, which is required to interact with NS5A for viral replication to occur (Wang, Gale et al. 2005). Therefore, CIDEB's regulation of the cholesterol pathway may be an additional pathway that can be manipulated to inhibit the HCV lifecycle.

The formation of VLDL is highly dependent on the presence of phospholipids such as phosphatidylcholine (PC), therefore enzymes regulating the synthesis of PC also influence the VLDL pathway. With this being said, CTP:phosphocholine cytidylyltransferase α (CCTα) is considered the rate limiting and regulated enzyme in the CDP-choline pathway for PC synthesis, and the activity of this enzyme is regulated in part by cellular localization, as it possesses nuclear localization signals, in addition to membrane binding domains. Moreover, the hepatocytes of CCTα deficient mice have impaired VLDL secretion and assembly, and accordingly larger cellular LDs (Jacobs, Lingrell et al. 2008). It is hypothesized that the observed increase in LD size is caused by the fusion of smaller LDs, similar to what occurs during CIDEB overexpression, as PC normally acts as a surfactant which limits LD fusion. Therefore, future work should investigate if the LD fusion properties of CIDEB are influenced or regulated in part by CCTα localization or activity by quantifying CCTα's product, CDP-choline, during CIDEB overexpression.

As knocking out CIDEB was shown to upregulate intracellular and secreted levels of core protein, the interaction of CIDEB and core should be investigated further using particle tracking studies. As core has been demonstrated to influence LD movement towards the perinuclear region of the cell during HCV infection (Boulant, Douglas et al. 2008; Lyn, Hope et al. 2013), and as core binding to LDs has been shown to downregulate the levels of other host LD binding proteins, such as

ADRP (Sato, Fukasawa et al. 2006; Boulant, Douglas et al. 2008), future studies should investigate how the presence of core influences CIDEB levels, CIDEB LD binding and LD dynamics. As I have demonstrated that CIDEB can impair HCV replication, I hypothesize that CIDEB will partially abrogate the core mediated LD movement as a secondary way to influence the HCV lifecycle. LD dynamics should be investigated using Differential interference contrast (DIC) microscopy and LD dynamics should be compared in Huh7, Huh7-SGR or Huh7.5-FGR cells overexpressing CIDEB. Moreover, the effect of CIDEB overexpression on regulating levels of other host LD binding proteins such as ADRP and TIP47 should also be investigated.

2.6.2 Conclusion

CIDEB plays an important role in human and murine lipid homeostasis. CIDEB overexpression upregulated lipogenesis in human hepatoma cells resulting in an accumulation of hepatic TAG and the formation of visually larger LDs. CIDEB appears to be a key host factor capable of modulating the HCV lifecycle. Human hepatoma cells stably expressing sub-genomic HCV genomes and treated with HS had upregulated CIDEB protein levels and CIDEB overexpression inhibited HCV replication in hepatoma cells stably expressing the sub-genomic or full-genomic HCV replicons. Moreover, of CIDEB's dual regulatory roles in hepatic lipid homeostasis and apoptosis, its ability to induce cell death was essential for its impairment of HCV replication. I propose that CIDEB expression is upregulated during HCV infection to enhance its apoptotic activity, thereby impairing viral replication and proliferation (Figure 29). Interestingly, CIDEB's inhibition of HCV and induction of cell death occurred in a caspase-independent manner. Overall, CIDEB's role as a modulator of the HCV lifecycle may have implications in HCV pathogenesis during chronic infection.

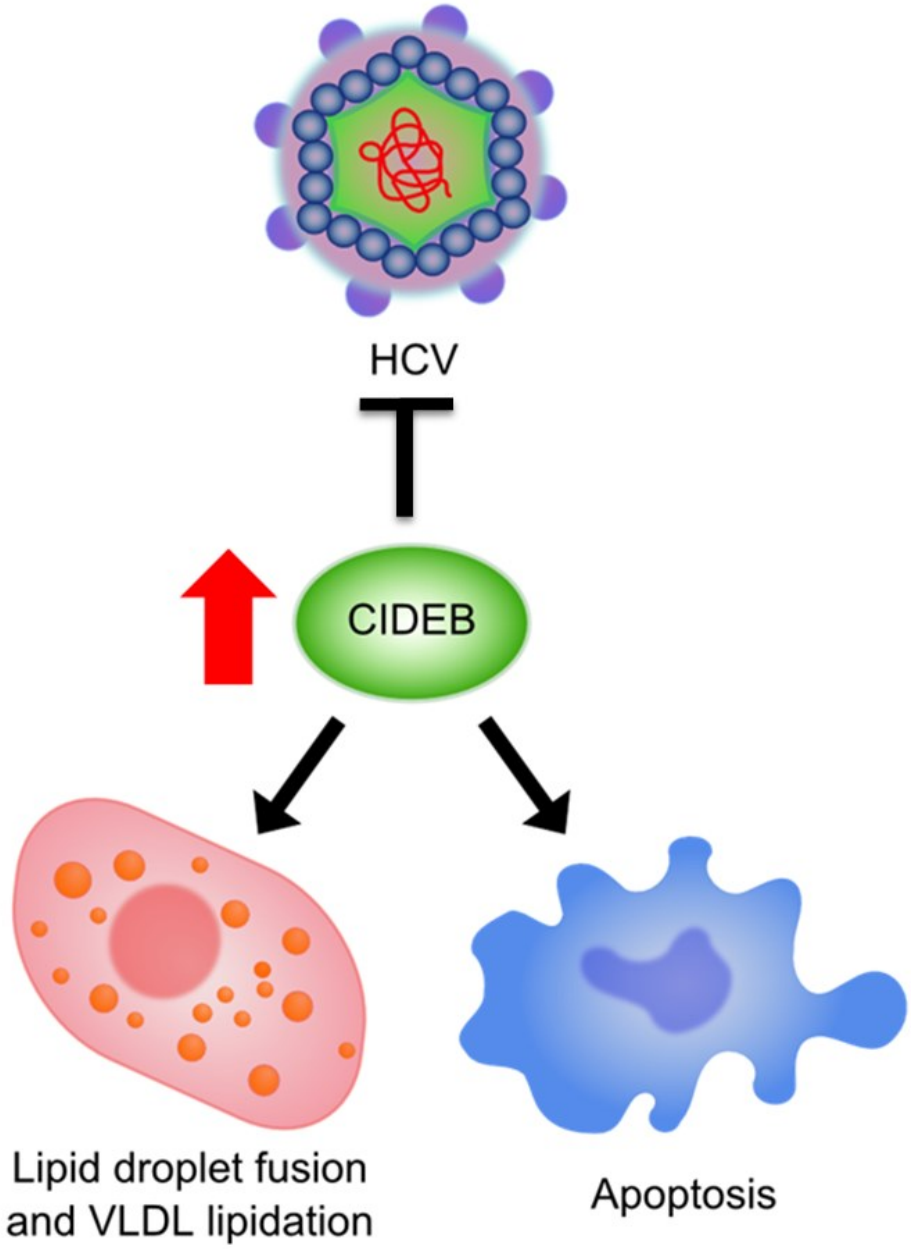

Figure 29. CIDEB is host factor that modulates the HCV lifecycle. CIDEB expression is upregulated during HCV infection as a means to stimulate CIDEB's apoptotic function which impairs HCV replication.

3.0 Chapter 2:

Elucidating Host Factors in HCV Infection Using Unnatural Amino Acid Technology

3.1 Abstract

Like many viruses, Hepatitis C virus (HCV) manipulates host cell metabolism on many levels to facilitate viral propagation. In order to interrogate the multitude of host-viral interactions necessary for this altered metabolic state of the host, a system for the identification of virus-interacting proteins was optimized using unnatural amino acid (UAA) technology. The photo-cross-linking UAAs p-azido-phenlyalanine (AZF) and 3'-azibutyl-N-carbamoyl-lysine (Abk) were incorporated into the viral proteins NS5A and core by expanding the genetic code of the host organism and the virus. This confers diverse physicochemical and biological properties to these viral proteins that can be exploited to investigate protein structure and function *in vitro* and *in vivo*. Here, the amber suppressor codon (TAG) was reassigned as the site of UAA incorporation, and an orthogonal tRNA ($tRNA_{CUA}$) and cognate aminoacyl–tRNA synthetase (aaRS) were employed to specifically and efficiently incorporate UAAs at the location of interest. The incorporated UAAs bear photo-reactive side chains that undergo cross-linking reactions when exposed to UV light, thereby effectively linking viral proteins to interacting host proteins, allowing for the isolation of host-viral protein complexes and the identification of interacting proteins. Overall, gaining insight into the numerous host-viral interactions that take place during HCV infection will both advance our understanding of HCV pathogenesis and uncover potential therapeutic targets.

3.2 Introduction

Proteins are essential macromolecules which serve as the structural and functional units of the cell, and possess the ability to carry out a remarkable range of functions. However, proteins contain a limited set of functional groups as they are biosynthesized from 20 canonical amino acids that are conserved across the majority of organisms, with rare exceptions in selenocysteine and pyrrolysine (Bock, Forchhammer et al. 1991; Srinivasan, James et al. 2002; Lukashenko 2010). With this being said, one innovative method to report on, and rationally control protein function is to incorporate unnatural amino acids (UAAs) into proteins of interest. UAAs possess distinct structures from the 20 standard amino acids with novel side chains that confer diverse physicochemical and biological properties into proteins of interest. To date, UAAs with a wide variety of functionalities have been described, including fluorophores, metal ion chelators, photo-caged and photo-cross-linking moieties, uniquely reactive functional groups, and NMR, IR, and X-ray crystallographic probes (Young and Schultz 2010). Overall, UAA technology can be exploited as a tool to explore protein structure and function *in vitro* and *in vivo*.

