Identification and Characterization of Transferrin Receptor 1 and other Novel HCV Entry Factors

BY

DANYELLE NICHOLE MARTIN

Bachelor of Science, University of Illinois at Urbana-Champaign, Urbana, 2004

THESIS

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Defense Committee:

Susan Uprichard, Chair and Advisor

Alan McLachlan

Nancy Freitag

William Walden

Scott Cotler, Medicine

I would like to dedicate this thesis to my mother, Debra B. Martin, whose unconditional love and encouragement made this accomplishment possible.

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LIST OF ABBREVIATIONS

aa	Amino Acid
ATP	Adenosine Triphosphate
cDNA	Complementary Deoxyribonucleic Acid
CHO	Chinese Hamster Ovary
CLDN	Claudin
DNA	Deoxyribonucleic Acid
ECMV	encephalomyocarditis
ER	Endoplasmic Reticulum
EC	Extracellular Loop
FRET	Fluorescence resonance energy transfer
GAG	Glycoaminoglycans
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GT	Genotype
HCC	Hepatacellular Carcinoma
HCV	Hepatitis C Virus
HCVcc	Hepatitis C Virus Cell Culture
HCVpp	Hepatitis C Virus Pseudotype Particle
HDL	High Density Lipoprotein
HIV	Human Immunodeficiency Virus
HSC	Hepatic Stellate Cell
IFN	Interferon
IL	Interleukin

LIST OF ABBREVIATIONS (continued)

IMPDH	Inosine Monophosphate Dehydrogenase
IRE	Iron Response Element
IRES	Internal Ribosomal Entry Site
IRP	Iron Regulatory Protein
ISG	Interferon Stimulating Genes
JFH	Japanese Fulminant Hepatitis
Kb	Kilobases
LEL	Large Extracellular Loop
LVP	Lipoviroparticle
mAb	Monoclonal Antibody
MLV	Murine Leukemia Virus
NANBH	Non A Non B Hepatitis
NEO	Neomycin
NI	Nucleoside Inhibitor
NNI	Non-nucleoside inhibitor
NPC1L1	Niemann Pick C 1 Like 1
NS	Nonstructural
NTR	Nontranslated Region
OCLN	Occludin
P.I.	Post Infection
PEG	Polyethylene Glycol
PPAR	Peroxisome Proliferator-Activated receptors

LIST OF ABBREVIATIONS (continued)

qPCR	Quantitative Polymerase Chain Reaction
RBV	Ribavirin
ROS	Reactive Oxidative Stress
RIG-I	Retinoic Acid Inducible Gene I
RNA	Ribonucleic Acid
RT	Reverse Transcription
SRB1	Scavenger Receptor class B I
SOC	Standard of Care
SREBP	Sterol Regulatory Element Binding Protein
STAT	Signal Transducer and Activator of Transcription
SVR	Sustained Virological Response
TEM	Tetraspanin Enriched Domains
TfR1	Transferrin Receptor 1
TTP	Transferrin Receptor Trafficking Protein
UT	Untreated
VLDL	Very Low Density Lipoprotein

SUMMARY

Hepatitis C virus (HCV) is a liver-tropic, enveloped, positive-sense, single-strand RNA virus. HCV chronically infects ~170 million people worldwide. With no vaccine available and current treatment options effective in only a subset of patients, long term HCV infection is associated with a wide variety of liver pathologies such as steatosis, fibrosis, cirrhosis, iron overload and hepatocellular carcinoma.

Like all viral infections, HCV infection begins upon entry into a target cell. Because enveloped viruses are surrounded by a lipid bilayer, their entry involves fusion of the viral envelop with the host membrane. This process typically occurs through the recognition and binding of viral surface glycoproteins to specific cellular host receptors leading to direct fusion at the plasma membrane or receptor-mediated endocytosis into an endocytic vesicle where fusion occurs. In terms of simple classification, HCV belongs to the latter clathrin-mediated endocytosis group, but HCV entry is proving to be a very complex, multistep process. Prior to the thesis work described herein, four cellular factors required for HCV entry had already been identified; the tetraspanin CD81, the scavenger receptor B-I (SRBI), claudin-1 (CLDN1) and occludin (OCLN). However, the molecular details of how these cellular factors mediate HCV entry into hepatocytes remains poorly defined. Likewise, expression of these four receptors is unable to confer HCV permissiveness to non-permissive cells suggesting that another receptor(s) remains to be identified. Because understanding the molecular mechanisms that mediate HCV entry could help identify novel antiviral targets and potentially reveal critical determinants of HCV restricted species tropism, elucidating the factors and steps of HCV entry that could facilitate not only therapeutic drug development, but also provide insight necessary to create

much needed small animal experimental HCV infection model. For these reasons, my thesis work focused on expanding our knowledge of HCV entry.

The first project, described in Chapter 3, identifies transferrin receptor 1 (TfR1) as a novel HCV entry factor. TfR1 is one of two cell surface proteins responsible for cellular iron uptake. Since HCV is associated with hepatic iron overload, initially we tested whether HCV cell culture (HCVcc) infection affected expression of iron metabolic genes. After initial experiments indicated TfR1 gene and protein expression is down regulated during infection we decided to further explore how TfR1 functions in HCVcc infection. In TfR1 RNAi knockdown studies, HCV RNA was inhibited suggesting TfR1 expression is required for HCV infection. Time of antibody addition experiments blocking cell surface TfR1 with either antibodies or a pharmaceutical inhibitor reduced HCVcc infection initiation and HCV pseudotype particle (HCVpp) entry demonstrating TfR1 functions during HCV entry. However, further functional analysis suggests that TfR1 is not critical for subsequent cell-to-cell spread. In terms of when TfR1 functions during the entry process, addition of TfR1 antibody or TfR1 inhibitor exhibited effective blocking of HCV infection until approximately 4 hours post virion binding. This inhibition was observed longer than anti-CD81 antibody inhibitory activity leading to the conclusion that TfR1 is acting post initial HCV binding and virion-CD81 engagement. Since TfR1 is acting post-attachment, we assessed whether TfR1 may be involved in HCV internalization by silencing an endocytic protein that has been shown to be specifically and uniquely required for TfR1 endocytosis, the TfR1 Trafficking Protein (TTP). Importantly, knockdown of TTP inhibited HCVcc infection and HCVpp entry to the same extent as TfR1

knockdown suggesting that TTP-mediated internalization of TfR1 is required for HCV uptake. Finally, we investigated whether the HCV virion binds to TfR1 by using the HCV non-permissive Chinese hamster ovary (CHO) cell-based binding assay. CHO cell lines stably expressing the human cellular HCV entry factors, SRBI, CD81 or TfR1 on their cell surface were inoculated with HCVcc at 4^{o}C and the level of encapsidated HCV RNA specifically bound was measured. Consistent with binding of HCV to TfR1, increased levels of cell-associated HCV RNA was detected in CHO cells expressing human TfR1.

Chapter 4 describes a second project identifying another novel HCV entry factor, Niemann Pick C1 Like 1 (NPC1L1) with a focus on the analysis illustrating the therapeutic potential of this entry factor as an antiviral target. NPC1L1 is the main cellular receptor responsible for dietary cholesterol uptake and homeostasis. While NPC1L1 is expressed on enterocytes across all species, in humans and non-human primates NPC1L1 is also expressed on hepatocytes. This intriguing correlation with HCV species tropism combined with the fact that infectious HCV particles are tightly associated with lipoproteins and specifically enriched in cholesterol which have been shown to be important for HCV entry, led our group to test whether NPC1L1 is required for HCV entry. Consistent with the hypothesis that NPC1L1 is involved in HCV entry, silencing or blocking of cell surface NPC1L1 inhibited HCVcc infection initiation, but not steady-state chronic HCVcc infection or HCV replicon replication. Antibody mapping studies suggest the cholesterol binding domain may be the region involved in HCV infection. Using the FDA-approved NPC1L1 cholesterol uptake inhibitor, ezetimibe, which binds to and inhibits NPC1L1 internalization, we performed time of addition studies demonstrating that ezetimibe was able to inhibit HCV infection initiation when added prior to infection

or up to 5 hours post viral attachment indicating that NPC1L1 functions relatively late in the HCV entry process. Further mechanistic studies confirmed that NPC1L1 acts at a post-attachment, pre-fusion step and is effective against all major HCV genotypes. Importantly, studies showing that 2 week pretreatment with ezetimibe inhibited HCV infection initiation in a hepatic xenopopulated uPA-SCID mouse model confirmed that NPC1L1 is involved in HCV entry *in vivo*. Particularly promising from a therapeutic perspective, studies examining the effect of combination IFN and ezetimibe treatment on chronically HCV infected cultures revealed a high level of synergy. Although still ongoing, initial testing of ezetimibe in chronically infected uPA-SCID mice confirmed that while ezetimibe alone has no inhibitory effect on chronic HCV infection, it enhances the inhibition achieved when administered in combination with IFN. These finding suggest this inexpensive, readily-accessible FDA-approved drug may be a promising new anti-HCV therapeutic that can be combined with current standard of care regimens to improve HCV treatment response.

1. INTRODUCTION

1.1 Overview of Hepatitis C Virus

1.1.1 General Overview

Hepatitis C virus (HCV) has become a major global health concern affecting more than 170 million people worldwide (6). It is estimated that approximately 80% of infected individuals fail to clear the virus, which then persists to establish a chronic infection that can result in severe liver disease (6). Currently there is no vaccine available and therapy options are only effective in a fraction of patients (3, 53, 60). As a result, HCV is the leading cause of liver transplantation in the United States. HCV is a blood borne pathogen. It is spread by activities that involve blood to blood contact including, but not limited to, intravenous drug use, the use of non sterile procedures in hospital settings, long term dialysis treatment, tattooing/piercing, and sexual activity (7). The development of stringent testing procedures to detect HCV in the blood supply along with education and knowledge of the disease has significantly limited the rise of new infections in the developed world. However, based on the large numbers of individuals already chronically infected HCV associated liver disease is expected to increase through at least 2020 (175).

HCV is classified as a member of the family *Flaviviridae* and the genus Hepacivirus. Because of the high genetic diversity among isolates recovered from infected patients from different parts of the world, HCV has been divided into 6 major genotypes which consist of multiple subtypes (22, 31). HCV is an envelope, positive-strand RNA virus with a genome of 9.6 kilobases (kb) that specifically targets the main cell type found in the liver, the hepatocyte. The genome encodes a single open reading frame polyprotein which is processed by host and viral proteases resulting in 3 structural proteins; E1, E2 and core and 7 non-structural (NS)

proteins; p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Fig 1). The viral life cycle occurs completely in the cytoplasm with replication being associated with large ER-derived membrane structures referred to as the "membranous web" (48, 121). The viral genome replicates via a RNA double-strand intermediate, which is a likely activator of the observed innate immune response against HCV. The RNA-dependent RNA polymerase, NS5B has high infidelity and no proof reading mechanism and therefore many viral quasispecies are produced within a single infection (22). This is thought to be a possible explanation for the remarkable ability of HCV to evade the host immune response and establish the chronic infection that puts patients at increased risk of numerous liver pathologies.

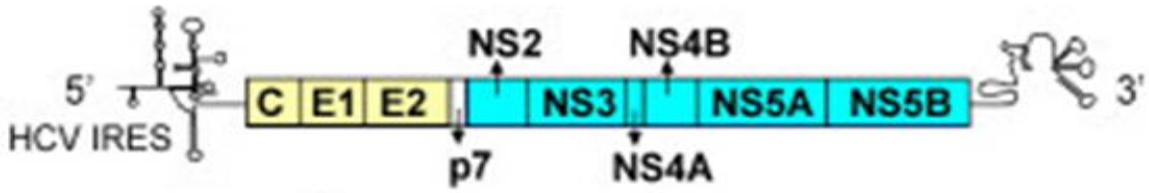

Figure 1. HCV genome. Structural viral proteins are shown in yellow and nonstructural proteins are shown in blue. The 5' end contains the HCV IRES. Figure adapted from Uprichard et al. (164).

1.1.2 **HCV Associated Liver Disease**

Early HCV infection is often asymptomatic and can go undiagnosed for many years. Several liver pathologies may occur as a result of years of chronic HCV replication within the liver, these include steatosis (fatty liver disease), fibrosis, cirrhosis, hepatocellular carcinoma and hepatic iron overload (6, 107, 156). While it is speculated that a variety of direct and indirect factors related to how the host cell responses to infection may play a role in HCV-induced pathogenesis (e.g. chronic innate immune signaling, oxidative stress, ER stress, changes in lipid metabolism, and iron accumulation), our understanding of the molecular mechanisms by which HCV causes liver disease still remains limited.

Steatosis, characterized by accumulation of lipid droplets within the cytoplasm of the hepatocyte, is associated with chronic HCV infection and happens to be particularly prevalent among patients infected with HCV genotype 3 (43, 125). Many hypotheses have been suggested concerning the cause of HCV induced steatosis. One proposed mechanism is inhibition of lipoprotein secretion resulting in accumulation of triglycerides and cholesterol in the hepatocyte. This was proposed because HCV infected patients tend to have lower cholesterol and lipoprotein serum levels than non-infected patients and this phenotype is reversed in patients who respond to treatment and have a sustained virological response (SVR). The viral protein core has also been implicated in the development of steatosis. Transgenic mice expressing core had triglyceride accumulation in the hepatocyte (123). The enzymatic activity of microsomal triglyceride transfer protein (MTP), the enzyme responsible for very low density lipoproteins (VLDL) assembly, was reduced potentially causing the lipoprotein accumulation (134). Over expression of core protein in hepatoma cells resulted in a down-regulation of the nuclear receptor, peroxisome proliferator activated receptor-α (PPAR-α), a transcription factor responsible for regulating genes involved in fatty acid degradation (43, 44). HCV infection has also been shown

to up-regulate enzymes involved in fatty acid synthesis like ATP citrate lyase (178). Therefore, HCV may not only inhibit fatty acid degradation but also induce fatty acid synthesis leading to the development of steatosis.

Fibrosis and later development of cirrhosis is generally believed to be the result of the long term inflammation associated with chronic HCV. However, other factors like endoplasmic reticulum (ER) or oxidative stress may also be involved in the development of fibrosis (30). Production of fibrotic tissue within the portal tracts and centrilobular area is a consequence of an extracellular collagen matrix synthesized by activated hepatic stellate cells (HSC) (14, 116). Because the presence of fibrosis and cirrhosis markedly reduces the livers ability to perform its normal functions and restricts portal blood flow, serious clinical consequences often develop. Notably, increases in HCV-associated cirrhosis have driven an increase in hepatocellular carcinoma (HCC) subsequently increasing the need for liver transplantation (151). Since the HCV life cycle occurs strictly in the cytoplasm, it is unlikely that the virus incorporates its genome into the host DNA. However, it has been suggested that not only cirrhosis but the viral structural core protein and/or the viral nonstructural proteins such as NS3, NS5A, and NS5B may act more directly as oncogenes affecting factors involved in cell cycle regulation (106, 122).

Mild to moderate hepatic iron deposition has also been implicated in the development of liver pathology observed in chronic HCV patients (63). Notably, these patients also have a lower response rate to treatment (63). In excess, iron can be deleterious and cause harmful side effects through the production of oxidative stress leading to liver damage and hepatocellular carcinoma. Specifically, excess iron results in free hydroxyl radicals via Fenton chemistry reaction. In this reaction, ferrous iron reacts with oxygen to generate hydroxyl radicals, a reactive oxygen species (ROS), which leads to oxidative stress. Hydroxyl radicals cause accumulation of 8-hydroxy-2' deoxyguanosine (8-OHdG), which induces G-C to T-A conversions in DNA leading to genomic

instability and potential carcinogenesis (89). Notably, oxidative stress markers like 8-OHdG, a marker for DNA damage caused by oxidative stress, has been shown by immunohistochemistry to be present at significantly high levels in hepatocytes of HCV chronic infected patients (88). Likewise, 8-OHdG has been shown to be associated with HCC. Oxidative stress also causes inflammation which has been associated with carcinogenesis. Oxidative stress has also been shown to be responsible for the activation of HSCs inducing collagen production and fibrogenesis, thus linking excess iron to the development of fibrosis and cirrhosis as well (116). Expression of some of the key genes involved in iron homeostasis, particularly the hepatocyte-secreted small peptide hormone hepcidin, which has been referred to as the master iron regulator, have been shown to be altered in HCV infected patients (65, 127, 161). Consistent with this, microarray analysis of liver tissue from infected patients has also revealed differential regulation of some of the genes involved in iron metabolism (77). Curiously, although HCV induces chronic inflammation and hepcidin levels are normally up regulated by inflammatory cytokines, hepcidin levels are unexpectedly decreased in HCV patients (61, 62, 161). Together these data suggest that HCV infection is either directly or indirectly disrupting iron regulation.

1.1.3 **Standard Care of Treatment for HCV Infection**

In the absence of a vaccine to prevent HCV infection, development of therapeutic options to try and cure those who become infected has been a major research focus. In 1989, recombinant interferon-α (IFN-α) was used in clinical trials and found to eliminate the virus in 15% of patients. Since then studies have shown that lengthening the duration of IFN-α treatment, in conjugation with inert polyethylene glycol (PEG) to IFN-α to increase the stability of the compound, and the additional co-treatment with oral ribavirin further increased sustained virological response (SVR) (60). IFN-α inhibits HCV indirectly by inducing interferon

stimulated genes (ISGs) which consequently promote viral clearance by boosting the host antiviral response (53). Interestingly, while ribavirin acts synergistically with IFN-α to inhibit HCV, it has been shown to have little efficacy when administered alone. Structurally, ribavirin is a guanosine analog and has been reported to be an inhibitor of inosine monophosphate dehydrogenase (IMPDH), an enzyme necessary for *de novo* guanosine synthesis (129). Significant controversy exists over the mechanism by which ribavirin inhibits HCV and as a result several hypotheses have been proposed concerning ribavirin's mechanism of action. For example, it has been speculated ribavirin may serve as a substrate for the viral RNA-dependent RNA-polymerase and thus becoming incorporated into the viral genome where it would act as a mutagen and lead to "error catastrophe" by decreasing HCV replication fitness (25). It has also been suggested ribavirin modulates the immune system, enhances ISG expression and inhibits eIF4E, a component of the translation initiation complex (93).

Several limitations are associated with PEG-IFN-α/ribavirin combination therapy. Notably, the percent SVR achieved in response to this interferon-based therapy is genotype dependent. Even in ideal candidates, SVR is only achieved in approximately 50% of patients infected with genotype (GT) 1, the predominate GT in North America accounting for 70% of infections. In contrast, a higher response rate, 76% to 82% SVR is achieved in patients infected with GT 2 or 3, the predominate GT in Asia (71). This striking difference in response rates based on GT difference demonstrates that better therapeutic approaches need to be investigated. In addition to GT, host factors such as age, ethnicity, weight, viremia load, and level of fibrosis also contribute to efficacy of PEG-IFN-α/ribavirin treatment. For instance, response rates in African Americans are significantly lower compared to other racial groups (57, 139). Therefore the decision to treat and duration of treatment is often dependent on GT and other host factors. For those who initiate treatment, virtually all experience severe side effects such as influenza like

symptoms such as fever, fatigue, headache, myalgias, and neuropsychiatric symptoms, and these can persist for the duration of the 24 to 48 week treatment (1). As a result dose reduction or earlier termination of therapy may be required furthermore impacting the efficacy of treatment.

Because the efficacy of the current standard of care (SOC) is variable among patients and is limited by different host and environmental factors, novel therapeutic approaches are currently being developed. Specifically targeted antiviral therapy for HCV (STAT-C) aims to develop antiviral therapies targeting HCV viral proteins or specific host factors. Small molecular compounds such as Telapravir and Boceprevir target the viral HCV protease NS3/4a inhibiting its function and consequently blocking viral replication (38, 40). Both these inhibitors increase SVR when used in combination with PEG-IFN-α/RBV. They have been FDA approved and are currently being administered in the clinic as adjunct therapy to PEG-IFN-α/RBV (40, 59, 117). The HCV polymerase, NS5B, has also been shown to be a promising drug target with both nucleoside analog inhibitors (NI) and non-nucleoside analog inhibitors (NNI) in development. Phase III clinical trials are underway and the first polymerase inhibitors are expected to be approved in 2012. The emergence of drug resistant mutations against direct acting antivirals continues to be a concern and has been observed during monotherapy with the protease inhibitors. However, treatment with these inhibitors in combination with IFN-α /RBV prevents escape. In effort to eventually avoid the use of IFN-α, more small compound molecule drugs targeting different stages of the viral life cycle are needed to so that effective drug cocktails that prevent viral escape can be designed.

1.2 Development of Experimental Model Systems

1.2.1 The Discovery of HCV

In 1989 HCV was discovered as the causative agent for non-A, non-B hepatitis (NANBH) providing the first information about the viral genome and insight regarding the viral life cycle (31). RNA isolated from NANBH infected chimpanzee plasma was used to generate cDNA which was inserted into the bacteriophage expression vector lambda gt 11. Using a blind immunoscreening approach, the cDNA library was screened using plasma from NANBH infected patients which resulted in the isolation of a positive clone called 5-1-1 (31). Southern blot analysis indicated the clone was not a host gene derived from the chimpanzee or human genome and that it bound only to antibodies in NANBH infected patients (31). This clone and other overlapping clones mapped a single stranded RNA molecule of approximately 10kb nucleotides. This first genomic clone was named H77 and the RNA virus it represents was thereafter referred to as HCV.

Although H77 and other subsequently derived consensus genomes were shown to be infectious in chimpanzees and therefore viable, it was determined the consensus clones synthesized from full length HCV RNA were unable to replicate and produce infectious virus in an *in vitro* setting hindering early HCV research (15, 84, 96). However, intraheptic inoculation of the HCV consensus clone into chimpanzees could produce a similar course of infection along with comparable disease characteristics as seen in humans, although milder (16, 17, 102). While the chimpanzee HCV model was instrumental in revealing the host immune response and pathogenesis related to HCV infection, the high costs, ethical concerns, and restricted accessibility to an animal facility approved to work with non-human primates limited the opportunities for many researchers to study the virus. Thus, the strict species tropism for humans and non-human primates which is characteristic of HCV combined with the lack of cell

culture systems limited progress of early HCV research. However, over the last 10 years, several experimental systems have been developed finally allowing investigation of HCV infection at every step of the viral life cycle.

1.2.2 **HCV Replicon System**

Ten years after the cloning of the HCV genome, Lohmann et al. succeeded in establishing efficient HCV replication *in vitro* using selectable subgenomic replicons (112). Analogous to the strategy used for other viruses, this system consists of a recombinant HCV genome in which the viral structural proteins were replaced by a selectable antibiotic resistant marker, in this case the neomycin phosphotranferase (Neo) gene. In this bicistronic construct the 5' non translated region was flanked by a T7 RNA polymerase promoter, a selection marker translated by the HCV internal ribosome entry site (IRES), the nonstructural proteins from an HCV GT 1b consensus clone translated via an inserted encephalomyocarditis virus (ECMV) IRES and finally the 3' end contained an engineered restriction site that allowed for run off RNA transcripts (112). Following *in vitro* transcription, the RNA was transfected into a human hepatoma cell line, Huh7, and antibiotic resistant clones were selected and expanded. HCV replication was confirmed by detection of negative-strand RNA synthesis by Northern blot. It was later found that the HCV replicons autonomously replicating within these cell clones acquired cell culture adaptive mutations that were required for the robust replication obtained(112). Notably, when the these adaptive mutations were introduced back into the wild type HCV genomic clone it rendered it unable to establish infection in chimpanzees following intrahepatic inoculation (23). This suggested that although these adaptive mutations were necessary to support robust replication *in vitro*, they were deleterious for other aspects of HCV infectivity. Since the development of the first HCV GT 1b subgenomic replicon system, replicons have been produced

for many of the HCV genotypes and the replicon system has proven to be an invaluable tool for studying the process of HCV replication. In particular the replicon has been used in the testing of antiviral compounds such as those targeting the viral protease protein, NS3 or the viral polymerase, NS5B. Replicon cells have also been used as reporter viruses allowing the study and visualization of the replicon complex (154).

1.2.3 **HCV Pseudotype Particle System**

While the development of the replicon system triggered the first wave of molecular HCV research, it only allows for the study of a limited aspect of viral life cycle. In 2003, two groups reported generating HCV pseudotype particles (HCVpp) that contained HCV glycoproteins E1 and E2 in their envelope and were capable of infecting permissive cell lines (13, 86). HCVpp is produced by transfecting a plasmid encoding the capsid protein of retroviruses such as human immunodeficiency virus (HIV) or murine leukemia virus (MLV) and a reporter gene flanked by the retrovirus LTR along with a plasmid expressing the E1 and E2 HCV glycoproteins into 293T cells. The reporter gene is packaged in to viral capsids and during capsid budding from the plasma membrane, the pseudotype virus acquires an envelope which contains the glycoproteins expressed on the cell surface consequently producing a reporter viral particle that superficially resembles HCV at the surface but is replication incompetent. HCVpp infected certain hepatoma cell lines and entry was dependent on both E1 and E2 glycoproteins. Furthermore, HCVpp entry was inhibited by anti-E2 monoclonal antibodies and HCV patient sera (12). This data was taken as evidence that HCVpp entry at least to some extent mimics HCV entry. Hence, the development of the HCVpp system has provided a tool to identify cellular receptors involved in HCV entry and aided in the screening of HCV entry inhibitors.

1.2.4 **HCV Cell Culture Infection System**

Finally, after several years of studying HCV with valuable yet limited cell culture systems a huge breakthrough in the field occurred. This was made possible when new a HCV consensus clone was generated from HCV RNA derived from the serum of a Japanese male patient with fulminant hepatitis. Sequence comparisons with other HCV clones revealed a clustering around genotype 2a. A subgenomic replicon of this clone, called Japanese Fulminant Hepatitis-1 (JFH-1), was constructed and replicated efficiently in Huh7 cells and non hepatic cells such as HeLa and HEK293 cells (90, 91). Importantly, efficient replication of the JFH-1 subgenomic replicon occurred independent of adaptive mutations.

Based on this unique lack of adaptive mutations, three groups investigated the infectivity of this clone in vitro and in 2005 reported the first HCV infection cell culture system that recapitulated the entire HCV life cycle including viral entry, replication and secretion of infectious HCV viral particles (109, 168, 186). The full length JFH-1 GT 2a consensus clone or chimeras containing structural proteins from clone H77 GT 1a or clone J6 GT 2a were *in vitro* transcribed and transfected into Huh7 or Huh7.5 cells. The Huh7.5 cell line is derived from Huh7 cells previously supporting HCV replicon replication and later cured by IFN treatment (20). This cell line supports higher levels of HCV RNA replication as compared to naïve Huh-7 cells most likely due to a single point mutation in the dsRNA sensor retinoic acid-inducible gene-I (RIG-I) rendering RIG-I signaling defective (20). Cells transfected with the HCV RNA were serially passaged without loss of infectivity and medium taken from these cultures was shown to infect naïve cells and viral titers were reported to be between 10^4-10^6 foci forming units/mL. Importantly, HCV produced in cell culture (HCVcc) is also infectious in chimpanzees and chimeric mice with livers repopulated with human hepatocytes. To confirm HCVcc had characteristics similar to HCV isolated from patients, neutralizing antibodies such as anti- E2 and

CD81 antibodies were shown to inhibit HCVcc entry and IFN-α inhibited replication. The ability to study the full life cycle has tremendously advanced HCV research allowing investigators to examine every aspect of the viral life cycle.

Although the development of the HCVcc infection system was a huge advancement in HCV molecular virology, some limitations of the system still restrict the study of HCV. For example, due to its strict tissue tropism, it was not a surprise that infection would be limited to human hepatoma cells. However, permissiveness for robust HCVcc infection is restricted to primary hepatocytes or the single transformed undifferentiated hepatic cell line, Huh7 and its derivatives (e.g Huh7.5 cells). Hence, we are able to study the viral life cycle, but are limited in the ability to accurately study HCV-host cell interactions particularly how HCV infection alters hepatocyte physiology. Notably however, variability in the degree of infectivity observed among different Huh7 cell lines suggests these cells may to some extent be useful for identifying host cell factors required for optimal HCV infection. Another major limitation of the current HCVcc infection system is that thus far, the JFH-1 GT 2a clone is the only isolate discovered with the ability to produce sufficient infectious virus in an *in vitro* setting. While chimeras constructed using the structural proteins of other HCV GTs and the nonstructural proteins from JFH-1 GT have been cloned, the study of the entire life cycle is limited to a single HCV GT and clone. This is less than ideal considering different HCV GT are associated with differing pathologies, have different infection kinetics and respond differently to IFN.

1.3 **HCV Life Cycle**

The development of the experimental model systems described above, particularly the full infection HCV cell culture system (10, 109, 186), has finally enabled investigation into the molecular details of the HCV viral life cycle. While our picture is still incomplete, the HCV life

cycle, originally illustrated in Lindenbach et al. (Fig 2), is made up of several steps beginning with the virion entering the hepatocyte. After initial attachment to the cell surface, which is believed to be mediated by heparan sulfate proteoglycans (11) and the low density lipoprotein receptor (LDL-R) (2, 119), several cellular factors are required for entry including scavenger receptor B-I (SRB1) (26, 153, 183), the tetraspanin CD81 (136, 184), and tight junction proteins claudin-1 (CLDN1) (50) and occludin (OCLN) (138). The virion is then internalized via clathrin-dependent, receptor-mediated endocytosis into the early endosome (18). Upon acidification of the endosome the viral particle membrane fuses with the host endosomal membrane inducing uncoating and release of the viral genome into the cytoplasm (163). After the viral positive-strand RNA genome is released into the cytoplasm it is translated by the host cell ribosomes into a single polyprotein of approximately 3000 amino acids via a cap-independent HCV IRES located in the 5' untranslated region (157). The polyprotein is then co- and post translationally cleaved by host and viral proteases into 3 mature structural proteins and 7 NS proteins (Fig. 1).

