INVESTIGATION OF THE ROLE OF NONSTRUCTURAL 4B PROTEIN IN
HEPATITIS C VIRUS REPLICATION COMPLEX FORMATION AND
INFECTIOUS VIRUS PRODUCTION

A Dissertation in
Biochemistry, Microbiology, and Molecular Biology

by

David P. Manna

© 2011 David P. Manna

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 2011
The dissertation of David P. Manna was reviewed and approved* by the following:

Kouacou V. Konan  
Assistant Professor of Biochemistry and Molecular Biology  
Dissertation Advisor  
Chair of Committee

Craig Cameron  
Paul Berg Professor of Biochemistry and Molecular Biology  
Associate Department Head for Research and Graduate Education

Joseph Reese  
Professor of Biochemistry and Molecular Biology

Avery August  
Professor of Veterinary Science

Anthony Schmitt  
Associate Professor of Molecular Virology

* Signatures are on file in the Graduate School.
Abstract

The Hepatitis C Virus (HCV) NS4B protein is one of the least understood viral proteins in terms of contributions to the virus lifecycle. NS4B has many functions related to formation and function of the viral genome replication complex (RC), most notably for its role in inducing a novel membrane structure termed the membranous web which houses the RC. The membranous web is largely believed to be derived from ER membranes, and recent data has also suggested membranes from the early endosome (EE) compartment are part of this membrane structure. This was due to the observation that Rab5, a regulator of EE membrane transport, was required for HCV RNA replication and that Rab5 co-localizes with NS4B foci in replicon cells. NS4B foci are considered to be an immunofluorescence marker equivalent to the membranous web. Recent data has also implicated a new role for the C-terminal domain (CTD) of NS4B in facilitating infectious virus production. Both point mutations and entire CTD sequence swaps between different genotype viruses have highlighted this new and exciting role for NS4B in virus production.

To test the hypothesis that cellular Rabs are involved in NS4B-mediated HCV RC formation, we employed two main methods. First, we modified an existing protocol for NS4B antibody directed immunoisolation of a subcellular fraction enriched in the HCV RC components, and used this fraction for immunoblot analysis. Secondly, we examined Rab co-localization with NS4B foci in replicon cells to compare to the profile in the isolated RC fraction, which was competent for HCV RNA synthesis. Rabs from every cellular compartment tested were present at different levels in the isolated RC fraction, but only the endocytic Rab5 and 7 were found to co-localize with NS4B foci. Dominant
negative mutants of Rab5 and 7 disrupt NS4B foci, and silencing of Rab5 or 7 resulted in a significant decrease in HCV RNA replication. Taken together, our data implicate endocytic Rabs as being important for NS4B-mediated HCV RC formation.

To test the hypothesis that the NS4B CTD is involved in virus assembly, we created chimeric virus genomes where the NS4B CTD from JFH1 virus (genotype 2a, gt2a) was replaced with sequences from Con1 (gt1b) or H77 (gt1a) virus. These chimeric genomes had little impact on RNA replication, but showed a significant defect in virus production, likely at the level of virus assembly. Investigation into the causes of lower virus titers showed that the chimeric viruses have lower NS5A protein levels and a decrease in the NS5A p58/p56 ratio relative to JFH1. Cell culture adapted viruses were generated that had similar titer levels to that of JFH1, showed higher levels of NS5A protein, but interestingly did not change in their NS5A p58/p56 ratio relative to JFH1. Sequencing of the adapted viruses revealed four mutations, two in the chimeric NS4B CTD region, one in domain III of NS5A and one adjacent to the cytosolic loop of the p7 protein. Introduction of these mutations back into the parental chimeric viruses rescued virus production, highlighting a genetic interaction between NS4B, NS5A and p7 proteins. These studies illuminate a role for the NS4B CTD in virus production, possibly through interaction or modification of other HCV proteins.
# Table of Contents

List of Figures.............................................................................................................................................vii

## Chapter 1. INTRODUCTION

Hepatitis C Virus Epidemiology..................................................................................................................1
Hepatitis C Virus Life Cycle.........................................................................................................................2
Hepatitis C Virus Structural Proteins........................................................................................................4
Hepatitis C Virus Nonstructural Proteins.................................................................................................5
Nonstructural 4B Protein.............................................................................................................................8
Regulation of Cellular Membrane Traffic by Rabs.....................................................................................10
Hepatitis C Virus Model Systems: Replicon Cells and JFH1 Cell Culture Virus.............................12
Hypothesis and Objectives.........................................................................................................................14

## Chapter 2. MATERIALS AND METHODS

Cells.............................................................................................................................................................20
Immunosolization of a Subcellular Fraction Containing HCV Replicase Proteins..........................20
Quantitative Real-Time PCR of HCV RNA in Bound and Unbound Fractions..........................21
In Vitro RNA-Dependent RNA Polymerase Assay...........................................................................22
SYPRO Ruby Protein Staining of Immunosolization Fractions.........................................................22
Antibodies.................................................................................................................................................23
Immunoblot of HCV and Host Proteins.................................................................................................23
Protein Identification by Capillary LC-ESI-MS/MS.......................................................................24
Rab Plasmids.............................................................................................................................................25
DNA Transfection.....................................................................................................................................25
Indirect Immunofluorescence..................................................................................................................25
siRNA Transfection.................................................................................................................................26
Construction of Viral cDNA Plasmids.................................................................................................27
*In Vitro* Transcription and Electroporation of Viral RNA into Huh7.5 cells..............................28
Luciferase Assay.......................................................................................................................................29
Titration of HCV Infectivity.....................................................................................................................30
Cell Lysate Preparation to Obtain Intracellular Virus........................................................................30
Sucrose Cushion Pelleting of HCV Particles.........................................................................................30
Analysis of Adapted Virus Nucleotide Sequence................................................................................31

## Chapter 3. IDENTIFICATION OF HCV REPLICATION COMPLEX-ASSOCIATED RABS BY IMMUNOISOLATION AND FLUORESCENCE MICROSCOPY

The Isolated HCV Replication Complex Fraction is Competent for NS5B-mediated *In Vitro* HCV RNA Synthesis................................................................................................................32
Endocytic and Secretory Rabs are Present in the Bound Fraction..................................................33
Rab5 and Rab7 Co-localizes with NS4B Foci.........................................................................................36
Rab5 Effectors Show Differential Replication Complex Association.............................................38
Dominant Negative Rab6 Disrupts Rab5 Co-localization With NS4B Foci...................................39
Dominant Negative Rab5 or 7 Disrupts NS4B Foci...........................................................................40
Rab2, 5 or 7 siRNA Silencing Decreases HCV RNA Synthesis.......................................................40
Differences in Rab-NS4B Association: Immunoisolation Versus Fluorescence...41
Possible Mechanisms of HCV Replication Complex Formation Via NS4B-Rab
Association..................................................................................................43

Chapter 4. THE NS4B C-TERMINAL DOMAIN PARTICIPATES IN VIRUS
PRODUCTION..................................................................................................64
Chimeric NS4B C-Terminal Domain Viruses are Defective in Virus Assembly..65
NS4B C-Terminal Domain Chimera Decrease Levels of NS5A Protein and
Phosphorylation...............................................................................................66
Cell Culture Adaptive Viruses Express High Levels of NS5A Protein............68
Suppressor Mutations Differentially Complement Virus Production............70
Some NS4B N216 Amino Acid Substitutions Result in Non-Viable Virus........73
Possible Roles for the NS4B C-terminal Domain in Virus Assembly, NS5A
Phosphorylation and NS5A Stability.............................................................73
Potential NS4B C-terminal Domain Interactions with p7 and NS5A to Promote
Virus Production..........................................................................................77

Chapter 5. CONCLUSIONS AND DISCUSSION.................................................99
Significance to the Field................................................................................99
Future Directions- NS4B and Endocytic Rabs.............................................100
Future Directions- NS4B CTD and Virus Production....................................102

References.....................................................................................................103
LIST OF FIGURES

FIGURE 1.1: HEPATITIS C VIRUS GENOME ORGANIZATION AND LIFE CYCLE………………………………………………………………………………………………………16

FIGURE 1.2: PREDICTED NS4B TOPOLOGY………………………………………………………………………………………………………………………………………17

FIGURE 1.3: NS4B AMINO ACID SEQUENCE ALIGNMENT……………………………………………………………………………………………………………………..18

FIGURE 1.4: VISUALIZATION OF NS4B-INDUCED MEMBRANOUS WEBS BY MICROSCOPY……………………………………………………………………………19

FIGURE 3.1: HCV REPLICATION COMPLEX IMMUNOISOLATION PROTOCOL……………………………………………………………………………………45

FIGURE 3.2: THE ISOLATED HCV RC CONTAINS RNA AND IS COMPETENT FOR RNA SYNTHESIS……………………………………………………………………46

FIGURE 3.3: HCV-SPECIFIC INHIBITORS DECREASE RDRP ACTIVITY IN THE BOUND FRACTION…………………………………………………………………………48

FIGURE 3.4: THE BOUND FRACTION CONTAINS CELLULAR MARKERS AND HCV REPLCATION PROTEINS………………………………………………………….49

FIGURE 3.5: THE BOUND FRACTION CONTAINS DIFFERENTIALLY ENRICHED RABS………………………………………………………………………………………50

FIGURE 3.6: RAB5 AND 7 CO-LOCALIZES WITH NS4B FOCI…………………………………………………………………………………………………………………………51

FIGURE 3.7: RAB5 AND 7 CO-LOCALIZES IN C5B CELLS……………………………………………………………………………………………………………………………54

FIGURE 3.8: RAB5 EFFECTORS ARE DIFFERENTIALLY ASSOCIATED WITH THE HCV RC………………………………………………………………………………………………57

FIGURE 3.9: DOMINANT NEGATIVE RAB6 DISRUPTS RAB5-NS4B ASSOCIATION…………………………………………………………………………………………59

FIGURE 3.10: SOME DOMINANT NEGATIVE RABS DISRUPT NS4B FOCI…60

FIGURE 3.11: RAB2, 5 OR 7 SIRNA SILENCING REDUCES HCV REPLICATION LEVELS……………………………………………………………………………………………62

FIGURE 3.12: MODEL OF ENDOCYTIC RAB RECRUITMENT INTO NS4B FOCI…………………………………………………………………………………………………………63

FIGURE 4.1: CHIMERIC NS4B CTD CONSTRUCTS………………………………………………………………………………………………………………………….80
FIGURE 4.2: J/C1-C AND J/H-C CHIMERIC VIRUSES DISPLAY DECREASED VIRUS PRODUCTION