Several approaches have been previously employed to incorporate UAAs into proteins, including solid phase peptide synthesis (Scott, O'Donnell et al. 2002), chemically aminoacylated tRNAs (Noren, Anthonycahill et al. 1989), UAA substitution in auxotrophic strains and global modifications of natural amino acids (van Hest and Tirrell 1998). Although each of these methods is successful in conferring new abilities into proteins of interest, each of these approaches demonstrates unique obstacles. For example, solid phase peptide synthesis requires the use of protecting group chemistry and there are restrictions as to the size of the protein that can be synthesized, as well as the sites of ligation that can be used with this technique. Other difficulties, such as constraints on protein folding, and the inherent extracellular nature of the majority of these techniques, are also a limiting factor (Liu and Schultz 2010). Moreover, during the large-scale replacement of common amino acids with a UAA, for example when auxotrophic strains are employed, cells can no longer sustain

exponential growth and non-selective incorporation of the UAA of interest will always occur, which is a major drawback of this approach (van Hest and Tirrell 1998). However, in 1997, the Schultz lab reinvented these methods by developing a technique that manipulates the cells own translational machinery to site-specifically incorporate UAAs into proteins of interest (Liu, Magliery et al. 1997). This method offers several advantages over the previously described techniques as genetically encoding UAAs should occur with the same fidelity, efficiency, and genetic manipulability of natural protein synthesis, thereby overcoming many of the previous problems encountered using other techniques (Liu and Schultz 2010).

To carry out the incorporation of a UAA at a defined site in a protein using this methodology, four components are required. First, a unique codon that does not code for one of the natural amino acids must be assigned as the site of UAA incorporation. As the 20 common amino acids are encoded by 61 degenerate triplet codons, this leaves the three stop codons (TAG, amber; TAA, ochre; and TGA, opal) as potential candidates (Young and Schultz 2010). Because the amber stop codon (TAG) is the least used stop codon in the *E. coli* genome (93% of E. coli genes end with TAA or TGA), it is reassigned to the UAA with the expectation that TAG suppression will have little impact on the *E. coli* native proteome (Young and Schultz 2010). Along with this unique amber codon, incorporation of an UAA also requires an orthogonal tRNA ($tRNA_{CUA}$, the amber suppressor tRNA) constructed such that it is not recognized by the endogenous aminoacyl–tRNA synthetases (aaRS) of the host. Additionally, a cognate aaRS, which aminoacylates the orthogonal tRNA with only the desired UAA, is also required. In order to minimize cross reactivity, a heterologous aaRS/tRNA pair from a different organism is employed. For example, for UAA incorporation in *E. coli,* aaRS/tRNA pairs from archea or eukaryotes are employed as a starting point, and then mutagenesis and selection are carried out to improve the orthogonality (Liu and Schultz 2010). Finally, the UAA must be efficiently transported into the cytoplasm when it is added to the growth medium or it must be biosynthesized by the host, and it must be stable in the presence of endogenous metabolic enzymes (Young and Schultz

2010). Overall, this technique results in the expansion of the genetic code of the host organism by adding a 21st amino acid, and the efficient incorporation of UAAs into the proteins of interest.

3.2.1 Photo-Cross-Linking UAAs

Previously, UAA technology has been demonstrated to be a significant method to characterize protein-protein and protein-nucleic acid interactions (Kim, Axup et al. 2013; Pless and Ahern 2013). Moreover, in order to elucidate novel host-virus interactions during HCV infection, several UAAs with different functionalities were selectively incorporated into the viral proteome. The first group of UAAs contain photo-reactive side chains, namely p-azido-phenlyalanine (AZF) and 3'-azibutyl-N-carbamoyl-lysine (Abk) that have been developed in mammalian systems (Figure 30A). These UAAs are of particular importance as they undergo cross-linking reactions when exposed to UV light, thereby effectively linking viral proteins that have incorporated these UAAs to interacting proteins.

AZF is an aryl azide (Figure 30A), and upon irradiation with short wavelengths of UV light ($\lambda < 310$ nm), this UAA forms short-lived nitrenes capable of reacting with C−H and N−H bonds to form covalent adducts. Moreover, these nitrenes may rearrange to form dehydroazepines which react rapidly with amines, again resulting in the formation of a covalent linkage. Incorporation of this UAA into *E. coli* proteins has been shown to be effective in cross-linking incorporated proteins, demonstrating between 10 and 30% cross-linking efficiency after 5 min of UV exposure (Ai, Shen et al. 2011). Previously, AZF has been employed to probe protein−peptide interactions after its incorporation into synthetic growth hormone-releasing peptides, which allowed for the identification of novel pituitary receptors for GH release (Ong, McNicoll et al. 1998). Moreover, AZF incorporation also allowed for the identification of novel peptide binding sites in rat heart that are involved in the regulation of coronary vascular tone (Bodart, Bouchard et al. 1999). AZF incorporation has also been successfully employed to probe protein−protein interactions between

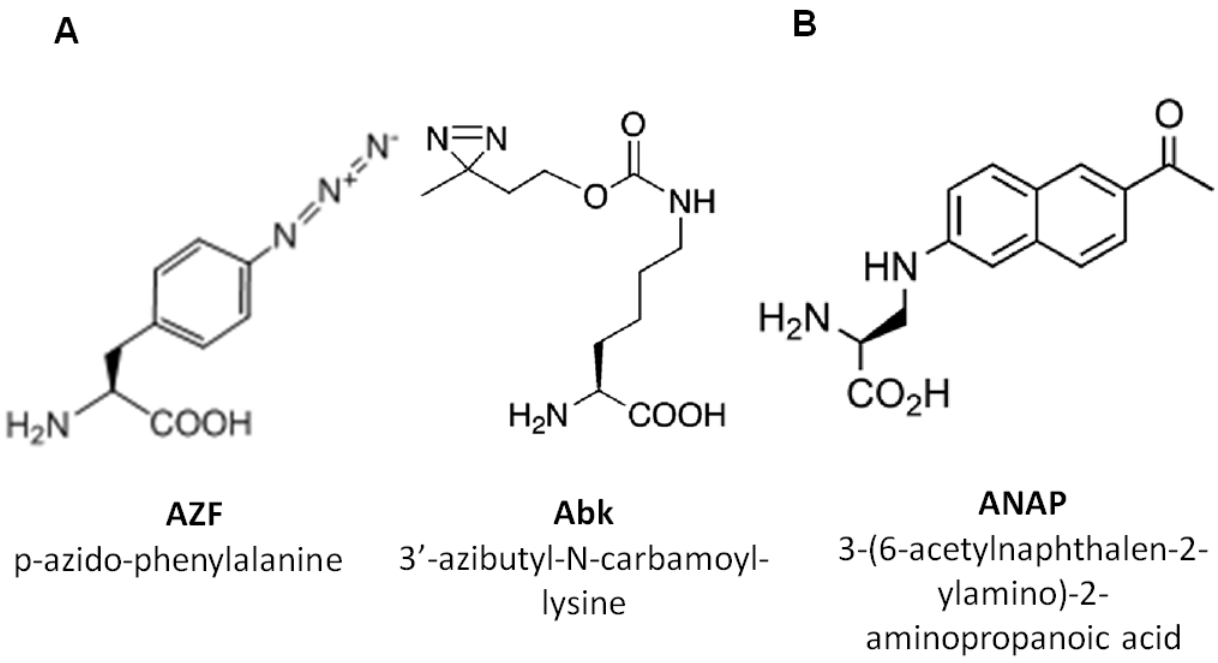

Figure 30. Chemical structures of unnatural amino acids (UAAs) used in this study. (A) Photo-crosslinking UAAs. (B) Fluorescent UAA.

RNA polymerase and its activator proteins, as well as between preprolactin and its ER targeting signal recognition particle (Krieg, Walter et al. 1986; Chen, Ebright et al. 1994). In order to site-specifically incorporate AZF into proteins of interest, an orthogonal tRNA/aaRS based on the archael *Methanococcus jannaschii* (*Mj)* tyrosyl-tRNA synthetase was initially evolved in *E. coli,* and this system was successful at incorporating AZF into glutathione-*S*-transferase in *E. coli*, which resulted in dimer cross-linking after UV exposure (Chin, Martin et al. 2002). More recently, a polyspecific aaRS initially derived from the *Mj* tyrosyl aaRS to incorporate *p*-cyanophenylalanine (Schultz, Supekova et al. 2006) was demonstrated to be capable of selectively incorporating 18 different UAAs, including AZF, into proteins of interest in *E. coli* (Young, Young et al. 2011). Importantly, a system for the incorporation of AZF into mammalian cells was recently described based on an *E. coli* aaRS endogenously used to charge the bacterial amino acid *p*-O-methyltyrosine, allowing for efficient AZF incorporation into mammalian proteins (Chatterjee, Xiao et al. 2013).

The aliphatic Abk UAA is another photo-reactive amino acid (Figure 30A), which offers better photo-stability than phenyl azides, and is considered to be more stable under physiological conditions. Upon excitation by approximately 350 nm light, it forms a reactive carbene that readily forms covalent bonds through non-specific addition reactions (Tippmann, Liu et al. 2007). Previously, an Abk-containing synthetic calmodulin-binding domain was used to investigate the regulation of Ca^{2+} pumps in the plasma membrane (Falchetto, Vorherr et al. 1992). Moreover, a pyrrolysyl tRNA/tRNA sythetase pair from *M. barkeri* has been evolved that efficiently incorporates Abk into proteins in *E. coli* and mammalian cells (Ai, Shen et al. 2011). Using this model, when Abk was site-specifically introduced into the active site of Cdk5, this UAA was able to photo-cross-link the kinase to its substrate, Pak1 (Ai, Shen et al. 2011). In summary, the host-viral protein complexes that result from incorporating these photo-reactive UAAs into proteins of interest can then be pulled down by immunoprecipitation, allowing any novel interacting proteins to be elucidated by mass spectrometry.