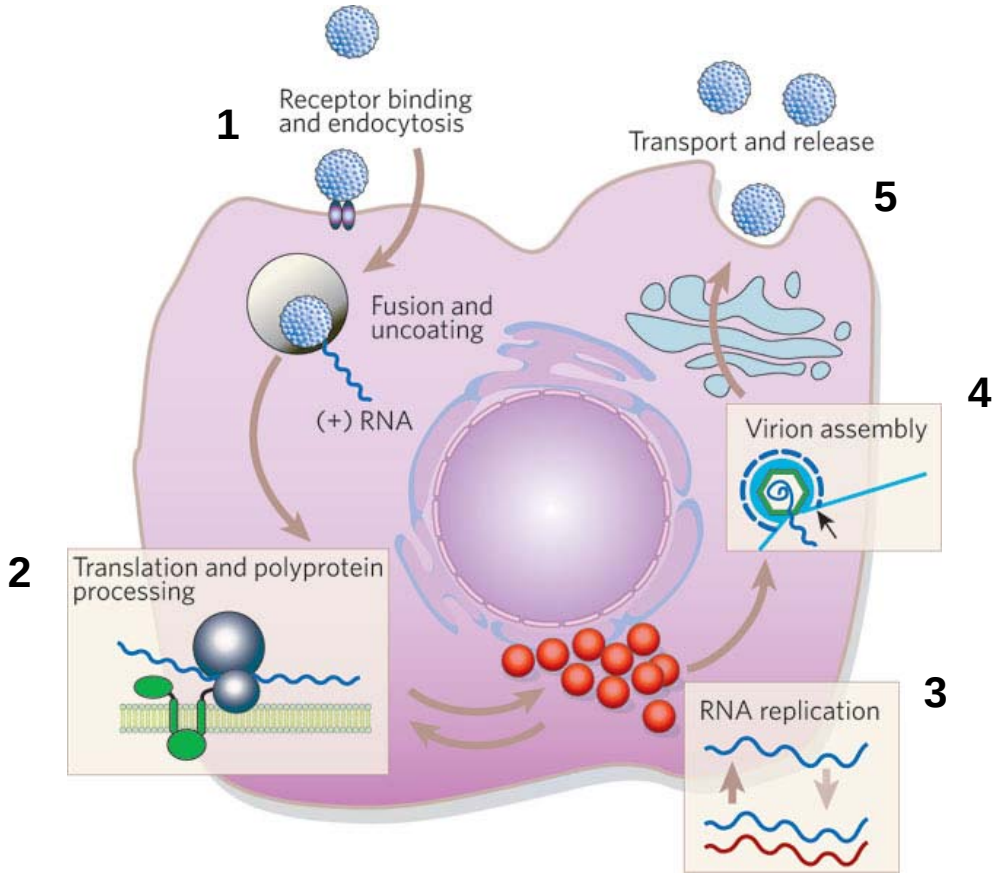

Figure 2. The HCV life cycle. 1) The virion attaches to the hepatocyte and sequential binds several host receptors. The virion is internalized via clathrin mediated endocytosis into the early endosome where fusion and uncoating occur and the genome is release into the cytosol. **2)** The genome is translated into a single polyprotein encoding an open reading frame which is then processed by host and viral proteases. **3)** Genome replication begins via the viral RNA dependent RNA polymerase which produces negative strand to be used as a replication template. **4)** Nascent viral genomes are then assembled and packaged into nascent virions. **5)** The virion is then transported and released from the cell. Figure adapted from (110)**.** Reprinted by permission from Macmillian Publishers Ltd: Nature, Lindenbach, B.D and Rice, C.M., 2005.

HCV replication begins after sufficient accumulation of the viral replication proteins, including the RNA-dependent, RNA polymerase (RdRp) viral protein NS5B, which synthesizes a complementary negative-strand RNA using the positive RNA genome as its template. These negative RNA strands are then utilized as templates by the same RdRp to produce more positive-strand RNA. Synthesis of positive-strand RNA genomes occurs at a level of 5 to 10 fold higher than that of negative-strand synthesis. The HCV replication complexes (RC) are contained in a membranous structure (aka the membranous web), which is in close proximity to and derived from the endoplasmic reticulum (ER) (48, 172). Transfection experiments have shown that expression of viral protein NS4B alone can induce the extensive ER membrane alteration required to form these vesicular structures (48, 49, 74). Using subgenomic replicons several groups have demonstrated that viral RNA replication occurs in crude membrane fractions and that all viral NS proteins are associated with the ER membrane (124, 135).

Notably, the positive-strand viral RNA that accumulates in the cell can either be translated to produce more viral proteins, used as a template to make more negative-strand RNA, or be encapsidated into newly assembled viral capsids to produce progeny virus. Because the HCVcc infection system was only recently developed, the details concerning how/if the use of the viral RNA in these various processes is temporally regulated is not known. Likewise, the process of viral assembly and secretion remain poorly defined. It is believed nucleocapsid formation is triggered when the viral core protein and positive-sense viral RNA interact and induce capsid oligomerization (101). After nucleocapsid formation, the virion buds into the ER where it acquires an envelope and the E1/E2 glycoproteins. Although much less is known about HCV assembly and secretion, assembly is dependent on the components of host very low density lipoproteins (VLDL) pathway. Several components of the VLDL such as ApoB, MTP, and

ApoE have been found to be involved in HCV assembly (27), with the regulation of all these independently being reported to prevent secretion of infectious viral particles.

1.4 HCV Entry

HCV entry is a dynamic, complex process that involves a multitude of steps. It is initiated when the viral particle attaches to the cell surface bringing it in closer proximity to its target receptors. Next, similar to other envelope viruses, HCV glycoproteins bind to a host cellular receptor(s). However, unlike most viruses which utilize only a primary and co-receptor to enter into their target cell, HCV utilizes many host cell surface receptors in a sequential pattern for entry into hepatocytes illustrated in Pietschmann et al. (Fig 3). It is thought that interaction with each cellular receptor likely induces some kind of change in the virion components and/or conformational change in the viral glycoproteins that prime the virion for its next engagement (118). The viral particle is then internalized via clathrin mediated endocytosis into early endosomes (18). The low pH of the early endosomes induces fusion of the viral membrane to the endosomal membrane. The viral genome is then released into the cytoplasm completing the entry process.

1.4.1 HCV Virion Components Involved in HCV Entry

To better understand the mechanisms underlying HCV entry, the viral envelope proteins have been thoroughly studied. E1 and E2 are both type I membrane proteins containing large N-terminal ectodomains and a C-terminal transmembrane domain. Both envelope proteins contain signal sequences within the C-terminus which lead to their anchoring in the ER membrane (34). They subsequently form non-covalent E1/E2 heterodimers (33). Residues within the

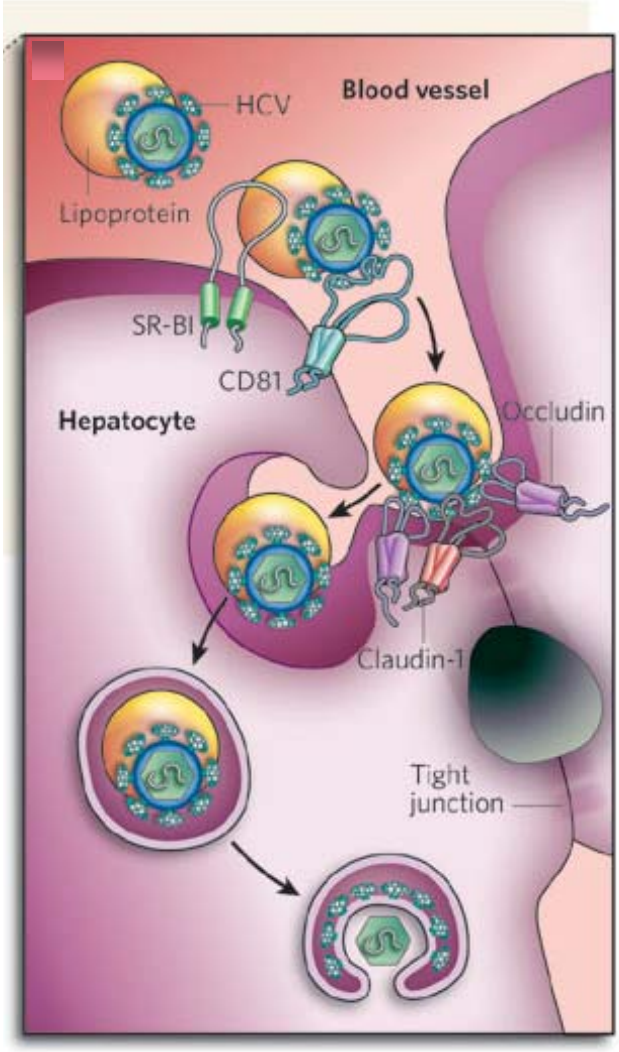

Figure 3. Diagram of current model of HCV entry. GAGs and LDL-R (not shown) mediate attachment of the HCV virion to the hepatocyte. After attachment to the host cell, the virion interacts with host cellular receptor SRBI and CD81 and then is transferred to the tight junction proteins CLDN and OCLN. Finally, the virion is internalized via clathrin dependent endocytosis into the early endosome where it fuses with the host cell membrane, uncoats and releases its genome into the cytosol. Reprinted by permission from Macmillian Publishers Ltd: Nature, Pietschmann et al., 2009.

hypervariable region 1 region have been shown to be important in E2 interaction with the HCV entry factor SRBI while blocking the CD81 binding site (9). Deletion of HVR1 significantly reduced binding of soluble recombinant E2 (sE2) protein to Chinese hamster ovary (CHO) cell lines expressing human SRBI but increased binding to CD81 in Molt 4 cells, a human T-cell leukemia cell line. These findings suggest the glycoproteins must bind to host receptors in a specific temporal process (i.e. the E1/E2 complex must first interact with SRB1 which induces a conformational change within the glycoproteins subsequently exposing its CD81 binding site). Domains within the E2 glycoprotein have also been found to be critical in virus escape from the immune response (82) . The HVR1, located in the N-terminus of E2, exhibits the highest degree of genetic heterogeneity and serves to masks conserved epitopes therefore protecting them from neutralizing antibodies. Virus particles in which this region is deleted display attenuated infectivity but are still infectious (141) .

Glycosylation has been found to be important for proper folding and heterodimer formation of functional E1/E2 complexes with highly conserved glycosylation sites found at 4 residues in the E1 protein and 11 residues in E2 (33-35, 72). Site directed mutagenesis of specific glycan sites on E1 and E2 prevented proper incorporation of E1 and E2 into HCVpp particles suggesting these sites were necessary for proper protein folding. The level of HCVpp entry is also affected after mutation of specific glycan sites that do not prevent glycoprotein incorporation suggesting glycosylation at certain sites may specifically be important for HCV entry (51). Finally, glycans have also been implicated in playing role in immune evasion. Glycosylation sites on E2 mask the CD81 binding site reducing HCV entry and reduce its sensitivity to neutralizing antibodies (82).

The E1/E2 glycoproteins also are believed to be necessary for viral fusion (103). Viral fusion proteins are classified into two major categories, either class I or class II (94). Because

HCV has been classified in the Flaviviridae family, the current belief is HCV envelope proteins are class II fusion proteins (132). Unlike class I fusion proteins which are synthesized as a precursor and the then cleaved by host proteases, class II fusion proteins are synthesized as a complex with one glycoprotein acting as the fusion protein and the other as a chaperone. The fusion peptide is released after cleavage of the chaperone protein which induces a conformational change in the fusion protein causing release of the fusion peptide and insertion into the plasma membrane. There is controversy over which glycoprotein acts as the fusion protein and which acts as the chaperone. Evidence for both glycoproteins containing sequence homology to other class II fusion proteins and potential fusion activity has been reported (103, 133).

Although technically derived from the host cell, lipoproteins represent another HCV virion component involved in HCV entry into host cells. HCV particles are enriched with cholesterol, low density lipoproteins and VLDLs leading them to be referred to as lipoviroparticles (8). Components of VLDL such as ApoB and ApoE play an important role in viral entry. Treatment with anti-ApoB or Apo-E antibodies inhibited HCVcc entry leading investigators to examine the role of lipoprotein receptors. The low density lipoprotein receptor (LDL-R) was discovered to be necessary for HCVcc but not HCVpp entry. Since HCVpp is not associated with lipids, it is proposed the LDL-R interaction with the viral particle is through the apolipoproteins associated with the virus. Most likely LDL-R serves to mediate primary attachment of the viral to the cell surface. High density lipoproteins (HDL) have also been shown to facilitate HCV entry (45, 167). However, this enhancement was not due to association with the viral particle but dependent on cholesteryl transfer to its receptor SRBI. SRBI has also been identified as an HCV entry factor and will be discussed in more detail below. As a whole, it has become apparent that lipids and cholesterol play a critical role in HCV infectivity.

1.4.2 **Previously Identified Host Cell Factors Involved in HCV Entry**

HCV has been found to use numerous host cellular proteins during the viral entry process beginning with the glycosaminoglycan (GAG) heparin sulfate and the LDL-R mediating attachment of the viral particle to the cell surface. After the viral particle is anchored to the cell it is then thought to be better able to engage more specific host cell binding factors. The first entry factor discovered to bind the HCV E2 glycoprotein was the tetraspanin CD81 (136). Next, the lipoprotein receptor SRB1 was shown to be required for HCV entry followed by the tight junction proteins claudin 1 (50) and occludin (138) . Each protein will be discussed below in the order of discovery.

CD81 is a 26kD protein and consists of four transmembrane helices, a small extracellular loop (EC1), a large extracellular loop (EC2) and short N and C terminal tails. CD81 is located on the cell surface and is ubiquitously expressed on all cell types except platelets. Tetraspanins interact with one another and form homo- or heterodimers complexes called tetraspanin enriched microdomains (TEMS) and are involved in biological processes such as adhesion, morphology, proliferation, differentiation and cell signaling. CD81 was first discovered to interact with sE2 through the use of cDNA library screening (136). A region within the CD81 EC2 was determined to be the binding site for E2 and was further confirmed after binding assays were performed using CD81-CD9 chimeras. Conserved residues within the E2 protein important for CD81 recognition have also been described. The role of CD81 in HCV entry has been further confirmed using the HCVpp and HCVcc infection systems. Furthermore, expression of CD81 in the normally CD81-deficient non permissive hepatoma cell line HepG2 rendered them permissive to HCVpp and HCVcc infection (184). Characteristic of tetraspanin membrane proteins, CD81 associates with several different partner proteins. One of its partner proteins

EWI-2, a member of a family of immunoglobulins is cleaved and produces EWI-2wint. Its cleavage product, which also interacts with CD81, has been demonstrated to block E2-CD81 binding (184). Inhibition of HCVpp entry and HCVcc infection is observed when EWI-2wint is ectopically expressed. Interestingly, while the cleavage product EWI-2wint is found in most cells it is absent in hepatocytes making this a potential factor for the hepatotropism seen with HCV infection (120, 146).

SRBI is a lipoprotein receptor that mediates selective cholesteryl ester uptake from high density lipoprotein (HDL) (37). The 85kD glycoprotein receptor is located on the cell surface, consists of 509 amino acids (aa) and contains a single large extracellular domain flanked by cytoplasmic N and C terminal domains. SRBI is expressed mainly in the liver and other steroidogenic tissues like adrenal glands and the ovary. Other known ligands of SRBI include LDL, oxidized LDL (oLDL) and VLDL (160) . Binding studies using a soluble form of E2 identified SRB1 as a putative entry receptor (153). The role of SRBI was later confirmed after observing inhibition of HCVcc and HCVpp infection following siRNA knockdown and blocking the cell surface protein using specific antibodies (76, 167, 183). Several groups have mapped the regions in the E2 glycoprotein that mediate SRBI binding and found that the HVR1 is required with deletion of this region resulting in significantly reduced HCV entry (9) . Recent studies utilizing human/mouse chimeras of SRBI in E2 binding assays found that aa 70-87 were important for E2 recognition (26). A point mutation in the receptor at E210A also showed a significant effect on E2 binding (26). As expected, mutants with decreased E2 binding also had a negative effect on HCV infection, however did not affect SRBI oligomerization or HDL ligand binding suggesting E2 and HDL do not compete for the same binding site. Additionally, Evans et al. (50) expressed human SRBI on CHO cell lines and observed HCVcc viral particle binding. The effect of SRBI ligands on HCV infection has also been investigated and an increase in HDL

was observed to enhance infection (45, 167). The time at which the virion interacts with SRBI has been determined by observing the kinetics of HCV infection inhibition seen when performing time-of-antibody addition experiments using anti-SRBI antibodies. Anti-SRBI was shown to retain inhibitory activity up to 60 minutes post binding demonstrating SRBI acts early in the viral life cycle at a post binding step, likely before CD81 (26, 183) .

More recently two tight junction proteins were identified as HCV entry factors, CLDN1 and OCLN (50, 138). Claudin-1 is a 23kD four transmembrane tight junction protein belonging to a family consisting of 24 members responsible for tight junction formation. CLDN1 is expressed in all epithelial tissues but predominantly in the liver and functions to regulate paracellular permeability and polarity. CLDN1 was identified as an HCV entry factor, after screening a cDNA library derived from the highly permissive Huh7.5 cell line for genes that conferred susceptibility of 293T cells to HCVpp (50, 138). It was the first receptor identified to confer HCV susceptibility to a non hepatic cell line. Similar experiments such as siRNA knockout and antibody blocking were performed to confirm the role of CLDN1 in HCV infection (50). Although no direct binding between the HCV glycoproteins and CLDN1 has been observed, mapping studies found residues, specifically I32, D38, and E48 in the first extracellular loop are important in mediating HCV entry. Evans et al. hypothesized an earlier engagement between the glycoproteins and SRBI or CD81 may be required to induce a conformational change in the E1/E2 heterodimer exposing the necessary epitopes for CLDN1 binding (50). This hypothesis was further supported after kinetics of antibody inhibition showed the CLDN1 antibody maintained its inhibitory activity longer than CD81 suggesting it was a late acting entry factor (50).

OCLN, another four transmembrane domain protein, was identified in a similar cDNA library screen as that used to identify CLDN (138). However, unlike in the CLDN1 screen,

genes that rendered the non permissive mouse embryonic fibroblast NIH3T3 cell line susceptible to HCVpp infection were the chosen candidates. OCLN enhanced HCVpp infection in NIH3T3 cells by approximately 120 fold. Silencing of OCLN inhibited HCVpp and HCVcc infection. Like CLDN1, a direct interaction with the glycoproteins was not seen but residues critical in HCV entry has been mapped to the second part of the second extracellular loop (138). Furthermore, similar to CLDN1, kinetic studies indicate OCLN acts at a post binding step and is a late acting entry factor as well. The discovery of OCLN also advanced our understanding of HCV species tropism as expression of the human OCLN and CD81 rendered the mouse fibroblast cell line NIH3T3 permissive to HCVpp infection (138). However, entry of HCVcc into NIH3T3 cells could not be achieved suggesting that there was an E1/E2-independent host cell HCV entry factor required for HCVcc entry into mouse cells yet to be identified.

1.4.3 **HCV Internalization and Fusion**

The mechanism of enveloped virus uptake upon contact with the cell surface has been well studied. Two different strategies have been determined to facilitate virus internalization. For instance, retroviruses deliver their genome into the cytoplasm by directly fusing with the plasma membrane after their glycoproteins contact its cognate receptors, while influenza A internalization is mediated by glycoprotein binding to its receptor which then triggers uptake into endocytic vesicles. The change in pH triggers the fusion machinery which facilitates fusion of the viral membrane with the endocytic membrane in turn leading to uncoating and genome release into the cytoplasm. While the specific interaction required to induce HCV internalization is still unknown, HCV internalization has been demonstrated to occur via receptor-mediated, clathrin-dependent endocytosis. The importance of clathrin was demonstrated after treatment with chlorpromazine, a drug which causes clathrin to assemble on endosomal membranes

therefore preventing assembly on coated pits at the plasma membrane, significantly reduced subsequent intracellular expression levels of HCV viral proteins (18). Evidence to further support the role of clathrin was observed after siRNA knockdown of the clathrin heavy chain also resulted in a decrease in HCV infection as measured by a decrease in intracellular viral protein expression.

HCV is internalized into endocytic vesicles suggesting the viral particle must fuse with the endosomal membrane to allow viral genome escape into the cytoplasm thereby completing the entry process. After treating cells with either NH_4Cl or Concanamycin A, inhibitors of endosomal acidification, entry of HCV reporter viruses was significantly reduced suggesting fusion is pH dependent (163). Generally when enveloped viruses that enter in a pH dependent manner are exposed to acidic pH a conformational change is induced in the glycoprotein releasing the fusion peptide causing premature fusion at the cell surface, but interestingly, HCV is relatively insensitive to low pH treatment suggesting that HCV may require an additional trigger or priming event to become acid sensitive. Recently, Sharma et al. suggested the E2 interaction with CD81 is the event needed to prime HCV for fusion (155).

1.4.4 **Modes of HCV Spread**

On a cellular level, once HCV has infected one cell, it can spread to naïve cells through at least two different pathways, either via cell-free virus or cell-to-cell spread. The strategy of infecting cells by cell-to-cell contact enables the virus to avoid the humoral immune response making this mode of transmission very advantageous in the context of an immunocompetent host (21). This means of spread is observed when naïve cells are co-cultured with HCV infected cells in the presence of neutralizing anti-E2 antibodies (158). Under these conditions, the naïve cells immediately surrounding the infected cells become positive for HCV. Because the cellular

factors required for cell-to-cell spread are not necessarily the same as those required for cell-free virus entry, the role of previously identified cell-free HCV entry factors in cell-to-cell spread has become of interest. Knowing which HCV cell-free entry factors are required for cell-to-cell spread is relevant not only in terms of identifying the most effective antiviral targets, but may help us to further understand the molecular details of HCV entry.

Not surprisingly, the tight junction proteins, claudin 1 and occludin, are necessary for HCV cell-to-cell spread (158, 176). Naïve target cells either treated with anti-CLDN1 antibodies or transfected with shRNAs targeting OCLN and then cocultured with HCV infected cells were completely resistant to cell-to-cell HCV transmission demonstrating that both tight junction proteins are necessary for this mode of transmission. SRBI, a factor believed to act early in the entry process and bind to the E2 glycoprotein, has also been implicated in facilitating cell-to-cell spread (21). While not as absolute, in the presence of SRBI antibodies or SRBI inhibitors cell-to-cell HCV transmission was reduced while over expression of SRBI promoted cell-to-cell spread . Furthermore, when using a mutant virus, JFH-1 G451R whose entry has been shown to be less dependent on SRBI less cell-to-cell spread was observed. The role of CD81 is more controversial with some reports saying HCV cell-to-cell transmission is CD81 dependent while others have observed the CD81 is not required (21, 158, 176).

1.5 **Significance of This Study**

HCV entry is a complex multi-step process that involves numerous host cell receptors at different steps. Previous studies have shown host cellular receptors, SRBI, the tetraspanin, CD81, and two tight junction proteins, CLDN and OCLN are required for HCV entry. While the discovery of the involvement of these cellular factors has advanced our knowledge in the area of HCV entry, the expression of these proteins on non permissive cell lines does not confer robust permissiveness to cell culture produced HCV (HCVcc) entry or clinical HCV isolates suggesting more entry factors have yet to be identified.

In this study we aimed to further our understanding of HCV entry. We identified two host cell proteins, TfR1 and NPC1L1, required for HCV infection and demonstrated that they both act at the level of HCV entry. This study, therefore, advances the knowledge in the field regarding the molecular event involved in the dynamic, complex HCV entry process. More specifically, the HCV entry factors we identified may answer critical questions that explain the mechanism of virion internalization and provide answers concerning species tropism that may finally lead to the development of a mouse model. The identification of TfR1 may provide insight into the iron overload phenotype seen in HCV patients. Furthermore, NPC1L1 appears to have therapeutic potential for which there is already and FDA approved drug which may prove to be an effective entry inhibitor.

2. MATERIALS AND METHODS

2.1 Cells

Huh7 human hepatoma cells (186) were obtained from Dr. Francis Chisari at The Scripps Research Institute, La Jolla, CA and were cultured as described below. 293T species type cells and CHO species type cells were purchased from the American Type Culture Collection (Manassas, VA). The Clone B HCV genotype 1b sub-genomic replicon (sg1b) Huh7.5 cells were obtained from Dr. Charles Rice (Rockefeller University, NY) through the NIH AIDS Research and Reference Reagent Program and have been previously described (19). The HCV sg2a replicon was established as previously described (99, 164), using the HCV genotype 2a JFH-1 subgenomic replicon encoding vector pSGR-JFH1 (90) (kindly provided by T. Wakita, National Institute of Infectious Diseases, Tokyo, Japan).

Huh7-based cell lines and 293T cells were cultured in Dulbecco's modified Eagle's medium (cDMEM) (Hyclone, Logan, UT) and CHO cells were cultured in Ham's F-12 medium. Both mediums were supplemented with 10% fetal bovine serum (FBS) (Hyclone), 100 units/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (Gibco Invitrogen, Carlsbad, CA). In addition HCV replicon cells were maintained in 500 µg/ml geneticin (Invitrogen), CHO-hTfR1 clones were maintained in 500 µg/ml zeocin (Invitrogen), and CHO-hCD81 and hSRBI clones were maintained in 600 µg/ml geneticin (Invitrogen).

2.2 HCVcc Generation

The plasmid containing the full-length JFH-1 HCV genome (pJFH1) was kindly provided by T. Wakita (National Institute of Infectious Diseases, Tokyo, Japan) and has been previously described (168). The plasmids containing the JFH-1-based inter-genotypic HCV clones were kindly provided by J. Buhk (National Institutes of Health, Bethesda, MD) and have been

previously described (75, 90, 91). Protocols for genomic JFH-1 RNA in vitro transcription and electroporation in to Huh7 cells have been described elsewhere (181). The HCVcc viral stocks were generated by infection of naïve Huh7 cells at a multiplicity of infection (MOI) of 0.01 focus forming units (FFU)/cell using medium collected from Huh7 cells electroporated with *in vitro* transcribed JFH-1 RNA as previously described (181).

2.3 **Reagents and Antibodies**

The mouse anti-human CD81 monoclonal antibody (MCA1847, clone ID6) was purchased from AbD Serotec (Raleigh, NC), the mouse anti-human SR-BI monoclonal antibody was purchased from BD Biosciences (Franklin Lakes, NJ), the rabbit anti-human CLDN1 monoclonal antibody was purchased from AbCam (Cambridge, MA) and the mouse anti-human OCLN monoclonal antibody was purchased from Invitrogen. The mouse anti-human TfR1 monoclonal antibody (clone M-A712) used for antibody blocking experiments was purchased from BD Biosciences (Franklin Lakes, NJ), and the mouse anti-human TfR1 monoclonal antibody (clone 66IG10) used for flow cytometry was purchased from Hycult Biotech (Plymouth Meeting, PA). The rabbit anti-human NPC1L1 polyclonal antibodies targeting extracellular loops 1, 2 and 3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), Cayman Chemicals (Ann Arbor, MI) and Dr. Yiannis Ioannou (Bsn4052 (41), Mount Sinai School of Medicine, NY), respectively. The rabbit anti-human NPC1L1 polyclonal antibody used for western blot analysis was purchased from Cell Signaling Technology (Danvers, MA). The human anti-HCV E2 glycoprotein monoclonal antibody (C1), a gift from Drs. Dennis Burton and Mansun Law at The Scripps Research Institute, La Jolla, CA, and the mouse anti-HCV NS5A monoclonal antibody (E910), a gift from Dr. Charles Rice at The Rockefeller Institute, New York, NY, have both been previously described (104, 109, 186). The monoclonal mouse anti-

HCV NS3 antibody (Clone 9-G2) was obtained from ViroGen (Watertown, MA). The HRP-conjugated anti-human, anti-mouse and anti-rabbit secondary antibodies were purchased from Pierce (Rockford, IL). The anti-mouse Alexa-555- and anti-rabbit Alexa-488-conjugated antibodies were from Invitrogen. Negative control mouse and rabbit IgG antibodies were from Santa Cruz Biotechnology. The cell-tracking fluorescent probe 5-chloromethylfluorescein diacetate (CMFDA) was purchased from Invitrogen and used to label live Huh7 cells (5μM) as described by Krieger *et al*. (100). Purified ezetimibe used for *in vitro* studies was purchased from Sequoia Research Products (Pangbourne, United Kingdom). For *in vivo* mouse studies, 10mg tablets of Zetia® (Schering Corporation, Kenilworth, NJ) were powderized and resuspended in corn oil. The nucleoside polymerase inhibitor NM107 (a gift from Michael J. Otto, Pharmasset, Inc., Princeton, NJ), the TfR1 inhibitor NSC306711 (National Cancer Institute) and the SRBI inhibitor ITX5061 (iTherX) were resuspended to concentrations of 100 mM, 10 mM, 10mM, and 1mM respectively in DMSO (Sigma) and stored at -20°C.

2.4 **DNA Constructs**

The human SR-BI expression plasmid (pZeo_huSR-B1) was cloned by PCR amplifying the human SR-BI ORF with primers 5'AGG CAA GCT TGC CGC CAT GGG CTG CTC CGC CAA AGC GCG CTG GG 3' (sense) and 5' CCA GTC TAG ACT ACA GTT TTG CTT CCT GCA GCA CAG AGC CC 3' (anti-sense). This product was cloned as a HindIII/XbaI fragment into HindIII/XbaI-digested pZeoSV2(-) (Invitrogen). The JFH-1 E1/E2 glycoprotein expression plasmid (pCDNA3.1_JFHcE1/E2) was cloned by PCR amplifying the polyprotein residues 167-751 of pJFH-1 with primers 5'-GAA TTC ATG GGG AAC CTA CCC GG-3' (sense) and 5'-CTC TAG ACT ATG CTT CGG CCT GG-3' (anti-sense). This product was cloned as an EcoRI/XbaI fragment into EcoRI/XbaI-digested pCDNA3.1 (Invitrogen). The vesicular

stomatitis virus (VSV) G glycoprotein expression plasmid (pCDNA3.1_VSVG) was cloned by PCR amplifying the VSVG coding sequence from pET-3c (Novagen, Gibbstown, NJ) with primers 5'- GAA TTC ATG AAG TGC CTT TTG TAC TTA GCC -3' (sense) and 5'-CTC TAG ATT ACT TTC CAA GTC GGT T-3' (anti-sense). This product was cloned as an EcoRI/XbaI fragment into EcoRI/XbaI-digested pCDNA3.1 (Invitrogen). The human TfR1 expression plasmid (pZeo_TfR1) was cloned by PCR amplifying the human TfR1 ORF with primers 5'TAA GAA TTC ATG ATG GAT CAA GCT AGA TCA G 3' (sense) and 5' TAA GGA TCC TTA AAA CTC ATT GTC AAT GTC 3' (anti-sense). This product was cloned as a BamHI/EcoRI fragment into BamHI/EcoRI digested pZeoSV2(-) (Invitrogen).

2.5 **Pseudotype Particle Production and Infection**

Pseudotyped viruses were produced as previously described (149). Briefly, pseudotyped viruses were generated by co-transfection of DNA vectors encoding the HCV E1/E2 or VSV G envelope glycoproteins with an Env-deficient HIV vector carrying a luciferase reporter gene (pNL4-3-Luc-R^--E^-) into 293T producer cells. Supernatants were collected 48h post transfection, filtered through a 0.45 μm-pore-size filter (BD Biosciences), aliquoted, frozen and subsequently p24 titers were determined using the QuickTiter Lentivirus Titer Kit (Cell Biolabs, Inc., San Diego, CA) according to the manufacturer's instructions. Infectivity titers were determined 72 h p.i. by lysing infected cultures in 20 μl of lysis reagent to measure luciferase activity (Promega, Madison, WI) using a FLUOstar Optima microplate reader (BMG Labtechnologies Inc, Durham, NC).