FIGURE 4.3: CHIMERIC CTD VIRUSES HAVE NO VISIBLE DEFECT IN VIRUS ASSEMBLY

FIGURE 4.4: CHIMERIC VIRUSES DISPLAY LOWER NS5A PROTEIN LEVELS AND PHOSPHORYLATION

FIGURE 4.5: CELL CULTURE ADAPTED CHIMERIC VIRUSES DISPLAY INCREASED NS5A LEVELS

FIGURE 4.6: SUPPRESSOR MUTATIONS HAVE MINIMAL EFFECT ON RNA REPLICATION

FIGURE 4.7: SUPPRESSOR MUTATIONS INCREASE CHIMERIC VIRUS TITER AND NS5A LEVELS

FIGURE 4.8: CHIMERIC VIRUSES CONTAINING SINGLE SUPPRESSOR MUTATIONS ARE MORE INFECTIOUS THAN PARENTAL VIRUSES

FIGURE 4.9: SOME NS4B N216 MUTATIONS RESULT IN NON-VIABLE VIRUS

FIGURE 4.10: MODEL OF NS4B CTD MEDIATING NS5A PHOSPHORYLATION AND STABILITY
Chapter One

Introduction

Hepatitis C Virus Epidemiology

Hepatitis C Virus (HCV) is a member of the Flaviviridae family of viruses, and is one of only two members of the Hepacivirus genus, the second being the newly discovered Canine Hepatitis Virus (CHV) (65). The Flaviviridae family of viruses all have positive strand RNA genomes and are exemplified by viruses such as classical flaviviruses (for example, Yellow Fever Virus, West Nile Virus, Dengue Virus and Tick-Borne Encephalitis Virus), animal pestiviruses (for example, Bovine Viral Diarrhea Virus) and GB Viruses A, B and C (131). HCV was originally identified from serum of a patient with non-A, non-B hepatitis in 1989 (26). HCV is grouped into six genotypes, based on a nucleotide sequence identity of about 30-35% between genotype members, and among these genotypes there are subtypes (a, b, c, etc) classified by a nucleotide sequence identity of about 20-25% (119). Approximately 170 million people worldwide are chronically infected with HCV. Roughly 80% of infections will result in chronic hepatitis, 20% of these patients will develop liver cirrhosis as a result of HCV infection, and 1-5% of these patients will go on to develop hepatocellular carcinoma (HCC) (95, 136). The current treatment for HCV is a combination of pegylated interferon-alpha and ribavirin. This treatment has varying efficacy depending on the genotype of HCV that the patient is infected with, ranging from 20-50% of patients who do not respond to treatment. There are also many harsh side effects of this treatment, including flu-like symptoms, anemia and psychiatric problems in the patient.
**Hepatitis C Virus Life Cycle**

HCV is an enveloped virus, containing a positive strand RNA genome of roughly 9600 nucleotides. The genome is composed of a 5′-untranslated region (UTR), a long single open reading frame encoding the HCV polyprotein, and a 3′-UTR (Figure 1.1A). HCV enters cells when the E1 and E2 glycoproteins bind to cellular receptors CD81, Scavenger Receptor B-1 (SRB-1), occludin and Claudin-1 co-receptors (33, 39, 104, 115). It is also known that HCV particles in the blood associate with low-density lipoproteins, and entry of the virus may also be facilitated by low-density lipoprotein receptor (LDLR) and glycosaminoglycans (29). The receptor bound viruses then enter the cell via clathrin-mediated endocytosis (11). Once internalized, HCV uses acidification of the endosome in order to drive conformational changes that facilitate viral envelope fusion with the endosomal membrane, and allows the viral genome to enter the cytoplasm (134).

Once in the cytoplasm, the HCV genome is directly translated into a polyprotein of about 3000 amino acids by cellular ribosomes under the direction of the HCV internal ribosome entry site (IRES) located in the 5′ end of the genome (Figure 1.1B). The endoplasmic reticulum (ER) resident signal peptidase co-translationally cleaves the structural proteins and p7 into the mature proteins core, E1, E2 and p7. The nonstructural proteins are cleaved by the NS2-3 autoprotease and the NS3-4A serine protease into the mature proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B (98).

The mature HCV proteins NS3-NS5B then must organize a RNA replication complex (RC) in order to amplify the viral genomes in the cell. This RC is formed in association with intracellular membranes, which is a characteristic shared by all positive
strand RNA viruses (113). In the case of HCV, NS4B protein is responsible for rearranging these RC-associated membranes into a structure termed the membranous web (35, 70). This structure is derived from ER membranes, and possibly also from Early Endosome (EE) or autophagic membranes (35, 44, 49, 70, 121, 122). The membranes used by other RNA viruses to form a RC are derived from many sources including ER, Golgi apparatus, mitochondria and lysosomes (85, 113). There are other instances of membrane rearrangement in the Flaviviridae family such as Kunjin virus “vesicle packets (86). In addition, the flavivirus dengue virus and the picornavirus poliovirus use ER-associated vesicles to house their RC (25, 28, 111). The structures and origins of these membrane associated RCs are different from the HCV membranous web, but highlight a common theme in RNA virus replication. There are multiple reasons for these viral RNA RC’s to associate with membranes. Membranes may act as scaffolds that can concentrate the components of the RC in specific subcellular locations to enhance replication. These membranes also may shield the viral RNA and double stranded RNA intermediates from the interferon response that can be triggered by RNA helicases that recognize double stranded RNA. Lastly these membranes may protect the viral RNA from cellular nucleases or the viral proteins from cellular proteases.

Positive strand RNA genomes that have been copied by the HCV RC are next assembled into infectious virus particles and exit the cell. Assembly of new virions occurs on lipid droplets (LD) that are closely associated with HCV RC membranes, perhaps allowing for a linkage between virus RNA replication and virus assembly complexes (96). Indeed, HCV virions can be visualized budding from membranes close to LDs by three-dimensional reconstruction of electron microscopy images (108). There
is evidence to support roles in virus assembly for E1, E2, p7, NS2, NS3 and NS5A proteins, as well as three new reports suggesting an involvement of the C-terminal domain (CTD) of NS4B (discussed below). The assembled virus particles are then thought to bud out of an ER-derived compartment and are secreted out of the cell, but this process remains poorly understood. There is a possible link between cellular lipoprotein metabolism and virus release, but little overall is known regarding HCV exit from the cell (4).

**Hepatitis C Virus Structural Proteins**

The membrane associated HCV structural proteins encoded in the 5’ end of the genome are comprised of the core, E1, E2 and p7 proteins. However, the exact classification of the p7 protein as a structural protein remains controversial, as it is unknown if p7 is found in virus particles. The core protein is the virion nucleocapsid protein that encapsidates and protects the genome. Mature core protein is formed by the removal of a signal peptidase cleavage sequence in the C-terminus of core (127). Core can be localized at the membranous web as well as on the surface of LD’s, which has further suggested a role in virus assembly for core protein (96). Core also may play a role in HCV genome replication through an interaction with DDX3X, a DEAD-box RNA helicase that is essential for the HCV lifecycle (124).

The envelope glycoproteins E1 and E2 extend out of the virus membrane with their N-terminal ectodomains containing the receptor binding sites, and the C-terminus houses the transmembrane domains and signals for E1-E2 heterodimerization (27). It is believed that after attachment to target cell receptors and endocytosis into the cell, the
lower pH of the endosome promotes a conformational change for type II fusion to the endosome membrane and release of the genome into the cytoplasm (52).

The small, hydrophobic membrane protein p7 is a member of the viroporin family of proteins and functions as an ion channel, as well as participating in virus production (47, 112). The p7 protein is comprised of two transmembrane domains separated by a short cytoplasmic loop (23). Assembly and release of infectious virus requires functional p7 and its ion channel activity, and the viroporin inhibitor amantadine blocks p7 ion channel activity and HCV virion production (47, 120).

**Hepatitis C Virus Nonstructural Proteins**

The HCV nonstructural (NS) proteins are membrane-associated proteins responsible for the majority of the enzymatic activities in polyprotein processing and RNA replication during the virus lifecycle, as well as participating in virus production. NS2 is a cysteine protease that cleaves the NS2/NS3 junction, while also functioning in virus assembly by interacting with both structural and nonstructural proteins (82). In fact, NS2 is an essential gene required for production of infectious virus, and functions at a late step in HCV particle assembly (61, 140). NS2/NS3 is an autoprotease due to its autocatalytic cleavage at the NS2/NS3 junction, and exists as a dimer in its active form (80).

The multifunctional NS3 protein contains N-terminal serine protease activity and C-terminal DExH/D-box helicase and ATPase activity (68, 76, 114). NS3 has been shown to oligomerize, but its helicase activity may not depend on oligomerization depending on which reaction conditions are used (59, 67). Also, NS3 and NS4B have a genetic interaction that is involved in HCV RNA replication, shown by compensatory
NS3 mutations rescuing HCV replicon RNA replication containing point mutations in NS4B (100). NS3 also has a biochemical interaction with NS4B, as they are able to bind to each other (3, 32). NS4A is a NS3 cofactor and is known to mediate NS3 attachment to membranes (138). The NS3/4A serine protease complex cleaves the HCV polyprotein at the NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B junctions. Also, NS4A contains a C-terminal acidic domain that when mutated reduces RNA replication, possibly by mediating NS3 RNA binding while bound to ATP (9, 75). NS3 also participates in early virus assembly, as shown by mutations in the helicase domain that rescue a defect in early virus assembly, at a stage following the recruitment of NS5A to the surface of lipid droplets (LD) (83). The NS3 protease is a potential target for antiviral drugs, such as the inhibitors telaprevir and boceprevir, which have recently been FDA approved (40, 117).

NS5A is a HCV phosphoprotein with diverse roles, including mediation of viral RNA replication, modulation of the infected cells physiology and assembly of virions. Membrane association of NS5A is mediated by an N-terminal amphipathic alpha helix (18). NS5A contains three domains separated by two short low complexity linkers (129). Domain I coordinates a zinc atom and dimerizes to form a RNA-binding groove (130). This RNA binding groove is able to bind to the 3’ end of plus and minus strand HCV RNA (54). This perhaps allows NS5A to aid in recruiting other replication proteins to the proper sites on the HCV RNA for genome replication. Domains II and III are less structured, and domain III is involved in both RNA replication and virus assembly (5, 55, 91, 128). Differential phosphorylation of NS5A yields the basally phosphorylated p56 form and the hyperphosphorylated p58 species, and p58 phosphorylation is modulated by NS3, NS4A and NS4B proteins (69). Domain II and III both contain phosphorylation
sites targeted by kinases such as CKII and CKIα, and phosphorylation of NS5A domain III has been implicated in virus production (106, 107, 128). NS5A interacts with both core and LDs in order to promote infectious virus production, and phosphorylation in Domain III seems to play a role in this process (91). NS5A is also a target for potential antiviral drugs, as the recently discovered NS5A inhibitor BMS-790052 has recently entered phase II clinical trials (41).