3.2.2 Fluorescent UAAs

Fluorescent UAAs are also a valuable tool for interrogating protein function *in vitro* and *in vivo*. Fluorescent UAAs serve as small biophysical probes for the direct visualization of protein localization and intermolecular interactions. They also offer an advantage over green fluorescent protein (GFP) and its derivatives by virtue of their smaller size and the fact that they can be introduced anywhere in a protein rather than just at the N or C terminus (Xie and Schultz 2006). To date, fluorescent UAAs have been incorporated into proteins to serve many applications, such as allowing for the direct localization of the molecular chaperone GroEL in *E. coli* cells (Charbon, Wang et al. 2011), acting as molecular probes to study the activity of G protein-coupled receptors (Naganathan, Grunbeck et al. 2013) and nicotinic receptors (Pantoja, Rodriguez et al. 2009), and to site-specifically label tubulin (Mukherjee and Bane 2013) and histone proteins (Chatterjee, Guo et al. 2013). Recently, an expression system for the fluorescent UAA ANAP (3-(6-acetylnaphthalen-2-ylamino)-2-aminopropanoic acid) has been developed in mammalian systems, enabling its site-specific incorporation into mammalian proteins with excellent selectivity and efficiency (Chatterjee, Guo et al. 2013) (Figure 30B). As ANAP is an amino acid derivative of the widely used fluorophore 6-propionyl-2-(N,N-dimethyl)aminonaphthalene (prodan) (Macgregor and Weber 1986), it can be used to investigate protein localization by acting as an environmentally sensitive fluorescent reporter. Previously, an ANAP-specific $tRNA_{CUA}^{EcLeu}$/aaRS pair was evolved from the *E. coli* leucyl-tRNA/LeuRS using a series of positive and negative selections in *S. cerevisiae* (Lee, Guo et al. 2009), which was demonstrated to also be orthogonal in mammalian cells due to their similar tRNA identity elements (Chatterjee, Guo et al. 2013). Accordingly, the incorporation of the UAA ANAP into viral proteins of interest provides an additional approach to interrogate the complex host-viral interactions that are taking place during HCV infection.

3.2.3 Studying Viruses with UAA Technology

The utility of UAA technology towards the study of viruses has previously been demonstrated by Strable *et al.* when they incorporated azide and alkyne-containing UAAs into the capsid proteins of HBV and bacteriophage Qβ capsid, using a methionine auxotrophic strain of *E. coli* and a methionine sense codon as the site of UAA incorporation (Strable, Prasuhn et al. 2008). Similarly, cancer-targeting peptides were site-specifically coupled to the viral capsid of bacteriophage MS2 after aminophenylalanine residues were site-specifically incorporated into the surface residues of the viral capsid via stop codon suppression in *E. coli* (Carrico, Romanini et al. 2008). This protocol was largely based on a previously reported method to display peptide-containing UAAs in a M13 bacteriophage using an orthogonal aaRS/$tRNA_{CUA}$ pair in *E. coli* (Feng, Tsao et al. 2004). Although all of these studies were successful at site-specifically incorporating and subsequently reacting UAAs in virus-like particles, mature, infectious virus was never produced**.** Recently, Lin *et al.* demonstrated that UAA technology can be applied to the study of an infectious viral lifecycle by site-specifically incorporating UAAs into the surface proteins of intact human hepatitis D virus (HDV) (Lin, Yan et al. 2013). This study carried out pyrrolysine-based genetic code expansion within HepG2 human hepatocytes, which resulted in the incorporation of six pyrrolysine analogues with different functionalities into the viral envelope proteins. Select mutants were optimized to retain almost wild-type infectivity and viability, and infectivity could be maintained after incorporated UAAs were further reacted with labeling probes under live cell conditions (Lin, Yan et al. 2013). Furthermore, another study demonstrated that infective adenovirus with selectively incorporated UAA azidohomoalanine (Aha) could be produced by supplementing HEK293 cells with Aha in methionine deficient media (Banerjee, Ostapchuk et al. 2011). This allowed for chemical modification of surface exposed azides, allowing for the attachment of a wide range of functional groups (Banerjee, Ostapchuk et al. 2011). Overall, these studies demonstrate the feasibility of UAA technology as a tool to study viral lifecycles. These results provide evidence that UAA incorporation

into viral proteins and infectious viral particles should also be possible for HCV, which would be an influential technique to examine the dynamics, localization and intermolecular interactions of viral particles during HCV infection.

3.3 Hypothesis and Objectives

I hypothesize that UAA technology can be applied to the study of the HCV lifecycle as a means to identify novel host-viral interactions and gain a more comprehensive understanding of HCV's manipulation of host cell metabolism. In order to investigate this hypothesis, photo-cross-linking UAAs were site-specifically incorporated into viral proteins NS5A and core in order to covalently cross-link viral proteins to their interacting host proteins. The aim of this study was to identify the residues in these viral proteins that are optimal for tolerating UAA incorporation, optimize the expression of UAA incorporated viral proteins and to optimize cross-linking of viral proteins to interacting host proteins. Long term goals of this study are to identify novel virus interacting proteins, incorporate UAAs with additional functionalities, such as fluorescent UAAs, into viral proteins, and to create a strain of infectious virus that relies on UAA incorporation for synthesis. This would allow for UAA incorporation at defined sites in viral particles, which would facilitate the manipulation of infectious virus and provide an avenue to interrogate the less-elucidated steps of the viral lifecycle.

3.4 Materials and Methods

3.4.1 Constructs and Cloning

The amber stop codon (TAG) was inserted into control and viral proteins at locations tolerable to mutations using the QuickChange(R) Lightning Site-Directed Mutagenesis Kit following the manufacturer's specifications and using the primers listed in Table 1 (Appendix). A mammalian expression vector encoding GFP with a TAG insertion into tyrosine 66 was obtained from the Schultz lab (Wang, Xie et al. 2003). The HCV NS5A protein was cloned into the pCMVβ mammalian

expression vector from the genotype 2a JFH-1 Subgenomic Replicon (AB114136). Initially, the amber codon was cloned into the C terminal unstructured region of NS5A, between the 418 and 419 amino acid, which was previously found to tolerate GFP insertion as a means to directly visualize replication complexes (Moradpour, Evans et al. 2004). Six additional NS5A constructs were created with varied TAG insertion sites to evaluate the efficiency of incorporation at other locations. Two residues critical for NS5A dimer formation (Tyr149, Tyr161) were mutagenized to TAG based on interacting residues in the crystal structure of the dimeric NS5A complex (Love, Brodsky et al. 2009). Moreover, four residues conserved among HCV genotypes and previously shown to tolerate mutation in the proline-rich motifs necessary for NS5A-phosphoinositide III-kinase (PI3K) binding (Pro29, Pro343, Pro351, Arg355) were additionally mutagenized to TAG (Tan, Nakao et al. 1999; Street, Macdonald et al. 2004; Macdonald, Mazaleyrat et al. 2005). The HCV core protein was also subcloned into the pCMVβ mammalian expression vector (genotype 3a) from a construct received from Dr. John McLaughlin (MRC Virology Unit, Institute of Virology, Glasgow, United Kingdom). Residues in domain II of the core protein were mutagenized to TAG based on their ability to tolerate change and the ability of mutations here to alter LD movement (Leu119, Lys121, Cys128, Gly161) (Boulant, Montserret et al. 2006). All cloning was carried out as described in Chapter 1.

3.4.2 UAA Incorporation

In order to incorporate UAAs in mammalian cells, vectors containing the orthogonal tRNA/aaRS pairs for each UAA were co-transfected with the mammalian expression plasmid encoding the TAG inserted control or viral protein. For AZF incorporation into GFP, the tRNA/aaRS expression plasmid containing a CMV driven aaRS and an H1 driven $EctRNA_{CUA}$ was employed. A vector encoding the *E. coli* TyrRS (EcTyrRS) variant previously evolved in yeast to charge *p*-O-methyltyrosine (pOMeY; OMeYRS) (Liu and Schultz 2010) driven by the CMV-IE promoter, as well as four copies of the suppressor $tRNA_{CUA}^{Tyr}$ were used for AZF incorporation in mammalian cells. A pyrrolysyl tRNA/tRNA sythetase pair from *M. barkeri* was previously evolved to efficiently

incorporate the aliphatic Abk amino acid into proteins in *E. coli* and mammalian cells (Ai, Shen et al. 2011). For Abk incorporate in mammalian cells, I cloned a pCMV-AbK plasmid that expressed the AbKRS under the CMV promoter and $tRNA^{Pyl}$ under the U6 promotor (Ai, Shen et al. 2011). The orthogonal tRNA/aaRS pairs were obtained from the Schultz laboratory, The Scripps Research Institute, La Jolla, California.

For *in vitro* incorporation, 3.5×10^5 Huh7 cells or 5.0×10^5 Huh7.5-FGR cells were seeded per well of a 6-well plate. Cells were transfected using FuGENE®HD transfection reagent as per the manufacturer's protocol 24 hours post-seeding once they had reached approximately 70% confluency and incubated for 48 hours post transfection. Following transfections, 1 mM final concentration of UAA was added to the desired wells. After the incubation period, cells were heated in lysis buffer to 95 ^{o}C, proteins were extracted and quantified as above.

3.4.3 Cross-linking

Cross linking was carried out using a handheld UV lamp (Spectroline E-Series ENF-240C UVA/UVC Hand Held UV Lamp). Cells or cell lysates were irradiated under a 365 nm for Abk incorporation, and 254 nm for AZF cross-linking for the specified times.