2.6 **Treatment of Cells and Infection**

Naïve Huh7 and Huh7 cells harboring HCV subgenomic replicons were seeded and cultured as described above. RNA silencing experiments were performed by reverse transfection (Lipofectamine™ RNAiMAX, Invitrogen) of Huh7 cells with indicated siRNAs. For infection experiments, transfected cells were infected with HCVcc at indicated times post-transfection. For NPC1L1 antibody experiments, cells were treated with 36 μg/ml of indicated antibodies prior to and during infection. For ezetimibe inhibition experiments, cells were vehicle-treated or treated with increasing concentrations of ezetimibe prior to infection (PRE), during the time of virus inoculation (CO), and/or following virus inoculation. Ezetimibe concentrations used in this study, 3.125 - 30 μM (i.e. 1.5 – 12.28 μg/ml culture medium) are consistent with previous published reports and are additionally in line with patient daily intake concentrations of 10mg/day (i.e. 2.0 - 3.3 μg/ml serum) (28, 68, 174). For TfR1 antibody and inhibitor experiments, cells were treated with 25 μg/ml of indicated antibodies and 50uM of inhibitor prior to and during infection. For RTqPCR analysis, total cellular RNA was extracted in 1X Nucleic Acid Purification Lysis Solution (Applied Biosystems, Foster City, CA) at indicated times post-infection. For HCV E2-positive foci analysis, infected cells were fixed with 4% paraformaldehyde (w/v) 72 hours p.i., and immunocytochemical staining for HCV E2 was performed (as described in section 2.8).

2.7 **Indirect Immunofluorescence Staining**

As previously described in Sainz et al. (149), Huh7 cells were fixed with 4% PFA (Sigma) at indicated times. Fixed cultures were then rinsed three times with 1X PBS, further permeabilized with 50% Methanol/50% acetone (v/v) (Fisher) and subsequently blocked for 1 hour with 1X PBS containing 3% (w/v) bovine serum albumin (BSA) (Sigma) and 10% (v/v)

FBS. Cells were stained with a 1:750 dilution of indicated primary antibody overnight at 4°C, followed by incubation with a 1:750 dilution of an anti-mouse Alexa-555 or anti-rabbit Alexa-488 conjugated secondary antibody (Molecular Probes) for 1 hour at room temperature. Cell nuclei were stained by Hoechst dye. Bound antibodies were visualized via confocal microscopy (63X, Zeiss LSM 510, Germany) and compared to negative control samples stained with irrelevant mouse or rabbit IgG control antibody (Santa Cruz Biotechnology) and appropriate Alexa-555- or Alexa-488-conjugated secondary antibody. Images were analyzed using Zeiss LSM Alpha Imager Browser v4.0 software (Zeiss). Brightness and contrast were adjusted using Adobe®Photoshop® (San Jose, CA).

2.8 Immunocytochemical Staining of HCV Foci

As previously described in Sainz et al. (149), for immunocytochemical staining of HCV E2-positive foci fixed cells were first incubated with 1X PBS containing 0.3% (v/v) hydrogen peroxide (Fisher, Fairlawn, NJ) to block endogenous peroxidase. Following three rinses with 1X PBS, cells were blocked for 1 hour with 1X PBS containing 0.5% (v/v) Triton X-100 (Fisher), 3% (w/v) bovine serum albumin (BSA) (Sigma) and 10% (v/v) FBS. The HCV E2 glycoprotein was bound with antibody by incubation at room temperature with 1X PBS containing 0.5% (v/v) Triton X-100 and 3% (w/v) BSA and a 1:500 dilution of the human monoclonal anti-HCV E2 antibody C1. Bound C1 was subsequently detected by a 1 hour incubation with a 1:1000 dilution of an HRP-conjugated anti-human antibody followed by a 30 minute incubation with an AEC detection substrate (BD Biosciences). Cells were washed with dH_2O and foci were quantified and photographed using a Zeiss Axiovert microscope (Carl Zeiss, Germany).

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2.9 **RNA Interference**

Pools of small interfering RNA oligonucleotides (siRNAs) targeting human NPC1L1 (Silencer® Select siRNAs s26623, s26633, and s26634) were purchased from Ambion (Austin, TX). Pools of siRNAs targeting human TTP (Silencer® Select siRNAs s24313, s24314, and s24315) were purchased from Ambion (Austin, TX). Individual siRNAs directed against human TfR1, human NPC1L1, human CD81, human SR-BI and EGFP (Control siRNA) were purchased from Ambion or Qiagen (Valencia, CA). Huh7 cells, seeded in 75-cm^2 BioCoat™ flasks (BD Biosciences) and differentiated in the presence of cDMEM supplemented with 1% DMSO (Sigma) for 20 days or seeded in 96 well plates (BD Bioscience) and cultured in DMEM were reverse transfected using Lipofectamine™ RNAiMAX Transfection Reagent as per the manufacturer's instructions. Briefly, in growing or non growing cells a transfection mix consisting of 1µl RNAiMAX and 70 nM or 12nM siRNA in OptiMem (Invitrogen) was placed in each well and incubated for 20 minutes at room temperature. Sixty thousand Huh7 non growing cells or five thousand growing cells were then seeded with the transfection mix in 96-well BioCoat™ plates. Twenty-four hours post-seeding 200 µl of fresh cDMEM supplemented with 1% DMSO (Sigma) was added to each well. At indicated times post-transfection, cultures were either mock-inoculated or inoculated with JFH-1 HCVcc at an MOI of 0.05 FFU/cell, and total cellular RNA was extracted in 1X Nucleic Acid Purification Lysis Solution (Applied Biosystems) from duplicate or triplicate wells at the indicated time points p.i. for RTqPCR analysis. To assess protein knockdown, at indicated times post-transfection cultures were either trypsinized for flow-cytometric analysis, fixed in 4% PFA (v/v) for indirect immunofluorescence analysis or lysed in 1.25% Triton X-100 lysis buffer for western blot analysis.

2.10 **Flow Cytometry**

Untreated, vehicle-treated, ezetimibe-treated, HCVcc infected or transfected CHO or Huh7 cells were resuspended in 150 µl of FACS buffer (1X PBS containing 2% (v/v) FBS, 0.3% (w/v) NaN_3 and 1mM EDTA) and incubated for 60 min at 4°C with a 1:100 dilution of antibodies specific for CD81 (AbD Serotec), SR-BI (BD BioSciences), CLDN1 (AbCam), OCLN (Invitrogen), TfR1 (Hycult Biotech) or NPC1L1 (Santa Cruz Biotechnology). Following three rinses with FACS buffer, bound antibodies were detected by incubation for 1 hour at 4°C with phycoerythrin (PE)-conjugated anti-mouse (BD Pharmingen) (for TfR1, CD81, SR-BI and OCLN) or anti-rabbit (Santa Cruz Biotechnology) (for NPC1L1 and CLDN1) antibodies at a dilution of 1:200. Cells stained with irrelevant immunoglobulin G antibodies and respective PE-conjugated secondary antibody served as negative controls. Cells were washed three times, fixed in FACS buffer containing 4% (w/v) PFA, and analyzed by flow cytometry using the DakoCytomation CyAn system (Dako, Carpinteria, CA) and Summit Software v4.3 (Dako).

2.11 **RNA Isolation and RT-qPCR**

As previously described in Sainz et al. (149), total intracellular RNA was purified using an ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems), as per the manufacturer's instructions. One µg of purified RNA was used for cDNA synthesis using the TaqMan reverse transcription reagents (Applied Biosystems), followed by SYBR green RTqPCR using an Applied Biosystems 7300 real-time thermocycler (Applied Biosystems). Thermal cycling consisted of an initial 10 minute denaturation step at 95 °C followed by 40 cycles of denaturation (15 seconds at 95 °C) and annealing/extension (1 minute at 60 °C). HCV, HIV, human GAPDH, murine GAPDH, NPC1L1, SR-BI, and CD81 RNA levels were determined relative to standard curves comprised of serial dilutions of plasmids containing the JFH-1 HCV

cDNA, the Env-deficient HIV backbone containing a luciferase reporter gene (pNL4-3-Luc-R$^-$ -E$^-$) or the human GAPDH, murine GAPDH, human NPC1L1 or human SR-BI coding sequences, respectively. The PCR primers used to amplify each respective amplicon were: Universal HCV primers (97) 5'-GCC TAG CCA TGG CGT TAG TA -3' (sense) and 5'- CTC CCG GGG CACTCG CAA GC-3' (anti-sense), HIV (32) (sense) 5'-AGT TGG AGG ACA TCA AGC AGC CAT GCA AAT-3' and (anti-sense) 5'-TGC TAT GTC AGT TCC CCT TGG TTC TCT-3', human GAPDH (186) 5'- CAA GAT CAT CAG CAA TGC CT -3' (sense) and 5'-AGG GAT GAT GTT CTG GAG AG-3'(anti-sense), murine GAPDH (148) 5'-TCT GGA AAG CTG TGG CGT G-3' (sense) and 5'-CCAGTGAGCTTCCCGTTCAG-3' (antisense), human NPC1L1 (55) 5'-TAT GGT CGC CCG AAG CA-3' (sense) and 5'-TGC GGT TGT TCT GGA AAT ACT G-3' (anti-sense), human SR-BI (138) 5'-TCG CAG GCA TTG GAC AAA CT-3' (sense) and 5'-CTC CTT ATC CTT TGA GCC CTT TT-3' (anti-sense), human TfR1 5'-CAG CCC AGC AGA AGC ATT-3' (sense) and 5'-CCA AGA ACC GCT TTA TCC AG-3' (anti-sense), and human TTP (sense) 5'- ACC ATC TCT ACG TCT TGG AC-3' and (anti-sense) 5'- TAT CAA TCA TAC CGC TGT CAC-3'.

2.12 **Western Blot**

As previously described in Sainz et al. (149), cells were harvested in 1.25% Triton X-100 lysis buffer (Triton X-100, 50mM Tris-HCl, pH7.5, 150mM NaCl, 2mM EDTA) supplemented with a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Fifty micrograms of protein was resolved by SDS-PAGE and transferred to Hybond nitrocellulose membranes (Amersham Pharmacia, Piscataway, NJ). Membranes were sequentially blocked with 5% nonfat milk, incubated with a 1:1000 dilution of a polyclonal rabbit anti-human NPC1L1 antibody (Cell Signaling Technology) or a 1:1000 dilution of monoclonal mouse anti-HCV NS3 antibody

(Clone 9-G2, ViroGen), washed 3 times with 1X PBS containing 0.05% Tween20 (v/v), incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody, and washed again to remove unbound antibody. Bound antibody complexes were detected with SuperSignal chemiluminescent substrate.

2.13 Cell Proliferation and Cytotoxicity Bioluminescence Assays

As previously described in Sainz et al. (149), the ViaLight® Plus Cell Proliferation assay kit (Lonza, Walkersville, MD), which incorporates bioluminescent detection of cellular ATP as a measure of cell viability and proliferation, was used according to the manufacturer's instructions. Briefly, vehicle treated and ezetimibe-treated cultures were lysed in Cell Lysis reagent for 10 min. One hundred µl of culture medium was transferred to white 96-well plates (BD Biosciences) containing ATP detection reagent, and luminescence, expressed as relative light units (RLU), was measured (FLUOstar OPTIMA, BMG Labtech). To assess drug-induced cellular toxicity, a bioluminescence-based assay (The Toxilight BioAssay Kit, Lonza) that measures adenylate kinase (AK) released from damaged cells was used as per the manufacturer's instructions. Briefly, 20 µl of supernatant was collected on indicated days and transferred to white 96-well plates (BD Biosciences). One hundred µl of AK detection reagent was then added to each well, and luminescence (RLU) was measured (Fluostar OPTIMA).

2.14 Binding Assay

Untransfected CHO cells and CHO cell lines transfected with human SRBI, human CD81, or human TfR1 expression plasmids were seeded in 12 well plates and inoculated with 400 µls of HCVcc. Plates were incubated at 4º C for 1 hour, removed and washed 3 times with

1X PBS to remove any non specific bound virus. Cellular and surface bound viral RNA was extracted in 1X Nucleic Acid Purification Lysis Solution (Applied Biosystems, Foster City, CA) for RTqPCR analysis (described above in section 2.11).

2.15 **HCV Infection in Chimeric Mice**

As previously described in Sainz et al. (149), all mouse studies were conducted with protocols approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University. Male uPA-SCID mice transplanted with human hepatocytes (BD Biosciences) were purchased from PhenixBio. Mice were treated daily with 10 mg per kg body weight ezetimibe via oral gavage of a 0.02 mg ml^{-1} solution of ezetimibe resuspended in corn oil (100 µl $20g^{-1}$) for a total of 3 weeks, with treatment initiation beginning 2 weeks, 1 week or 2 d before infection. Control mice were treated via oral gavage with corn oil alone (100 µl per 20 g body weight). A total of four to seven mice were included in each group. On day 0, we intravenously inoculated mice with serum from HCV-infected humans containing 1.0×10^5 copies of HCV genotype 1b. Mouse serum samples were obtained for HCV RNA or human albumin determination by RT-qPCR and Alb-II Kit (Eiken Chemical), respectively.

3. IDENTIFICATION AND FUNCTIONAL ANALYSIS OF TfR1 AS AN HCV ENTRY FACTOR

3.1 Background on TfR1

TfR1 is a well characterized protein first described in 1959 (87). Its primary function is cellular iron uptake and it is therefore important for many biological functions such as DNA synthesis and regulation of cell growth. Consequently, embryonic development is dependent on TfR1 with knockout mice embryos failing to survive past day 12.5 due to erythopoiesis and neurological defects (108). TfR1 is a ubiquitously expressed type II transmembrane homodimer cell surface glycoprotein found on the basolateral side of polarized cell (illustrated in Fig 4A). It is expressed at higher levels in proliferating and cancer cells. Each monomer is approximately 95kD and consists of 3 domains, an intracellular domain, a transmembrane domain and a large extracellular domain. The extracellular domain of each monomer is further divided into 3 subdomains an apical domain, a protease-like domain resembling carboxy- and amino-peptidases but with no evident protease activity and a helical domain required for noncovalent subunit dimerization. In addition, monomers are joined by two disulfide bonds. Post translational modifications are critical for TfR1 functioning. Specifically, N-linked glycosylation is necessary for proper protein folding and protein transport to the cell membrane and palmitylation of residues within the transmembrane domain aids in anchoring TfR1 within the cell membrane. No known function has yet been identified for the apical domain.

The mechanism of TfR1 mediated iron uptake has been extensively investigated and is diagramed in Fig. 4B. Cellular iron uptake is initiated when iron binds transferrin (Tf), a serum protein containing two binding sites for ferric iron (Fe^{3+}), binds TfR1 at the cell surface. The iron-Tf complex, also called holo-Tf which has a higher binding affinity to TfR1 at physiological pH, is internalized with the TfR1 via clathrin mediated endocytosis into an early endosome.

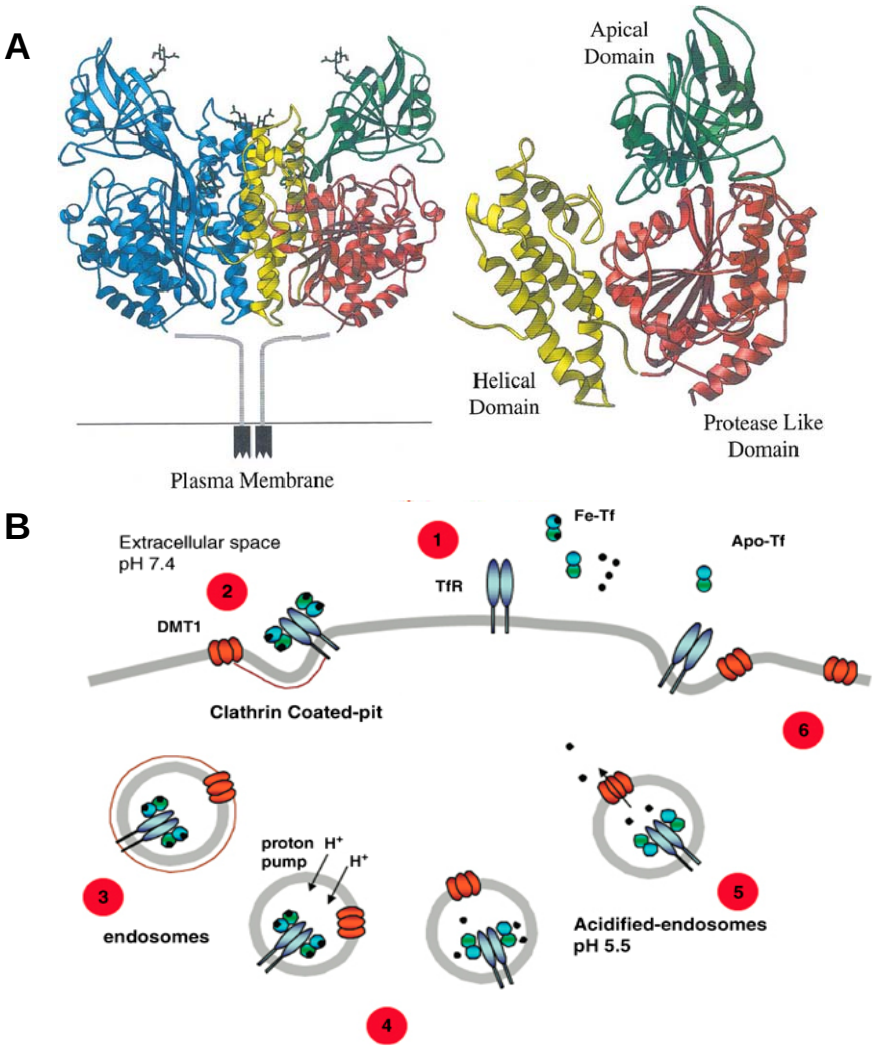

Figure 4. Structure and Function of TfR1. A) Crystal structure of exocytic recombinant human TfR at 3.2 Å resolution. Despite the absence of disulfide bonds formed in the intramembranous region *in vivo* the molecules spontaneously dimerize in the crystal as shown in the left structure. One monomer is shown on the right (adapted from Lawrence et al., 1999 (105)). Reprinted from Transferrin receptor 1, 2004, with permission from Elsevier. **B)** Endocytosis of TfR-1 induced by Fe-Tf. At physiological pH, membrane TfR-1 binds to Fe-loaded (holo)-transferrin (1). Endocytosis starts through clathrin-coated pits (2) that invaginate and give rise to vesicles containing ligand-receptor complexes (3). Following endosomal acidification (pH 5–5.5) iron is released from transferrin (4) and pumped to cytosol through the DMT1 transporter (5). TfR-1-apo-transferrin complexes are recycled to the cell membrane and dissociate at physiological pH (6). Reprinted by permission from Macmillan Publishers Ltd: Leukemia, (24), 2007.

The endocytic protein, TfR1 Trafficking protein (TTP) (159), is required for TfR1 internalization. A decrease in pH within the early endosome facilitates the release Fe^{3+} from Tf, where it is first reduced to ferrous iron (Fe^{2+}) and then transported across the endosomal membrane through a divalent metal transporter (DMT1) for release into the cytoplasm . TfR1 and iron free-Tf complex, also called apo-Tf, is recycled back to the cell surface where it is released from TfR1 due to its low binding affinity at physiological pH. Depending on cell type, the Tf-TfR1 iron delivery cycle is completed within 5-20 minutes (92). Studies have shown holo-Tf affinity for TfR1 is 10-100 fold greater than that of apo-Tf (145). Using techniques such as cryo-electron microscopy, single particle averaging and information from X-ray crystal structures of Tf and the TfR1 extracellular domain, a density map of the holo-Tf/TfR1 complex was constructed giving insight into the domains within TfR1 involved in the interaction with Tf (29, 79, 105, 113). Specifically, holo-Tf interacts with the helical domain, which contains a conserved RGD sequence, critical for Tf binding to TfR1 (46). However, results from mutagenesis studies have suggested the protease-like domain is also important for Fe-Tf binding (69). Upon internalization of the holo-Tf/TfR1 complex and exposure to a low pH, Tf undergoes a conformational change thought to be necessary for release of the iron molecules from the protein.

Because excess iron can be toxic causing synthesis of free hydroxyl radicals and oxidative stress consequently being detrimental to normal cellular function, genes involved in iron metabolism are tightly regulated. TfR1 is regulated post-transcriptionally via mRNA stabilization by cellular iron levels and more specifically by the iron regulatory proteins 1 and 2 (IRP1 and IRP2). Under low iron conditions the IRPs recognize and bind the iron response elements (IRE) located within the 3'untranslated region (157) of the TfR1 transcript stabilizing the mRNA and enabling protein translation. In contrast, under high iron conditions the IRP-1

protein binds assembled iron-sulfur clusters acting as an aconitase and the IRP-2 protein is targeted to the proteosome for degradation (78). Therefore, the IRPs are not available to bind the IRE and stabilize the TfR1 mRNA making it accessible to endonuclease degradation.

Interestingly, several groups have demonstrated the use of TfR1 in the entry of diverse types of viruses. Radoshitzky et al. (142) showed TfR1 is the entry receptor for New World arenaviruses. Arenaviruses are enveloped single stranded bi-segmented RNA viruses. On the viral surface they bear a glycoprotein (GP) that is processed into two subunits GP1 and GP2. GP1 has been shown to be the subunit responsible for interacting with the host cell receptor and mediating entry. Characterization of truncated variants of the Machupo virus (MACV) glycoprotein 1 (GP1) found residues 79-258 were sufficient to mediate binding to Vero African green monkey kidney cells (142). Following precipitation of this yet unidentified protein, mass spectrometry revealed the GP1 was bound to TfR1. In binding assays, MACV GP1-IgG bound CHO cells expressing human TfR1 (CHO-hTfR1) but not the parental CHO cell line. After being transfected with a hTfR1 expression plasmid, previously non-permissive CHO and baby hamster kidney (BHK-S) cell lines were infectable when challenged with MACV pseudotype particles (MACVpp) confirming that not only is TfR1 the entry receptor but the human form is required. Soluble TfR1 and TfR1 specific antibodies significantly reduced entry of MACVpp and Junin pseudotype virus (JUNVpp) infection in 293T cells but did not affect entry of Lassa virus or lymphocytic choriomeningitis virus, Old World arenaviruses, suggesting TfR1 is the entry receptor specific for New World arenaviruses.

In addition to New World arenaviruses, TfR1 has also been demonstrated to be the viral receptor for feline panleukopenia virus (FPV), canine parvovirus (CPV) and mouse mammary tumor virus (MMTV) (170). FPV and CPV both infect cells using the feline TfR1, however, CPV has acquired an additional glycosylation site which now allows it to recognize the canine

TfR1 apical domain permitting the use of canine TfR1 for viral entry (73). MMTV TfR1 recognition is also species specific and viral entry is restricted to mouse and rat cells (170). Using mouse/human TfR1 chimeras, TfR1 residues important for MMTV were mapped to the apical domain and the membrane distal region of the protease like domain (169). Interestingly, although the apical domain of the TfR1 has no known function for holo-Tf binding or receptor cycling, it appears to be a critical region for viruses that utilize TfR1 for entry.

3.2 Identification of TfR1 as a HCV Entry Factor

3.2.1 TfR1 is Down Regulated During HCV Infection

Because previous clinical studies have shown chronic HCV patients have mild to moderate hepatic iron overload suggesting HCV infection may be affecting expression of iron metabolic genes (39, 63, 64, 80, 88), we initially were interested in using the HCVcc infection system to investigate the affect of HCV infection on expression of host cell genes involved in iron metabolism. Therefore, Huh7 cultures were either mock infected or infected with HCVcc at an MOI of 0.5 and the expression of different iron genes was monitored over time in these parallel cultures. Of the genes analyzed, we observed the most significant change in TfR1 mRNA levels which at 10 hours post infection were consistently reduced (Fig. 5A). Since TfR1 is predominantly regulated post-transcriptionally, we directly looked at TfR1 protein levels during HCVcc infection. Parallel Huh7 cell cultures were either mock infected or infected with HCVcc at an MOI of 1. At the indicated time points, protein lysates were harvested for Western blot analysis. Infection was confirmed by immunoblotting for the viral NS3 protein. β-actin levels were measured as a loading control. Notably, TfR1 protein levels were reduced in HCV infected cells compared to mock infected cells by 5 days p.i. and remained down until the end of the experiment at day 14 p.i (Fig. 5B). TfR1 protein constantly recycles from the cell surface to

the cytoplasm in vesicular bodies. Because western blot measures total cellular protein, we aimed to determine if HCV infection affected changes in TfR1 localization. Therefore, we mock infected or infected Huh7 cells at an MOI of 1 and performed IF staining for TfR1 and E2 protein. By day 8 p.i. TfR1 staining was less apparent on the cell surface of infected cells compared to uninfected cells (Fig. 5C). To directly assess whether cell surface TfR1 levels were down during HCV infection, cells were alternatively processed for flow cytometry analysis. Again, we observed a decrease in TfR1 cell surface levels in HCV infected cells by day 5 p.i. compared to uninfected cells (Fig. 5D). Notably, down regulating expression levels of genes involved in viral entry is characteristic of many viruses. Analogously, in the context of HCV infection, down regulation of the HCV entry factors CD81, CLDN1 and OCLN has been observed (111, 162).

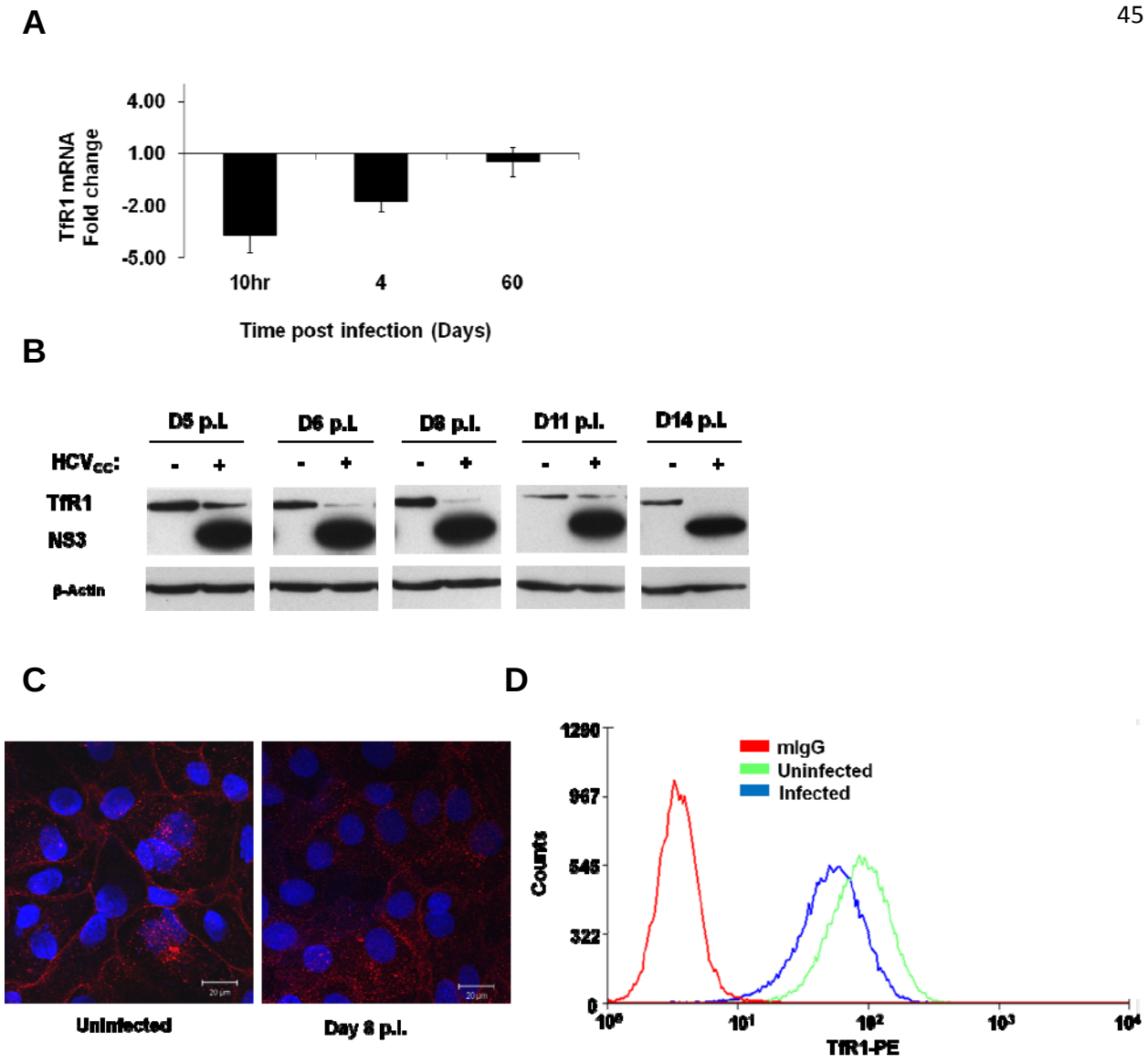

Figure 5. TfR1 is down regulated during HCV infection. A) Huh7 cells either mock infected or infected with HCVcc and cellular RNA was harvested at the indicated times. TfR1 mRNA levels were measured by RT-qPCR and are expressed as fold change compared to uninfected TfR1 mRNA. **B**) TfR1 and NS3 and protein levels analyzed by western blot in Huh7 cells mock infected or infected with HCVcc. β-actin levels measured as a loading control. **C**) Confocal analysis of TfR1 protein expression in Huh7 cells mock infected or infected with HCVcc. Fixed cells were stained with TfR1 antibody and counterstained with Hoechst stain (131) and Alexa 555-conjugated secondary antibody (red). The scale bar = 20µm. **D**) Flow cytometric analysis of cell surface TfR1 in Huh7 cells mock or HCVcc infected for 5 days. Cells were stained with mouse antibody against TfR1 followed by anti-mouse secondary antibody conjugated with PE.

3.2.2 **TfR1 siRNA Knockdown Inhibits HCVcc Infection at a Step Prior to Viral Replication**

After observing that TfR1 expression was altered in response to HCV infection, we decided to determine if TfR1 was functionally involved in HCV infection by analyzing the effect of TfR1 knockdown. Huh7 cells were reverse transfected with 12nM of an irrelevant control siRNA or a TfR1-specific siRNA. Four days after Huh7 cells were transfected with the TfR1-specific siRNA, we observed greater than 95% knockdown in TfR1 mRNA levels compared to scrambled siRNA transfected cells and this knockdown lasted until at least day 8 post-transfection (Fig.6A). Based on these knockdown kinetics, siRNA-transfected cells were inoculated with JFH-1 HCVcc at 4 days post-transfection at an MOI of 0.05 and HCV infection kinetics were assessed by monitoring intracellular HCV RNA levels by RT-qPCR from day 2 through 4 p.i. Consistent with TfR1 playing a role in HCV infection, we observed greater than a two log decrease in HCV RNA levels at 2 days p.i. in TfR1 knockdown cells compared to control cells (Fig 6B). Notably, amplification of the reduced level of HCV RNA present in the TfR1 knockdown cells at day 2 paralleled the amplification kinetics of the HCV RNA in scrambled siRNA transfected control cells suggesting that inhibition occurred early and that subsequent intracellular viral RNA replication was not affected by TfR1 knockdown.