The RNA-dependent-RNA polymerase (RdRp) NS5B is attached to membranes via its C-terminal transmembrane domain (97). The primary function of NS5B is to mediate RNA replication by synthesizing a negative strand intermediate RNA, which then can be used by NS5B as a template to amplify more positive strand RNA genomes. NS5B has the common viral RNA polymerase structure where the active site contains the fingers, palm and thumb domains (79). However, NS5B has more functions than RNA replication, as illustrated by evidence for NS5B altering the RNA helicase activity of NS3 and its ability to bind to and cause the degradation of the retinoblastoma tumor suppressor (58, 92, 99). The current HCV treatment regimen uses NS5B as an antiviral drug target, specifically with the nucleoside analog ribavirin. NS5B nonspecifically incorporates ribivirin triphosphate into the HCV RNA, causing random RNA mutagenesis and a decrease in virus replication (84, 143). Other drugs such as benzothiadiazine and related analogs are also being tested as NS5B targeted inhibitors (30).
**Nonstructural 4B protein**

NS4B is a very hydrophobic 261 amino acid protein that is localized to the ER and ER-derived membranes. Topology predictions describe at least four transmembrane domains, with the N- and C-terminal domains (NTD and CTD) likely oriented toward the cytosol (Figure 1.2), however the exact NTD orientation remains controversial (81). The majority of sequence diversity in NS4B between different HCV genotypes is found in the NTD and CTD of the gene (Figure 1.3). The NTD contains an amphipathic alpha helix that contributes to NS4B insertion into the ER membrane, and the CTD contains two alpha helical structures, the second amphipathic α-helix of them being able to mediate membrane association (38, 45). NS4B induces formation of the membranous web when expressed alone (Figure 1.4A), and can be visualized by fluorescence microscopy as NS4B foci (Figure 1.4B). These structures are seen in HCV replicon cells as well as HCV infected cells (35, 70, 109). The membranous web clearly is the site of genome replication, as HCV RNA and all HCV NS proteins involved in RNA replication are localized there (44, 109). This observation is confirmed by the fact that when membranous web vesicle structures are disrupted by NS4B mutations there is a resulting decrease in HCV genome replication (3, 101).

The various functions and interactions of NS4B implicate it in HCV RC formation and function. NS4B contains a nucleotide-binding motif between residues 129-135 that has GTPase and ATPase activity that mediates HCV RNA replication (36, 132). This activity perhaps allows NS4B to simultaneously bind to membranes and viral RNA, which would aid in tethering HCV RNA to the RC. NS4B interacts with all of the other HCV NS proteins, but has only been shown to interact with two cellular factors,
ATF6β and Rab5 (32, 121, 133). Binding of NS4B to Rab5 likely aids in formation of the HCV RC by aiding in the membrane fusion activities necessary to form the membranous web. NS4B is also reported to form oligomers (141). Both the NTD and CTD of NS4B have been implicated in oligomerization, specifically by heterotypic (i.e. NTD-CTD) and homotypic (i.e. CTD-CTD) intermolecular interactions, as well as palmitolylation at the C-terminus of NS4B (46, 101, 141). Recently, our lab has also demonstrated a role in HCV replication for GxxxG motifs contained in the NS4B transmembrane domains, likely through mediating oligomerization and interaction with viral or host factors (51). Oligomerization and interaction with viral and host proteins likely are assisting NS4B with formation of the HCV RC, but the mechanism by which NS4B alters host membranes into membranous web vesicles is unknown. It is possible that the amphipathic α-helices in the N and C-termini can induce membrane curvature in an analogous manner to the cellular proteins epsin, amphiphysin and the small GTPase Sar1 (144). Also interaction with host factors such as the early endosome (EE) vesicular traffic regulator Rab5, cytoskeletal proteins, and phosphatidylinositol 4-kinase II α are implicated in formation of the RC, although NS4B has a biochemical interaction with only Rab5 of these factors (10, 17, 71, 121, 137).

Many studies have illustrated an essential role for NS4B in HCV RNA replication. Amphipathic α-helices in both the N and C-termini of NS4B are involved in HCV RC formation, as shown with helix disrupting point mutations that abrogate RNA replication (38, 45). NS4B influence on RNA replication is also genotype specific as swapping NS4B domains between different genotype sequences alters RNA replication (12). There are many instances of point mutations in the NS4B CTD that disrupt RNA
replication and RC formation or localization (3, 13, 45, 62, 100). Interestingly, specific CTD mutations have also been shown to disrupt membranous web vesicle organization using electron microscopy (3). There is also an interaction between basic residues in the NS4B CTD and the 3’ end of negative strand viral RNA that may be involved in RC formation (37). Recent data has also suggested a role in the NS4B CTD in virus assembly and release. Three specific mutations in the NS4B CTD (N216A, H217V and H250A) enhance HCV production without altering RNA replication levels in the JFH1 cell culture system (discussed below) (62, 101). Also, our lab has recently shown that a chimeric JFH1 virus that has the NS4B C-terminal domain sequences changed to that of genotype 1b Con1 virus is defective in virus production (51).

**Regulation of Cellular Membrane Traffic by Rabs**

The Rab family of proteins are members of the Ras superfamily of small GTPases, and are represented by over 50 members. Rabs are master regulators of cellular membrane trafficking, and are responsible for regulation of vesicle budding, transport and fusion with target membranes (22, 48, 89). Like all Ras family members, Rabs cycle between GTP-bound active states and GDP-bound inactive states (19, 53). It is important to note that Rabs are regulating but not performing the actual functions in membrane traffic, it is their cognate effector proteins such as tethering factors and SNAREs, which are the membrane fusion machinery of the cell. GTP-bound active Rabs undergo a conformational change that allows their C-terminus to become bound to target membranes via a prenylation motif (43). Rab-GTP hydrolysis requires the activity of their respective GTPase-activating proteins (GAPs), while guanine nucleotide exchange factors (GEFs) catalyze GDP disassociation and Rab binding to a new GTP molecule.
Various Rab mutants have been useful in the study of membrane trafficking, such as constitutively active Rabs which are locked in the Rab-GTP bound state, and dominant negative (DN) Rabs are locked in the Rab-GDP state and sequester the GEFs required for activation (72, 105). DN Rabs have been a valuable tool in understanding how intracellular parasites alter host membrane traffic. Using DN Rabs as tools, Rab5 was shown to participate in HCV and foot-and-mouth disease entry while Rab1 was shown to mediate *Legionella pneumophila* replication in vacuoles (31, 60, 93).

The various cellular Rabs are all associated with specific membrane compartments of the cell, thus they are ideal cellular markers. Multiple domains in Rabs work together to result in proper membrane targeting, such as the C-terminal hypervariable region, the RabF motif and the RabSF motif (2). However, Rabs also targeted to their membranes with the help of their cognate effector proteins, including GAPs, GEFs, Rab escort proteins (REPs), and Rab GDP dissociation inhibitors (RabGDIs) to name a few (116). There are many examples of this specific membrane targeting in Rabs, and the Rabs that are used in the following study provide an illustration of this specificity. For instance, Rab5 is localized to the Early Endosome (EE) and mediates endocytosis and EE vesicle fusion (48). Rab7 is bound to the Late Endosome (LE) and Lysosome (LYS) compartments, and regulates transport from the LE to the LYS (50, 57). Rab6 is a regulator of EE recycling through the Golgi, as well as intra-Golgi traffic (87, 90). The secretory Rab1 and Rab2 are largely found in the ER and Golgi, and mediate both anterograde and retrograde traffic between the ER and the Golgi (48).
Hepatitis C Virus Model Systems: Replicon Cells and JFH1 Cell Culture Virus

For many years after its discovery, investigation of HCV was limited by the lack of a reliable, infectious cell culture virus with which to perform in vitro studies. The first major breakthrough was the development of the replicon system, in which autonomously replicating, HCV genome-derived RNA molecules in human hepatoma (Huh7) cells allow for the study of HCV RNA replication (14, 78). These replicons were constructed as bicistronic RNAs (Figure 3.1B), with a selectable marker (neomycin phosphotransferase, Neo) in the first cistron. The Neo marker is fused to the HCV IRES, which drives translation of the Neo gene. The second cistron was placed under the control of the IRES from encephalomyocarditis virus (EMCV), and encodes either a subgenomic polyprotein containing only the HCV proteins involved in RC function (NS3-NS5B) or a full length genome (56, 78). Upon transfection into Huh7 cells and neomycin selection, replicon-harboring colonies were expanded and characterized. The Neo resistance was conferred after the NS3-5B proteins were translated from the replicon RNA, organized a HCV RC and replicated the replicon RNA. This resulted in amplified levels of Neo expression in the cell that was sufficient to confer Neo resistance. The subgenomic replicons are the replicon of choice for most RNA replication studies as smaller RNAs were able to replicate more quickly than full length RNAs (8, 78).

During the subsequent continuous culturing of HCV replicons many interesting findings were revealed. Immunoelectron microscopy was used to show the HCV NS proteins and HCV RNA association with the ER derived membranous webs, and strengthened previous theories of the HCV RC being housed in the membranous web (35, 44). A strong link between HCV RNA replication and the cell cycle was displayed, as
actively growing cells are required for high levels of HCV RNA replication, and cells reaching senescence show a large decrease in HCV RNA levels (103). Continuous culture also allowed for the discovery of cell culture adaptive mutations in the replicon RNA that increased levels of RNA replication. A large portion of the adaptive mutations appeared in the NS5A gene. Most of these were specifically in a cluster of serine residues in the middle of NS5A (S2197, S2201, S2204 in polyprotein numbering), and were involved in abrogating p58 hyperphosphorylation of NS5A (6, 8, 77, 126). However, the adaptive mutations were not solely limited to NS5A as all of the nonstructural proteins were found to harbor mutations. Important mutations were also described in the C-terminal helicase domain of NS3 and the C-terminus of NS5B, as well as 2 mutations in NS4B at residues K135T and V186L (NS4B protein numbering) (15, 77). K135T is located in the nucleotide-binding motif of NS4B and could increase genome replication activity. V186L is in the fourth transmembrane domain of NS4B, and mutation could benefit NS4B oligomerization. However, these adaptive mutations were of no assistance in generating infectious virus. This was shown by injecting chimpanzees with HCV samples that contained these adaptive mutations or contained reversion mutations back to the original HCV consensus sequence (Con1) used to construct the replicons (20). Work on the replicon system, however, did provide findings that were important for the advancement of the first infectious cell culture virus system. An example of this was when replicon cells were “cured” by treatment with IFN-α, a second replicon RNA transfection in these cured cells supported higher levels of RNA replication than in parental Huh7 cells (16). One specific clonal line, named Huh7.5, contained a mutation in the interferon-inducible cellular RNA helicase retinoic acid
inducible gene 1 (RIG-I), which would allow for more permissive cells to virus infection in cell culture by disrupting MAVS-induced transcription of Interferon (123).