3.5 Results

3.5.1 Control Experiment for GFP Elongation

In order to demonstrate proof of concept with UAA technology in our own lab, a plasmid encoding CMV driven Y66TAG GFP was co-transfected with the orthogonal tRNA/aaRS pair for AZF. When the TAG inserted gene, orthogonal tRNA/aaRS pair and AZF were all added to the well, GFP signal could be observed via confocal microscopy (Figure 31).

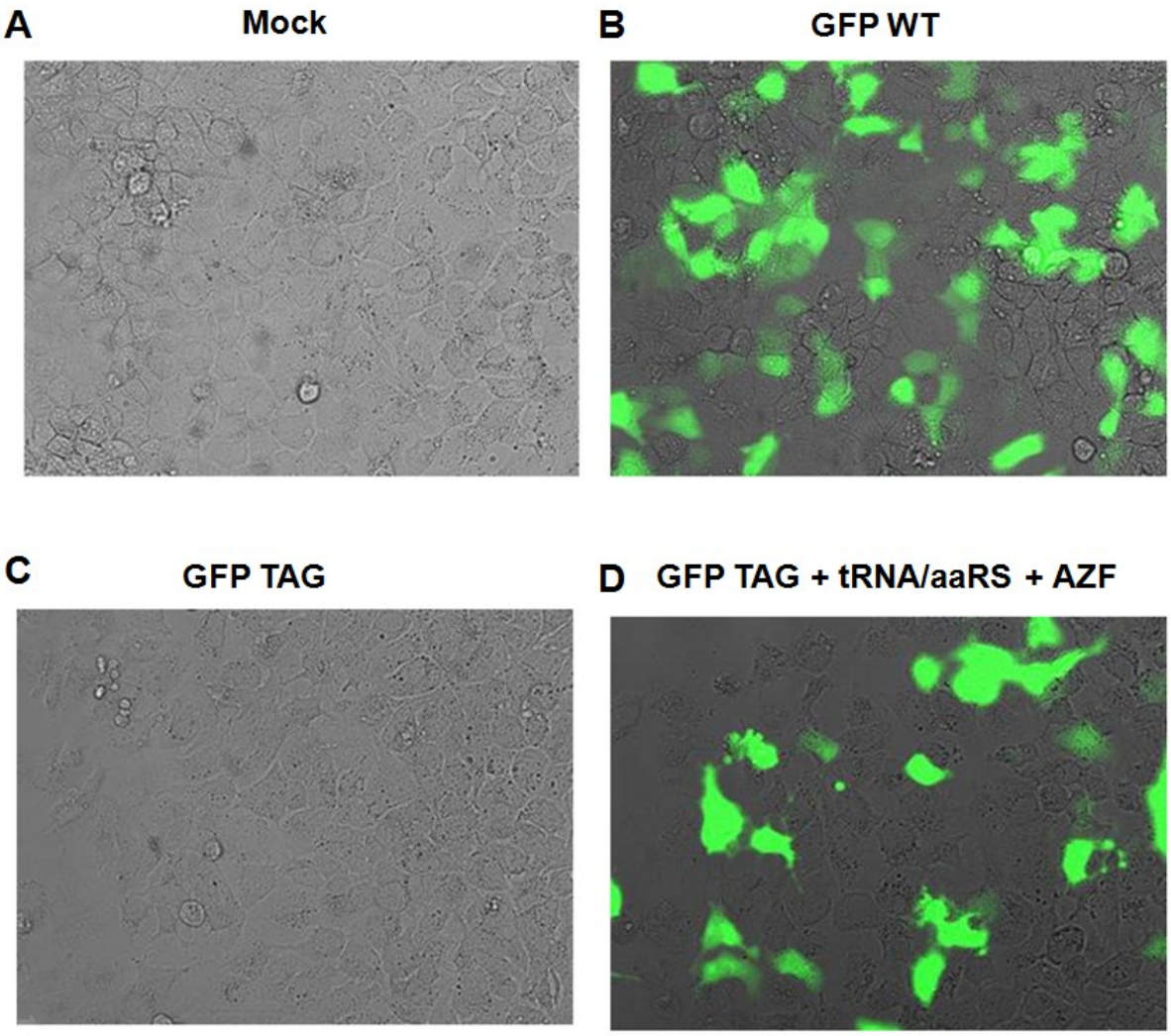

Figure 31. AZF incorporation into GFP in human hepatoma cells. Confocal images of live Huh7 cells transfected for 24 hours with (A) mock, (B) WT GFP, (C) TAG GFP, (D) TAG GFP + tRNA/aaRS. AZF (1 mM) was added to cell culture medium for sample D.

3.5.2 UAA Incorporation into NS5A

Initially, the TAG inserted NS5A construct was cloned (418-TAG-419 NS5A), and the incorporation of AZF into NS5A was observed (Figure 32A). When cells were double transfected with the 418-TAG-419 NS5A plasmid and the aaRS/tRNA plasmid for AZF, and media was supplemented with AZF, the presence of full length NS5A was observed (lane 6 Figure 32A), whereas full elongation was not observed when either AZF or the aaRS/tRNA plasmid was not added to the cells (lane 5 and 4 respectively, Figure 32A), which demonstrated the specificity of incorporation. An incorporation efficiency of approximately 20% was calculated using densitometry (Figure 32A). However, even after optimizing UAA concentration and UV exposure time, cross-linking was not seen (Figure 33). Moreover, AZF incorporation into the 418-TAG-419 NS5A construct was also carried out in Huh7.5-FGR cells, however, although incorporation was observed again, cross-linking was not detected via Western blotting (Figure 32B). Furthermore, the new NS5A constructs were tested for AZF incorporation. Of the six constructs tested, Tyr161TAG, Pro343TAG and Pro351TAG demonstrated the largest degree of specific NS5A elongation (Figure 34BC). Of particular interest, Pro351TAG had bands of higher molecular weight above the NS5A indicating that interacting proteins were potentially cross-linked to NS5A, although a small amount of cross-linking was also visible in the non-UV-exposed sample (Figure 34C). Pro29TAG seemed to demonstrate non-specific incorporation with elongated NS5A seen when the TAG construct alone was transfected (Figure 34A). Moreover, although specific elongation of Phe149TAG and Arg355TAG was detected, these constructs demonstrated a smaller amount of incorporation when compared to the other constructs tested (Figure 34A and C).

Abk incorporation into the 418-TAG-419 NS5A construct was detected at a higher degree than AZF with an efficiency of approximately 80% in naïveHuh7 or Huh7.5-FGR cells (Figure 35). However, cross-linking was not observed when whole cells or cell lysates were irradiated with UV light, even for prolonged exposure times (Figure 36).

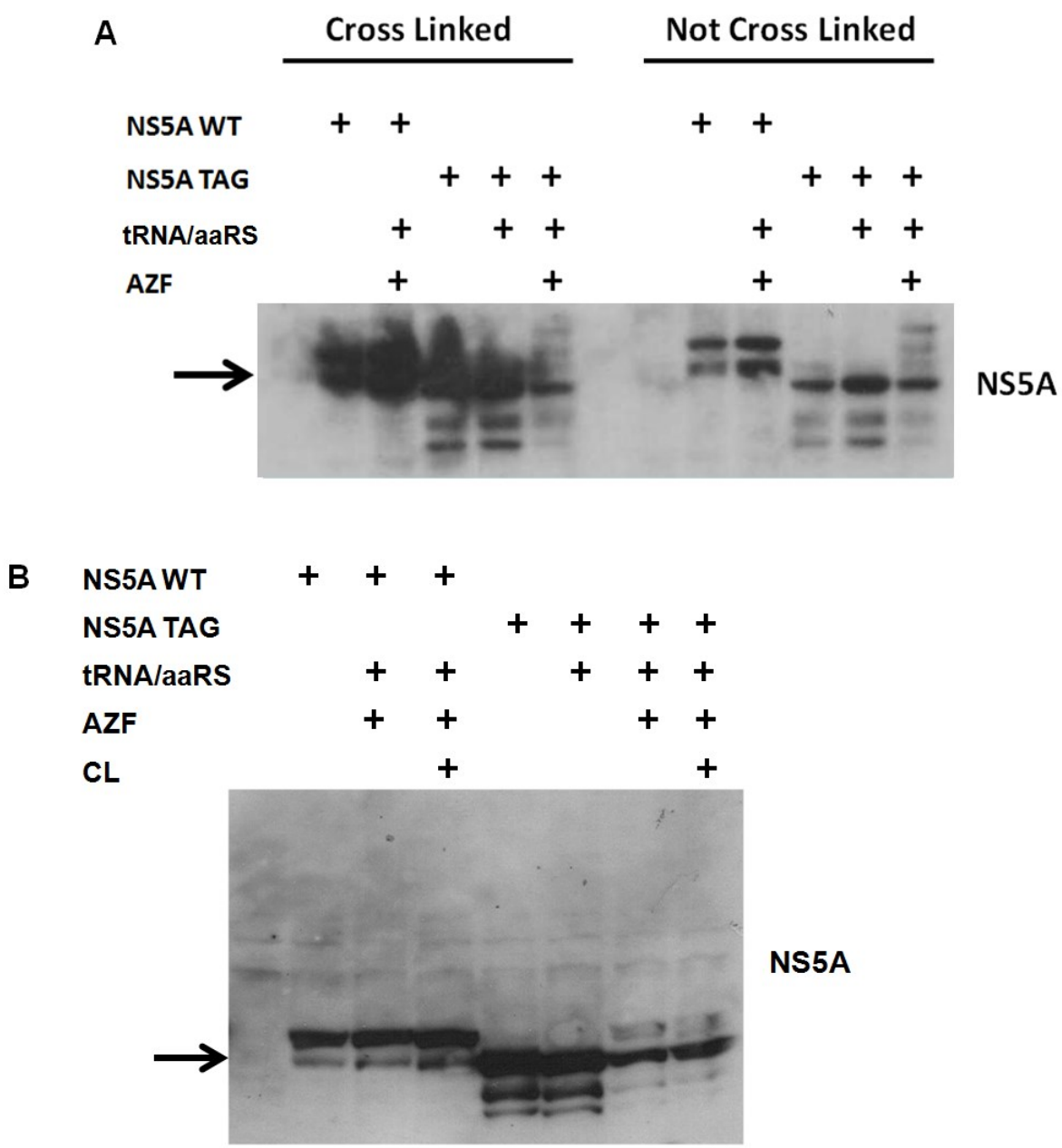

Figure 32. AZF incorporation into NS5A (418-TAG-419). (A) Western blot of Huh7 cell lysates and (B) Huh7.5-FGR cell lysates probed with anti-NS5A genotype 2a antibody. Cells were transfected for 48 hours with mock (lane 1), WT NS5A (lane 2), WT NS5A + tRNA/aaRS (lane 3), TAG NS5A (lane 4), TAG NS5A + tRNA/aaRS (lane 5 and 6). AZF (1 mM) was added to cell culture medium for cells in lanes 3 and 6. Samples were crossed linked by exposure to a 254 nm UV light for 5 minutes or prepared in the dark (not cross-linked). Arrow represents lower band of NS5A dimer (56 kDa). 60 ug of protein is loaded per well.