To directly determine if TfR1 knockdown affects HCV replication we performed similar siRNA knockdown experiments with HCV subgenomic replicon cells. HCV subgenomic JFH-1 (sgJFH-1) replicon cells were transfected with the respective siRNAs and again TfR1 mRNA levels were reduced by 95% compared to controls by day 4 post-transfection (Fig 7A). Intracellular sgJFH-1 HCV RNA levels were assessed 4 and 5 days post-transfection by RT-qPCR and were not altered in the TfR1 knockdown cells relative to controls providing evidence that TfR1 knockdown does not affect HCV replication (Fig. 7B).

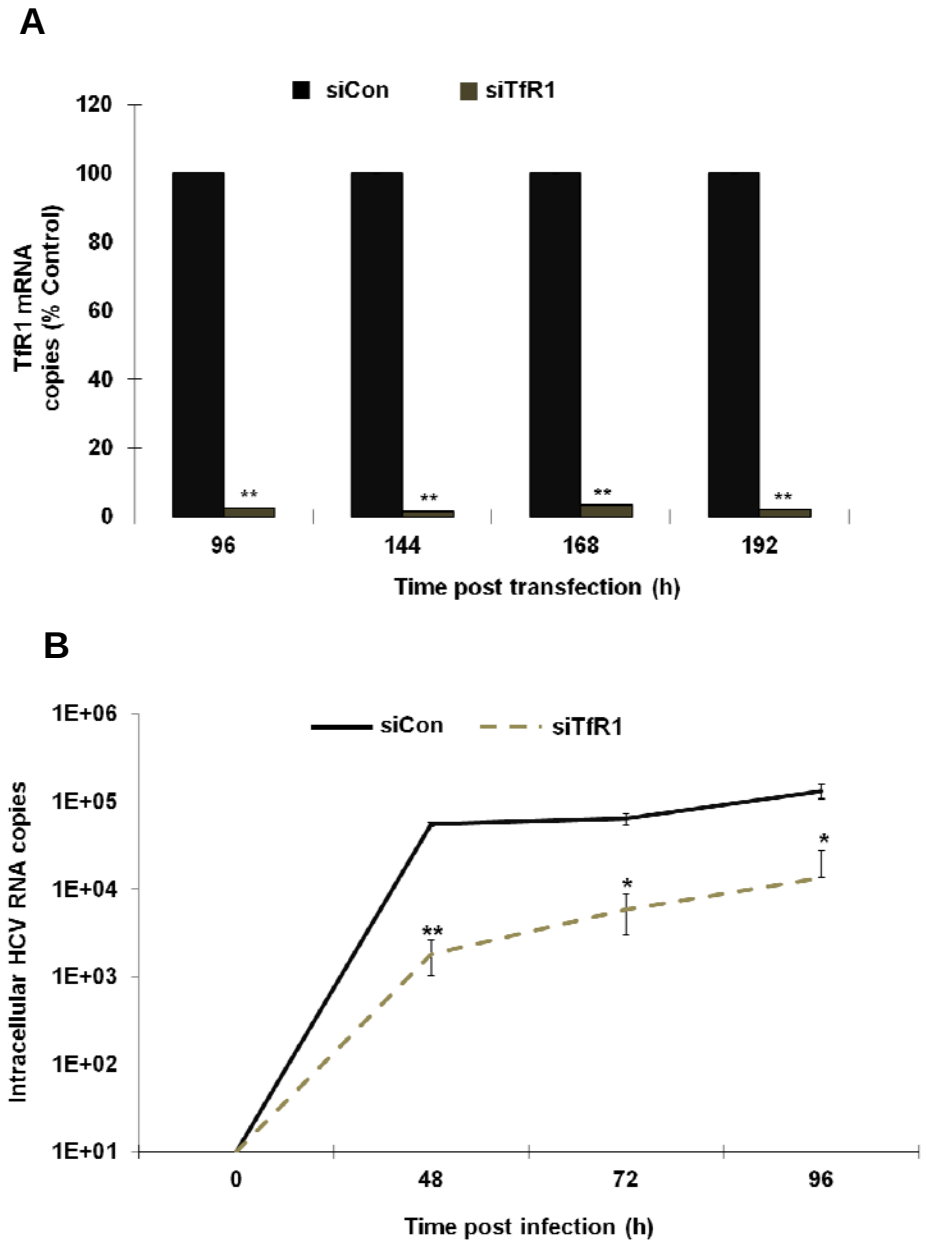

Figure 6. TfR1 knockdown inhibits HCVcc infection. A). Huh7 cells were transfected with 12nM control or TfR1 specific siRNA. At the indicated time points post-transfection TfR1 mRNA was measured by RT-qPCR, normalized to GAPDH and graphed as a percentage of the maximum numbers of copies determined in siCon transfected cultures. **B**) Control and TfR1 siRNA transfected Huh7 cells were infected with HCVcc 4 days post-transfection at an MOI of 0.05. At the indicated times post-infection total cellular RNA was harvested and TfR1 mRNA and HCV RNA levels were quantified Black lines represent siCon transfected Huh7 cells and gray dashed lines represent siTfR1 transfected Huh7 cells. In all cases, significant differences relative to controls (one-way analysis of variance (ANOVA) and Tukey's post-hoc *t* test) are denoted as $*P < 0.05$ or $**P < 0.01$.

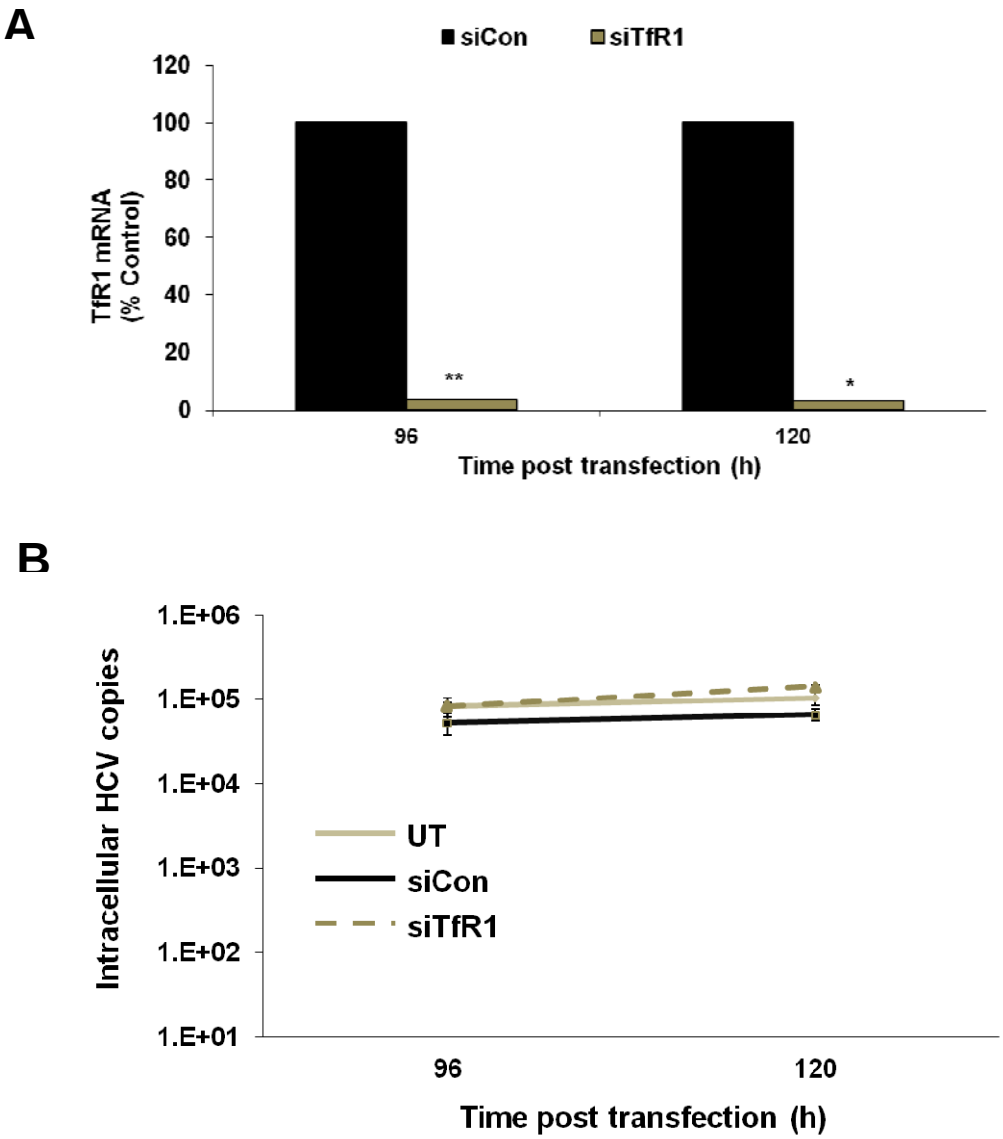

Figure 7. TfR1 knockdown has no affect on HCV replication. A) HCV subgenomic 2a replicon cells were transfected with 12nM control or TfR1 specific siRNA. At 96 and 120 hours post-transfection TfR1 mRNA was measured by RT-qPCR, normalized to GAPDH and graphed as a percentage of the maximum numbers of copies determined in siCon transfected cultures. **B**) HCV replicon RNA levels were determined by RT-qPCR and normalized to GAPDH at the indicated times after transfection with control or TfR1 specific siRNA. Light gray line represents untransfected cultures, black line represents siCon transfected cultures, dark gray dashed line represents siTfR1 transfected replicon cells. In all cases, significant differences relative to controls (one-way analysis of variance (ANOVA) and Tukey's post-hoc *t* test) are denoted as $*P < 0.05$ or $**P < 0.01$.)

3.2.3 **Blocking or Down Regulation of Cell Surface TfR1 Inhibits HCV Infection Initiation**

Since siRNA knockdown only examines whether expression of the gene is required but does not give insight into which aspect of the viral life cycle the gene may function and TfR1 knockdown had no effect on HCV replication, we next investigated whether blocking cell surface TfR1 inhibited HCV infection initiation. As an initial approach, we performed antibody blocking experiments in which antibodies were added to cells before or after infection initiation. Huh7 cells were either pre-incubated for 1 hour with an irrelevant mouse IgG control antibody, anti-CD81, or anti-TfR1, and then infected with HCVcc or alternatively the same antibodies were added when the viral inoculum was removed from the cells at 10 hours post-infection. Twenty-four and 48 hours p.i., total cellular RNA was harvested and intracellular RNA was measured by RT-qPCR. As expected HCV RNA levels were similar in cells pre-incubated with the control isotype antibody compared to cells left untreated (Fig 8A). Notably, however, cultures pre-incubated with anti-TfR1 showed significantly less intracellular HCV RNA levels relative to controls and similar to the reduction observed in cells pre-incubated with an antibody to the known HCV entry receptor, CD81 (Fig. 8A). In contract, HCV RNA levels were comparable to the IgG-treated control when the same antibodies were added to cultures 10 hours post-infection (Fig 8B). Although the lack of HCV inhibition in cultures treated with antibody 10 h post-infection already suggested that antibody treatment was not affecting HCV replication, to confirm the TfR1 antibody treatment was not initiating intracellular signaling that effects HCV replication, we performed antibody blocking experiments using the HCV subgenomic JFH-1 (sgJFH-1) replicon cell line in which HCV RNA levels were measured by RT-qPCR at 24 and 48 hours after cells were either mock untreated or incubated with anti-CD81 or anti-TfR1

antibodies. There was no effect on HCV replicon RNA levels after incubation with any of the antibodies confirming TfR1 antibody treatment was not affecting HCV replication (Fig. 8C).

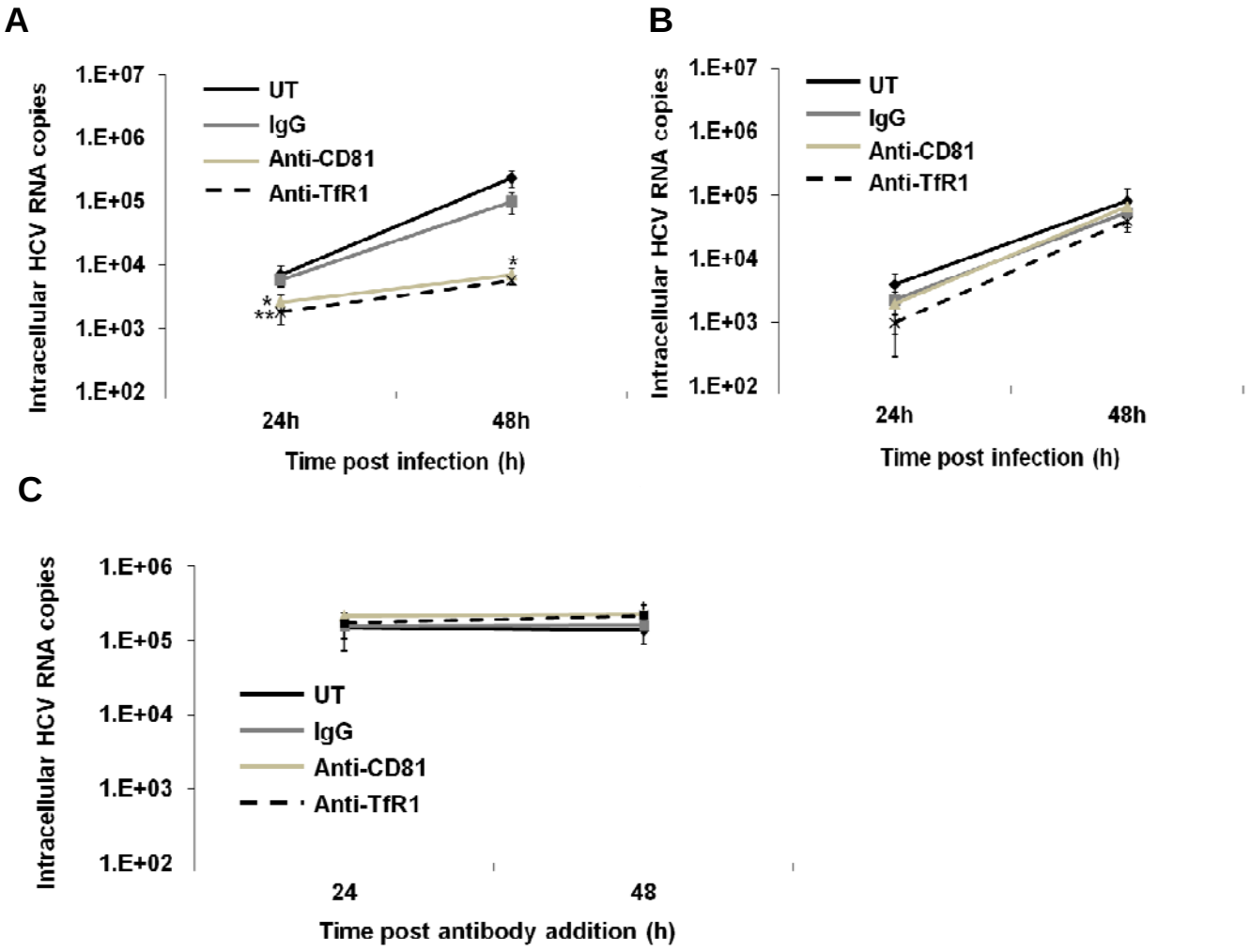

Figure 8. Inhibition of HCV infection initiation but not HCV replication by anti-TfR1 antibodies. A) Huh7 cells were preincubated for 1 hour with irrelevant isotype control, anti-CD81 or anti-TfR1 or left untreated (UT) and then infected with HCVcc at an MOI of 0.01. **B)** Huh7 cells were infected with HCVcc at an MOI of 0.01. At 10 hours post inoculation virus was removed and either left UT or incubated with irrelevant isotype control, anti-CD81, or anti-TfR1 **C)** HCV 2a subgenomic replicon cells were left UT or incubated with irrelevant isotype control, anti-CD81, or anti-TfR1. **A-C)** Total cellular lysate was harvested 24 and 48 hours post HCVcc infection and HCV RNA levels were determined by RT-qPCR and data normalized to GAPDH. Solid black line represents untreated cells, dark gray line represents isotype control treated cells, light gray line represents anti-CD81 treated cells and dotted line represent anti-TfR1 treated cells. In all cases, significant differences relative to controls (one-way analysis of variance (ANOVA) and Tukey's post-hoc *t* test) are denoted as $*P < 0.05$ or $**P < 0.01$).

To further confirm the reduction in HCV infection observed following pre-incubation with TfR1 antibody was specific to blocking of TfR1, we also measured HCV infection levels in the presence of a TfR1 inhibitor, ferristatin, which causes internalization and degradation of cell surface TfR1 (85). To determine the optimal dose required to inhibit HCVcc infection, cells were pre-incubated with three different doses of the inhibitor and then infected. A dose-dependent decrease in HCV RNA levels was observed when cells were pre-treated with 3 different concentrations (50μM, 75μM, and 100μM) of the TfR1 inhibitor. Since no toxicity was seen at the lowest dose used (50μM), inhibition of HCV infection was greater than 1 log by 48 hrs (data not shown) and this dose was used in previous reports characterizing the inhibitor, this concentration was used in future experiments. Huh7 cells were again pre-treated for 1 hour with 50μM and infected with HCVcc. HCV RNA was measured by RT-qPCR and normalized to GAPDH. Similar to results observed in the antibody blocking experiments, we observed inhibition of HCV RNA levels at 24 and 48 hours post infection after pre-treatment with the TfR1 inhibitor ferristatin (Fig 9A). To again confirm blocking TfR1 with ferristatin inhibits HCV infection initiation, we performed a foci reduction assay to directly measure the effect of the inhibitor on HCV initiation events. Cells were pre-treated for 1 hour prior to HCVcc infection, treated at time of infection or treated 20 hours post-infection with 25μM or 50μM ferristatin. A methylcellulose overlay was added to cultures 24 hours post-infection to prevent cell-free spread. Seventy-two hours after HCVcc infection, cells were fixed and stained with an anti-E2 antibody to quantify the number of unique HCV infection events that occurred in each culture (Fig 9B). A significant decrease occurred in the percentage of foci quantified in cultures either pre- or co-treated with the TfR1 inhibitor. In contrast, post-treatment with the inhibitor did not significantly reduce the total foci formed relative to that observed in the untreated wells (Fig 9B).

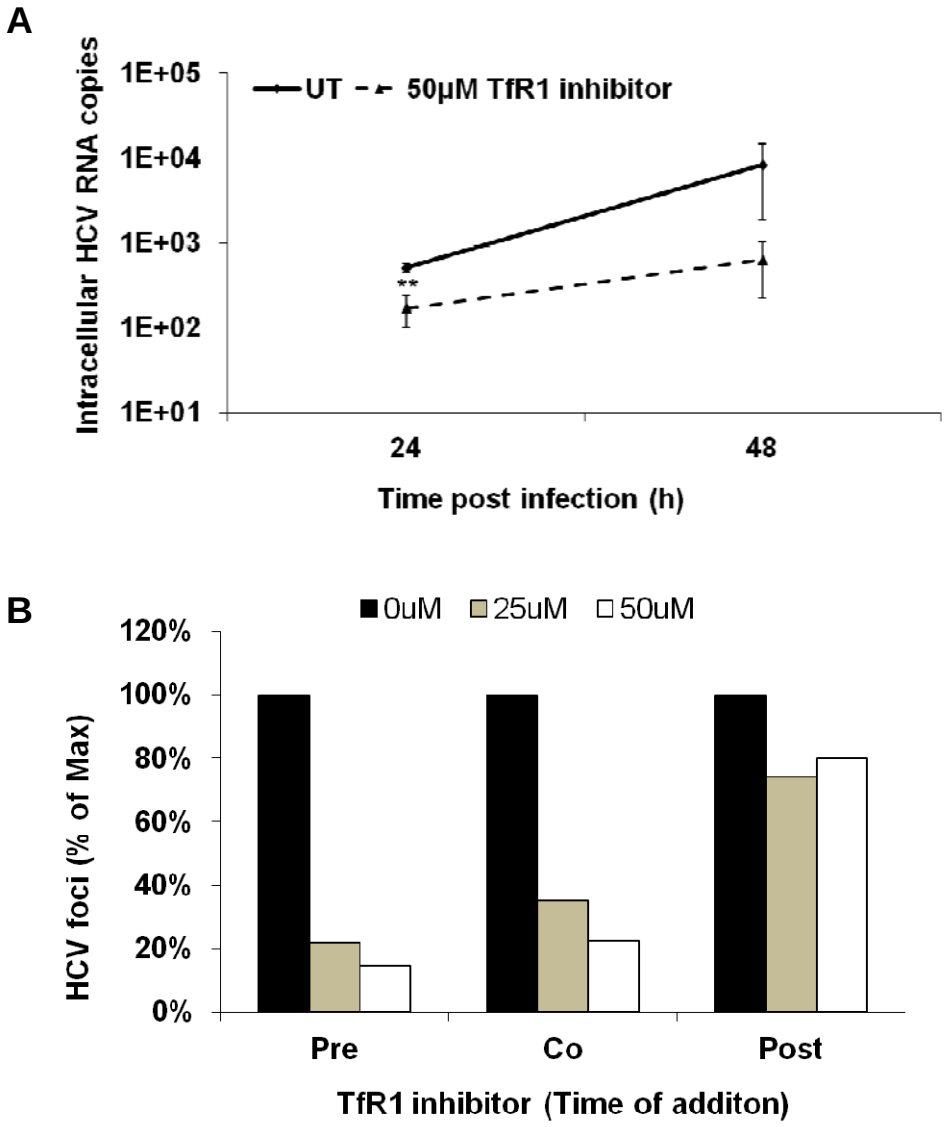

Figure 9. Inhibition of HCV infection initiation by TfR1 inhibitor. A) Huh7 cells were pre-incubated for 1 hour with 50μM TfR1 inhibitor or left untreated (UT) and then infected with HCVcc. HCV RNA levels measured by RT-qPCR and normalized to GAPDH expressed as intracellular HCV RNA copies per microgram total RNA. Significant differences relative to controls (one-way analysis of variance (ANOVA) and Tukey's post-hoc *t* test) are denoted as **P* < 0.05 or ***P* < 0.01. **B**) Huh7 cells treated with 25μM or 50μM TfR1 inhibitor either 1 hour pre-, co-, or 20 hours post HCVcc infection. Cells were fixed 72 hours post-infection and HRP stained with anti-E2 antibody. Foci were counted and are expressed as a percentage of the total foci detected in the control mock-treated wells

3.2.4 **TfR1 Participates in E1/E2-Dependent HCVpp Entry**

To determine whether TfR1 acts at the level of E1/E2-mediated entry we next performed TfR1 inhibition experiments using a pseudoparticle system which consists of recombinant HIV luciferase reporter particles pseudotyped with the E1 and E2 glycoproteins from our HCV JFH-1 virus or the glycoproteins from control viruses. Since previous studies have demonstrated that the New World arenavirus, Machupo virus (MACV), utilizes TfR1 (142) as an entry receptor while entry of the Old World arenavirus Lymphochoriomeningitis virus (LCMV) is independent of TfR1, these pseudotyped viruses were used as positive and negative controls for TfR1-dependence, respectively in addition to the standard vesicular stomatitis virus (VSV)-Gpp negative control. For these experiments, Huh7 cells were either left untreated or pre-incubated with a control mouse isotype antibody, anti-CD81, or anti-TfR1 and then parallel cultures were infected with the different pseudotype viruses. Seventy-two hours following pseudotype virus inoculation, cells were lysed and luciferase activity was measured as the readout for entry. As expected, there was no decrease in entry of any of the pseudotyped viruses following pre-incubation with the isotype control antibody while anti-CD81 inhibited entry of HCVpp, but not the LCMVpp, MACVpp or VSV-Gpp. Pre-incubation with anti-TfR1 inhibited entry of MACVpp, but not LCMVpp or VSVGpp indicating the antibody blocking was working as expected. Importantly, similar to the TfR1-dependent MACVpp, entry of HCVpp was reduced by 1 log following pre-incubation with anti-TfR1 analogous to the inhibition observed after pre-incubation with anti-CD81 (Fig.10A). Next we tested whether blocking TfR1 inhibited HCVpp expressing E1/E2 from different HCV genotypes (GT1a clone H77; GT1b clone Con1). Like the GT2a HCV JFHpp, H77pp and Con1pp entry was inhibited to the same extent when either TfR1 or CD81 was blocked (Fig. 10B). These results demonstrate that TfR1 is involvement in HCV entry is not specific to the HCV GT2a but is required for entry of other genotypes as well.

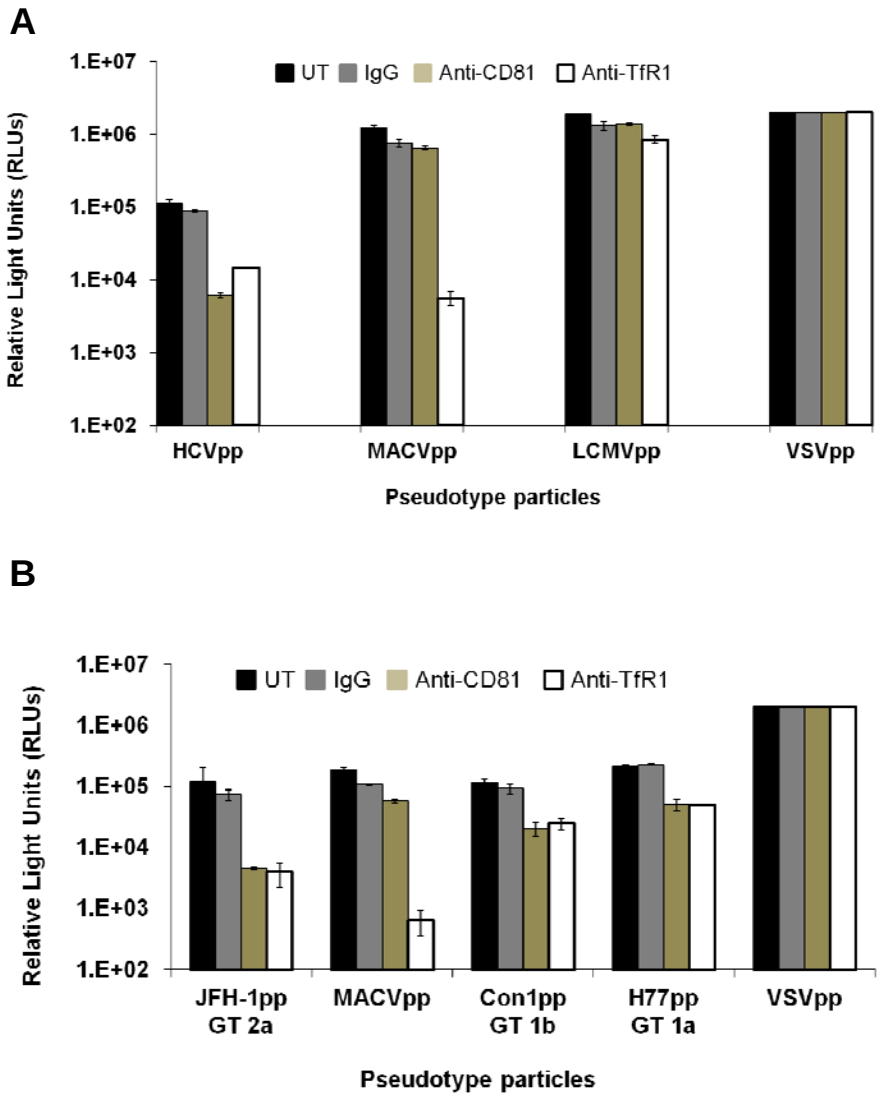

Figure 10. TfR1 inhibits E1/E2-dependent HCVpp entry. A and **B**) Huh7 cells were left untreated or pre-incubated with 25µg/ml irrelevant isotype control, anti-CD81 or anti-TfR1 for 1 hour and then infected with the indicated pseudotype viruses. At 72 hours post infection total celluar lysate was collected, luciferase activity was measured and is expressed as relative light units (RLUs). Black bars represent untreated cells, Dark gray bars represent isotype control antibody treated cells, light gray bars represent anti-CD81 treated cells and white bars represent anti-TfR1.

3.3 Functional Analysis of the Role of TfR1 in HCV Entry

3.3.1 Inhibition of TfR1 Does Not Prevent HCV Cell-to-Cell Entry

As described above, the HCV entry process can be initiated by extracellular virions binding to the cell surface receptors. While this cell-free entry mechanism is the first event required to initiate a primary infection, following this event HCV can also enter uninfected neighboring cells via a direct cell-to-cell spread mechanism. It has been shown that the cellular factors required for these two entry pathways are not necessarily identical. Hence, as part of initial efforts to determine the role TfR1 might play in HCV cell entry, we assessed if TfR1 is required for HCV cell-to-cell spread by performing foci formation assays. An initial round of HCV infection was initiated by inoculating Huh7 cultures with 0.01 ffU/ml of HCVcc. Before the completion of one viral life cycle (20 hours post-infection) antibodies recognizing CD81, CLDN1, TfR1 or an isotype control antibody were added to the culture medium. In addition, all cultures were co-incubated with an antibody against viral glycoprotein E2 at a concentration that we predetermined was sufficient to neutralize extracellular virus and preventing cell-free viral spread. Cells were fixed after 72 hours post-infection and HCV-positive foci were detected by staining for HCV viral protein E2. The number of E2 positive cells per foci was counted (Fig. 11). Under these conditions in which cell-free virus spread is inhibited, we consider the presence of foci containing 4 or more E2 positive cells as evidence of cell-to-cell spread. In contrast the presence of foci containing less than 3 E2 positive cells was considered as evidence that cell-to-cell spread had been inhibited (Of note foci containing 2 cells could result from cell division during the assay). As expected when cultures were left untreated or incubated with the anti-isotype control antibody more than 80% of HCV foci contained 4 or more E2 positive cells. Similar to untreated and isotype control treated cells, addition of anti-CD81 antibody did not have a negative effect on HCV cell-to-cell spread with more than 80% of foci containing 4 or

more E2 positive cells. In contrast, we observed 60% of foci containing less than three E2 positive cells in cultures incubated with anti-CLDN1 suggesting cell-to-cell spread requires CLDN1, a conclusion consistent with previously published reports. Though not as dramatic, a detectable inhibition of HCV cell-to-cell spread was observed in anti-TfR1 and ferristatin-treated cultures suggesting TfR1 may enhance HCV cell-to-cell spread but it is not necessarily required.