The answer to the infectious virus system was provided by a genotype (gt) 2a HCV strain isolated from a Japanese patient with fulminant hepatitis (JFH1), that was shown to be able to replicate RNA to high levels without adaptive mutations and produce infectious virus after transfection into Huh7 cells (66, 73, 135, 142). However, the initial JFH1 system is limited by a relatively low efficiency of virus production and infectivity. A major advancement in the search for higher virus production was the use of cured replicon cells (Huh7.5) for JFH1 RNA transfection, which resulted in higher levels of infectious virus production compared to Huh7 cells (142). Another strategy involved the creation of chimeric JFH1 viruses, which contained the JFH1 nonstructural proteins to maintain the high RNA replication levels, and the structural proteins from the genotype 2a virus J6 to increase virus production (102). This chimeric virus, Jc1, showed a roughly one log increase in virus titer compared to parental JFH1 virus, and was infectious in vivo when injected into chimpanzees (21, 74, 102). Genotype 1a infectious virus from Hutchinson strain (H77) as also been produced, and H77 sequences can also make successful chimeras with genotype 2a virus (73, 102, 139). The advent of the JFH1 virus system allowed the discovery of many aspects of the virus lifecycle, such as entry into the cell and assembly of virus particles that were not possible in the replicon system.

Hypothesis and Objectives

The purpose of this dissertation is twofold. First is to investigate the link between NS4B and cellular Rabs in HCV RC formation. Our lab has previously shown that the EE regulator Rab5 is involved in HCV replication, likely at the stage of membrane
rearrangement to form the RC (121). In order to study the role of the EE in HCV RC formation, a method of immunoisolation of the HCV RC using NS4B specific antibody was employed (Figure 1.3). We used this isolated subcelluar fraction, which is competent for *in vitro* NS5B-mediated RNA synthesis, to discover which types of Rab GTPases were present in the HCV RC membranes, and confirmed this with fluorescence microscopy in full length replicon cell line C5B. We hypothesized that disruption of these NS4B associated Rabs with dominant negative (DN) mutants or siRNA knockdown can decrease HCV replication, highlighting their role in HCV RC formation.

The second purpose is to test the role of the NS4B CTD in JFH1 virus production. New data in the field has suggested an involvement for the NS4B CTD in virus production (51, 62, 101). We hypothesized that the creation of chimeric JFH1 viruses, which could potentially generate suppressor mutations, would allow us to begin to gain insight into the mechanisms behind the role of the NS4B CTD in virus production. The sequences for the NS4B CTD of gt 1a H77 virus or gt 1b Con1 virus were inserted into JFH1 and tested for defects in RNA replication and virus production. Chimeric viruses with virus production defects then could be cultured continuously to generate second site mutations. These mutations can reveal genetic interactions between NS4B and other viral proteins to be further investigated.
Figure 1.1 Hepatitis C Virus Genome Organization and Life Cycle. A. The HCV genome is comprised of approximately 9600 nucleotides, and contains 5’- and 3’- untranslated regions (UTR) with a single polyprotein encoded between them. B. The HCV lifecycle is exclusively in the cytosol. HCV enters the cell via receptor mediated endocytosis (a), and the genome is uncoated after endosome acidification (b). The genome is directly translated (c) into a polyprotein which is cleaved by host and viral proteases on the ER membrane. The mature HCV NS proteins then organize a RNA replication complex (d), where negative strand templates are used to amplify new genomic RNA. New virions are then thought to be secreted from the cell (f).

“Used with Permission from: Moradpour et al., Replication of hepatitis C virus, Nature Reviews in Microbiology, 2007. License number 2784830859818.”
Figure 1.2 Predicted NS4B Topology. The localization of the NS4B domains in relation to the ER membrane are displayed, as predicted by topology models. There are alpha helical structures in both the cytosolic NTD and CTD regions, which mediate membrane association. The NS4B nucleotide-binding motif is also located in a cytosolic loop. Image provided by Jason Aligo.
Figure 1.3 NS4B Amino Acid Sequence Alignment. The amino acid sequence of four different HCV isolates is displayed with the genotype 1b Con1 (consensus sequence 1) as the reference. Conserved residues are highlighted in green. Notice that the majority of differences are located in the cytosolic N- and C-terminal regions of NS4B.
Figure 1.4 Visualization of NS4B-induced Membranous Webs by Microscopy.  A. NS4B fused with GFP was expressed in Huh7 cells and visualized by transmission electron microscopy (TEM). The circled area is enlarged for visualization of the characteristic NS4B-induced membranous web structure. Bars = 1 micrometer. B. NS4B-GFP was expressed in Huh7 cells and visualized by fluorescence microscopy to visualize the characteristic NS4B foci pattern. TEM image is courtesy of Jason Aligo.
Chapter 2
Materials and Methods

Cells

Cells were grown as monolayers in advanced Dulbecco's modified Eagle's medium (DMEM) supplemented with 1.5% fetal bovine serum (FBS), L-glutamine, 100 units/ml penicillin and 100 mg/ml of streptomycin (Invitrogen, Carlsbad, CA) at 37° C in a 5% CO₂ incubator. For replicon cells, the media also contained 0.3–0.5 mg/ml G418 (Geneticin). Parental Huh7 and full-length C5B replicon cells were generously provided by Stanley Lemon (University of North Carolina) (56). Huh7.5 cells, a highly permissive cell line for HCV replication were provided by Apath, LLC (St. Louis, MO) (16).

Immunoisolation of a Subcellular Fraction Containing HCV Replicase Proteins

Initially, 4–6 × 100 mm dishes (1×10⁶ cells/dish) each of parental (Huh7) and replicon cells (C5B) were grown for 48 h. The cells were collected and resuspended in phosphate-buffered saline (PBS) containing 0.25 M sucrose (PBS/sucrose) and protease inhibitors (1 mM PMSF and 1 tablet of Complete Mini, EDTA-free; Roche, Nutley, NJ). The cells were then lysed with 7 passages in a ball-bearing homogenizer to ensure approximately 90% lysis (7). Cell lysates were spun at 2500 ×g for 10 min at 4° C to pellet cellular debris and nuclei. This supernatant is referred to as the crude lysate. Purified NS4B antibody at a 1:200 dilution (Covance, Denver, PA) was added to 4 mg of protein from either parental or replicon cells, and the 1.6 ml mixture was incubated overnight at 4° C with constant rotation. The antibody and lysate mixture was overlaid on a discontinuous iodixanol (Sigma-Aldrich, St. Louis, MO) gradient (4%, 8%, 12%, 20%, 30%, and 35%) in PBS/sucrose and the gradient was spun
at 120,000 × g for 1 h 45 min at 4°C in an Ti80 rotor. A total of 12 fractions (630 µl each) were collected from top to bottom. Fractions 7 and 8 are endoplasmic reticulum (ER)-enriched, as determined by Calnexin-positive bands. In replicon cells these fractions also contain viral replicase proteins including NS3, NS4B, NS5A and NS5B. For the membrane floatation assay, fractions 7 and 8 were combined and mixed with 60% iodixanol. A discontinuous iodixanol gradient (5%, 25% and 30%) was layered on the top of the combined fractions and the samples were spun at 120,000 × g for 4 h 25 min at 4°C in a Ti80 Rotor. A total of 8 fractions (867 µl each) were collected from top to bottom. Typically, the floatation gradient fraction 2 contained most of the HCV replicase proteins. Secondary anti-rabbit antibody-coated magnetic Dynabeads M-280 (4.3×10⁷/ml; Invitrogen, Carlsbad, CA) were added to the floated membrane fraction 2 with 3% final BSA. After overnight incubation at 4°C with constant rotation, the mixture was placed in a magnetic rack and the resulting supernatant was collected and labeled as “Unbound”. NS4B positive membrane-bound beads were labeled as “Bound”. The bound fraction was washed four times in PBS to get rid off loosely bound proteins or membranes, whereas the unbound supernatant microsomes were spun twice at 100,000 × g for 1 h at 4°C. Bound and unbound fractions were resuspended in 4× sample buffer (240 mM Tris pH 6.8, 4% SDS, 40% glycerol, 4% β-mercaptoethanol, 0.01% bromophenol blue) for immunoblot. For HCV RNA-dependent RNA polymerase activity, Bound and unbound fractions were resuspended in diethylpyrocarbonate (DEPC)-treated PBS.

**Quantitative Real-Time PCR of HCV RNA in Bound and Unbound Fractions**

Total cellular RNA was prepared from bound and unbound fractions by the TRIZOL method (Invitrogen, Carlsbad, CA), and resuspended in DEPC-treated water.
First strand cDNA was synthesized from the RNA using random primers and the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Triplicate samples of cDNA were mixed with a Taqman probe and a set of forward and reverse primers specific for either HCV NS4B (genotype 1b, strain N) or GAPDH and the mixture was subjected to real-time quantitative PCR using the ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA).

**In vitro RNA-Dependent RNA Polymerase Assay**

An equal volume (25 µl) of crude lysate, bound or unbound fraction was resuspended in a 50 µl reaction containing 1 mM ATP, GTP and UTP (CTP at 40 µM), 1 mCi of (α$^{32}$P) CTP per ml, 1× buffer (50 mM Tris–Cl pH 7.8, 50 mM KCl, 5 mM MgCl$_2$, 10 mM DTT, 5 mM creatine phosphate and 25 µg/ml creatine kinase), 10 µg/ml actinomycin D and 800 U/ml RNasin. The reaction was incubated at 37° C for 1 h, with gentle mixing every 30 min, followed by RNA extraction using the TRIZOL method (Invitrogen, Carlsbad, CA). The RNA was resuspended in DEPC-treated water and separated on a 1% formaldehyde denaturing gel. After drying, the gel was exposed to a phosphor screen and the RNA was visualized with a phosphorImager (Typhoon 8600, Molecular Dynamics, Sunnyvale, CA).