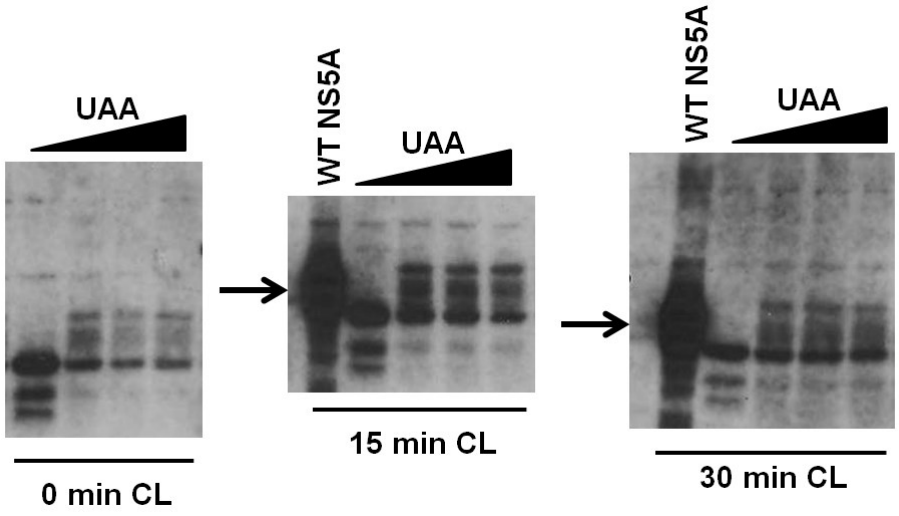

Figure 33. AZF cross-linking optimization in NS5A (418-TAG-419). Western blot of Huh7 cell lysates probed with anti-NS5A genotype 2a antibody. Cells were transfected with WT NS5A or TAG NS5A + tRNA/aaRS for 48 hours. AZF was added to cell culture medium at increasing concentrations (1, 5 and 10 mM). Samples were crossed linked (CL) by exposure to a 254 nm UV light for15 or 30 minutes or prepared in the dark (0 min CL). Arrow represents lower band of NS5A dimer (56 kDa).

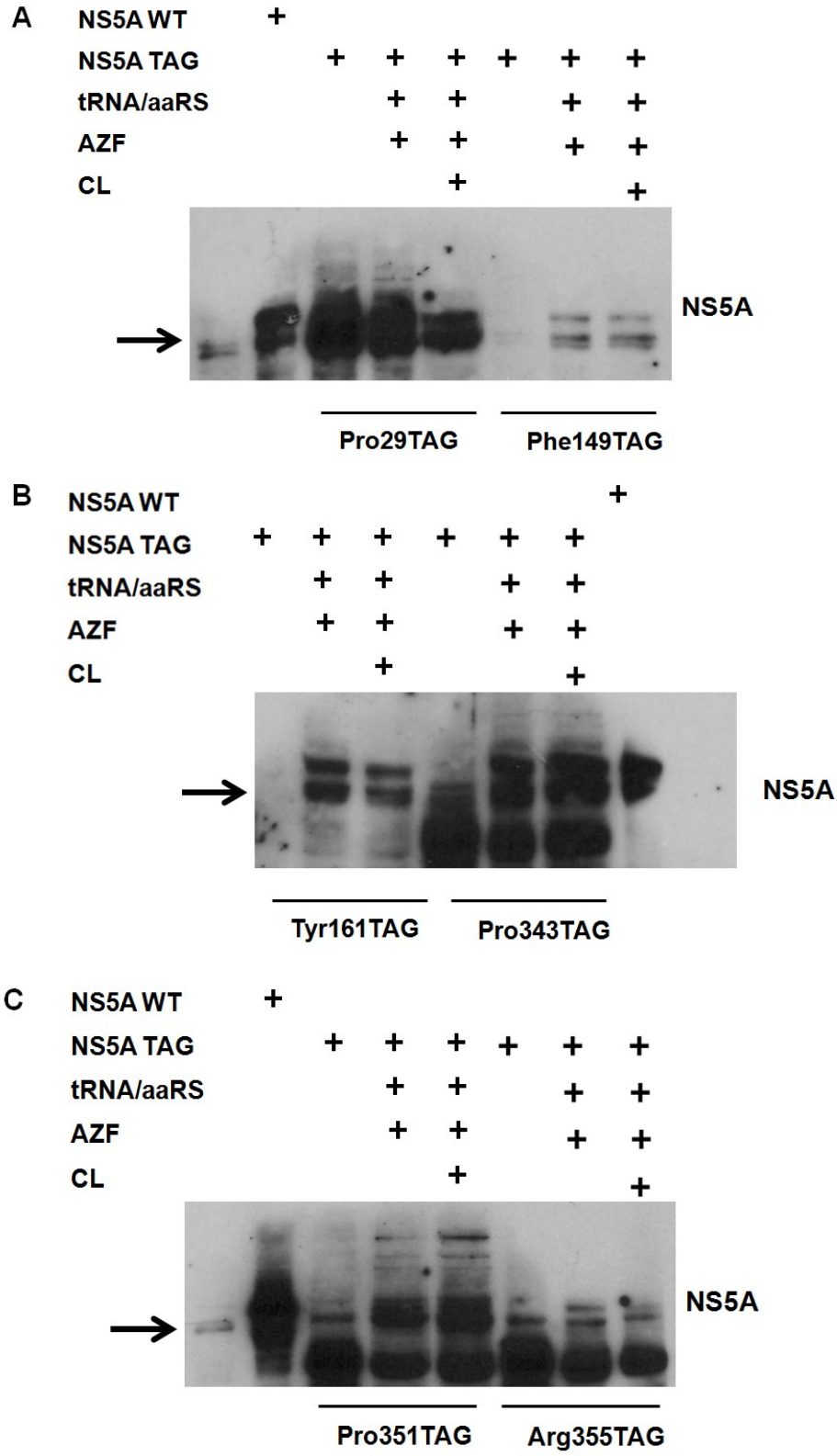
A
NS5A WT
NS5A TAG
tRNA/aaRS
AZF
CL
NS5A
Pro29TAG
Phe149TAG
B
NS5A WT
NS5A TAG
tRNA/aaRS
AZF
CL
NS5A
Tyr161TAG
Pro343TAG
C
NS5A WT
NS5A TAG
tRNA/aaRS
AZF
CL
NS5A
Pro351TAG
Arg355TAG

Figure 34. Optimization of AZF incorporation into NS5A mutants in human hepatoma cells. Western blot of Huh7 cell lysates probed with anti-NS5A genotype 2a antibody. Mutants tested for incorporation were (A) Pro29TAG and Phe149TAG (B) Tyr161TAG and Pro343TAG (C) Pro351TAG and Arg355TAG. (A and C) Cells were transfected for 48 hours with mock (lane 1), WT NS5A (lane 2), TAG NS5A (lane 3 or 6) TAG NS5A + tRNA/aaRS (lane 4-5, 7-8). AZF (1 mM) was added to cell culture medium for cells in lanes 4, 5, 7, 8. Samples 5 and 8 were crossed linked (CL) by exposure to a 254 nm UV light for 5 minutes while remaining samples were prepared in the dark (not cross-linked). (B) Cells were transfected for 48 hours with TAG NS5A (lane 1 or 4) TAG NS5A + tRNA/aaRS (lane 2-3, 5-6), WT NS5A (lane 7), mock (lane 8). AZF (1 mM) was added to cell culture medium for cells in lanes 2, 3, 5, 6. Samples 3 and 6 were crossed linked (CL) by exposure to a 254 nm UV light for 5 minutes while remaining samples were prepared in the dark (not cross-linked). Arrow represents lower band of NS5A dimer (56 kDa).