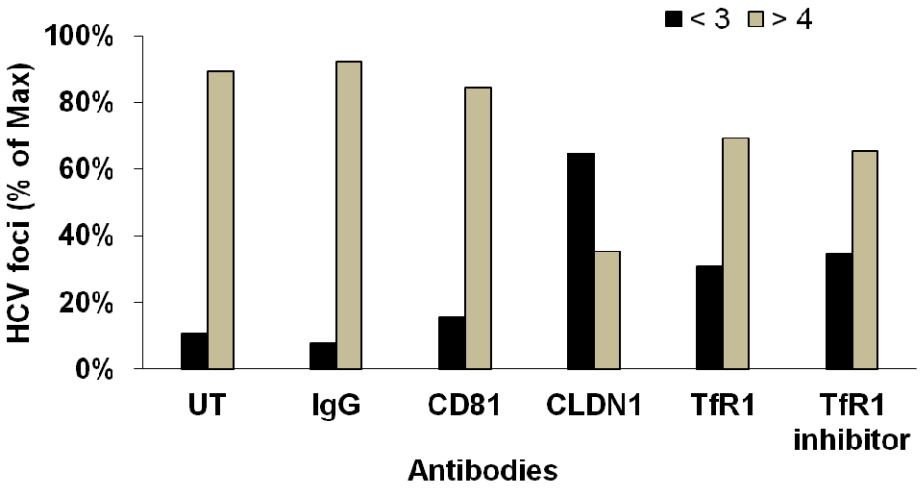

Figure 11. TfR1 enhances, but is not required for HCV cell-to-cell spread. Huh7 cells were infected with HCVcc and 25μg/ml of the indicated antibody or 50μM of TfR1 inhibitor was added 20 hours post infection. At 72 hours post-infection cells were fixed and stained using an anti-E2 antibody. E2 positive cells per foci were counted and the number of E2 positive cells are expressed here as a percentage of total number of foci.

3.3.2 **TfR1 Acts at a Post-Binding Step in the HCV Entry Process**

After demonstrating TfR1 is an HCV entry factor, we also assessed the time at which TfR1 acts during the entry process. The order in which the HCV viral particle interacts with specific host cellular factor during the entry process has been determined previously by adding specific antibodies at different times during the course of infection to determine when that antibody loses its inhibitory activity and thus when the required function of the targeted protein has already occurred. Thus, cells were inoculated with HCVcc at 4 degrees Celsius to allow for virus binding without internalization. Cells were then moved to 37 degrees Celsius to allow entry to proceed. Antibodies to the cellular receptor CD81 or TfR1 or isotype control IgG were added prior to virus binding or after virus binding at hour intervals post-temperature shift. As previous groups have observed, the anti-CD81 antibody lost its inhibitory effect at a post-binding step early during the entry process. Specifically, CD81 inhibitory activity was less than 50% by 2 hours post-binding. The addition of the anti-TfR1 inhibited HCV by greater than 50% until 4 hours post-temperature shift indicating that TfR1 functions in HCV entry at a post-binding step after CD81 (Fig. 12).

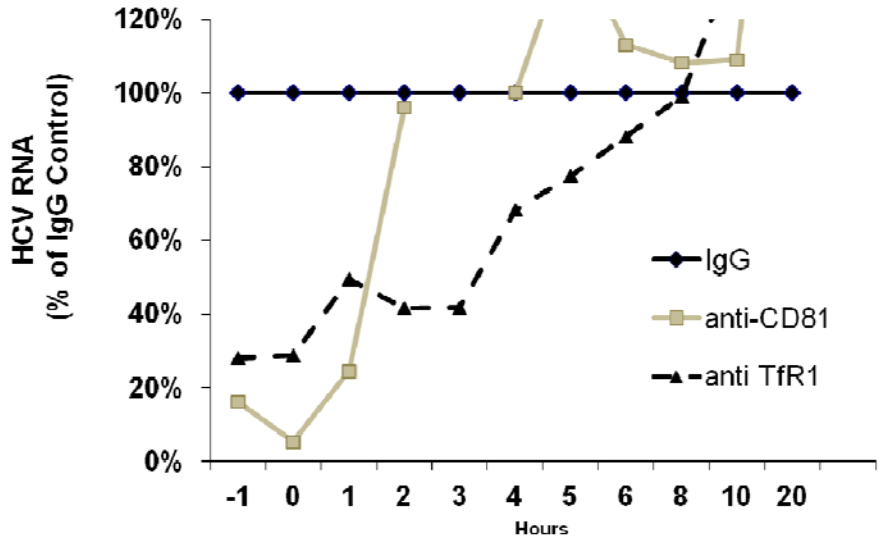

Figure 12. TfR1 acts at a post-binding step after CD81 in HCV entry. Huh7 cells were mock treated or pre-treated with an IgG control isotype, anti-CD81 or anti-TfR1 for 1 hour. Cell were inoculated with HCVcc and placed at 4ºC for 1 hour to synchronize infection. After 1 hour cells were washed with 1X PBS and placed at 37º C and antibodies were added at the indicated times post-temperature shift. HCV RNA levels were measured 30 hours p.i. by RT-qPCR and normalized to GAPDH.

3.3.3 **Blocking TfR1 Endocytosis Inhibits HCVcc Internalization**

As mentioned earlier TfR1 is internalized via the clathrin mediated pathway; however, Tosoni et al. (159) identified a TfR1 Traffiking Protein (TTP) that that is reported to be specifically and uniquely required for TfR1 endocytosis. Performing siRNA knockdown studies they found TTP knockdown inhibited TfR1 internalization but had no effect on clathrin-mediated endocytosis of other proteins such as LDL-R or EGFR. Furthermore, in TTP knockdown cells, the number of TfR1 loading clathrin coated pits was reduced, however a reduction in EGFR loading clathrin coated pits was not observed. This suggested TTP functions specifically in TfR1 endocytosis. Therefore, we tested whether inhibiting TfR1 internalization by knocking down TTP affected HCVcc entry. Huh7 cells were transfected with an irrelevant GFP siRNA control or siRNAs specific for TfR1 or TTP. We observed greater than 90% TTP knockdown efficiency in cells transfected with the specific siRNAs compared to control (Fig. 13A). Four days post-transfection, we inoculated with HCVcc. When intracellular HCV RNA levels were measured by RT-qPCR analysis 24 and 48 hours post-infection, we observed that HCV levels were reduced in TTP knockdown cells to the same extent as observed in the TfR1 knockdown cells (Fig 13B).

Likewise, we tested the effect of TTP knockdown on HCVpp entry. Four days after transfecting cells with 12nM of control, CD81, TfR1 or TTP specific siRNA (Fig. 14A), cells were inoculated with HCV JFHpp, MACVpp or VSVpp-G. As expected, CD81 and TfR1 knockdown inhibited HCVpp entry and had no effect on VSVpp (Fig. 14B). A decrease in HCVpp also occurred in TTP knockdown cells suggesting disrupting TfR1 internalization affected HCVpp entry. Likewise, TfR1 knockdown resulted in a decrease in MACVpp entry, however, TTP knockdown did not appear to effect MACV entry.

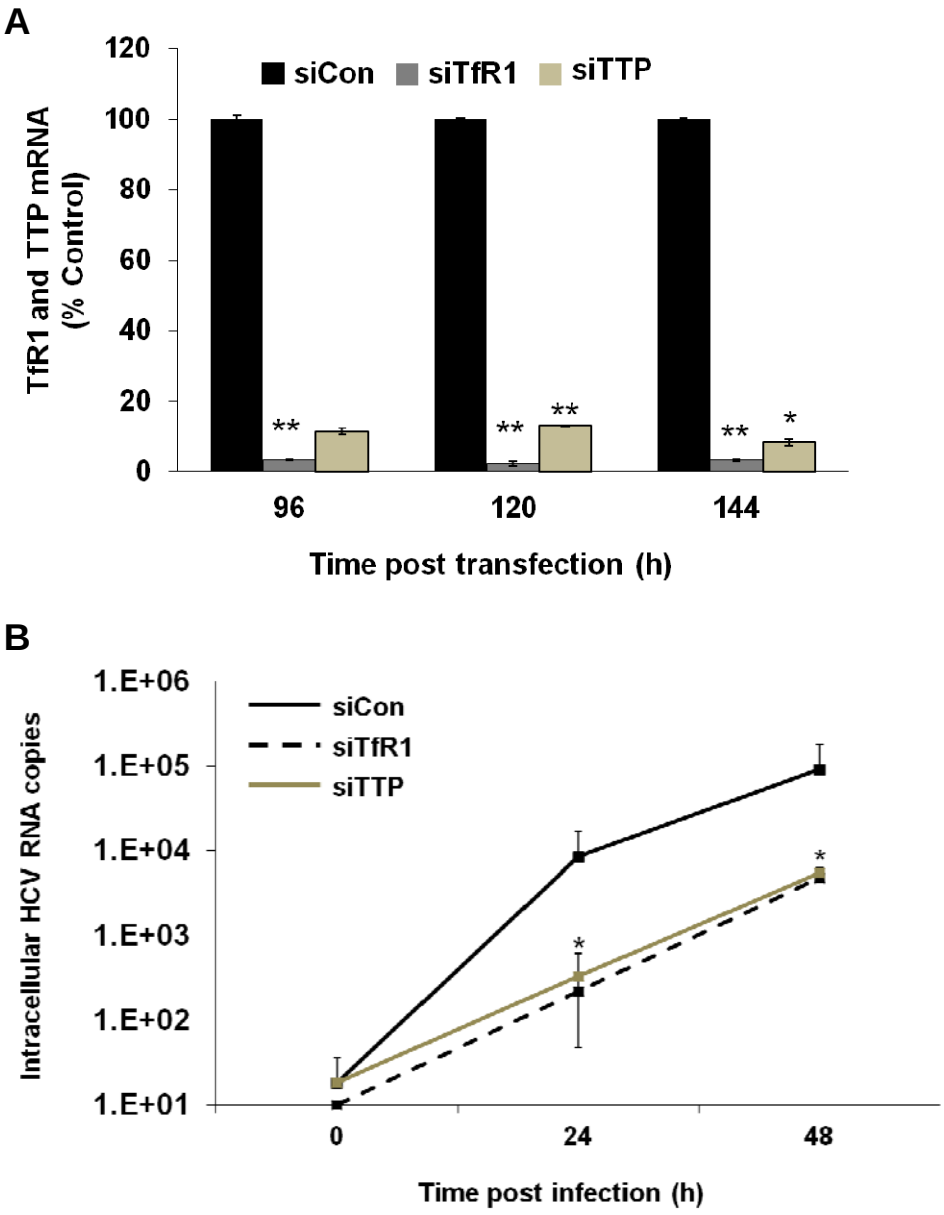

Figure 13. TTP Knockdown inhibits HCVcc infection. A) TfR1 and TTP mRNA levels measured by RT-qPCR at the indicated times post-transfection with 12nM irrelevant GFP control, TfR1 or TTP specific siRNA. mRNA levels are expressed as a percentage of the mRNA levels in the cells transfected with control. **B**) HCV RNA copies in Huh7 cells transfected with TfR1 and TTP siRNA. Black line represents siCon transfected cells, dotted line represents siTfR1 transfected cells and gray line represents TTP cells. In all cases, significant differences relative to controls (one-way analysis of variance (ANOVA) and Tukey's post-hoc *t* test) are denoted as $*P < 0.05$ or $**P < 0.01$.

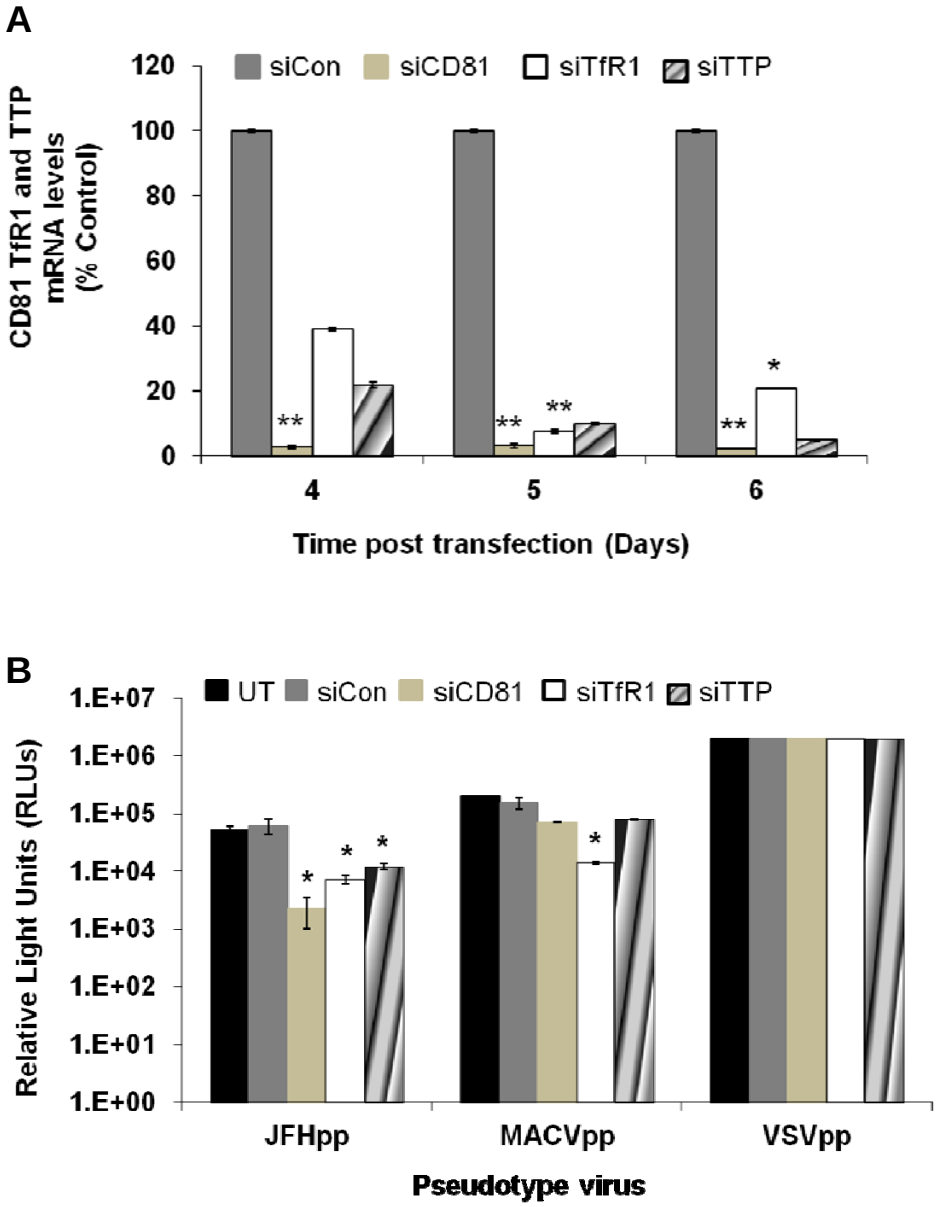

Figure 14. TTP Knockdown inhibits HCVpp entry. A) TfR1 and TTP mRNA levels measured by RT-qPCR at the indicated times post transfection with 12nM irrelevant control, CD81, TfR1 or TTP specific siRNA. mRNA levels are expressed as a percentage of the mRNA levels in the cells transfected with control. **B)** Untreated, control, CD81, TfR1 and TTP knockdown cells were infected with the indicated pseudotype viruses. At 72 hours post infection luciferase activity was measured expressed as RLUs. Black bars represent untreated cells, dark gray bars represent siCon cells, light gray bars represent siCD81, white bars represent siTfR1 and diagonal bars represent siTTP cells. In all cases, significant differences relative to controls (one-way analysis of variance (ANOVA) and Tukey's post-hoc *t* test) are denoted as $*P < 0.05$ or $**P < 0.01$.

3.3.4 **TfR1 Binds HCV E1/E2 Glycoproteins**

While the above data suggests TfR1 may be involved in the endocytosis of HCV, the observed inhibition of E1/E2-dependent HCVpp entry after blocking cell surface TfR1 suggest that function may be preceded by a direct binding of TfR1 to one of these HCV glycoproteins. Therefore, we performed binding studies to determine if the HCV particle binds to TfR1. For this, non permissive CHO cells were co-transfected with a pcDNA3.1 plasmid and an expression plasmid encoding either the human SRBI, or CD81 genes or transfected with a TfR1 expression plasmid containing a zeocin selection marker. After transfection, cells were placed under G418 or zeocin selection and resistant colonies were expanded. Clones were screened by RT-qPCR for high mRNA levels of SRBI, CD81 or TfR1 and then chosen for HCV binding studies based on detectable surface expression of the respective human receptor (Fig.15 A-C). Binding experiments were performed by inoculating each cell line with HCVcc at 4ºC for 1 hour to allow virus binding. Cells were washed with cold PBS to remove any nonspecific bound virus. Lysis buffer was added to cultures to harvest cellular RNA and viral RNA from the virus bound to cell surface. HCV binding was analyzed by RT qPCR and HCV RNA levels from each clone were compared to the HCV RNA levels measured from untransfected CHO cells. This CHO cell based binding assay is not robust, but analogous to previous reports we observed an approximately three fold increase in HCVcc binding to CHO cells expressing human SRBI than to the parental CHO cells and this binding was more pronounce than that detected on CHO cells expressing CD81. Notably, CHO cells expressing TfR1 exhibited greater than a three fold increase in HCVcc binding (Fig 15D).

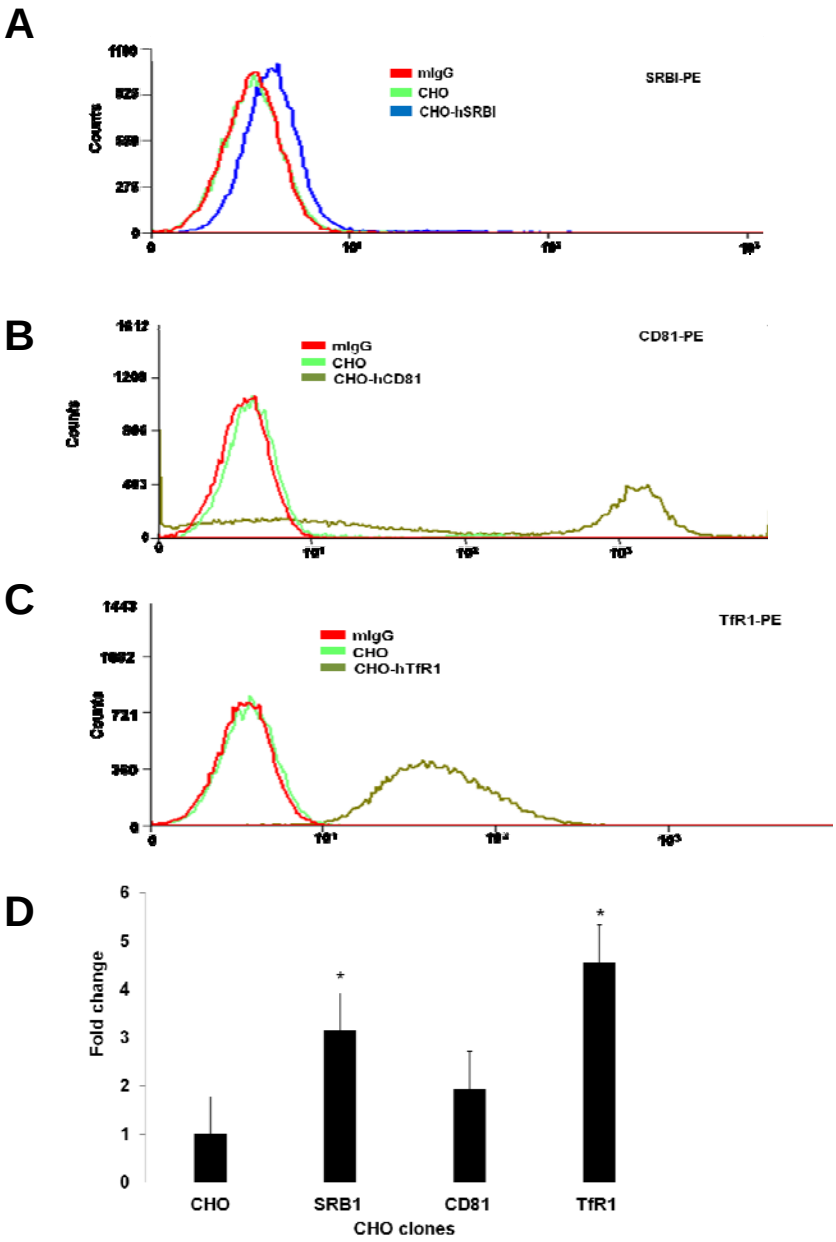

Figure 15. HCVcc binds CHO cells expressing human TfR1. A-C) Flow cytometric analysis of HCV receptors expressed on CHO cells. Cells were stained with mouse antibody against SRBI, CD81 or TfR1 followed by a anti-mouse secondary antibody conjugated with PE. CHO cell PE background signal (red line), parental CHO cells (light green), transfected CHO cells (dark green or blue). **D**) Binding assay in which indicated CHO cell lines were inoculated with HCVcc for 1 hour at 4º C. Cells were lysed to recover cell surface bound HCV and RNA was analyzed by RT-qPCR and normalized to GAPDH. Fold change was calculated by comparing HCV RNA levels bound to human receptor CHO cell lines compared to HCV RNA bound to parental CHO cells. Significant differences relative to controls (one-way analysis of variance (ANOVA) and Tukey's post-hoc *t* test) are denoted as $*P < 0.05$ or $**P < 0.01$.

3.4 **Discussion**

Since the discovery of HCV, several cellular host proteins have been demonstrated to be involved in HCV entry suggesting it is a very complex multistep process. However, the entry process remains ill-defined with additional entry factors still being discovered. In this study, we observed a down regulation in cellular TfR1 mRNA and protein surface levels during HCV infection (Fig. 5A-D), which lead us to eventually identify TfR1 as an HCV entry factor (Figs. 8 and 10). Following siRNA knockdown and cell surface blocking of TfR1 we observed inhibition of HCVcc and HCVpp infection demonstrating TfR1 plays a role in E1/E2-dependent HCV entry. Initial studies indicate that while TfR1 mediates cell-free HCV entry, it does not appear to be required for HCV cell-to-cell viral spread (Fig.11). Kinetic analysis indicated that TfR1 acts at a post-binding step after CD81, which has been defined as an early HCV entry factor (Fig. 12). Further mechanistic studies suggest that TfR1 acts during HCV entry by binding to the viral particle (Fig.15) possibly mediating viral TTP-dependent endocytosis (Fig. 13 and 14).

Previously identified receptors SRB1 and CD81 have both been shown to interact with sE2, while a direct interaction between the viral glycoproteins and CLDN1 and OCLN has not been observed (50, 138). Although CD81 has been shown to bind sE2, Evans et al. (50) observed enhanced HCVcc binding to CHO cells expressing cell surface SRBI compared to both normal CHO cells and CHO cells expressing cell surface CD81, a result that appears to be consistent with a previous engagement between the E1/E2 glycoprotein complex and SRBI being necessary for efficient binding to CD81 (9). Using the same CHO cell binding assay, we asked whether TfR1 binds to the HCV particle. In our study, HCVcc binding to CHO cells expressing human TfR1 on the cell surface was enhanced suggesting a direct interaction with the viral particle and TfR1. Notably, the relative degree of binding detected suggests that the viral particle can bind TfR1 efficiently without the need for previous engagement with another

receptor to physically prime the interaction. However, additional studies are needed to confirm and define the nature of the interaction between HCV and TfR1. In particular, it would be of interest to determine if TfR1 binding to the HCV particle is mediated specifically by one of the viral glycoproteins.

Regardless of the viral components involved in the interaction, future mapping studies to determine the functional domain within the TfR1 protein required for HCV entry would be informative and perhaps identify a specific HCV antiviral target. The anti-human TfR1 antibody used in our blocking experiments has been shown to recognize a mouse-human TfR1 chimera containing only human residues 187-383 within the apical domain, but not a mouse-human TfR1 chimera containing human residues 187-207 or 213-383 within the apical domain (143). This suggests the apical domain, particularly residues 208-212 is where this antibody binds. Since both HCVcc and HCVpp were inhibited using this anti-TfR1 antibody, the apical domain may represent a promising place to begin initial mapping studies.

TfR1 is internalized via clathrin mediated endocytosis. The protein TTP has been identified as a cargo specific endocytic protein required for TfR1 internalization. To date, TTP is thought to be TfR1-specific as it directly binds TfR1 via its SH3 domain. Its role in directing TfR1 for endocytosis is presumably mediated by its interaction with clathrin and dynamin. An interaction between TTP and EGFR is not seen and knockdown of TTP does not affect LDL-R or EGFR internalization further supporting the conclusion that TTP functions specifically in TfR1 endocytosis. This combined with the fact that the decrease in HCV RNA levels seen after TTP knockdown was equivalent to that seen following TfR1 knockdown suggests that TfR1 endocytosis is required for HCV infection. Interestingly, although MACV uses hTfR1 for viral entry and enters the cell via clathrin-mediated endocytosis, it was relatively insensitive to TTP knockdown. This is similar to MMTV, which uses mouse TfR1 to enter cells, but has been

reported to be TTP-independent (170). This suggests that dependence on TTP is somewhat unique to HCV and that TTP in a sense is yet another factor involved in HCV entry.

It would be expected that a molecule involved in virion internalization would act later in the entry process and based on our results TfR1 does appears to act with similar kinetics to the late entry factor NPC1L1 (149). Recently, Farquhar et al. (52) reported CD81 and CLDN1 endocytosis is induced by HCV with a significant increase in intracellular CD81 and CLDN1 in the presence of infectious HCV particles but not heat-inactivated HCV. While this was interpreted to suggest that a CD81/CLDN1 complex might be involved in HCV entry, down-regulation of the surface expression of these viral receptors may be independent of viral uptake and instead related to the fact that HCV like many viruses down regulates surface expression of its receptors post-infection. A role of CD81 in HCV endocytosis would also be inconsistent with the many reports indicating that CD81 is an early HCV entry factor (98) . Hence, while kinetic studies are suggestive, further mechanistic studies are needed to clarify the roles of all the different cellular factors involved in HCV entry. In this regard, using virion labeling techniques previously employed by the Randall group (36), it would be interesting to investigate which host cell entry factors are internalized with the viral particle.

Since holo-Tf is the ligand for TfR1 it might be interesting to examine whether holo or apo-Tf binding to TfR1 modulates HCV entry, however, since TfR1 is constitutively endocytosed independent of the presence of holo-Tf (173), there is no reason to assume these known TfR1 ligands would impact TfR1 uptake rates and thus indirectly be required for HCV uptake. This may be why New World arenaviruses entry was not found to be affected by the addition of holo or apo-Tf. Likewise, we would not predict a competition for TfR1 binding if our hypothesis about HCV binding to the apical domain of TfR1 is correct as it is the helical

domain which mediates holo-Tf binding. However, it would be interesting to empirically test whether the presence of holo-Tf functionally competes for HCV binding or inhibits HCV entry.

In terms of whether changes in cellular iron homeostasis can directly impact cellular permissiveness to HCV infection, it has been shown that intracellular iron levels regulate TfR1 expression. Therefore, adding a non-transferrin dependent iron source like ferric ammonium citrate (FAC) or an iron chelating agent such as deferoxamine (DFO) to cell cultures has been shown to cause a down regulation or up regulation in TfR1 expression, respectively. In previous studies it has been shown that the addition of FAC induced a decrease in TfR1 expression and subsequently reduced TfR1-dependent entry of New World arenaviruses and that entry was increased in DFO treated (i.e. TfR1 up regulated) cells . While these experiments were performed simply as a way to manipulate cellular TfR1 levels and confirm TfR1 is required for viral entry, this also demonstrates that changes in cellular iron homeostasis can potentially modulate viral entry efficiency. It would be interesting to examine whether cellular regulation of TfR1 expression significantly affects HCV entry. Preliminary experiments to examine the effects of these agents on HCVcc infection initiation were inconclusive because TfR1 expression did not respond to treatment as expected, but treatment optimization is underway. Notably however, because TfR1 does not appear to be essential for HCV cell-to-cell spread, it is likely that changes in TfR1 expression do not influence the spread of previously established HCV infections.

In this study we demonstrate that TfR1 and TTP are required for cell-free HCV entry. This discovery further sheds light on the complex HCV entry process. Our data suggests the virion binds TfR1 and that no conformational change within the viral glycoproteins needs to occur to expose the recognition site. Although not absolutely required for HCV cell-to-cell spread, this may reveal a promising target for the development of therapeutics that could be used

in combination with other inhibitors of HCV spread. In particular, more extensive studies on the role of TfR1 in HCV entry may contribute to better understanding of the mechanisms involved in virion internalization.

4. IDENTIFICATION OF THE NIEMANN-PICK C1 LIKE 1 AS A NEW HCV ENTRY FACTOR AND ANTIVIRAL DRUG TARGET

4.1 **Background on Niemann Pick C 1 Like 1 (NPC1L1)**

The NPC1L1 cellular cholesterol uptake receptor is a homolog of the intracellular cholesterol trafficking NPC1 protein. NPC1L1 is highly N-glycosylated, contains a signal peptide, an N-terminal "NPC1 domain," and 13 transmembrane domains 5 of which comprise a sterol sensing domain (SSD) (Fig.16) (171, 179). NPC1L1 is the primary receptor that mediates unesterified cholesterol uptake from the diet. It exhibits tissue specific localization on intestinal enterocytes across all species. However, in humans and non-human primates NPC1L1 is also uniquely expressed on hepatocytes in the liver. NPC1L1 is located predominantly on the apical membrane of enterocytes and hepatocytes. Cholesterol induces NPC1L1 translocation with low cholesterol levels resulting in the accumulation of NPC1L1 on the apical membrane.

Since NPC1L1 has been demonstrated to be the main receptor for uptake of dietary cholesterol, studies have examined the cholesterol binding site on NPC1L1. Information from the crystal structure of it homolog NPC1 indicated a sterol binding pocket in cysteine rich N-terminal domain (NTD). Similarly, NPC1L1 contains a cysteine rich NTD, referred to as the large extracellular loop 1 (LEL1), leading researchers to speculate that this may be the region for cholesterol binding. This was confirmed when Zhang et al. (185) determined NPC1L1 indeed binds cholesterol via its N-terminal domain and binding was prevented after mutation of leucin 216 within the NTD.

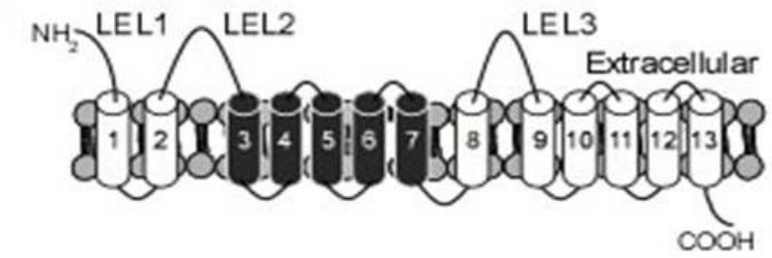

Figure 16. NPC1L1 topology. Schematic of the NPC1L1 13 transmembrane protein. The sterol sensing domains are designated in black and the large extracellular loops are depicted as LEL1-3. Originally published in Sainz et al., Nature Medicine, 2012 (149) Original illustration by Bruno Sainz Jr.