**SYPRO Ruby Protein Staining of Immunoisolation Fractions**

In order to examine the total protein profile in each step of the isolation procedure, one microgram of protein was separated by SDS-PAGE and the gel was fixed in 50% methanol and 7% acetic acid. The gel was then stained overnight with 75% SYPRO Ruby Protein Gel Stain (Invitrogen, Carlsbad, CA). After two washes with 10%
methanol and 7% acetic acid, the proteins were visualized on a phosphorImager (Typhoon 8600, Molecular Dynamics, Sunnyvale, CA).

**Antibodies**

Rabbit polyclonal antibody to HCV NS4B was obtained from Covance (Denver, PA). Rabbit polyclonal antibodies to HCV NS3, NS5A and NS5B were kindly provided by Craig Cameron (Penn State, University Park, PA). Mouse monoclonal antibody to HCV NS5A was a gift from Charles Rice (The Rockefeller University, New York, NY). Antibodies to Rab1A, 2, 4A, 5A and B, 6, and Rabaptin5 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rab7 antibody was from Cell Signaling (Danvers, MA). Mouse monoclonal antibody to GAPDH was purchased from Fitzgerald (Concord, MA). Mouse monoclonal antibodies to calnexin, calreticulin and syntaxin13 were from Stressgen (Victoria, Canada). Mouse monoclonal antibodies against clathrin and golgin97 were purchased from Abcam (Cambridge, MA). Antibodies to EEA1 and Sar1 are from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal to Rabex5 and Hsp70 were purchased from BD Biosciences (San Jose, CA) and Affinity Bioreagents (Golden, CO) respectively. Horseradish peroxidase conjugated secondary antibodies (used in chemiluminescence) were obtained from Vector laboratories (Burlingame, CA). Alexa Fluor 594- or 488- conjugated secondary antibodies (used in immunofluorescence) were from Invitrogen.

**Immunoblot of HCV and Host Proteins**

Protein samples were resuspended in 4x sample buffer (240 mM Tris pH 6.8, 4% SDS, 40% glycerol, 4% -mercaptoethanol, 0.01% bromophenol blue) and boiled for 5 min. The proteins were separated by SDS-PAGE, followed by transfer onto an
Immobilon-P membrane (PVDF, Millipore). After binding with the respective primary antibody and HRP-conjugated secondary antibody, proteins were visualized by the enhanced chemiluminescence (ECL) detection method (Pierce, Rockford, IL).

**Protein Identification by Capillary LC-ESI-MS/MS**

Protein bands that were enriched in the Bound fraction, but not the Unbound fraction were cut from a gel and subjected to in-gel digestion overnight with sequencing grade trypsin (Promega, Madison, WI). Samples were analyzed by capillary liquid chromatography-tandem mass spectrometry system with a nanospray ionization source (capLC-nanoESI-MS/MS). The system consisted of a Surveyor Micro AS autosampler, a Surveyor MS pump, and an LTQ linear ion trap mass spectrometer (Thermo Electron Corp, Waltham, MA). The digestes were desalted and eluted with a 30 min linear acetonitrile gradient (2% to 40% acetonitrile in 0.1% formic acid) at a rate of ~0.3 µl/min obtained by pre-column splitting of a flow of 200 µl/min. The PicoFrit analytical column (New Objective) was packed with 5 µm wide pore C18 particles (Supelco Co, Bellefonte, PA). From a survey scan over a m/z range of 550-1500 Tandem MS (e.g. collision induced dissociation or CID) was triggered for precursor ions over an intensity threshold determined from the baseline. Raw tandem MS spectra were processed and searched against the protein sequence database using the Mascot 2.2 search engine. Mass tolerance was 3 amu for precursor ions and 2 amu for product ions. Two missed cleavages were allowed for trypsin digestion, and methionine oxidation was considered as a variable modification. A minimum of two unique peptides with a Mascot score higher than 40 were required to positively identify a protein.
**Rab Plasmids**

CFP-fused WT Rabs (1, 2, 6, 7 and 11), their dominant negative (DN) versions (Rab1A-S25N, Rab2-S20N, Rab6A-T27N and Rab7-T22N), and GFP-fused Rab5A-S34N were provided by Won Do Heo (Korea Advanced Institute of Science and Technology, Korea). WT GFP-Rab5 was provided by Brian Knoll, Baylor College of Medicine (Houston, TX). To construct the YFP-Rab5 vector, pEYFP-C1 (Clontech, Mountain View, CA) was digested with NheI and NotI. The purified YFP fragment was subcloned into NheI- and NotI-cleaved GFP-Rab5 vector, thus replacing GFP.

**DNA Transfection**

Huh7 or C5B replicon cells were trypsinized and grown overnight in 6-well plates to obtain 60% confluent monolayer cells. 30 min prior to transfection, the cells were washed in PBS and fed with 2 ml of complete medium. Cells were transfected with the TransIT-LT1 lipofection reagent, according to the manufacturers protocol (Mirus, Madison, WI). The DNA mixture was added to each dish and incubated at 37° C for 24 or 48 h. The transfection efficiency was usually 70–90% using this procedure.

**Indirect Immunofluorescence**

Cells were seeded onto coverslips and at 72 h posttransfection the cells on coverslips were washed with PBS and fixed for 10 min in 4% formaldehyde/PBS. The cells were permeabilized for 5 min at room temperature in 0.05% Triton-X-100/PBS, and blocked for 30 min with 3% BSA in PBS buffer. Primary antibodies were diluted in 3% BSA and incubated with the cells for 1 h at room temperature. After three washes, Alexa fluor conjugated secondary antibody in 3% BSA was incubated on the cells for 1 h at room temperature. After three washes in PBS, the cell nuclei were stained with DAPI for
10 min at room temperature, followed by three more washes in PBS. The stained coverslips were mounted on glass slides in Vectashield (Vector Laboratories, Inc., Burlingame, CA) and sealed with nail polish. The samples were then examined either with a confocal laser-scanning microscope (Olympus Fluoroview FV1000, Munster, Germany) or with an epifluorescence microscope (Zeiss Axiovert 200M, Thornwood, NY) at x630 magnification. Confocal image analysis was performed using Olympus Fluoroview version 1.7c software. Pearson's co-efficient (PC) for colocalization is calculated on a pixel by pixel basis; it is from a scale of −1 to 1 and is based on two variables (x and y) and the linear relationship between these variables. Numbers below 0.5 suggest little to no co-localization, whereas those above 0.5 indicate moderate to strong association. All images were saved as TIFF files, imported to and processed in Adobe Photoshop. Co-localization of green (FITC) and red (cy3) signals produces yellow, whereas co-localization of green, red, and blue produces white.

**siRNA Transfection**

One day prior to transfection, C5B cells were seeded in 6-well plates to yield a roughly 60% confluent monolayer the day of transfection. The cells were washed with PBS and fed with 1 ml of complete medium. The C5B cells were transfected with 50 nM siRNA according to the TransIT-TKO protocol (Mirus, Madison, WI). 50 nM of siCONTROL nontargeting siRNA pool was used as a control. Transfected cells were incubated for 48 h in complete media before processing for RT-PCR analysis. The siGENOME SMART-pool siRNA was purchased from Dharmacon (Lafayette, CO).
**Construction of Viral cDNA Plasmids**

pJFH1-WT and pJFH1-GND vectors encoding wild-type and replication defective JFH1 genomes, respectively, were kindly provided by Dr. Takaji Wakita (Tokyo Metropolitan Institute for Neuroscience) (135). These vectors were used to generate the various JFH1 chimera and point mutants in the full-length genome and the subgenomic luciferase (Luc)-expressing replicons. The plasmid pLuc-JFH1 was constructed according to methods previously described and contains the T7 promoter sequence fused to nucleotides 1 to 389 of the JFH1 consensus sequence, followed by the firefly luciferase (Luc) gene (66). Next is the encephalomyocarditis virus (EMCV) IRES fused to the HCV nucleotides spanning from the beginning of the NS3 gene to the 3’NTR of JFH1. Overlap extension PCR was used to construct the full-length genomes containing chimeric C-terminal domain of the NS4B gene, where the JFH1 sequences were replaced with either Con1 or H77 sequences, and was constructed according to the methods previously described by Han et al (51). The subgenomic Luc replicons were constructed by directly replacing the fragment between the EcoRI site and SpeI site of pJFH1 or its derived chimeric vectors with EcoRI- and SpeI-cut fragment from pLuc-JFH1. The EcoRI- and SpeI-cut fragment from pLuc-JFH1 contains the HCV 5’ UTR followed by Luc reporter, the EMCV IRES and the beginning of NS3 sequence (nt 3431-4106).

Blunt end ligation was used to introduce the single point mutations into JFH-1, J/C1-C or J/H-C genomes as previously described (51). Since the single mutations were engineered by the same approach, pJ/C1-C N216S (NS4B) vector will illustrate how the constructs were made. First, a F1 PCR product spanning the NsiI(NS3) to NS4B N216S region (nt 5281-6148) was amplified from the pJ/C1-C vector using the forward primer
NsiI-Fwd (5’-CACCCTCACACACCCTGGG-3’) and the phosphorylated reverse primer N216S-Rev (5’-CGAAGCGAACGCTATCAGCC-3’). Next, a F2 PCR product spanning the NS4B N216S to RsrII-NS5A region (nt 6149-7692) was amplified from the pJ/C1-C vector using the phosphorylated forward primer N216S-Fwd (5’-CGGGGTAGTCACGTCTCCC-3’: underlined nucleotides correspond to the NS4B N216S mutation) and the reverse primer RsrII-Rev (5’-GTATGACATGGAGCAGCACG-3’). The F1 and F2 fragments were digested with NsiI and RsrII, respectively, and the resulting fragments were ligated into NsiI- and RsrII-cut pJFH1 vector.

The multiple point mutations in J/C1-C (or J/H-C) were engineered by using the previous vectors containing single mutations. To construct pJ/C1-C containing p7, NS4B and NS5A mutations for instance, the p7 mutation (H31L) was removed from the pJ/C1-C H31L plasmid by digestion with EcoRI (in 5’ UTR) and NsiI (in NS3), and ligated into an EcoRI- and NsiI-digested pJ/C1-C N216S (NS4B) vector, thereby combining the p7 and NS4B mutations into the resulting vector pJ/C1-C H31L/N216S. Next, the NS5A mutation (C465S) was removed from the pJ/C1-C C465S plasmid by digestion with RsrII (in NS5A) and EcoRV (in 3’ UTR), and ligated into a RsrII- and EcoRV-digested pJ/C1-C H31L/N216S vector. The resulting recombinant DNA was sequenced to confirm the presence of the three mutations.