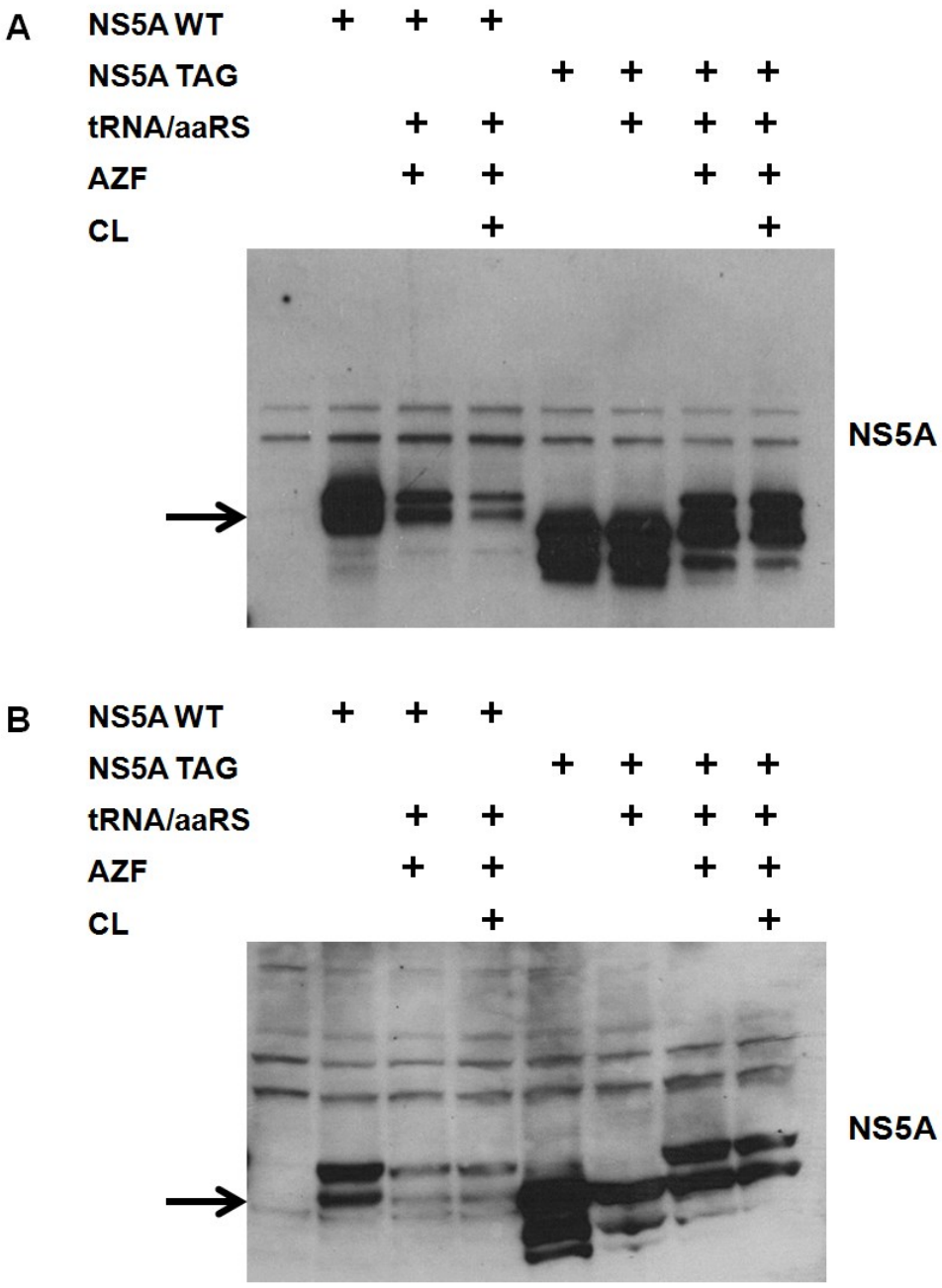

Figure 35. Abk incorporation into NS5A (418-TAG-419). (A) Western blot of Huh7 cell lysates and (B) Huh7.5-FGR cell lysates probed with anti-NS5A genotype 2a antibody. Cells were transfected for 48 hours mock (lane 1), WT NS5A (lane 2), WT NS5A + tRNA/aaRS (lane 3-4), TAG NS5A (lane 5), TAG NS5A + tRNA/aaRS (lane 6-8). AZF (1 mM) was added to cell culture medium for cells in lanes 3, 4, 7, 8. Samples 4 and 8 were crossed linked (CL) by exposure to a 365 nm UV light for 2 minutes while remaining samples were prepared in the dark (not cross-linked). Arrow represents lower band of NS5A dimer (56 kDa). 60 ug of protein is loaded per well.

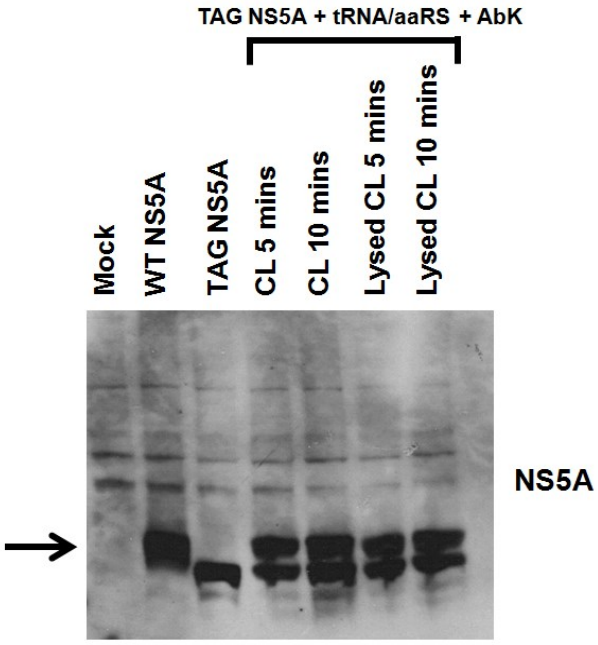

Figure 36. Abk cross-linking optimization in NS5A (418-TAG-419). Western blot of Huh7 cell lysates probed with anti-NS5A genotype 2a antibody. Cells were transfected with mock (lane 1), WT NS5A (lane 2), TAG NS5A (lane 3) or TAG NS5A + tRNA/aaRS (lanes 4-7) for 48 hours. Abk was added to cell culture medium at a concentration of 1mM. Samples were crossed linked (CL) by exposure to a 365 nm UV light for 5 or 15 minutes, in cells washed with PBS or cell lysates. Arrow represents lower band of NS5A dimer (56 kDa).

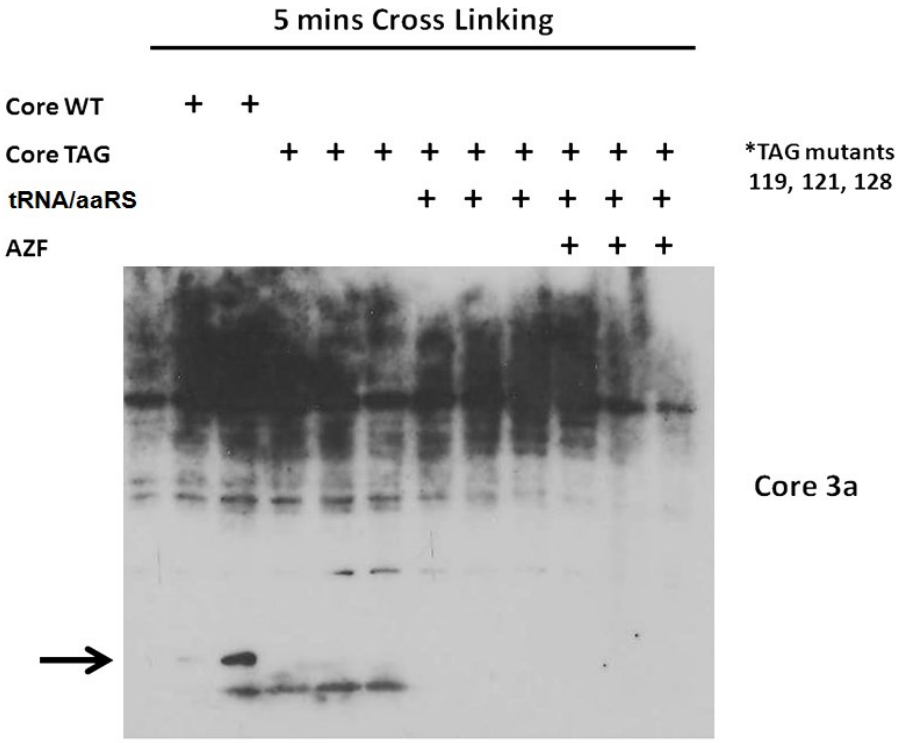

Figure 37. AZF incorporation into core in human hepatoma cells. Western blot of Huh7 cell lysates probed with anti-core genotype 3a antibody. Cells were transfected for 48 hours with mock (lane 1), WT NS5A (lane 2), WT NS5A + tRNA/aaRS + AZF (lane 3), TAG core (lanes 4-6), TAG core + tRNA/aaRS (lanes 7-12). AZF (1 mM) was added to cell culture medium for cells in lanes 9-12. Samples were crossed linked by exposure to a 254 nm UV light for 5 minutes. Arrow represents size of WT core.

3.5.3 UAA Incorporation into Core

TAG codons were inserted at four unique sites in the D2 domain of the core protein (Leu119, Lys121, Cys128, Gly161) based on the ability of these residues to tolerate change and the ability of mutations here to alter LD movement (Boulant, Montserret et al. 2006). Initial attempts to incorporate AZF into the core protein were not successful in Huh7 cells (Figure 37). Moreover, Abk incorporation was also not observed at any locations of TAG insertion into core in Huh7 cells. Again, incorporation of both AZF and Abk were carried out in Con1 cells so that UAA incorporation into core could be carried out in the presence of HCV viral proteins. However, core elongation could not be observed at any of the four TAG insertion sites (data not shown).

3.6 Discussion

Viruses are opportunistic pathogens that rely exclusively on the metabolic capabilities of the host to allow for their proliferation. With this being said, techniques to selectively label, monitor, and manipulate infectious viruses are largely inadequate, thus restricting our understanding of the molecular mechanisms of viral infection (Brandenburg and Zhuang 2007). The incorporation of UAAs into proteins of interest is a remarkable technique to interrogate protein structure and function, and provides an avenue for the engineering, labeling, and subsequent manipulation of proteins (Xie and Schultz 2006; Liu and Schultz 2010; Young and Schultz 2010). Moreover, several previous studies have demonstrated the utility of UAA technology towards the study of viral interactions and lifecycles (Carrico, Romanini et al. 2008; Strable, Prasuhn et al. 2008; Banerjee, Ostapchuk et al. 2011; Lin, Yan et al. 2013). Therefore, I have investigated the feasibility of applying UAA technology towards the study of the HCV lifecycle, by attempting to incorporate photo-cross-linking UAAs into the HCV proteins core and NS5A.