NPC1L1 is synthesized in the rough endoplasmic reticulum (ER) and transported to the plasma membrane via the golgi apparatus. Once at the plasma membrane, NPC1L1 uptake is cholesterol dependent and occurs via clathrin dependent endocytosis. The protein is then trafficked to endocytic recycling compartments and back to the cell surface or alternatively to the late endosome followed by the lysosome for degradation. During NPC1L1 trafficking, free cholesterol associated with NPC1L1 is released and carried by vesicles or carrier proteins to the ER for esterification and incorporation into chylomicrons.

NPC1L1 gene regulation is still not fully understood. Different nuclear receptors such as PPARδ, PPARα, liver X receptor (LXR) and retinoid X receptor (RXR) have been implicated in NPC1L1 regulation; however they may not be directly regulating NPC1L1 (144, 166, 180). Evidence suggests NPC1L1 may be regulated by cellular cholesterol content through the transcription factor sterol regulatory element binding proteins (SREBP-2) which regulates expression of cholesterol synthesis genes. SREBP-2 increases NPC1L1 transcription by binding two sterol regulatory elements within the NPC1L1 promoter (5, 140). However, the idea that cellular cholesterol content regulates NPC1L1 remains under debate because addition of cholesterol to mouse diet did not reduce intestinal NPC1L1 expression nor does ezetimibe treatment, an agent that causes a reduction in cellular cholesterol content , increase NPC1L1 expression (137, 165).

Ezetimibe is a member of the 2-azetidinone drug class that acts as a potent cholesterol absorption inhibitor by preventing the uptake of NPC1L1 (28, 66). It has been FDA approved and administered to treat hypercholesterolemia (commercially known as Zetia). NPC1L1 has been proven to be the molecular target for ezetimibe. Ezetimibe binds directly to NPC1L1 and the binding site has been mapped to NPC1L1's second large extracellular loop (LEL2) (174). Functionally, using knockout mice the systemic cholesterol lowering effect of ezetimibe was

shown to be mediated via NPC1L1. Specifically, cholesterol absorption was decreased to the same extent in NPC1L1 knockout as ezetimibe-treated mice; however ezetimibe-treatment had no effect in NPC1L1 knockout mice. Notably, although it had been originally reported that ezetimibe may target SRB1, SRB1 knockout mice did not show a decrease in cholesterol absorption and ezetimibe still inhibited cholesterol uptake in these mice in the absence of SRB1 (115).

As discussed in Chapter 1, the HCV viral particle is tightly associated with lipoproteins and cholesterol which play a crucial part in HCV infectivity (4, 177). Cell surface receptors, such as LDL-R and SRBI, which function in lipoprotein uptake have been identified as HCV entry factors. Because the HCV particle is highly enriched in cholesterol and virion-associated cholesterol has been shown to be involved in HCV entry and because HCV species tropism parallels that of hepatic NPC1L1 expression (i.e. is limited to only human and chimpanzees), we decided to investigate whether the recently identified cholesterol receptor, NPC1L1, is involved in HCV infection.

4.2 **Identification of NPC1L1 as an HCV Entry Factor**

4.2.1 **Silencing or Blocking Surface NPC1L1 Inhibits HCV Prior to Replication**

To determine whether NPC1L1 is involved in HCV infection, we transfected Huh7 cells with an irrelevant control siRNA or siRNAs targeting NPC1L1 or the known HCV entry factors CD81 or SR-BI. RT-qPCR and Western Blot analysis confirmed a reduction in NPC1L1 mRNA and protein siNPC1L1 transfected cells (Fig 17A). Compared to cells transfected with an irrelevant control siRNA, CD81-, SR-BI- and NPC1L1-silenced cells were significantly less susceptible to HCVcc infection at 48, 72, 96, and 120 hours post-infection and NPC1L1 knockdown was specific and not the result of off-target effects (Fig. 17C).

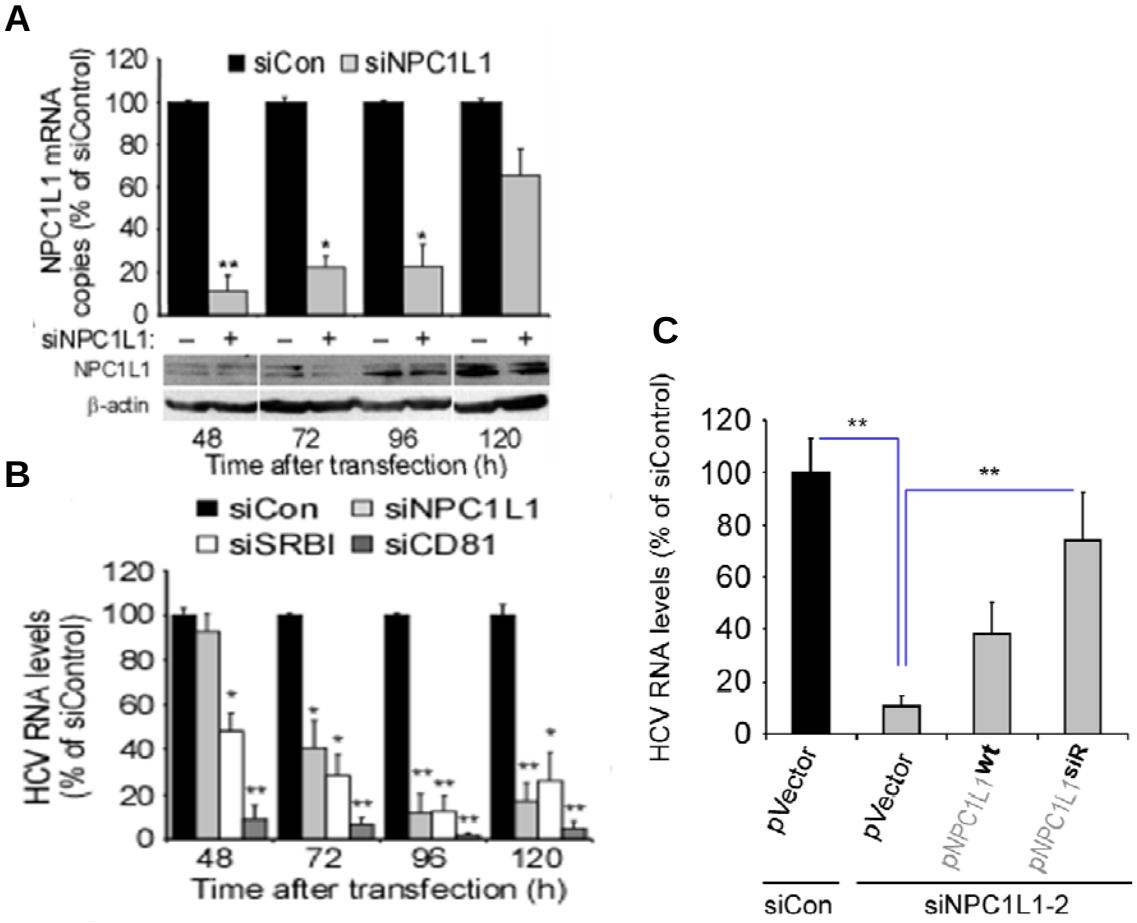

Figure 17. NPC1L1 knockdown inhibits HCVcc infection. A) NPC1L1 transcript levels were quantified by RT-qPCR, normalized to GAPDH and graphed as a percentage of the maximum number of copies determined in siCon-transfected cultures at each time point examined. Immunoblot of NPC1L1 and β-actin protein in siCon-transfected (−) and siNPC1L1-transfected cultures . **B)** HCV RNA measured by RT-qPCR in parallel Huh7 cultures at the indicated times after mock transfection or transfection with irrelevant control (siCon), SR-BI–specific, CD81-specfic or NPC1L1-specific siRNAs. Data are normalized to GAPDH are expressed as a percent of HCV RNA detected in siCon cells. **C)** Huh7 cells were reverse co-transfected with 70 nM siCon or an NPC1L1-specific siRNA (si-2) and a vector control (pVector), a plasmid encoding the wild-type NPC1L1 coding sequence (pNPC1L1wt) or a plasmid encoding an siRNA-resistant NPC1L1 coding sequence (pNPC1L1siRes). Seventy-two hours post-tranfection cells were HCVcc-infected at an MOI of 0.05 FFU cell-1 and 48 h p.i. total cellular RNA was harvested. HCV RNA was quantified by RT-qPCR, normalized to GAPDH and HCV RNA levels are graphed as a percentage of HCV RNA detected in siCon-transfected cultures. In all cases, significant differences relative to controls (one-way analysis of variance (ANOVA) and Tukey's post-hoc *t* test) are denoted as $*P < 0.05$ or $**P < 0.01$. All results are graphed as means ± s.d. for triplicate samples. Originally published in Sainz et al., Nature Medicine, 2012 (149).

To determine whether the inhibitory affect on HCV infection in NPC1L1 knockdown was due to a decrease in HCV replication, we used the HCV replicon system to test the affect of NPC1L1 knockdown on replication. Both HCV GT1b and 2a subgenomic replicon cell lines were transfected with control or NPC1L1-specific siRNA. At three days post transfection NPC1L1 mRNA levels were measured to confirm knockdown efficiency (Fig. 18A-B). NPC1L1 silencing had no effect on HCV subgenomic RNA replication tested in either HCV genotypes 2a and 1b (Fig. 18C-D).

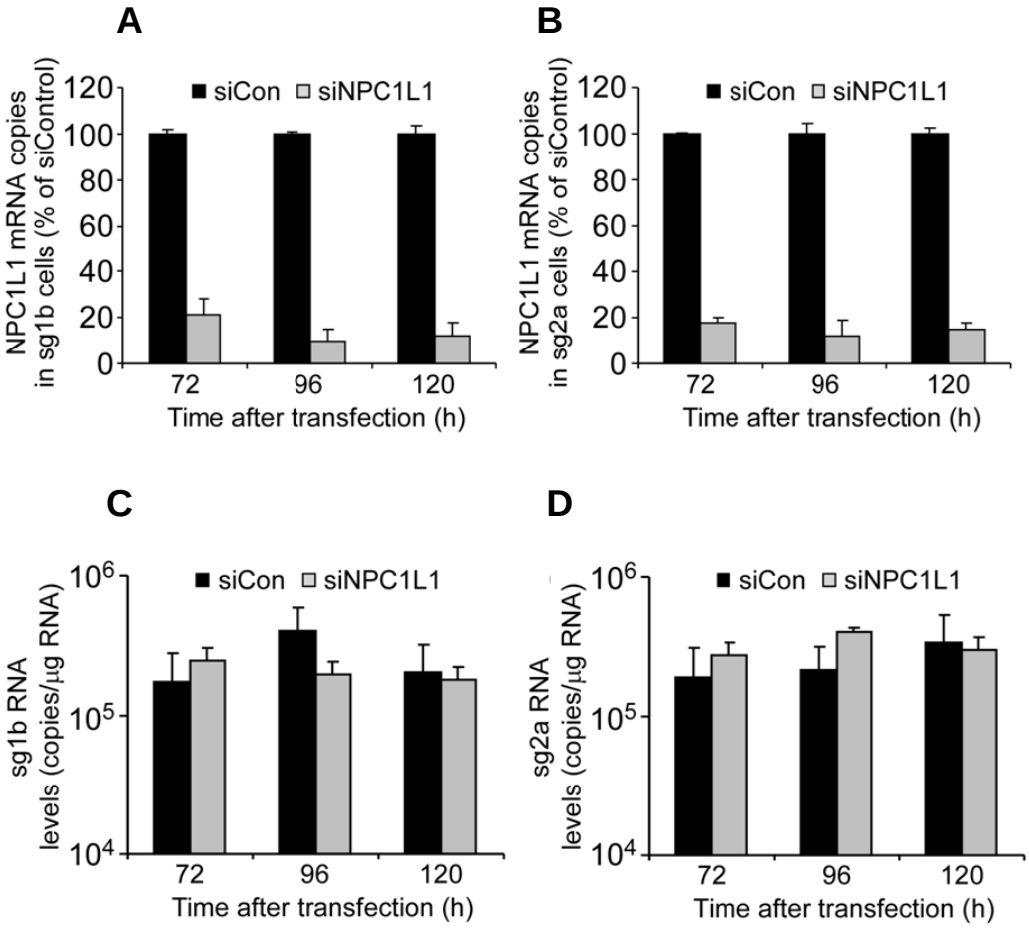

Figure 18. NPC1L1 knockdown does not inhibit HCV subgenomic RNA replication. HCV sg1b replicon (panels **A** and **C**) or HCV sg2a replicon (Panels **B** and **D**) cells were reverse transfected with 70 nM of a negative control siRNA (siCon) or siRNA targeting human NPC1L1 (siNPC1L1). At indicated times post-transfection total intracellular RNA was extracted. **A-B)** NPC1L1 mRNA levels and **C-D)** HCV RNA levels were quantified by RTqPCR and normalized to GAPDH. NPC1L1 mRNA levels are expressed as a percentage of that detected in siCon transfected cells. HCV RNA levels are displayed as HCV RNA copies/µg total cellular RNA. HCV RNA levels are displayed as HCV RNA copies/µg total cellular RNA. Significant reductions in HCV RNA levels relative to siCon-transfected cultures at each time point were assessed by one-way ANOVA and Tukey's post hoc *t* test. A reduction was noted in sg1b replicon cells 96 h post-transfection ($P = 0.08$) in this particular experiment but not in two other independent experiments. Originally published in Sainz et al., Nature Medicine, 2012 (149).

Because siRNA-mediated knockdown of NPC1L1 did not inhibit replication and we were interested to determine if NPC1L1 is involved in HCV entry, we next assessed whether HCV infection was susceptible to inhibition by antibody-mediated blocking of cell surface NPC1L1. Compared to Huh7 cells treated with irrelevant IgG control antibodies, HCVcc infection, as measured by intracellular HCV RNA levels at 24 and 48 hours post virus inoculation, was significantly reduced in cells pre-treated with an antibody specific for the known HCV cell entry factor CD81 (Fig 19A). When cells were pre-incubated with an NPC1L1-specific antibody, HCVcc infection was similarly reduced (Fig 19A). To determine which of the NPC1L1 LEL domains might be necessary for HCV entry, cells were treated with antibodies individually targeting each of the NPC1L1 LELs (Fig. 16). Of the three NPC1L1 LELs, only blocking of NPC1L1 LEL1, but not LEL2 or LEL3, reduced subsequent HCV infection (Fig 19B). Thus, NPC1L1 silencing and antibody-mediated blocking of NPC1L1 LEL1 reduced HCV infection as effectively as targeting other known HCV cell entry factors.

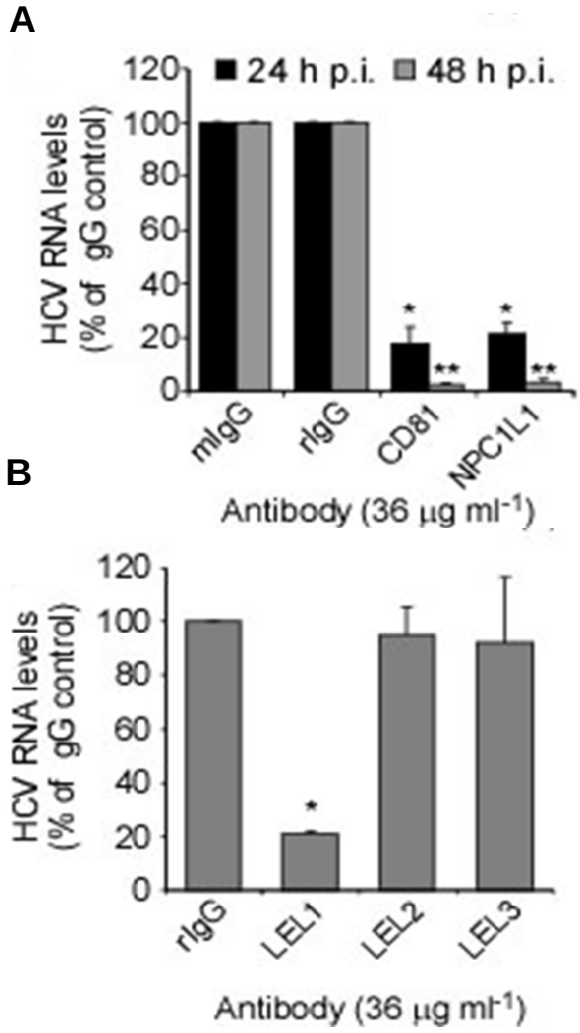

Figure 19. Anitbody blocking of NPC1L1 LEL1 inhibits HCV infection. A) Intracellular HCV RNA levels detected in parallel Huh7 cell cultures treated for 1 h before and during HCVcc infection with 36 μg/ml of a mouse (mIgG) or rabbit (rIgG) isotype control antibody, a CD81-specific antibody or NPC1L1 antibody **B)** Antibodies specific for each of the three LELs of NPC1L1 were incubated with cells for 1 h before and during HCVcc infection. Shown are HCV RNA levels, determined by RT-qPCR and normalized to GAPDH levels, 24 h (**A**) or 48 h (**A and B**) after infection. Results are graphed as a percentage of infection achieved in the respective IgG control-treated cultures. In all cases, significant differences relative to controls (one-way analysis of variance (ANOVA) and Tukey's post-hoc *t* test) are denoted as $*P < 0.05$ or $**P < 0.01$. Originally published in Sainz et al., Nature Medicine, 2012 (149).

4.2.2 **Blocking NPC1L1 Uptake Inhibits HCV Infection Initiation**

As ezetimibe has been shown to be a direct inhibitor of NPC1L1 internalization we next used this high-affinity, specific pharmacological agent as an alternate means of targeting NPC1L1 before, during or after viral inoculation (28, 174). For this, we performed in parallel an HCVcc foci-reduction assay. The foci assay consisted of quantifying HCV-positive foci (clusters of ≥5 HCV E2–positive cells) three days post viral inoculation as a measure of individual HCV infection initiation events. Ezetimibe reduced HCVcc foci formation in a dose-dependent manner when present before infection and then removed (pre-treatment) (Fig. 20A) or when present only during virus inoculation (co-treatment) (Fig. 20B). However, when we added ezetimibe to cells after viral inoculation (post-treatment), the initiation of HCV-positive foci was unaffected, as would be expected for a viral entry inhibitor (Fig. 20C). Notably, the highest dose of ezetimibe (25 μM) reduced the size of the HCV-positive foci observed from the typical ≥5 HCV E2–positive cells to only 1–3 HCV E2–positive cells per focus, which accounts for the lower number of foci with ≥5 HCV E2-positive cells being counted in those cultures. Notably this suggests that NPC1L1 may also be required for efficient HCV cell-to-cell spread.

Importantly, we confirmed that the antiviral effect of ezetimibe was not due to drug-mediated cytotoxicity (Fig, 21A), changes in cell proliferation (Fig. 21B) or inhibition of HCV RNA replication (Fig.21C). Finally, we also confirmed that NPC1L1 is involved in the entry of other HCV genotypes, by testing the ezetimibe sensitivity of a panel of HCVcc intergenotypic clones containing the structural regions of diverse HCV genotypes (1 – 7) (Fig. 22).

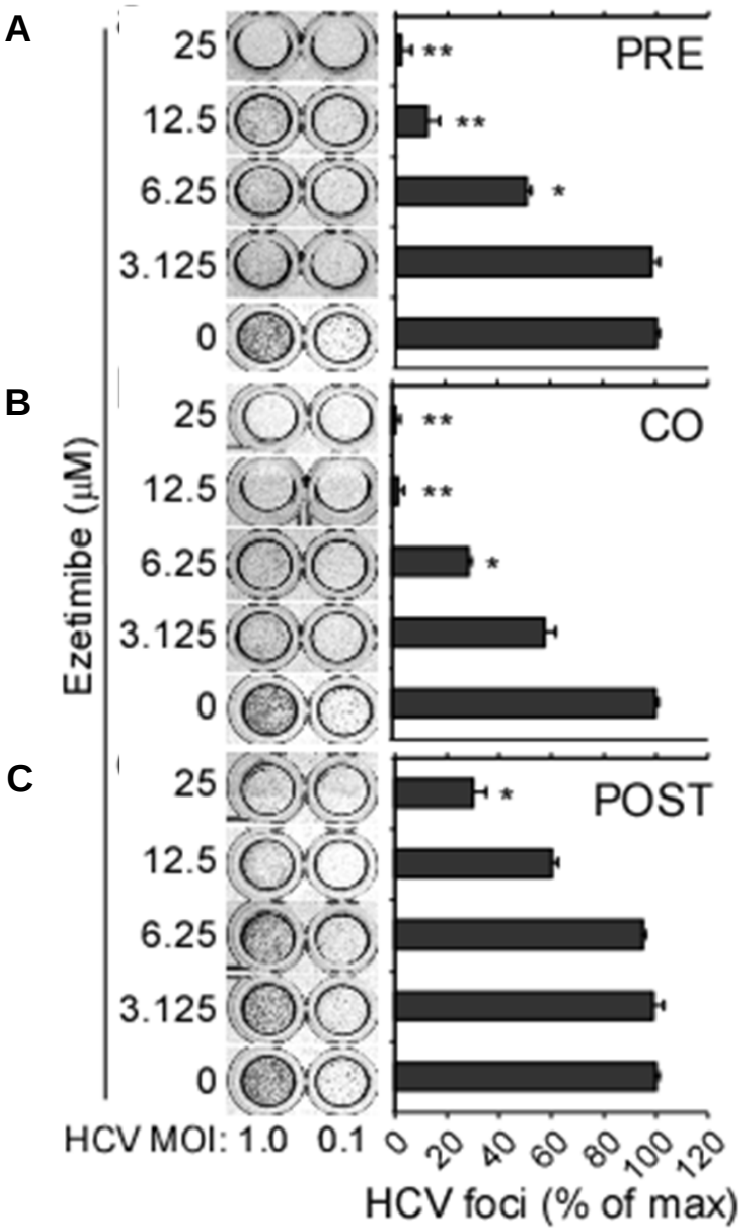

Figure 20. Ezetimibe inhibits HCV infection initiation. A) Quantification of HCV foci observed in Huh7 cultures treated with vehicle or increasing concentrations of ezetimibe for 6 h before infection and then removed (PRE), **B)** 12 h coincident with viral inoculation and then removed (CO), **C)** or after viral inoculation . HCV foci are expressed as a percentage of the foci obtained in vehicle-treated (0 μM). **P* < 0.05 or ***P* < 0.01 for HCV relative to vehicle-treated cultures (one-way ANOVA and Tukey's post-hoc *t* test). Originally published in Sainz et al., Nature Medicine, 2012 (149).

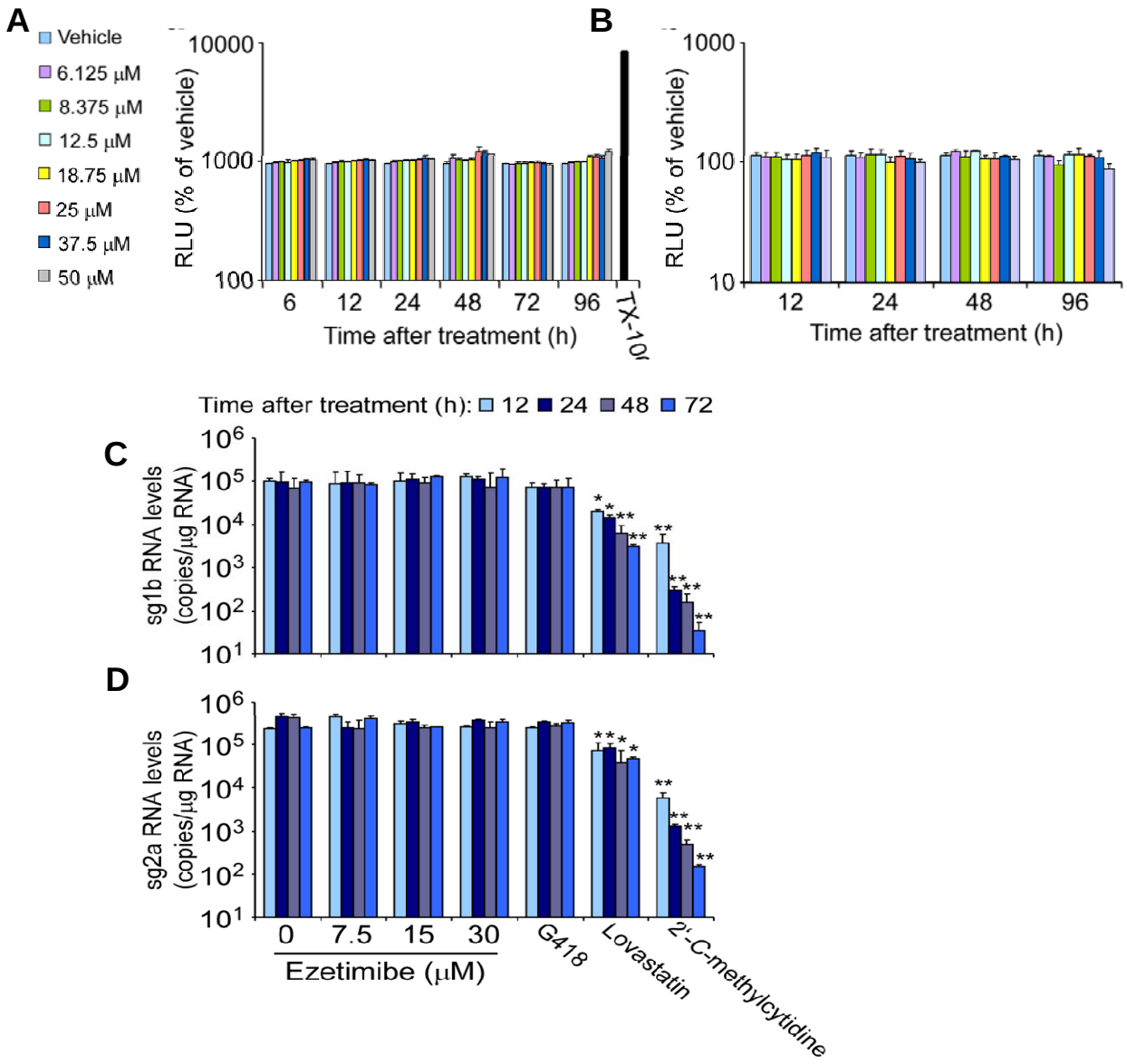

Figure 21. Ezetimibe is non-cytotoxic, does not affect cell proliferation or HCV subgenomic RNA replicaton. A) At indicated time post-treatment culture supernatant was harvested and ezetimibe-induced toxicity was determined by measuring cellular release of adenlyate kinase (AK) using the ToxiLight® Non-destructive Cytotoxicity luminescence assay kit. Lysed Huh7 cells served as positive control for maximum AK release from Huh7 cells. **B)** Cultures were lysed and bioluminescence detection of cellular ATP was performed using the ViaLight® Plus Cell Proliferation luminescence-based assay kit as a quantitative measure of cell viability. Results are presented as a percentage of relative luminescence (38) compared to vehicle-treated cultures. **C)** HCV sg1b replicon cells **D)** or HCV sg2a replicon cells were vehicle-treated or treated with the indicated concentrations of ezetimibe, G418 (500 µg ml^{-1}), Lovastatin (15 µM), or 2'-*C*-methylcytidine (12.5 µM) for 72 h. Intracellular RNA was collected from triplicate wells at indicated times post-treatment and HCV RNA was quantified by RTqPCR, normalized to GAPDH and is displayed as HCV RNA copies/µg total cellular RNA. Originally published in Sainz et al., Nature Medicine, 2012 (149).

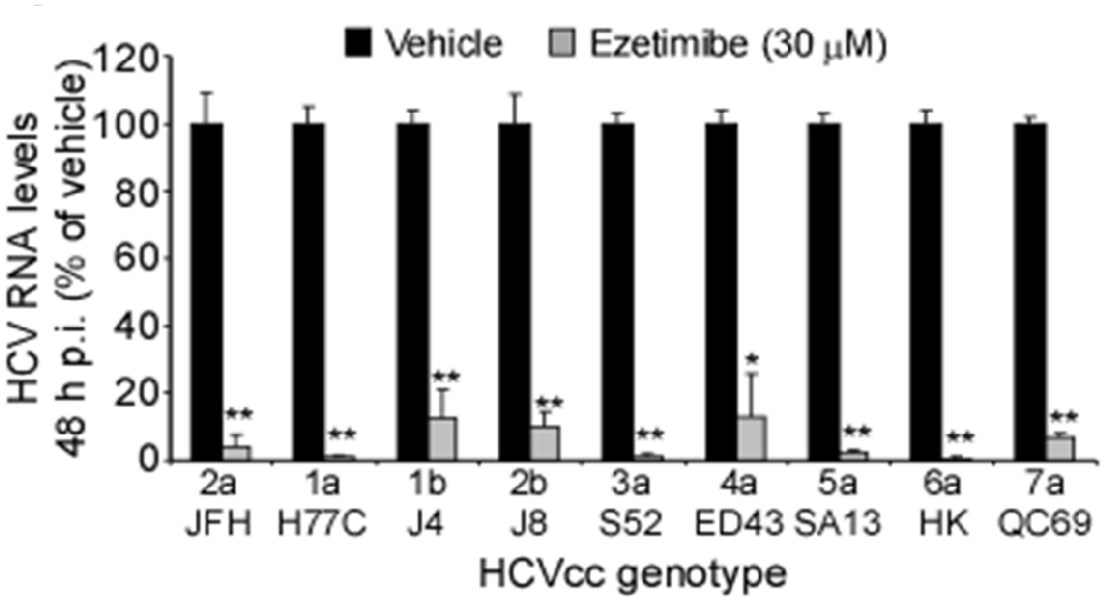

Figure 22. Ezetimibe inhibits entry of different HCV genotypes. Parallel Huh7 cultures were treated with vehicle or ezetimibe beginning 1 hour before and during infection with HCV chimeric viruses containing the structural region of the indicated genotypes and the nonstructural region of JFH-1. HCV RNA levels were determined by RT-qPCR and normalized to GAPDH at 48 hours post-infection. Results are graphed as a percentage of infection in vehicle-treated cultures. *$P < 0.05$ or **$P < 0.01$ for HCV relative to vehicle-treated cultures (one-way ANOVA and Tukey's post-hoc *t* test). Originally published in Sainz et al., Nature Medicine, 2012 (149).