**In Vitro Transcription and Electroporation of Viral RNA into Huh7.5 Cells**

Plasmid cDNA constructs containing WT or chimeric NS4B CTD genomes were linearized with XbaI and purified using the Cycle Pure Kit (Omega Bio-Tek, Norcross, GA). RNA was synthesized using the T7 RiboMAX Express Large Scale RNA
Production Systems Kit (Promega, Madison, WI) according to the manufacturer’s instructions. The RNA was then isolated using Trizol-LS (Invitrogen). Prior to electroporation, subconfluent Huh7.5 cells were trypsinized and resuspended in complete DMEM. The cells were then washed three times and resuspended at a concentration of 1.25 x 10^7 cells/ml in PBS. Then, 1 µg of viral RNA or 10 µg of SGR-Luc RNA was mixed with 2.5 x 10^6 Huh7.5 cells in 0.2 ml ice-cold PBS and electroporated with an ElectroSquarePorator (BTX) in a 0.2 mm gap cuvette. The electroporator was set at 820 V, 99 µs at 1.1 s intervals and 4 pulses. The cells were left to recover for 10 min at room temperature before being diluted into 10 ml of complete media. The cells were then seeded into a 10 cm dish and virus samples harvested every 3 days, or seeded into 24 well plates for Luc-expressing replicons and subsequently harvested at 4 h, 2 days (D2), D4 and D6 for Luc assay.

**Luciferase Assay**

At the indicated time points post transfection, media was removed from triplicate wells and the cells were washed twice in PBS. Fifty microliters of ice-cold 1x Cell Culture Lysis Reagent (CCLR) buffer (Promega) was added to each well of the 24 well plate, and the plates were gently rocked at room temperature for 15 min to lyse the cells. The lysate was removed from the plate and transferred to a 1.5 ml tube on ice. The lysate was vortexed and the supernatant was transferred to a fresh tube after a brief spin at 12,000 xg in a microfuge. Twenty µl of the lysate were then mixed with 20 µl of Luc assay susbtrate (Promega) and quickly vortexed prior to enzyme assay in a luminometer.
**Titration of HCV Infectivity**

Huh7.5 cells were seeded into 96 well plates at a density of $5 \times 10^3$ cells/well. Viral supernatant samples were serially diluted 10-fold in no serum DMEM and were used to infect the seeded cells. Following three days of incubation, the cells were immunostained with NS5A-specific antibody. Positive foci were counted, and the infectivity titer was calculated from triplicate wells. The viral titer was expressed as focus-forming units/ml (FFU/ml), or as total FFUs if the virus samples are collected in different volumes.

**Cell Lysate Preparation to Obtain Intracellular Virus**

Intracellular virus samples were generated as previously described (42). Briefly, cells were collected and washed with phosphate-buffered saline (PBS), followed by resuspension in cell culture media and four cycles of flash freezing and thawing in a 37°C water bath. The samples were centrifuged at 3500 rpm for 5 min at 4°C and virus-containing supernatants were collected.

**Sucrose Cushion Pelleting of HCV Particles**

Virus supernatants were collected from four dishes at day 6 post electroporation, pooled, and the total volume was concentrated to 18 ml by an Amicon Ultra-15 centrifugal filter (Millipore, Billerica, MA). Next, 7 ml of concentrated virus supernatant was overlayed onto 1.5 ml of a 20% sucrose cushion in TNE buffer (10 mM Tris pH 8, 150 mM NaCl, 2 mM EDTA), and pelleted by spinning at 112,000 xg for 1 h 30 min in a Ti80 rotor (Beckman Coulter, Brea, CA). The supernatant was removed and the virus pellet was resuspended in 140 µl PBS for extraction of viral RNA using the QIAamp Viral RNA mini kit (Qiagen).
**Analysis of Adapted Virus Nucleotide Sequence**

Total cellular RNA was extracted from J/C1-C-Ad1 or J/H-C-Ad1 virus-infected cells using the Trizol-LS reagent (Invitrogen). Five micrograms of total cellular RNA was used as a template for reverse transcription of HCV cDNA by using Superscript III (Invitrogen) and a specific primer 9470R, which binds at the HCV 3’ NTR. Using a series of overlapping primers, six PCR fragments which covered the whole HCV genome were then amplified and sequenced (110).
Chapter Three

Identification of HCV Replication Complex-Associated Rabs by Immunoisolation and Fluorescence Microscopy

A previous study in our lab showed that the Early Endosome (EE) regulator Rab5 co-localizes with NS4B foci in replicon cells, is required for HCV replication and that an isolated HCV replication complex (RC) fraction contained the EE factors Rab5 and EEA1 (121). These findings were confirmed by a study that showed a block of JFH1 virus replication or Con1 replicon replication after siRNA knockdown of Rab5 or EEA1 (10). Rabs are the central regulators of membrane traffic in the cell and could possibly be involved in membranous web formation. Therefore, we hypothesized that other cellular Rabs may be playing a role in HCV RC formation and function. In order to investigate the composition of the HCV RC we used a NS4B antibody-mediated immunoisolation procedure to isolate a HCV RC enriched subcellular fraction, as well as fluorescence microscopy to visualize NS4B-Rab association in replicon cells. The isolated HCV RC was competent for in vitro NS5B-mediated RNA synthesis and contained Rabs from every cellular compartment that was tested, but by fluorescence microscopy only the endocytic Rab5 and 7 co-localized with NS4B foci. Expression of dominant negative (DN) Rab5 or 6 caused a disruption in NS4B foci, and siRNA knockdown of Rab2, 5 or 7 decreased HCV replication levels. Together the data indicates a role for multiple Rabs in the formation of the HCV RC, especially the endocytic Rab5 and 7.
The Isolated HCV Replication Complex Fraction is Competent for NS5B-Mediated *In Vitro* HCV RNA Synthesis

We modified our original RC isolation protocol by adding a membrane flotation step to further enrich the RC fraction with membrane-bound NS4B (Figure 3.1A). We hypothesized that this isolated fraction would enable us to identify other cellular Rabs that are associated with the HCV RC. C5B replicon cells (Figure 3.1B) were gently lysed in a ball bearing homogenizer to preserve membrane structure, followed by addition of NS4B specific antibody. The lysates were subjected to subcellular fractionation, and NS4B enriched fractions were collected for membrane floatation. Then magnetic dynabead conjugated secondary antibody was added to the floated membrane fraction. The isolated RC fraction was named “Bound”, and the “Unbound” fraction contained the non-NS4B antibody bound vesicles. The Bound fraction contained the C5B replicon RNA (Figure 3.2A) and was competent for *in vitro* RNA synthesis by the HCV RNA-dependent RNA polymerase (RdRp) NS5B (Figure 3.2B). When rNTP’s with a $^{32}$P label were added to the isolated RC fraction, the newly synthesized RNA was from NS5B as the reaction contained actinomycin D, which blocks transcription from DNA templates, and the cellular RNA II polymerase inhibitor $\alpha$-amanitin had no effect on RNA synthesis (Figure 3.2C, lane 4). The isolated RC fraction also displayed characteristics consistent with it being a membrane associated complex. Treatment of the Bound fraction prior to the RdRp assay with Triton X-100 detergent abrogated *in vitro* RNA synthesis, but pre-treatment with protease or nuclease had no effect on RdRp activity (Figure 3.2D, lanes 4, 5, 8 and 9). Interestingly, when the micrococcol S7 nuclease was present during the RdRp assay, a decrease in RNA synthesis was seen that was absent in Bound fractions.
that were pre-treated with nuclease and then inactivated before the RdRp assay (Figure 3.2D, lane 7).

Specific inhibitors of HCV replication were also used to verify the relevance of the RC isolation procedure. These cellular drugs were added to the C5B replicon cells for 48 hours prior to collection of cell lysates. The first, BILN 2061, is an inhibitor of the NS3 protease, and the isolated RC fraction shows a decrease in HCV RNA synthesis with increasing amounts of BILN 2061 (Figure 3.3A). The second, 2’-C-methyl adenosine (2’ CMA), is targeted to the NS5B RdRp and acts as a chain terminator when incorporated into growing RNA strands. Again, a decrease in RNA synthesis is concomitant with an increase of 2’ CMA added to the C5B replicon cells (Figure 3.3B). These results are indicative of a decrease in C5B replicon RNA levels due to inhibition of RNA replication in the cell, and the lower RdRp synthesis levels are likely due to decreased amounts of template RNA being present in the isolated RC. An in vitro RNA chain terminator, 3’-deoxy GTP (3’ dGTP), was also tested to verify that RNA synthesis could be inhibited during the RdRp reaction with a specific inhibitor. As seen in Figure 3.3C, there is a decrease in RNA synthesis levels with increasing levels of 3’ dGTP present in the RdRp reaction. Together this data suggest that the active immunoisolated HCV RC fraction is suitable to use for identifying cellular factors that involved in RC formation.

**Endocytic and Secretory Rabs are Present in the Bound Fraction**

In order to verify that the isolated RC fraction was indeed enriched in specific factors, protein samples from the various RC isolation procedure steps were run on SDS-PAGE and visualized by SYPRO-Ruby protein staining. The input C5B lysate, subcellular fractionation fractions #7 and #8 (NS4B enriched fractions), the floated
membrane fraction, Unbound and Bound samples were visualized. Both the Unbound fraction (dots) and Bound fraction (arrowheads) show differences in specific protein abundance relative to each other, implying that the isolation protocol did enrich the Bound fraction with particular proteins (Figure 3.4A). Immunoblotting of various cellular markers displayed that the Bound fraction contained ER markers Calnexin, Calreticulin and Sar1 (Figure 3.4B). These ER factors, however, were not enriched compared to the Unbound fraction, suggesting that they could be present due to nonspecific membrane isolation during the procedure. The Golgi marker Golgin 97 was not seen in either the Bound or Unbound fraction, suggesting that Golgi membranes were removed during the RC isolation procedure, but the mitochondrial marker Hsp70 was unexpectedly present in both fractions (Figure 3.4B). This suggests that along with specific HCV RC isolation, there is also a nonspecific isolation of some cellular membranes along with the HCV RC. Immunoblotting of HCV proteins verified that as expected, the Bound fraction contained the HCV replication proteins NS3, NS4B and NS5B (Figure 3.4C). Interestingly, the HCV nucleocapsid protein Core was also present in the Bound fraction (Figure 3.4C). This implies that Core can be found at HCV RC membranes and provide a means by which to encapsidate newly synthesized viral RNA for assembly into virions.