Importantly, HCV proteins core and NS5A were chosen for UAA incorporation as they both have well documented roles in altering host cell metabolism through host-virus interactions. The core

capsid protein influences cellular metabolism on many levels through interactions with host proteins such as MTP (Perlemuter, Sabile et al. 2002), heterogeneous nuclear ribonucleoprotein K (hnRNP K) (Hsieh, Matsumoto et al. 1998), lymphotoxin b receptor (LT-bR) (Matsumoto, Hsieh et al. 1997), tumour necrosis factor receptor 1 (TNF-R1) (Zhu, Khoshnan et al. 1998) and DEAD box protein (DDX3) (Mamiya and Worman 1999). Moreover, core is known to play a significant role in the alteration of host cell lipid metabolism during HCV infection, where it directly interacts with LDs to influence their dynamics, and induces a redistribution of LDs towards the HCV replication complexes (Boulant, Douglas et al. 2008; Lyn, Kennedy et al. 2010). Furthermore, core is known to upregulate cellular lipogenesis, ultimately resulting in hepatic steatosis, a common pathology of HCV (Shrivastava, Manna et al. 1998; McLauchlan 2000; Perlemuter, Sabile et al. 2002; Boulant, Douglas et al. 2008). Similarly, NS5A also plays a vital role during HCV pathogenesis, being involved in both viral RNA replication and modulating host cell physiology (Macdonald and Harris 2004). NS5A has been reported to interact with several host proteins such as ApoE (Benga, Krieger et al. 2010), phosphatidylinositol-4 kinase III alpha (PI4KIIIα) (Reiss, Rebhan et al. 2011), PI3K (Street, Macdonald et al. 2004) and protein kinase R (PKR) (Gale, Korth et al. 1997), which allows NS5A to modulate membrane structure and fluidity, IFN-responsiveness and host cell lipid metabolism. In addition to performing UAA incorporation in viral proteins critical for the regulation of host cell metabolism, UAA incorporation was carried out for the first time in Huh7 human hepatoma cells through expansion of their genetic code, which is of particular relevance as this model can carry out the entire HCV lifecycle.

Initially, control experiments were performed in order to confirm that UAA incorporation could function in our HCV models. The amber codon (TAG) was incorporated into the DNA sequence of GFP using site-directed mutagenesis. Tyr66 was chosen as the site of TAG insertion in GFP as previous studies have shown that an aromatic amino acid is required at this position in order to generate fluorescence (Tsien 1998). On its own, the amber codon stalled protein elongation,

therefore inhibiting the synthesis of full length GFP, as expected (Figure 31C). However, when plasmids containing the TAG-GFP sequence and the orthogonal tRNA/aaRS pair were co-transfected into mammalian cells in the presence of AZF, full length GFP was detected via its fluorescent signal using confocal microscopy. This indicated that AZF was being successfully incorporated at the amber codon, allowing for a full length, functional protein to be synthesized (Figure 31D).

Furthermore, the viral protein NS5A was cloned out of the HCV genome (JFH1 isolate, genotype 2a) and its sequence was mutated to contain an amber codon (TAG) between residues 418 and 419 in the unstructured C terminal region of this protein. The NS5A sequence from the JFH-1 isolate was chosen in order to screen this genome for sites that tolerate TAG incorporation, so that infectious virus with UAAs incorporated at specific locations could be created in future studies. Again, when mammalian cells were co-transfected with plasmids containing the TAG-NS5A sequence and the orthogonal tRNA/aaRS pair, and were supplemented with AZF, AZF was incorporated into NS5A allowing for proper elongation (Figure 32). Incorporation was specific as it was not observed when the TAG-NS5A was added with the tRNA/aaRS alone, indicating that natural amino acids with similar structures (such as tyrosine and phenylalanine) were not being non-specifically incorporated to a large degree (Figure 32). Ideally, when UV light was shone on these cells, the side chain of AZF should have undergone a cross-linking reaction with surrounding proteins, linking AZF-incorporated NS5A to its host binding partners. However, cross-linking was not observed by Western blot analysis in naïve Huh7 cells (Figure 32A). As successful cross-linking requires close proximity of interacting proteins, it was hypothesized that cross-linking of the AZF-incorporated NS5A was not occurring as NS5A was not in its typical location during HCV infection, as no other viral proteins were present to form the replication complex. Therefore, AZF incorporation was carried out in Huh7.5-FGR cells which stably express all viral proteins; however again, no cross-linking was observed (Figure 32B). Moreover, although Abk incorporation into NS5A appeared to be more efficient (Figure 35), cross-linking was still not observed, even after optimizations (Figure 36).

Considering the new NS5A constructs tested, Tyr161TAG, Pro343TAG and Pro351TAG were the best candidates for future cross-linking and fluorescent UAA incorporation studies, as they demonstrated the largest degree of specific NS5A elongation (Figure 34BC). Of particular interest, Pro351TAG had several higher molecular weight bands above the NS5A band, indicating that interacting proteins were potentially cross-linked to NS5A (Figure 34C). The highest band detected had a molecular weight of approximately 150 kDa, which is very close to the predicted molecular weight of a NS5A-PI3K complex (approximately 140 kDa), and is expected as Pro351 is a residue required for PI3K binding (Macdonald, Mazaleyrat et al. 2005). Moreover, the lower cross-linking bands observed around 100 kDa in this lane may represent cross-linking of the NS5A dimer (Love, Brodsky et al. 2009). Although cross-linking was observed when cells were irradiated with UV light, bands of higher molecular weight above NS5A were also seen without exposure to UV light (Figure 34C). This may indicate that thermal cross-linking or cross-linking due to the presence of residual light is occurring, which should be investigated in future studies to diminish this effect. Additionally, the Pro343TAG mutant also displayed some light bands of higher molecular weight above NS5A and should be investigated further in future studies.

Future work should aim to optimize cross-linking and identify binding partners. Initially, the incorporation of intact, non-quenched AZF should be confirmed by reacting it with an Alexa Fluor® 488 DIBO Alkyne using a copper-free click reaction. Furthermore, Abk incorporation should be carried out with the new NS5A constructs to try and identify previously reported interactions between NS5A and itself, and NS5A and PI3K. Moreover, the best cross-linking candidates should undergo incorporation in Huh7.5-FGR cells so that NS5A will be localized to the HCV replication complex to increase the probability of cross-linking to its interacting proteins. Once substantial cross-linking can be observed via Western blot, Westerns should be probed with antibodies of known NS5A interacting proteins, such as PI3K and PI4K. Furthermore, immunoprecipitation should be carried out on cell lysates as a means to pull down NS5A-protein complexes, and identify interacting proteins using

mass spectrometry. Additionally, once sites that tolerate UAA incorporation have been identified, these sites should also be used to incorporate the fluorescent UAA ANAP as a means to interrogate NS5A subcellular location and movement dynamics.

Although UAAs were incorporated into the core protein at residues that were well conserved across HCV genotypes (Boulant, Vanbelle et al. 2005) and were previously shown to tolerate changes in amino acid composition (Boulant, Montserret et al. 2006; Lyn, Hope et al. 2013), full length protein elongation could not be observed for core using either AZF or Abk (Figure 37). Based on the study by Boulant *et al.*, all of the residues chosen for mutation were part of the second, amphipathic domain or the core protein that is involved in LD binding. Two of the residues chosen for mutation were hydrophilic and thought to be pointed towards the lipid droplet surface (L119 and G161), whereas the other two residues were hydrophobic and pointed away from the LD (K121 and C128). All four of these mutants were found to be stable when they were mutated to glutamic acid, and this was not found to have a large impact on the alpha helical structure of core or its ability to bind LD (Boulant, Montserret et al. 2006). Interestingly, this study found that none of the other hydrophobic residues in the D2 domain of core could tolerate mutations, which caused the author to hypothesize that these hydrophobic residues served a secondary purpose of preventing core degradation upon its maturation, probably by promoting correct folding of the protein through association with membranes (Boulant, Montserret et al. 2006). With this being said, I hypothesize that the two hydrophobic residues that were chosen as sites for UAA incorporation could not tolerate mutations to bulky hydrophilic residues of the UAAs as they are so close to either end of the D2 helix, which inhibited successful UAA incorporation. Additionally, I hypothesize that the hydrophilic residues could not tolerate incorporation of the hydrophobic UAAs as this would alter the structure of the amphipathic helix, which explains why proper elongation could not be observed for these core mutants either.

Moreover, although the remaining hydrophobic residues in the D2 domain of core could not tolerate mutation to glutamic acid, there is a chance that they could tolerate mutations to either AZF

or Abk due to their hydrophobic nature. Accordingly, future studies should focus on the incorporation of UAAs at hydrophobic residues of core that have similar structures to these UAAs, such as P130, which was also shown to alter LD binding when mutated, and therefore may be a critical residue for interacting with host proteins on the LD surface. Incorporation of the fluorescent UAA ANAP should also be carried out for core as this would be a powerful technique to interrogate the movement of core and the LD that it surrounds during HCV infection, due to the small size of this UAA fluorescent probe.

Overall, after identifying regions in the viral genome that are optimal for UAA incorporation, future studies should aim to create a strain of infectious virus that relies on the expanded genetic code for viral proliferation. The goal of this system is to efficiently incorporate either cross-linking or fluorescent UAAs into the viral capsid, which will permit the identification of viral interacting proteins and allow for the tracking of viral particles during HCV infection *in vivo* and *in vitro*. Overall, this unique approach provides an avenue to examine some of the poorly elucidated steps in the HCV lifecycle, such as initial infection and viral entry, which will be crucial in order to gain a complete understanding of HCV pathogenesis.