We next assessed whether ezetimibe inhibits HCVcc binding or a post-binding step by examining cell-associated HCV RNA versus intracellular protein expressed from internalized RNA in vehicle- and ezetimibe-treated HCVcc-infected cultures. At 10 hours after infection, a time before detectable HCV RNA replication occurs, ezetimibe did not affect cell-bound HCV RNA levels (Fig. 23A). In contrast, at later time points, HCV RNA levels (Fig. 23A) and *de novo* NS5A protein expression were reduced in ezetimibe-treated cultures(Fig. 23B-C), suggesting HCV can efficiently bind ezetimibe-treated cells, but a post-binding step required for HCV RNA to enter the cytoplasm and be translated is prevented.

To further test this hypothesis and determine whether ezetimibe acts prior to viral fusion with the cell a fluorescence-based HCVcc fusion assay was performed. Specifically, HCVcc was labeled with the hydrophobic fluorophore DiD (182), which incorporates into biological membranes and, at high concentrations, is self-quenching. Upon fusion of viral and target membranes, the DiD fluorophores diffuse away from each other, causing dequenching and allowing for the progression or inhibition of fusion to be measured in real time. Compared to NH_4Cl, an inhibitor of endosomal acidification (163) , ezetimibe more potently inhibited $HCVcc^{DiD}$ fusion, such that only ~10% $HCVcc^{DiD}$ of the dequenching observed in vehicle-treated controls was detected in ezetimibe-treated cultures (Fig. 24A). Analogously, antibody-mediated inhibition of both CD81 and NPC1L1 also reduced $HCVcc^{DiD}$ fusion, indicating that the inhibition observed in ezetimibe-treated wells was not drug specific (Fig. 24B), but that NPC1L1 prevents HCVcc cell entry at or before virion–host cell fusion. Time of addition experiments with ezetimibe added at various times after virus binding at 4° C further indicated that ezetimibe retained inhibitory activity after temperature shift to 37° C for up to 5 hours (half maximal inhibition at 4 hours), confirming that NPC1L1 functions after binding, probably late in viral entry (Fig. 25).

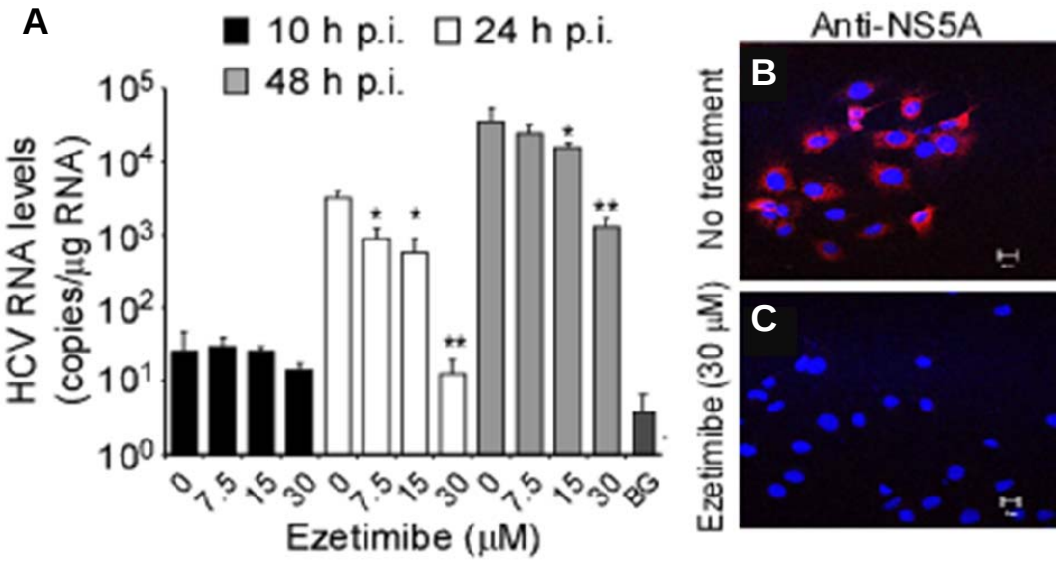

Figure 23. Ezetimibe does not inhibit HCV binding, but prevent productive entry. A) Parallel Huh7 cultures treated with vehicle or the indicated concentrations of ezetimibe beginning 1 hour before and during infection with HCV RNA levels determined by RT-qPCR and normalized to GAPDH, at the indicated times after infection. Results are graphed as mean HCV RNA copies per μg total cellular RNA ± s.d. Assay background (BG) is the HCV RNA level detected in uninfected samples. **B and C)** As readout of productive HCV entry into cells indirect immunofluorescence analysis of HCV NS5A was performed in vehicle-treated and ezetimibe-treated (30 μM) cultures 24 h after infection with HCVcc JFH-1. Scale bars= 20 μm *$P < 0.05$ or **$P < 0.01$ for HCV relative to vehicle-treated cultures (one-way ANOVA and Tukey's post-hoc *t* test). Originally published in Sainz et al., Nature Medicine, 2012 (149).

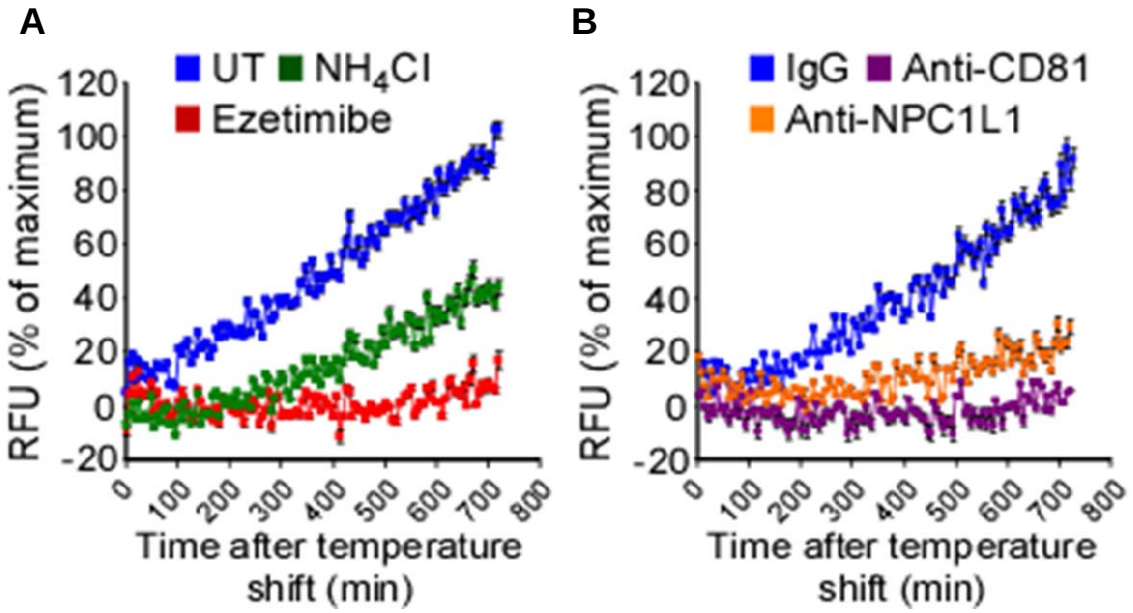

Figure 24. NPC1L1 acts at a pre-fusion step in HCV entry. A) HCV fusion measured by DiD fluorescence dequenching in Huh7 cells pre-treated with vehicle (UT), NH_4Cl (10 mM), ezetimibe (30 μM) or **B)** IgG control antibody (36 μg ml^{-1}), CD81-specific antibody (anti-CD81, 36 μg ml^{-1}), or an antibody to NPC1L1 LEL1 (anti-NPC1L1, 36 μg ml^{-1}). Results are graphed as a percentage of maximum background-corrected relative fluorescence units (RFU) achieved in vehicle-treated or IgG control-treated cultures. Originally published in Sainz et al., Nature Medicine, 2012 (149).

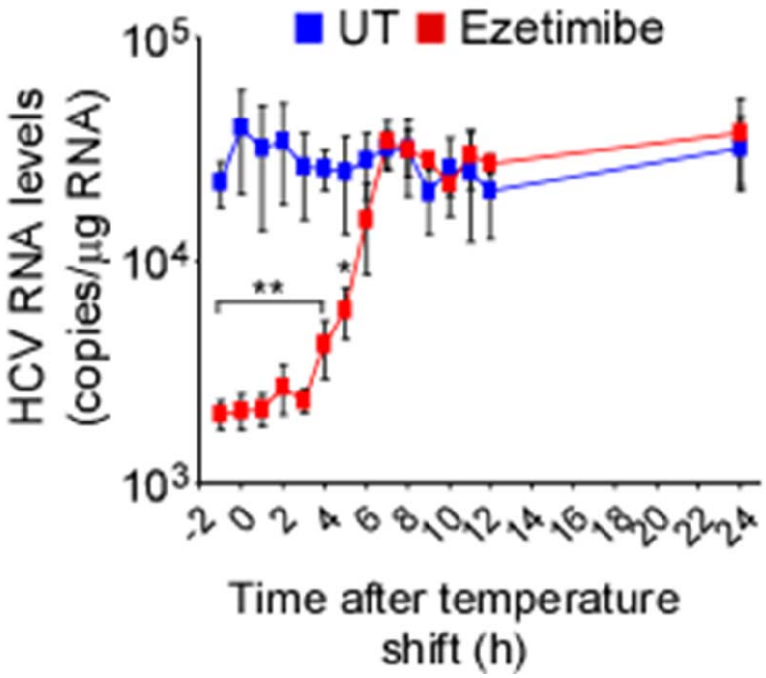

Figure 25. Ezetimibe inhibits HCV entry at a post binding step. Parallel Huh7 cultures were either treated with vehicle or ezetimibe (30 μM) before or during infection at the indicated times. Viral inoculum was added at 4 degrees for 1 hour to synchronize infections. Intracellular HCV RNA levels were detected by RT-qPCR and normalized to GAPDH 30 hours after infection. Results are graphed as mean HCV RNA copies per μg total cellular RNA. $*P < 0.05$ or $**P < 0.01$ for HCV relative to vehicle-treated cultures (one-way ANOVA and Tukey's post-hoc *t* test). Originally published in Sainz et al., Nature Medicine, 2012 (149).

4.2.3 **NPC1L1 is Down Regulated During HCV Infection**

To determine if HCV infection alters NPC1L1 protein expression, Huh7 cells were either mock infected or infected with HCVcc and cell lysates were harvested a different time points during infection. NPC1L1 protein levels in infected and mock infected cell were assessed by western blot and infection was confirmed by blotting for the viral protein NS3. We observed a down regulation of NPC1L1 in HCVcc-infected Huh7 cultures compared to uninfected cultures as early as day 4 after infection and NPC1L1 levels remained down regulated until the end of the experiment at day 12 after infection (Fig. 26). This, similar to what has been observed for other HCV entry factors (111), our data indicates HCV infection down regulates NPC1L1.

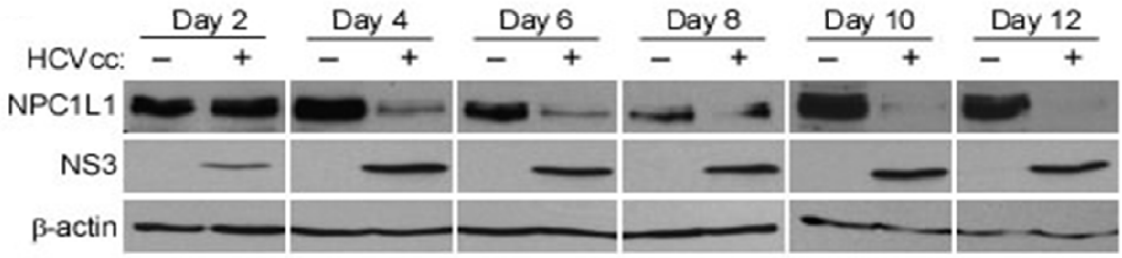

Figure 26. HCV infection down regulates NPC1L1 protein expression. NPC1L1 and NS3 protein levels analyzed by western blot over the course of 12 days in Huh7 cells either mock infected or infected with HCVcc at an MOI of 3 ffu/ml. β-actin levels measured as a loading control. Originally published in Sainz et al., Nature Medicine, 2012 (149).

4.2.4 **Ezetimibe Treatment Inhibits HCV Infection Initiation *In vivo***

To confirm the involvement of NPC1L1 in HCV cell entry *in vivo*, we evaluated the ability of ezetimibe to inhibit infection of a genotype 1 clinical isolate in a hepatic xenorepopulation mouse model of acute HCV infection (95). Specifically, in collaboration with Dr. Chayama's group at Hiroshima University, urokinase-type plasminogen activator–severe combined immunodeficiency (uPA-SCID) mice were repopulated with human hepatocytes and then treated via oral gavage with ezetimibe (10 mg per kg body weight per day) or diluent (i.e. corn oil) alone for a total of 3 weeks, with treatment beginning 2 weeks, 1 week or 2 d before challenge with HCV genotype 1b positive serum containing 1 x 10^5 genome equivalents of HCV (Fig. 27A).

As expected, 100% of the nine control diluent–treated mice were serum positive for HCV 1 week after challenge, whereas 71% (five out of seven) of mice treated with ezetimibe for 2 weeks before infection were HCV negative, respectively (Fig. 27B). While two of the five mice in the 2-week ezetimibe pretreatment group that were HCV negative at week 1 became HCV-positive by week 2 post-challenge (data not shown), two were completely protected and remained HCV negative at weeks 2 and 3 after infection (and one mouse died during gavage). Thus, similar to what was recently reported for another potential HCV entry inhibitor, erlotinib[30], ezetimibe was able to significantly ($P = 0.0192$) delay and in some cases prevent HCV infection *in vivo* indicating that as observed in cell culture, NPC1L1 plays a role in HCV infection initiation. Notably, however, when mice were pretreated for only 1 week before infection (Fig. 27C), ezetimibe only delayed infection in 43% (three out of seven) mice ($P = 0.062$) and it was completely ineffective when treatment was initiated only 2 d before challenge or after infection had been established (data not shown).

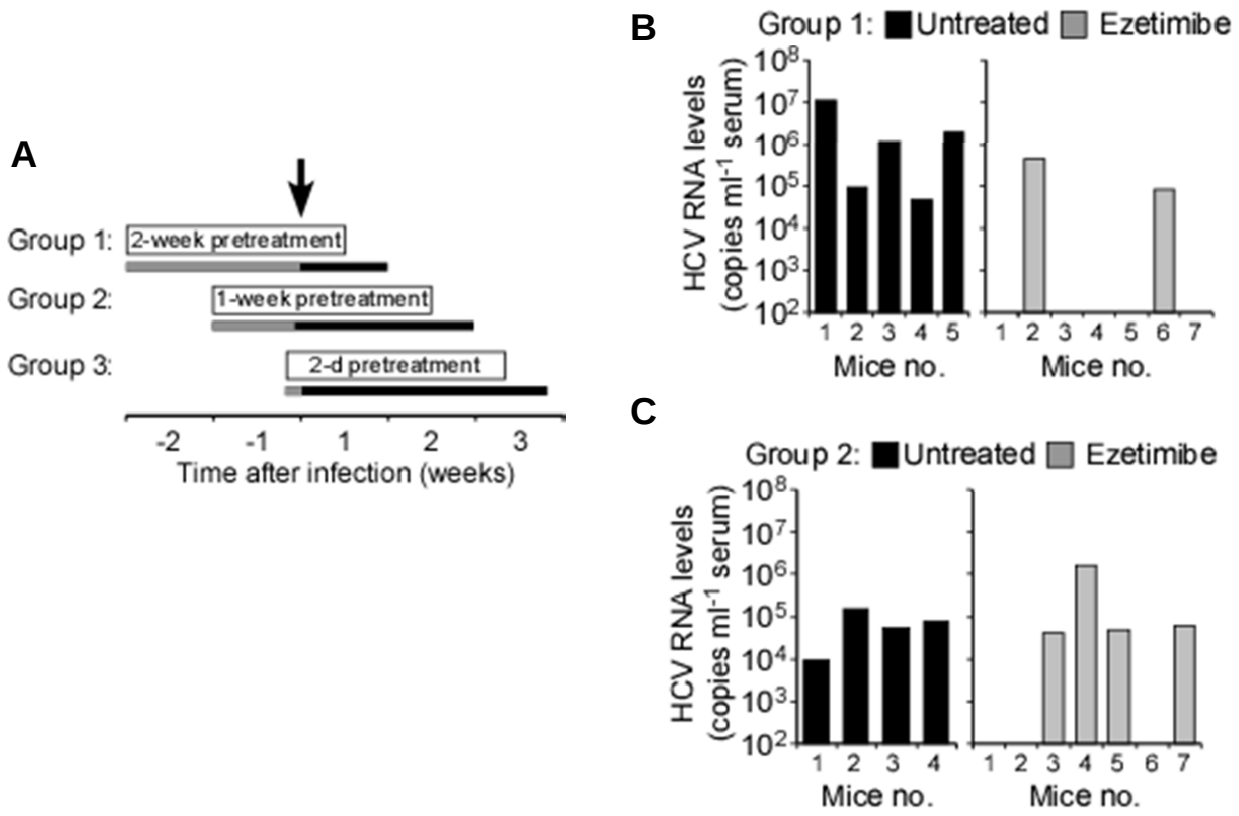

Figure 27. Ezetimibe delays the establishment of HCV infection in hepatic xenorepopulated mice. A) Schematic diagram of an experiment in which uPA-SCID mice transplanted with human hepatocytes were pretreated with diluent alone (n = 4 or 5) or ezetimibe (10 mg per kg body weight per day) (n = 7), via oral gavage, starting 2 weeks, 1 week or 2 d before infection (indicated by gray bars). The arrow indicates when the mice were intravenously inoculated on day 0 with HCV human serum containing 1.0×10^5 genome copies of HCV genotype 1b and the black bars indicate the time period post-HCV inoculation that treatments were continued. **B, C)** HCV RNA levels (genome copies per milliliter of serum) 1 week after infection from mice pretreated for 2 weeks **B)** or 1 week **C**). The lower limit of HCV RNA detection is equal to 100 genomic copies per milliliter of serum. A two-tailed Fisher's exact test was performed to compare categorical variables. In all cases, $P < 0.05$ was used to reject the null hypothesis that the distribution of HCV-positive and HCV-negative mice between ezetimibe-treated and nine diluent-treated mice at specific weeks after infection were the same. Originally published in Sainz et al., Nature Medicine, 2012 (143).

4.3 NPC1L1 as a Therapeutic Target During Chronic HCV Infection

4.3.1 Introduction

As discussed earlier, for the last 20 years the SOC for chronically infected patients has been pegIFN-α/RBV. PegIFN-α/RBV treatment is effective in approximately 50% of patients and response to treatment is heavily dependent on HCV genotype and the genetic background of the patient. Therefore, developing therapies either to replace or complement pegIFN-α/RBV to boost the rate of response is of particular interest. Most recently protease inhibitors, Telaprevir and Boceprevir, have been introduced in the clinic and administered in combination with pegIFN-α and RBV. In the near future we expect polymerase inhibitors will also be approved for use in combination with pegIFN-α and RBV. While ezetimibe and other entry inhibitors alone may not be expected have immediate effects on reducing HCV levels in already infected cells, we were interested to test if this FDA-approved, clinically available drug might be useful as part of a combination therapeutic approach against chronic HCV infection.

4.3.2 Synergy Between Ezetimibe and Type 1 Interferons

To test whether the NPC1L1 inhibitor, ezetimibe, had any effect on chronic steady state HCV infection when added in combination with IFN-α, Huh7 cells were infected with HCVcc and serially passaged for 10 days to establish a chronic infection. Cell lysates were harvested during split times to confirm steady state HCV RNA levels had been reached. At day 12 parallel cultures were left untreated or treated with 100U/ml IFN-α alone, 30uM ezetimibe alone, or a combination of both 30uM ezetimibe and 100U/ml IFN-α. Cells were then continually passaged for 70 days in the presence of the inhibitors. As predicted treatment with ezetimibe alone had no effect on chronic HCV RNA levels, which were similar to those seen in cells left untreated. As expected, treatment with IFN-α alone resulted in approximately a 2 log decrease in HCV RNA

by day 30 compared to the control untreated cultures (Fig. 28A). However, combination treatment with both IFN-α and ezetimibe caused a 4.5 log decrease in HCV RNA, a 1000-fold enhancement of inhibition compared to IFN-α alone (Fig. 28A).

We next determined if HCV infection had been cured by the combination treatment. On day 75 post-treatment initiation, we removed the 100U/ml IFN-α alone treatment and the combination 30uM ezetimibe and 100U/ml IFN-α treatment from one set of cultures and continued treating a second set. By day 80, five days after removal of treatment, HCV RNA in the cultures previously treated with 100U/ml IFN-α had rebounded to levels seen in untreated cultures. In contrast, after removal of the combination 30uM ezetimibe and 100U/ml IFN-α treatment, HCV RNA levels remained at the detection limit of our assay similar to levels in cells in which treatment remained consistent (Fig. 28B). Hence, ezetimibe acted synergistically with IFN-α to an extent that was able to eliminate HCV from the culture.

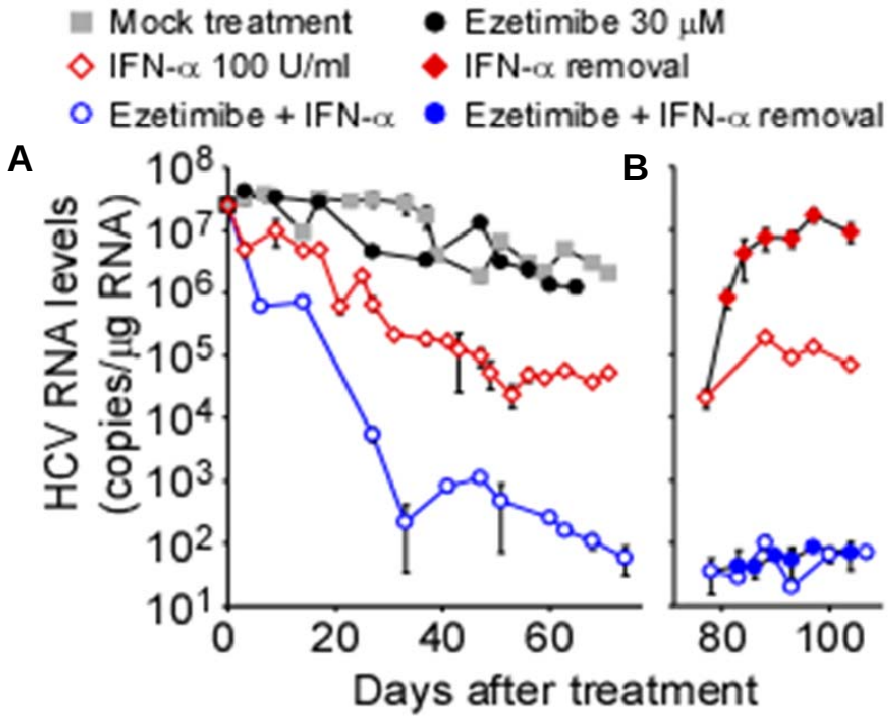

Figure 28. Ezetimibe behaves synergistically with Interferon-α. A) HCV RNA determined by RT-qPCR and normalized to GAPDH in chronically infected Huh7 cells either mock treated or treated with ezetimibe (30μM), IFN-α (100 U/ml), or both inhibitors. **B**) HCV RNA levels after removal of treatments. Mock treatment (gray squares), IFN-α (open red diamond), ezetimibe (black circle), ezetimibe and IFN-α (open blue circle), IFN-α removal (closed red diamond), ezetimibe and IFN-α removal (closed blue circle).

Because HCV typically infects differentiated, non-growing hepatocytes in the liver, we additionally tested the potential synergy between IFN-α and ezetimibe in non-dividing cell cultures chronically infected with HCVcc. For this Huh7 cells were plated on collagen in 96-well plates and cultured in medium supplemented with 1% DMSO for 20 days to induce cells to enter into a more differentiated, non-dividing state and were then infected with HCVcc (150). After fifteen days post viral inoculation, when HCV RNA levels reached steady state in representative wells, parallel wells were either mock treated or treated with 30uM ezetimibe alone, 100U/ml IFN-α alone or both 30uM ezetimibe and 100U/ml IFN-α. Although the cells were non-dividing and thus required no passaging, drug treatments were replenished when the medium was changed every 2 days. Cellular RNA was harvested from duplicate wells every week for eight weeks. Similar to what was observed in growing cells treated with IFN-α alone, by 60 days post treatments HCV RNA levels were reduced by at least 1.5 logs and 30uM ezetimibe alone had no inhibitory effect on HCV RNA levels compared to controls (Fig. 28A). However, again a synergistic reduction in HCV RNA was seen in cultures treated with 100U/ml IFN-α and 30uM ezetimibe (Fig. 29A). Similar experiments were performed using 20U/ml IFN-β and analogous results seen (Fig. 29B).

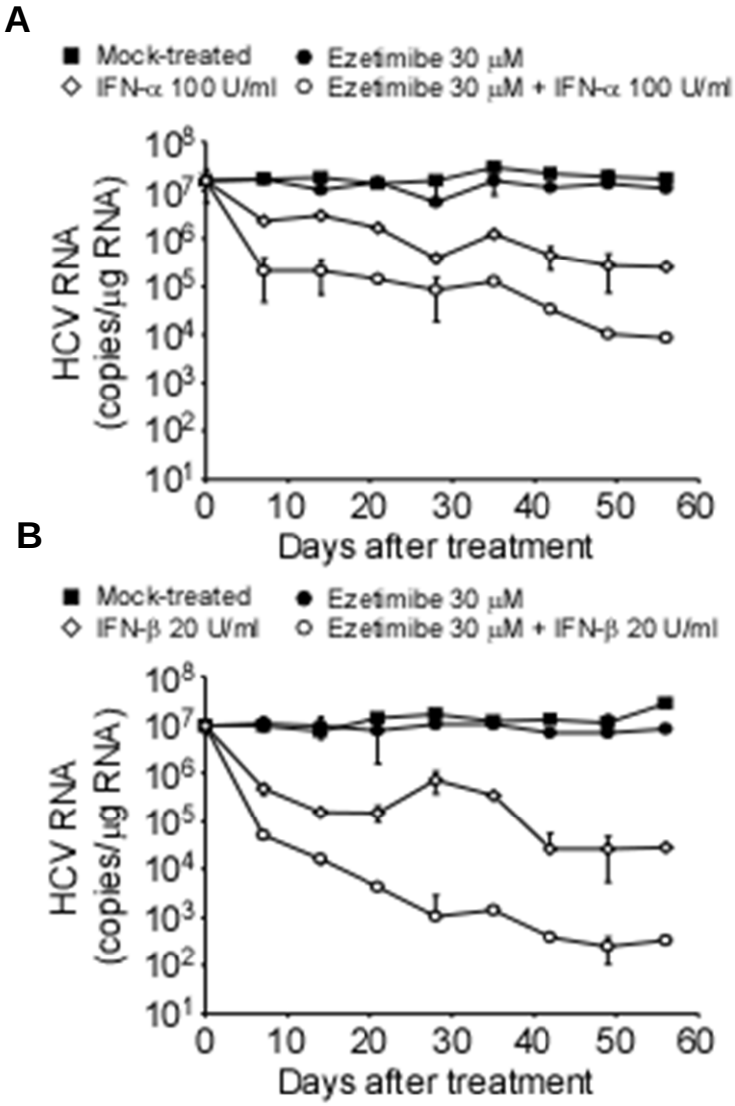

Figure 29. Ezetimibe behaves synergistically with Interferon in Non-dividing Huh7 cells. A) Non dividing Huh7 cells were either mock treated or treated with ezetimibe (30μM), IFN-α (100 U/ml), or both inhibitors Or **B)** Ezetimibe (30μM), IFN-β (20 U/ml), or both inhibitors. HCV RNA levels were determined by RT-qPCR and normalized to GAPDH. Mock treatment (black squares), **A)** IFN-α **B)** IFN-β (open diamond), ezetimibe (black circle), **A)** ezetimibe and IFN-α (open circle), **B)** ezetimibe and IFN-β (open circle).

As a final preclinical assessment of ezetimibe as a potential HCV antiviral, we are testing whether ezetimibe has the same synergistic effects with IFN-α *in vivo*; results from the initial experiment are shown in Figure 30. Again, in collaboration with Dr. Chayama's group at Hiroshima University, uPA-SCID mice were repopulated with human hepatocytes and then infected with HCV genotype 1b positive serum containing 1 x 10^5 genome equivalents of HCV. Serum HCV RNA levels followed to assess establishment of a steady state infection. Mice were then either treated with ezetimibe alone (oral gavage), IFN-α alone (injection) or a combination of both ezetimibe and IFN-α. While treatment with ezetimibe alone has no effect on HCV infection, consistent with the observed *in vitro* results, combination treatment with ezetimibe and IFN-α resulted in a higher reduction in serum HCV RNA levels than IFN-α treatment alone (Fig. 30).

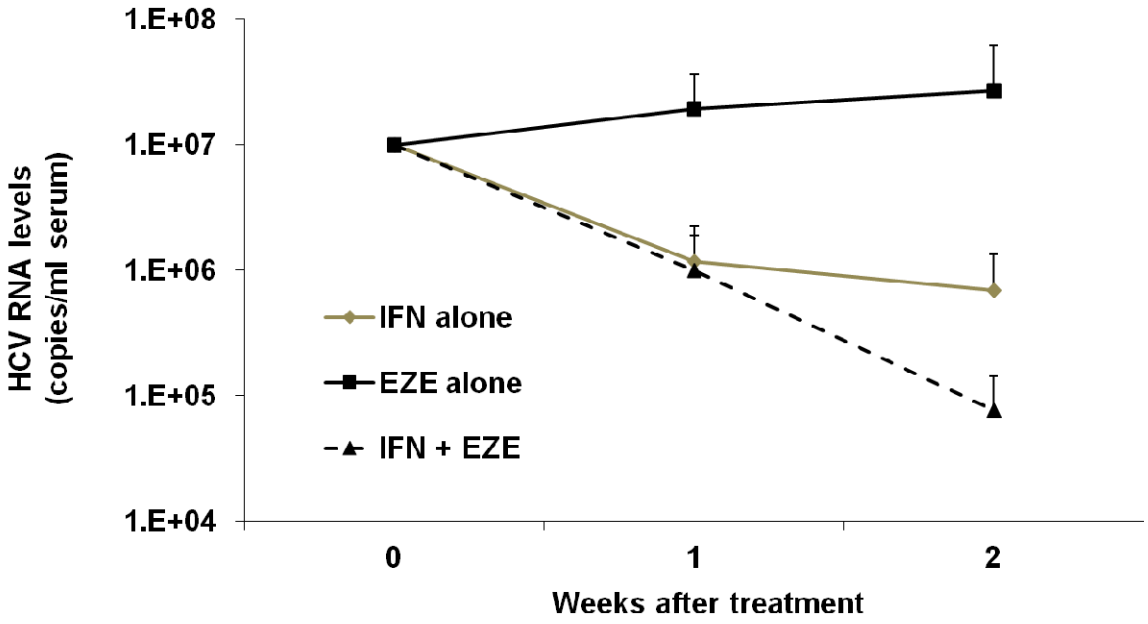

Figure 30. Ezetimibe enhances HCV inhibition when administered with Interferon in hepatic xenopopulated HCV infected mice. uPA-SCID mice transplanted with human hepatocytes were intravenously inoculated with HCV human serum containing 1.0×10^5 genome copies of HCV genotype 1b. Once steady state HCV serum levels were achieved, mice were treated with ezetimibe alone, IFN-α alone or ezetimibe and IFN-α and HCV RNA levels (genome copies per milliliter of serum) were measured 1 and 2 weeks post treatment. Ezetimibe treated (black line), IFN-α treated (gray line) and ezetimibe and IFN-α treated (dotted line).