We have previously displayed the presence of the EE regulating factors Rab4 and Rab5 in the Bound fraction by western blot, and preliminary mass spectrometry analysis of the Bound fraction showed that Rab2A, 6A, 11A and 11B were present (121). Immunoblotting of the Bound fraction was used to confirm these observations. Relative to crude lysate, the Bound fraction had differentially enriched levels of Rab1A, 2, 4A,
36

Rab5 and Rab7 Co-localizes with NS4B Foci

Two possible explanations for the differential association of Rabs in the Bound fraction are the nonspecific recruitment of a large population of ER- and LE/LYS membranes into the Bound fraction, or the preferential recruitment of specific Rabs into the Bound fraction. Fluorophore tagged Rabs expressed in both C5B replicon and parental Huh7 cells were used with confocal microscopy in order to examine the subcellular localization of the Rabs in question. Immunostaining of some of the cellular Rabs proved difficult, so for consistency the Rabs were all expressed with a N-terminal CFP, GFP or YFP tag. Fig 3.6 (panels i-v) shows that expression of the Rab constructs in Huh7 cells results in Golgi-like fluorescence for Rab1, 2 and 6, while the endocytic Rab5 and 7 results in the characteristic foci and perinuclear patterns typically seen with these proteins. Expression of CFP alone in Huh7 cells resulted in a typical cytosolic and nuclear localization pattern (Figure 3.6, panel vi). Next the Rabs were expressed in C5B replicon cells to test for colocalization with NS4B protein. Immunostaining of NS4B in C5B replicon cells typically results in a perinuclear foci pattern, and in Huh7 cells there is minimal background (Figure 3.6, panels vii-viii). Figure 3.6B shows that Rab1 (panels i-iii; a), Rab2 (panels iv-vi; b) and Rab6 (panels x-xii; c) display minimal co-localization
with NS4B, with a Pearson coefficient (PC) of less than 0.4 for each (Figure 3.6C). The EE Rab5 (Figure 3.6B, panels vii-ix; c) and LE Rab7 (Figure 3.6B, panels xiii-xv; d) showed much stronger NS4B co-localization, with a PC of at least 0.5 (Figure 3.6C). A PC of 0.5 or greater suggests strong co-localization of the two fluorescent signals. This suggests that EE and LE/LYS Rabs are selectively associated with NS4B foci. These are the Rabs previously identified as enriched in the Bound fraction relative to C5B lysate after RC isolation (Figure 3.5B).

Since singly expressed Rab5 and 7 co-localize with NS4B foci, we hypothesized that Rab5 and 7 would co-localize with each other in C5B cells. First, co-expression of the Rabs in parental Huh7 cells was examined. YFP-Rab5 was pseudo-colored red during image analysis and the other CFP-tagged Rabs (CFP-Rab1, 2, 6 and 7) were pseudo-colored green for any resulting co-localization to be more easily seen as a yellow color. Figure 3.7 indicates that co-expression of Rab5 with Rab1 (panels i-iii), 2 (panels iv-vi), 6 (panels vii-ix) or 7 (panels x-xii) results in minimal fluorescence co-localization, displaying their compartment specificity. Co-expression of Rab5 with the other Rabs in C5B replicon cells was then paired with immunostaining of NS4B foci. YFP-Rab5 (pseudo-colored green) and NS4B foci (red secondary antibody) will merge to display a yellow color. Any further co-localization of YFP-Rab5 and NS4B foci with CFP-Rab1, 2, 6 or 7 (not pseudo-colored) merges to result in white fluorescence. As seen in Figure 3.7, Rab5 co-expression with Rab1, 2 or 6 resulted in less Rab-Rab co-localization (panels i-xii; a-c) than with Rab5 and Rab7 co-expression (panels xiii-xvi; d). The PCs measured for these images confirm a stronger Rab5-Rab7 association, with a PC of well over 0.5 for Rab5-Rab7 co-localization (Figure 3.7C). Taken together, these images
indicate that Rab5 and 7 co-localize in C5B replicon cells but not in parental Huh7 cells. This suggests that NS4B may be mediating selective recruitment of Rab5 and 7 into the HCV RC.

**Rab5 Effectors Show Differential Replication Complex Association**

Since Rab5 is a master regulator of EE membrane transport and is required for HCV replication, we hypothesized that NS4B recruits Rab5 effector proteins into the HCV RC in order to perform the Rab5-regulated membrane transport functions such as vesicle tethering or membrane fusion. First the Bound fraction was used for immunoblotting to test for the presence of Rab5 effectors. We have previously shown that EE tethering factors Rabaptin-5 and EEA1 co-localize with NS4B foci in replicon cells (121). As expected, Rabaptin-5 and EEA1 can be found in the Bound fraction by immunoblotting (Figure 3.8A). Interestingly, the EE SNARE syntaxin 13 is highly enriched in the Bound fraction relative to crude lysate (Figure 3.8A). SNAREs are the proteins that perform the actual functions needed for vesicle-vesicle fusion. Rabex5, a Rab5 GEF, is not present in the Bound fraction (Figure 3.8A). Rabex5 is a soluble factor that depends on other Rab5 effectors in order to associate with EE fusion machinery, and may have dissociated from NS4B positive vesicles during the isolation procedure. Clathrin, the coat protein of endocytic vesicles, is also absent in the Bound fraction (Figure 3.8A). Since the Rab5 effectors Rabex5 and clathrin were not present in the bound fraction, we sought to confirm this observation by fluorescence co-localization with NS4B foci in C5B replicon cells. Contrary to expectations, Rabex5 was found to co-localize with NS4B foci in C5B replicon cells (Figure 3.8B, panels i-vi). Clathrin, however, did not co-localize with NS4B foci, confirming that it was not a part of the
isolated HCV RC vesicles (Figure 3.8B, panels vii-xii). The syntaxin 13 antibodies tested were unable to display immunofluorescence staining in C5B replicon cells, so it remains unanswered if syntaxin 13 co-localizes with NS4B foci.

**Dominant Negative Rab6 Disrupts Rab5 Co-localization With NS4B Foci**

We next hypothesized that the selective recruitment of endocytic Rabs into NS4B foci was due to ER-derived NS4B positive secretory vesicles being transported to the plasma membrane and retrieved back into the cytosol via the endocytic pathway, thereby allowing for incorporation of Rab5 and 7 into NS4B foci. Dominant negative (DN) Rab mutants were used as a tool to disrupt secretory pathway function in order to determine if this hypothesis was correct. The DN forms of CFP-Rab1 (Figure 3.9, panel i), 2 (Figure 3.9, panel v) and 6 (Figure 3.9, panels ix and xiii) display a more diffuse distribution than their WT counterparts. Figure 3.9 illustrates that DN Rab 1 (i-iv; a) and 2 (v-viii; b), which partially inhibit ER to Golgi traffic, had no appreciable effect on NS4B and Rab5 colocalization. DN Rab6, which inhibits LE-Golgi and intra-Golgi traffic, partially disrupted Rab5 and NS4B association, as seen by the increased red NS4B staining (Figure 3.9, panels ix-xvi, c and d). This implies that ER-Golgi traffic is not required for Rab5 to be recruited into NS4B foci, but we cannot completely rule out the secretory pathway because there would be preformed NS4B foci present in C5B replicon cells before transfection of the DN Rab constructs. The white fluorescence seen in Figure 3.9 (panels a, b, c and d) likely result from the diffuse DN Rab distribution that overlaps with NS4B and Rab5 colocalization.
**Dominant Negative Rab 5 or 7 Disrupts NS4B Foci**

Since Rab5 and 7 are recruited into NS4B foci, we postulated that these Rabs are necessary to form NS4B foci. We have previously shown that DN Rab5 expression disrupts NS4B foci in replicon cells (121). DN Rab6 expression was also tested since it disrupts NS4B-Rab5 colocalization (Figure 3.9, panels c and d). Typical NS4B foci staining pattern was unaffected with expression of WT Rab 5 (Figure 3.10, panels i-iii, a), 6 (panels vii-ix, c) and 7 (panels xiii-xv, e). However, expression of DN Rab5 (Figure 3.10, panels iv-vi, b) and 7 (Figure 3.10, panels xvi-xviii, f) caused a more reticular NS4B distribution. DN Rab6 (Figure 3.10, panels x-xii, d) expression caused a minor disruption in NS4B foci, suggesting that Rab 5 and 7 are involved in NS4B foci formation, with Rab6 also playing a lesser role. DN Rab5 or 7 expression partially disrupts the endocytic pathway, so we next tested whether endocytic recycling is involved in NS4B foci formation by expression of DN Rab11. For consistency, DN Rab1 and 2 were also tested for effects on NS4B foci. Figure 3.10B shows that all of these DN Rabs have no significant effect on NS4B foci formation (panels i-xviii, a-f), showing that Rabs in the secretory and recycling endosome (RE) compartments have little effect on NS4B foci formation.

**Rab2, 5 or 7 siRNA Silencing Decreases HCV RNA Synthesis**

Our lab and others have recently shown that Rab5 and 7 are required for HCV genome replication (10, 121). To confirm this and test the effect of the secretory pathway in HCV replication, siRNAs to Rab2, 5A and 7 were used to silence expression of these Rabs in C5B replicon cells. Expression of each of these siRNAs decreased HCV RNA levels by 50-60% (Figure 3.11A). Each siRNA was effective in reducing its target
Rab protein levels (Figure 3.11B) without nonspecific effects on levels of other Rab proteins or cellular GAPDH (Figure 3.11C). Therefore, Rab2, 5 and 7 play a role in HCV genome replication.