It is important to note that although previous studies have shown that reassignment of the TAG codon as the site of UAA incorporation does not yield significant cytotoxicity to bacterial and mammalian cell cultures (Liu, Brock et al. 2007; Wang, Takimoto et al. 2007; Arbely, Torres-Kolbus et al. 2012; Chatterjee, Guo et al. 2013), the heterologous expression of the suppression system will still result in the undesirable elongation of some host proteins that may cause non-lethal perturbations to cell physiology. To counteract this problem, several approaches are being used such as the construction of orthogonal ribosomes (Wang, Neumann et al. 2007; An and Chin 2009) and the use of quadruplet codons to delineate the sites of UAA incorporation (Anderson, Wu et al. 2004; Neumann, Wang et al. 2010; Wang, Schmied et al. 2012; Niu, Schultz et al. 2013), which are

expected to address the issue of cellular genetic code expansion. Some of these novel approaches may be employed in the future to continue the study of HCV using UAA technology.

3.6.1 Conclusion

In summary, I have demonstrated that UAA incorporation can be carried out in Huh7 cells, an important model for studying the HCV lifecycle. Moreover, I showed that photo-cross-linking UAAs can be incorporated into the viral protein NS5A where cross-linking to other cellular proteins was observed upon irradiation with UV light. Overall, I have demonstrated that UAA technology can be applied successfully to the study of the HCV lifecycle. Future work should aim to identify interacting proteins in order to gain insight into HCV's manipulation of host cell metabolism, and to create unnatural virus that relies on the incorporation of UAAs for successful viral proliferation, as a means to site-specifically incorporate unique functional groups into infectious viruses to facilitate their manipulation. Overall, demonstrating proof-of-principle with this project will pave the way for similar studies in other experimental models and disease pathways, which would be valuable for numerous fields of study.

4.0 General Discussion

Many studies have demonstrated that the complex array of host-virus interactions that occur during viral infection are critical for viral proliferation, as these interactions are necessary for viral manipulation of the host cell and thereby viral hijacking of host cell metabolic pathways (Ou, Duan et al. 2012; Devasthanam 2014). Examples of the significance of host-virus interactions to viral pathogenesis can be observed during HIV (Jager, Gulbahce et al. 2010), HBV (Thomas, Jacyna et al. 1988), HAV (Vaughan G 2014) and West Nile virus infection (Brinton 2001). Of particular interest, HCV is also well known to manipulate host factors as a means to facilitate its lifecycle (Tellinghuisen and Rice 2002; Chisari 2005; Gale and Foy 2005; Randall, Panis et al. 2007; Li, Brass et al. 2009). Due to CIDEB's role as a dual regulator of cellular apoptosis and lipid homeostasis, I hypothesized that CIDEB was a host factor involved in HCV pathogenesis, due to the profound reliance that the virus has on these two host pathways, which was further supported by the previously reported interaction between CIDEB and HCV protein NS2 (Erdtmann, Franck et al. 2003). My work supports a model where CIDEB is a host factor capable of modulating the HCV lifecycle, where CIDEB expression is upregulated during HCV infection to enhance its apoptotic activity, thereby impairing viral replication and proliferation (Figure 29). Moreover, CIDEB's role as a modulator of the HCV lifecycle may have clinical implications during chronic HCV infection when death of hepatic tissue is a common side-effect of HCV infection (Calabrese, Pontisso et al. 2000; Bantel, Lugering et al. 2001). Overall, it is evident that HCV manipulates host cell metabolic pathways to allow for viral replication, assembly and secretion.

To further investigate additional host factors and metabolic pathways that are involved in the HCV lifecycle, I optimized a model system to identify host-viral interactions in mammalian cells that are capable of recapitulating every step of the HCV lifecycle. Using the Schultz method, cross-linking UAAs were incorporated into viral proteins of interest at defined sites, allowing for host-viral

protein complexes to be covalently linked and isolated, as a means to identify novel interactions. The viral proteins core and NS5A were chosen for UAA incorporation as both of these proteins have been shown to be critical for the manipulation of host cell metabolism during the HCV lifecycle. For example, the core capsid protein is known to interact with LDs, which influences their dynamics and localization (Boulant, Douglas et al. 2008; Lyn, Kennedy et al. 2010). Furthermore, core is known influence lipid metabolism on many levels, ultimately resulting in hepatic steatosis that is a common pathology of HCV (Shrivastava, Manna et al. 1998; McLauchlan 2000; Perlemuter, Sabile et al. 2002; Boulant, Douglas et al. 2008). Moreover, NS5A has been previously confirmed to interact with several inositol kinases which causes alterations in membrane structure and fluidity at the HCV replication complexes, in addition to modulating viral particle secretion and host cell lipid metabolism (Ghosh, Steele et al. 1999; Gong, Waris et al. 2001; Shi, Polyak et al. 2002; Appel, Zayas et al. 2008; Tellinghuisen and Foss 2008). Overall, cross-linking was observed for the viral protein NS5A in human hepatoma cells, and future work should focus on optimizing cross-linking and identifying interacting proteins to advance our knowledge of HCV's modulation of host cell metabolism.

In conclusion, there is still much to learn about the intricate series of interactions taking place between host and virus during HCV infection. My thesis work characterized the role of the host factor CIDEB during HCV pathogenesis, and addressed the need to identify additional host factors necessary for the viral lifecycle by optimizing a relevant mammalian system for the incorporation of cross-linking UAAs into viral proteins. Overall, a more detailed molecular understanding of these interactions will be critical for the rational design of therapeutics and prognostic markers in the future.

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Statement of Contribution

Dr. John Paul Pezacki, Dr. Peter Schultz and Ragunath Singaravelu helped me with experimental design and implementation.

I performed all experiments described, with the exception of having Ragunath Singaravelu perform qRT-PCT on cell samples that I prepared.

The thesis was originally written by myself and was edited by my supervisor, Dr. John Paul Pezacki.

Table 1. List of oligonucleotides.

Name	Sequence (5' to 3')	Description
pCMVβ-CIDEB-F	GTTTTTGCGGCCGCATGGAGTACCTCTCAGCTC	NotI site
pCMVβ-CIDEB-R	AATATGGCGGCCGCTTAGTAGGAGTAGAGGCGG	NotI site
SDM-Y160F-F	CATTCTACGGGCTCTTCTCTATGAGTTGTG	Y160F SDM
SDM-Y160F-R	CACAACTCATAGAGAAGAGCCCGTAGAATG	Y160F SDM
pIRES2-EGFP-CIDEB-F	TATATAGAATTCATGGAGTACCTCTCAGCTCTG	EcoRI site
pIRES2-EGFP-CIDEB-R	TAAAATGGATCCTTAGTAGGAATGGAGGC	BamHI site
SDM-KA-F	CAAGGACTTGGCCCAGCGAAAGTACTCAGGG	KRR→ ARR
SDM-KA-R	CCCTGAGTACTTTCGCTGGGCCAAGTCCTTG	KRR→ ARR
SDM-KRA-F	CCAGCGAAAGTACTCGCGGAGCTCCTTCGTTG	ARR→AAR
SDM-KRA-R	CAACGAAGGAGCTCCGCGAGTACTTTCGCTGG	ARR→AAR
SDM-KRRA-F	GTACTCGCGGAGCTCCTTGCTTGGACCTCCACACTGC	AAR→AAA
SDM-KRRA-R	GCAGTGTGGAGGTCCAAGCAAGGAGCTCCGCGAGTAC	AAR→AAA
pTriEx4-CFP-F	AAGGAAAAAAGCGGCCGCAATGGTGAGCAAGGGCGAGG	NotI site
pTriEx4-CFP-R	CGACCATGGCTTGTACAGCTCGTCCATGC	XhoI site
pCMVβ-NS2-FLAG	GTTTTTGCGGCCGCATGGATTACAAGGATGACGACGATAAGGA CCGGGAGATGGCAGC	NotI site
pCMVβ-NS2-R	AATATGGCGGCCGCTTAGAGGAGTCGCCACCCCTC	NotI site
pCMVβ-NS5A-F	GTTTTTGCGGCCGCATGTCCGGATCCTGGCTCCGCGACG	NotI site
pCMVβ-NS5A-R	AATATGGCGGCCGCTTAGCAGCACACGGTGGTATCGTCCGCAG CACACGG	NotI site
418-TAG-419-NS5A-F	CTCTATGCCCCCCTAGCTCGAGGGGGAGC	TAG SDM
418-TAG-419-NS5A-R	GCTCCCCCTCGAGCTAGGGGGGCATAGAG	TAG SDM

SDM-core-119-F	GGAGGTCCCGCAATTAGGGTAAAGTCATCG	TAG SDM
SDM-core-119-R	CGATGACTTTACCCTAATTGCGGGACCTCC	TAG SDM
SDM-core-121-F	GGTCCCGCAATTTGGGTTAGGTCATCGATACCCTCACG	TAG SDM
SDM-core-121-R	CGTGAGGGTATCGATGACCTAACCCAAATTGCGGGACC	TAG SDM
SDM-core-128-F	CATCGATACCCTCACGTAGGGATTCGCCGATCTCATG	TAG SDM
SDM-core-128-R	CATGAGATCGGCGAATCCCTACGTGAGGGTATCGATG	TAG SDM
SDM-core-161-F	GTGAGGGCCCTTGAAGACTAGATAAATTTCGCAACAG	TAG SDM
SDM-core-161-R	CTGTTGCGAAATTTATCTAGTCTTCAAGGGCCCTCAC	TAG SDM