4.4 **Discussion**

In this study we identified NPC1L1 as an HCV cell entry factor and a potential HCV antiviral target. Specifically, we show that HCV infection is dependent on NPC1L1 expression and is inhibited after NPC1L1 silencing (Fig. 17A), antibody blocking (Fig. 19A), or inhibition with ezetimibe (Fig. 20). To confirm NPC1L1 was not negatively affecting replication we showed that neither NPC1L1 knockdown or ezetimibe inhibition had an effect on subgenomic HCV RNA replication (Fig. 17 and 21). Furthermore, ezetimibe inhibition was not specific to the JFH-1 2a GT but also inhibited 6 other HCV GTs suggesting NPC1L1 is a universal entry factor among the HCV genotypes (Fig. 22)

Antibody mapping experiments revealed that while blocking LEL2 and 3 had no effect on HCV infection, blocking of the NPC1L1 LEL1 dramatically reduced subsequent HCV infection suggesting this may be the domain important in HCV infection (Fig. 19B). Interestingly, the LEL1 is located in the NTD which has been proposed to be the cholesterol binding site and NPC1L1 internalization is induced by cholesterol binding. These findings suggest the virion associated cholesterol may be recognized by NPC1L1 possibly allowing the virus to utilize the receptor for uptake into the cell. Relevant to this hypothesis, additional data from our laboratory has shown that the relative sensitivity of different HCV particles to ezetimibe mediated inhibition (i.e. the relative dependence of their entry on NPC1L1) correlates with the varying amounts of cholesterol associated with their virion. The most striking example of this correlation was observed with HCV pseudoparticles (HCVpp), which has very minimal virion cholesterol and was resistant to NPC1L1 inhibition compared to a cell culture adapted HCVcc virus, $HCVcc^{G451R}$, which contains 50% more cholesterol per viral genome than the wild type JFH-1 HCVcc and exhibited 3-fold higher sensitivity to ezetimibe inhibition. Viewed in a different way, the lack of HCVpp dependence on NPC1L1 for entry into cells is also consistent

with the hypothesis that NPC1L1 does not interact with the HCV glycoproteins. Notably, there is precedent for HCV to interact with cellular factors via its lipid content rather than its viral glycoproteins. In particular, while it has been shown that the host cell LDL-R is involved in HCVcc entry, it also has been shown not to be required for HCVpp entry and is therefore believed to interact with HCV via its virion lipoprotein content. That being said, more conclusive studies need to be performed to assess of the interaction between NPC1L1 and the HCV virion as well as the virion components that might be involved.

Using ezetimibe in various HCV entry kinetic experiments indicated that NPC1L1 is acting at a post-binding, pre-fusion step during the entry process (Fig. 24A) with ezetimibe losing its inhibitory activity between 4-5 hours post-binding (Fig. 25). These results suggest NPC1L1 may be acting late during the entry process, which could be consistent with the hypothesis that NPC1L1 may be involved in virion internalization. However, it is also plausible that interaction with NPC1L1 may alter HCV virion cholesterol in a way that unmasks or causes a conformational change within the viral glycoproteins to prime them for their next interaction.

Because the *in vitro* cell culture environment does not necessarily recapitulate the cellular environment *in vivo*, the finding that ezetimibe treatment inhibits HCV infection in the uPA-SCID chimeric mouse model lends support to the theory that NPC1L1 is involved in HCV entry in vivo (Fig. 27). However, the need to pre-treat mice for two weeks to achieve significant inhibition of HCV infection raises questions regarding the efficiency of ezetimibe delivery to the liver. Since NPC1L1 is highly expressed on the apical surface of intestinal enterocytes, one can imagine that a considerable amount of orally administered ezetimibe might initially bind to these cells following oral administration, perhaps delaying accumulation in the liver. Thus, it is plausible that development of alternate non-oral delivery or drug-targeting methods might improve transport of ezetimibe to hepatocytes and increase its anti-HCV efficacy.

Small compound inhibitors targeting the viral protease and polymerase that work synergistically with or enhance pegIFN have been developed and are currently being used in the clinic. Our findings that ezetimibe can act synergistically with IFN treatment *in vitro* in growing and non dividing cells prompted us to test the effect of combination treatment in an in vivo setting. Although the effect seen *in vivo* was not as dramatic as that observed *in vitro*, the observation that ezetimibe enhanced IFN inhibitory effect provides evidence that ezetimibe might prove useful in combination with IFN in the clinical setting. As suggested earlier, NPC1L1 is highly expressed on the enterocytes (152, 185) and therefore initial ezetimibe treatment most likely binds to the NPC1L1 in the small intestine. Perhaps if duration of ezetimibe treatment combined with IFN was extended a more dramatic enhancement of inhibition would be observed. Currently mouse experiments are underway to confirm ezetimibe synergistic effect with IFN.

As discussed previously, many factors make IFN a less than desirable treatment option. Toxic side effects and limitations such as HCV GT, age, weight, and race affect IFN efficacy. Therefore, there is a desire to develop IFN free combination therapies. In theory the synergistic effect of ezetimibe should not be limited to combination treatment with IFN but should occur with any HCV inhibitor that has the ability to clear HCV from a subset of infected cells. *In vitro* studies done in our lab have demonstrated that ezetimibe acts synergistically with HCV protease inhibitors (data not shown). Currently, mouse studies are underway to test ezetimibe synergistic effect with the protease inhibitor currently being used in the clinic, Telaprevir (59, 117).

An ongoing innate and adaptive anti-viral response occurs in patients despite the failure to clear HCV. Ezetimibe treatment alone did not have an inhibitory affect on HCV RNA levels in an established infection in *in vitro* growing or non dividing cell cultures, nor was there an inhibitory affect observed in our *in vivo* mouse experiments. However, long term treatment with

ezetimibe in the presence of the host immune response may have an inhibitory effect on HCV. Long term studies would need to be done to determine whether ezetimibe treatment alone is able to reduce HCV infection in patients. Nevertheless, our finding that ezetimibe can delay the establishment of HCV infection in mice (Fig. 27A) and enhance IFN-α inhibition of previously established HCV infection in mice (Fig. 30) does suggest the therapeutic potential ezetimibe or other anti-NPC1L1 therapies for the treatment of HCV.

5. DISCUSSION AND FUTURE DIRECTIONS

5.1 **Updated Overview of HCV Entry**

Entry is the first step in the HCV viral life cycle and therefore it is a key determinant in establishing an HCV infection. Based on our current understanding, HCV entry appears more complex than many known viruses in that different components of the lipoviroparticle (LVP) appear to interact in a defined cascade with several host factors before it is eventually internalized via a clathrin dependent pathway. The sequential order of virion engagement with its host cell factors along with the role each of these factors in the entry process (e.g. binding, trafficking, internalization, fusion) as well as their contribution to the restrictive species tropism of the virus are all questions still being investigated. In this study we identify two novel HCV entry factors, TfR1 and NPC1L1. This discovery not only adds significantly to the known repertoire of factors involved in HCV entry, but individually the investigation of each of these factors has begun to provide insight concerning the molecular requirements of virion internalization (i.e.endocytosis), the determinants of HCV species tropism, and a new promising therapeutically amenable antiviral target.

Our tight kinetic antibody/inhibitor time of addition studies indicate that anti-TfR1 and the TfR1 inhibitor ferristatin retained inhibitory activity when added up to approximately 4 hours after initial viral binding notably longer than anti-CD81 suggesting TfR1 acts after CD81 in the HCV entry process (Fig 12; data not shown). Analogous data found that ezetimibe and anti-NPC1L retained inhibitory activity approximately 5 hours post-binding (Fig. 25; data not shown). Together this suggests that both TfR1 and NPC1L1 act relatively late during viral entry, with TfR1 likely proceeding NPC1L1. Furthermore, silencing the protein responsible for TfR1 internalization, TTP, inhibited HCV infection suggesting TfR1 may be functionally involved in

HCV uptake. Hence, we propose a model in which TfR1 is acting after CD81 but before NPC1L1 and that both these receptors may potentially be involved in viral particle uptake (Fig 31). Because SRBI binding is thought to be a prerequisite for CD81 binding, this model thus represents TfR1 and NPC1L1 acting after the early entry factors SRB1 and CD81. However, the details surrounding the sequence in which the HCV virion engages with these and other host cell entry factors remains undefined. and should be further investigated. In particular, our current model is vague on where the tight junction proteins CLDN1 and OCLN fit into the functional order of events with them being depicted as acting before, concurrently, or after TfR1 and NPC1L1.

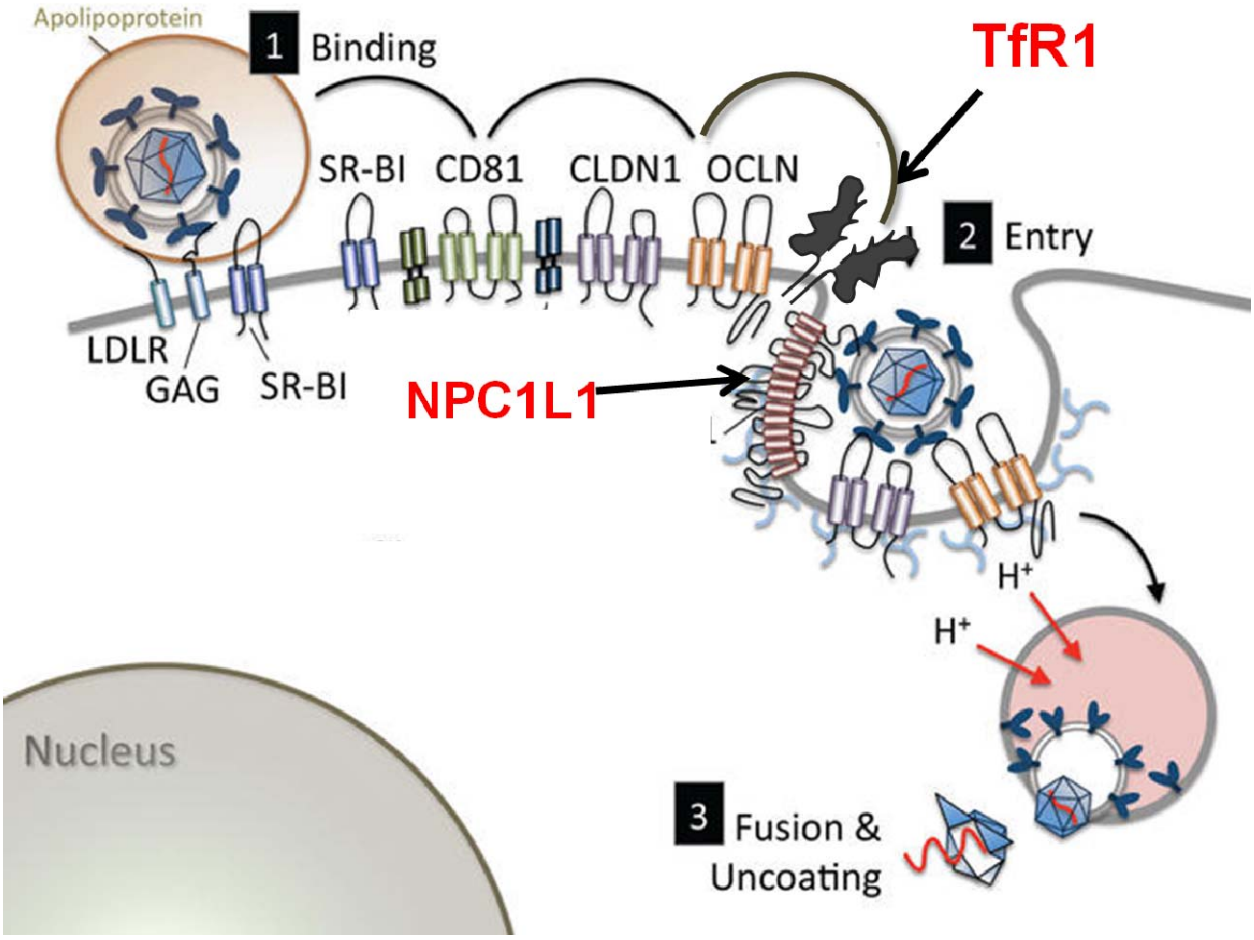

Figure 31. Proposed model for the role of TfR1 and NPC1L1 in HCV entry. Based on kinetic studies we propose TfR1 and NPC1L1 both act post virion binding late in the HCV entry process. Initially host cellular factors GAG and LDLR mediate virion attachment to the hepatocyte via the virions associated lipoproteins. Following attachment to the cell, the viral particle interacts specifically with SRB1 which primes its interaction with CD81. After the virion interacts with CD81 we propose the viral particle interacts with TfR1 prior to engagement with NPC1L1. Future kinetic studies need to be performed to determine when TfR1 and NPC1L1 act relative to the tight junction proteins CLDN1 and OCLN. (Figure adapted from Yu et al., manuscript in preparation. Original illustration by Bruno Sainz Jr,).

5.2 **Receptor-Receptor Interactions**

Regarding the order in which the various cellular HCV receptors act during the entry process and to potentially shed light on the role TfR1 and NPC1L1 play in HCV entry, it would be informative to investigate the receptor-receptor interactions that occur among the various HCV entry factors. Interactions between previously established HCV entry factors such as CD81 and CLDN1 have been reported (81, 100). Using FRET based systems, CD81 and CLDN1 were found to interact, but that this interaction was disrupted in the presence of antibodies targeting CLDN1 which negatively affected HCV infection suggesting these two proteins interact during HCV entry (81). Similar approaches could be employed to determine if TfR1 and NPC1L1 interact with any of the other previously identified HCV entry factors or with each other. NPC1L1 associates with lipid raft proteins resulting in the formation of cholesterol rich membrane microdomains (67) and plasma membrane cholesterol mediates the organization of tetraspanin microdomains (157). Because SRBI, CD81 and NPC1L1 localize to cholesterol enriched microdomains, potential interactions may be occurring between these HCV entry factors. Another potential interaction may be between our newly identified entry factors, TfR1 and NPC1L1. According to our kinetics data (Fig. 12 and 25) both receptors appear to be acting later in the HCV entry process. Furthermore, both receptors are endocytosed into early endosomes. Future co-localization and FRET based assay studies need to be carried out to determine if these proteins are interacting to coordinate HCV uptake.

5.3 **HCV Tropism**

For many years the narrow species and tissue tropism associated with HCV infection has hampered HCV research. Although tremendous advances have been made, including the discovery that CD81 and OCLN are the minimal human factors needed to render mouse cells permissive to HCVpp entry, the development of a mouse model susceptible to infectious HCV entry has not been attained. Some of the questions concerning the factors responsible for the strict species and tissue tropism seen with HCV may be addressed with discovery of these additional HCV entry factors. Since TfR1 is ubiquitously expressed and previous reports have shown human cell lines have been made permissive to HCVcc and non-human cell lines have been made permissive to HCVpp, most likely human TfR1 is not an absolute tropism requirement and does not explain the specific tissue or species tropism of HCV infection. However, there have been reports that viruses that use TfR1 as an entry receptor are restricted to specific species homologs. Although mouse and human TfR1 share 86% homology, MMTV entry is restricted to mouse TfR1 (170). Interestingly, the different New World arenaviruses have been shown to have adapted to the TfR1 of their respective rodent hosts but all can use human TfR1 for efficient transmission to humans (143). Thus, there is precedent to think that HCV may show some preference for human TfR1 and improve the efficiency of HCV into non-human cells. Of course, the unique presence of NPC1L1 on hepatocytes only in humans and chimpanzees strongly implicates NPC1L1 as a potential determinant of HCV species and tissue tropism and raises the exciting question of whether NPC1L1 is the missing link that finally allows efficient HCV entry into mouse hepatocytes. Specifically, in terms of HCVpp entry into mouse, only 30% of the HCVpp entry into Huh7.5 cells was detected as entering mouse when all four of the previously identified cellular HCV entry factors were expressed suggesting that additional entry factors may be required even for optimal HCVpp entry into mouse cells. Thus,

expression of human TfR1 on mouse cells may enhance HCVpp entry. Perhaps more importantly, the four previously identified cellular HCV entry factors are not sufficient to allow for detectable infectious entry of authentic HCV into non-permissive cells and expression of human TfR1 and/or NPC1L1 may be the final factor(s) necessary to confer HCV entry permissiveness to mouse hepatocytes. Ongoing studies in the laboratory are being performed to determine if NPC1L1 is the final human factor required for HCV entry into mouse cells.

5.4 **Relevance of HCV Down Regulating Critical Host Cell Factors**

Several groups have reported the down regulation of host cellular receptors involved in HCV entry. This event is not specific to HCV as many viruses typically down regulate their entry factors presumably to prevent superinfection. However, virus induced changes in expression of host cellular factors may have deleterious effects on cellular homeostasis. Tight junction proteins, CLDN1 and OCLN, are important in maintaining cell to cell integrity and mediating paracellular trafficking of molecules. Decreases in expression of CD81 and changes in localization of CLDN1 have been implicated in the development and metastasis of HCC (83). Notably, HCV infection also causes a down regulation in our newly identified entry factors TfR1 and NPC1L1.

Until we have a better understanding of the role of hepatic NPC1L1 (in contrast to intestinal NPC1L1 which has been the focus of virtually all NPC1L1 functional studies), it is difficult to speculate how HCV-induced down regulation of this protein might ultimately affect cholesterol homeostasis. While much more is known about the function of TfR1 on hepatocytes, the complexity of cellular and systemic iron homeostasis remains a challenge to decipher. As mentioned above, studies have shown chronically infected patients can develop mild to moderate hepatic iron overload which can be detrimental to liver health in general as well as limit response

to HCV antiviral interferon therapy. Going back to our initial experiments monitoring iron genes, we observed an early down regulation in TfR1 gene and protein expression in HCV infected cells (Fig 4). It is reasonable to speculate that this virus induced alteration in TfR1 expression may influence iron uptake levels and thus may cause local cellular changes to iron responsiveness which might ultimately alter systemic iron homeostasis.

TfR1 is normally regulated post-transcriptionally by body iron levels through the iron response element/iron response protein (IRE/IRP) system (47, 130). In this system, the IRP protein binds to IRE elements within the 3' end of the TfR1 mRNA transcript stabilizing it and resulting in increased TfR1 protein levels. During conditions in which body iron levels are elevated the IRP protein is released from the IRE region and the transcript is degraded causing a decrease in TfR1 protein levels. Of course in very simplistic terms, if one assumes a direct effect with no subsequent regulatory responses, the observation that TfR1 protein is down regulated during HCV infection may appear counterintuitive to the iron overload phenotype as the immediate effect of TfR1 down regulation would be reduced cellular iron uptake. However, this apparent "discrepant" effect could have several explanations. One explanation is that there may be a possible compensatory response to the initial down regulation of TfR1. Based on preliminary analysis of TfR1 levels in HCV infected patient hepatocytes (data not shown), it may be that the initial viral down regulation of TfR1 induces regulatory mechanisms that over compensate and ultimately lead to increased TfR1 expression in chronically infected patients. Studies to investigate TfR1 protein levels during a long term infection, preferable in vivo in the context of systemic iron regulation, would need to be carried out to determine if the pattern of TfR1 expression changes.

Another explanation may be that HCV independently alters other aspects of cellular and/or systemic iron regulation. For example, one study found enhanced IRP2 expression in

their HCV replicon cells compared to the parental Huh7 cells from which the replicon clone was derived (58). The replicon cells in general exhibited a disruption in their response to cellular iron changes. In particular, while IRP/IRE binding was appropriately enhanced in the presence of iron, it did not subsequently decrease to similar levels as seen in parental cells. As stated above, these studies were carried out in a replicon clonal population, but it would be interesting to determine if these effects are observed in the context of HCV infection where more comparable cell cultures could be compared +/- addition of HCV. Hence, studies examining IRPs expression and response to iron perturbations along with IRP/IRE binding in the HCV infection system should be performed to determine if HCV infection alters iron accumulation and/or longterm TfR1 regulation.

In terms of potential effects on systemic iron regulation, there are several reports suggesting chronic HCV patients show reduced hepcidin serum levels (62, 70). This small peptide hormone, which is secreted from the liver, has become accepted as the master regulator of iron homeostasis. Hepcidin directly binds the transmembrane transporter responsible for iron efflux, ferroportin, and induces its internalization and degradation (127). Consequently under low iron conditions, hepcidin secretion is low permitting iron release into the system, increasing serum iron levels. On the other hand, when iron levels are high hepcidin secretion is elevated preventing iron release into the system resulting in decreased serum iron levels. Because hepcidin expression is upregulated by proinflammatory cytokines such as interleukin IL-6 (42, 126, 128) and HCV is a proinflammatory disease which induces IL-6 expression (54, 114), it was very unexpected that hepcidin levels were found to be low in HCV patients. This suggests that the virus may be altering hepcidin regulation and secretion. Evidence to support this notion was shown after impaired hepcidin levels in chronic HCV patients were significantly increased after pegIFN/RBV treatment lead to SVR. In contrast, serum hepcidin levels in patients who failed to

achieve SVR remained low (62). This parallels the finds that iron overload becomes reduced in patients who achieved SVR. Interestingly, one study found hepcidin serum and mRNA levels increased in chronic HCV patients just 12 hours after initiation of pegIFN-α/RBV treatment (147). While *in vitro* data indicate that IFN-α treatment may be inducing hepcidin expression through phosphorylation of signal transducer and activator of transcription 3 (STAT3) protein (147), the rapid increase observed *in vivo* could also perhaps indicate that active HCV replication is required to suppress hepcidin under inflammatory conditions. While these studies do not explain how HCV is causing a decrease in hepcidin expression, they do elude to fact that HCV is actively causing this inhibition. Therefore, questions remain regarding how HCV is inhibiting hepcidin as well as whether this contributes to the iron overload phenotype seen in HCV patients.

Clearly, future studies are needed to elucidate the mechanism(s) explaining the iron overload phenotype seen during chronic HCV infection. Addressing questions concerning whether this phenotype results from HCV disruption of cellular iron genes like TfR1 and IRP, or is due to HCV disruption of systemic iron homeostasis through alteration in hepcidin may add insight in to the mechanism(s) responsible for iron overload in HCV patients.

5.5 **HCV Entry as a Potential Therapeutic Target**

Developing therapeutic strategies to combat HCV infection is a primary objective to treat currently infected individuals and lower the incidence of new infections. Elucidating the molecular mechanisms of each step of the viral life cycle facilitates defining potential antiviral targets. HCV entry is an attractive antiviral target because it is essential for initiation of infection and the viral spread that contributes to maintenance of infection.

Since HCV infection is typically diagnosed after the establishment of a chronic infection, in the majority of cases HCV entry inhibitors alone would not necessarily be optimally effective

in this context. However, when used in combination with other inhibitors that block intracellular viral replication (e.g. polymerase and protease inhibitors), the presence of an entry inhibitor has the potential to enhance viral clearance from the host as the cells in which HCV has been significantly reduced or "cured" by the replication inhibitor would be protected from potential re-infection. Aside from simply enhancing the efficacy of viral clearance another advantage of preventing infection/re-infection of cells during HCV antiviral treatment with a direct acting inhibitor is the prevention of spread of viral escape mutants that may arise against the direct acting antivirals.

Importantly, with the ability to prevent infection before it starts, entry inhibitors could potentially also be useful in the HCV-positive liver transplant setting. Severe liver pathologies associated with long term HCV infection can lead to end stage liver disease resulting in the need for liver transplantation, with HCV being the most common indication for liver transplantation in the United States. Unfortunately, re-infection of the new liver graft is universal resulting in much more rapid fibrosis and disease progression than observed during infection of the original liver (56). Because the use of entry inhibitors could potentially prevent or at least significantly slow infection of the new liver, there is the expectation that they could be very beneficial in post liver transplantation settings.

Finally, while life-long therapy is certainly not the ideal, in regions where HCV is endemic and in high risk patients, HCV entry inhibitors could provide an effective prevention strategy to limit the incidence of new infections. This would require the drug(s) to be sufficiently convenient and inexpensive, but if generically available drugs, such as ezetimibe, could be used to prevent the spread of HCV infection particularly in high risks populations (e.g. military combat personnel, prison systems) this would likely not only minimize transmission, but

also help alleviate the long term healthcare costs that accompany a long-term therapeutic regiment required for treating a chronic HCV patient.

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Title: Identification of the Niemann-Pick C1–like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor
Author: Bruno Sainz, Naina Barretto, Danyelle N Martin, Nobuhiko Hiraga, Michio Imamura, Snawar Hussain, Katherine A Marsh, Xuemei Yu, Kazuaki Chayama, Waddah A Alrefai, Susan L Uprichard
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Danyelle N Martin, PhD
850 W. Argyle St. Chicago, IL 60640
217-741-4113
Dnmartin08@gmail.com

SUMMARY: Strong research background in infectious disease with a specific focus on Hepatitis C virus. Capable of working independently and in group settings. Participated in collaborative team projects resulting in publication. Ability to orally present and communicate research concepts and data. Sharp interpersonal skills along with excellent critical thinking and analytical skills. Exceptional at multi-tasking as a result of honed time management skills.

EDUCATION:

2006- 2012: Ph.D., Department of Microbiology and Immunology, University of Illinois at Chicago, Chicago.

1999-2004: B.S. Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana.

PROFESSIONAL/RESEARCH EXPERIENCE:

8/2006-Present **Graduate research**, Department of Microbiology and Immunology, University of Illinois at Chicago, Chicago, Illinois. Advisor Dr. Susan Uprichard.
Focus: Identified and characterized the role of transferrin receptor 1 in Hepatitis C virus entry.

- Assisted in determining patient criteria and study guidelines for retrospective clinical study
- Collaborated with key opinion leader (KOL), Dr. Scott Cotler, and assisted in writing study protocol resulting in IRB approval to analyze expression patterns of iron genes in HCV patient's liver tissue samples.
- Designed experiments and developed protocols to answer questions pertinent to research goals.
- Successfully collected, analyzed and interpreted data and effectively presented results to audiences consisting of individuals from non-HCV backgrounds or thought leaders within the field.
- Collaborated with internal colleagues on projects which resulted in publication.
- Maintained thorough and up to date knowledge of HCV basic and clinical research.

2/2005-7/2006 **Researcher I, Neurobiology Laboratory in Department of Pharmacology**, SIU School of Medicine, Springfield, Illinois. Advisor Dr. Donald Caspary
Focus: Examined $GABA_A$ receptor subunit changes in noise exposed rats in the medial geniculate body (MGB)

8/2003-8/2004 **Undergraduate Research Assistant**, Neuronal Pattern Analysis Laboratory, Beckman Institute, Champaign. Advisor Dr. Michael Gabriel

Focus: Worked on determining the effects of excitotoxic lesions of the nucleus accumbens on avoidance behavior and learning related neuronal activity.

WRITTEN AND ORAL PRESENTATION EXPERIENCE:

- Presented experimental results at the 17th International Meeting on HCV and related viruses conference held in Yokohama, Japan.
- Interpreted and presented experimental data to Department of Microbiology and Immunology students, faculty and thesis committee.
- Presented published scientific results during departmental journal clubs.
- Grant and manuscript writing, editing experience

TEACHING EXPERIENCE: Laboratory teaching assistant instructing medical students enrolled in the Medical Microbiology Laboratory course at University of Illinois at Chicago, 2007.
Trained incoming lab members on proper use of Hepatitis C virus cell culture infection system.

PRESENTATIONS AT NATIONAL AND INTERNATIONAL MEETINGS:

1. Sainz, B. Jr, Barretto, N., **Martin, D.N.**, Hiraga, N., Imamura, M., Yu, X., Chayama, K., Alrefai, W.A., Uprichard, S.L. 2012. The Niemann-Pick C1-like 1 cholesterol absorption receptor: a novel hepatitis C virus entry factor and therapeutic target. 18th International Meeting on Hepatitis C Virus and Related Viruses, Seattle, Washington, September 2011.(Accepted for oral presentation)
2. **Martin, D. N.** and Uprichard, S.L. The role of transferrin receptor 1 in Hepatitis C virus entry. College of Medicine Research Forum, Chicago, Illinois, November 2010.
3. **Martin, D.N.** and Uprichard, S.L. The role of transferrin receptor 1 in Hepatitis C virus entry. 17th International Meeting on Hepatitis C Virus and Related Viruses, Yokohama, Japan, September 2010. **Accepted for oral presentation (Presenter)**.
4. **Martin, D.N.** and Uprichard, S.L. The role of transferrin receptor 1 in Hepatitis C virus entry. 2010 National Pre-doctoral Clinical Research Training Program Meeting, St. Louis, Missouri, May 2010.
5. Caspary , D., **Martin, D.**, Ling, L., Wang, H., Hutson, P., Turner, J., Reuschel, E., Hughes, L. GABA (A) receptor subunit changes in a noise-exposure model of tinnitus: Rat medial geniculate body, Assoc. Res. Otolaryngology. Ab.:159, Baltimore, Maryland, 2007.

HONORS AND AWARDS:

- Travel award, 17th International Meeting on Hepatitis C Virus and Related Viruses, September 2010.
- Pre-doctoral Education for Clinical and Translational Scientist (PECTS) Fellowship, University of Illinois at Chicago, 2009-2011
- Abraham Lincoln Fellowship, University of Illinois-Chicago, 2006-2007, 2011-2012.
- Dean's List, University of Illinois at Urbana-Champaign, 2004.

OTHER SKILLS:
Proficient in recombinant DNA techniques, virology techniques, cell culture techniques**,** gene expression analysis, immunohistochemistry, immunofluorescent staining and confocal microscopy

COMPUTER SKILLS: MS Word, Excel, Power Point, Adobe Photoshop

PUBLICATIONS:
Sainz, B. Jr, Barretto, N., **Martin, D.N.**, Hiraga, N., Imamura, M., Hussain, S., Marsh, K.A., Yu, X., Chayama, K., Alrefai, W.A., Uprichard, S.L. 2012. Identification of the Niemann-Pick C1-like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor. Nature Medicine. 18 (2):281-5.

Martin, D.N. and Uprichard, S.L. 2012. Identification of Transferrin Receptor 1 as a Hepatitis C Virus entry factor. In preparation.