**Differences in Rab-NS4B Association: Immunoisolation Versus Fluorescence**

There are several reasons why we chose to investigate the role of cellular Rabs in HCV RC formation and replication. Rabs are central regulators of membrane traffic in the cell that control vesicle transport, docking and membrane fusion in the cell (22, 48). Positive strand RNA viruses, with their characteristic membrane associated RNA RCs, would possibly be able to manipulate Rabs to facilitate viral RC formation. By association with Rabs, viral proteins could initiate membrane rearrangement by sequestering Rabs, the regulators of vesicle traffic. It is also important to remember that Rabs are associated with specific intracellular membranes, and therefore can be used as a marker to identify the sources of the intracellular membranes that comprise the HCV RC. NS4B foci are visualized by transmission electron microscopy as a cluster of vesicles which each have a diameter of roughly 200 nm (3). These vesicles are much larger than cellular COPII coated vesicles, which have a diameter of roughly 87 nm (94, 118). It is possible that NS4B is recruiting cellular Rabs in order to initiate fusion of ER-derived vesicles into the membranous web. In fact, the size of membranous web vesicles more resembles EE vesicles that have a diameter of 100-250 nm (24), which supports the theory that NS4B foci are derived from multiple membrane sources. Combined with the previous finding in our lab that Rab5 is involved in HCV replication and co-localizes with NS4B foci, we attempted to identify cellular Rabs that are implicated in the formation of the HCV RC (121). Immunoblot of an isolated subcellular fraction from
C5B replicon cells that is competent for in vitro RNA synthesis showed the presence of various cellular Rabs, such as Rab1, 2, 4, 5, 6 and 7. However, fluorescence microscopy examination of C5B replicon cells shows that only the endocytic Rab5 and Rab7 are strongly associated with NS4B foci. This finding could indicate that some Rabs were nonspecifically isolated with the NS4B positive Bound fraction. Rab5 and 7 were also shown by FM to co-localize with each other in NS4B foci. This suggests that NS4B interacts with both Rab5 and 7, or that NS4B foci contain membrane fusion proteins from the EE and LE/LYS compartments that would require Rab5 and 7 for their regulation. We also found that secretory Rabs do not seem to be required for Rab5 to co-localize with NS4B foci, suggesting that the Rab5-NS4B association occurs either directly on ER membranes, or by an alternate membrane trafficking pathway. Dominant negative (DN) Rab mutants that partially inhibit EE membrane fusion (DN Rab5), LE to LSY membrane fusion (DN Rab7), or LE to Golgi membrane fusion (DN Rab6) cause a disruption in the pattern of NS4B foci staining. This implicates these membrane trafficking pathways in the NS4B-mediated formation of the HCV RC membranous webs. Also, siRNA silencing of Rab2, 5 or 7 results in decreased HCV RNA replication levels. This would presumably be due to an inhibition of HCV RC formation after silencing of Rab protein expression. However, siRNA silencing of single Rabs does not cause a severe decrease in HCV RNA replication, again confirming the notion that multiple Rabs are required for NS4B foci formation. There was one endocytic Rab protein, the recycling endosome (RE) Rab11, which did not associate with NS4B foci by immunofluorescence. This could be due to specific signals in Rab5 and Rab7 or their respective effector proteins that are required for incorporation into NS4B foci. Conversely, the NS4B-Rab5 or 7
interactions could be occurring in membrane trafficking pathways that circumvent the RE compartment.

**Possible Mechanisms of HCV Replication Complex Formation Via NS4B-Rab Association**

New publications during this study showed that Rab5 and 7 are needed for HCV genome replication (10, 125). This can possibly be explained by our data that Rab5 and 7 play a role in NS4B foci formation, structures considered to be the site of the HCV RC. These Rabs are localized to the EE and LE/LYS compartments of the cell and are not normally found in the ER compartment after they have been synthesized and transported to their target membranes. As the ER is the site of NS4B protein synthesis, it could be where NS4B-mediated membranous web formation is initiated. Therefore, we proposed the following model to postulate two possible mechanisms by which these endocytic Rabs are included into NS4B foci (Figure 3.12). In the first scenario, NS4B-containing secretory vesicles are transported to the plasma membrane of the cell and retrieved back into the cytosol via the endocytic pathway, thereby recruiting Rab5 and 7 into NS4B-positive vesicles. The second scenario entails Rab5 and 7 being recruited into NS4B foci on the ER membrane during protein synthesis before Rab5 and 7 are transported to their destinations in endocytic compartment. This second hypothesis is more consistent with the data, as partial inhibition of ER to Golgi membrane traffic by DN Rab1 or 2 did not affect NS4B foci formation or their co-localization with Rab5. These DN Rabs would presumably inhibit NS4B-positive vesicles from trafficking to the plasma membrane. However, the secretory pathway may still play a role in NS4B foci formation, as disrupting LE to Golgi or intra-Golgi traffic by DN Rab6 disrupts NS4B foci staining.
pattern. There is the possibility that preformed NS4B foci found in C5B replicon cells are less sensitive to DN Rab expression by transfection, and would therefore complicate the data analysis. Knockdown of the secretory Rab2 by siRNA also reduces HCV genome replication, but it is possible that this reduction is due to a disruption in the trafficking of Rab5 and 7 to their target membranes. It is then possible that both models are partially correct, especially in light of a recent study that showed there are two different types of HCV RC structures. The smaller HCV RC structure is more motile and microtubule-associated, and the larger HCV RC structures are more static (71, 137), implying differential associations with membrane trafficking regulators.
Figure 3.1 HCV Replication Complex Immunoisolation Protocol. A. NS4B mediated RC immunoisolation is comprised of three main steps, including subcellular fractionation of C5B replicon lysate with primary NS4B antibody added, membrane floatation of two RC-enriched fractions, and isolation via secondary antibody-coated magnetic Dynabeads. B. Schematic of the full-length C5B replicon RNA construct that was used in this study.
Figure 3.2 The Isolated HCV RC contains RNA and is competent for RNA synthesis. A. RNA was extracted from the Bound and Unbound fractions and the relative amount of RNA was determined by real time PCR, and normalized to cellular GAPDH mRNA levels. B. RdRp activity was tested using samples from the various steps of the isolation protocol. *In vitro* transcribed C5B and NS5A-GFP replicon RNA was included as markers (lanes 7-8). Notice the RdRp activity in the crude lysate, floated membranes fraction and Bound fraction (lanes 2-4).
Figure 3.2 The Isolated HCV RC contains RNA and is competent for RNA synthesis. C. Crude lysates (lanes 1 and 2) and the Bound fraction were tested for RdRp activity without addition of rNTP’s (lane 3) or in the presence of the RNA pol II inhibitor alpha-amanitin (lane 4). D. Crude lysates (lanes 1 and 2) and the Bound fraction (lane 3) was tested for RdRp activity after pre-treatment with TX-100 (lane 4), proteinase K (lane 5), S7 micrococcal nuclease (lane 8) and RNase A (lane 9). Lane 6 displays RdRp activity in the absences of the RNase inhibitor RNasin, and lane 7 shows RdRp activity by the Bound fraction when the S7 micrococcal nuclease was not inactivated after pre-treatment. Notice the large decrease in RdRp activity after TX-100 treatment, consistent with the RC being a membrane-associated structure.
Figure 3.3 HCV-specific inhibitors decrease RdRp activity in the Bound fraction. A. C5B replicon cells were treated with increasing amounts of the NS3 protease inhibitor BILN 2061 (dissolved in DMSO) for 48hrs prior to RC isolation. Notice the dose-dependent decrease in RdRp activity in the Bound fraction. B. C5B replicon cells were treated with increasing amounts of the NS5B RdRp targeted chain terminator 2’-C-methyl-adenosine (2’ CMA) for 48hrs prior to RC isolation. Again notice the dose-dependent decrease in RdRp activity in the Bound fraction. C. The RdRp reaction had the indicated amounts of the in vitro chain terminator 3’ deoxy-GTP (3’ dGTP) added, and displays a dose-dependent decrease in RNA synthesis compared to the untreated Bound fraction.
Figure 3.4 The Bound fraction contains cellular markers and HCV replication proteins. A. One microgram of protein from each isolation step was separated by SDS-PAGE and stained with SYPRO Ruby. The dots and arrowheads indicate differentially enriched proteins between Bound and Unbound fractions. Image provided by Teresa Killick. B. Fifty micrograms of protein were separated by SDS-PAGE followed by immunoblotting with antibodies directed to various cellular markers. C. Fifty micrograms of protein was separated by SDS-PAGE followed by immunoblotting with antibodies directed to various HCV proteins. SYPRO Ruby gel image courtesy of Teresa Killick.
Figure 3.5 The Bound fraction contains differentially enriched Rabs. A. Fifty micrograms of protein was separated by SDS-PAGE followed by immunoblotting with antibodies specific to various cellular Rabs. B. Quantitation of band densitometry values in the Bound fraction relative to C5B lysate. Notice Rab2, 6 and 7 are preferentially enriched in the Bound fraction.
Figure 3.6 Rab5 and 7 co-localizes with NS4B foci. A. Huh7 cells were transfected with N-terminal fluorophore tagged Rab constructs. At 24h post transfection cells were processed for confocal microscopy (panels i-v). Controls for CFP expression alone in Huh7 (vi) as well as NS4B immunostaining in Huh7 (vii) and C5B cells (viii) are included. Notice the diffuse CFP fluorescence when not fused to a Rab protein, and the typical pattern of NS4B foci staining in C5B cells without staining in parental Huh7 cells. Bars = 10 micrometers.
Figure 3.6 Rab5 and 7 co-localizes with NS4B foci. B. C5B replicon cells were transfected with CFP-Rab1 (i-iii, a), CFP-Rab2 (iv-vi, b), GFP-Rab5 (vii-ix, c), CFP-Rab6 (x-xii, d) and CFP-Rab7 (xiii-xv, e). At 24h post transfection the cells were immunostained with NS4B antibody and processed for confocal microscopy. White boxes indicate magnified areas (a-e), and notice that the CFP-Rabs were pseudo-colored green for imaging purposes. Bars = 10 micrometers.
Figure 3.6 Rab5 and 7 co-localizes with NS4B foci. C. Quantitation of Rab-NS4B co-localization in C5B cells using the Pearson Coefficient (PC) method. Significant co-localization is indicated by PC values of 0.5 or greater. Quantitations are from at least 5 cells that are positive for Rab and NS4B expression. Notice the PC values indicating strong co-localization of NS4B with Rab5 and Rab7.
Figure 3.7 Rab5 and 7 co-localizes in C5B cells. A. Huh7 cells were co-transfected with YFP-Rab5 and CFP-Rab1 (i-iii), CFP-Rab2 (iv-vi), CFP-Rab6 (vii-ix) or CFP-Rab7 (x-xii). At 24h post transfection the cells were processed for confocal microscopy. Note that CFP-Rabs are pseudo-colored as green and YFP-Rab5 is pseudo-colored as red for imaging purposes, and Rab5 does not significantly merge with other Rab proteins. Bars = 10 micrometers.
Figure 3.7 Rab5 and 7 co-localizes in C5B cells. B. C5B cells were co-transfected with YFP-Rab5 and CFP-Rab1 (i-iv, a), CFP-Rab2 (v-viii, b), CFP-Rab6 (ix-xii, c) or CFP-Rab7 (xiii-xvi, d). At 24h post transfection the cells were immunostained with NS4B antibody and processed for confocal microscopy. YFP-Rab5 is pseudo-colored as green for imaging purposes, and magnified areas (a-d) are indicated by white boxes. Merging of all three colors results in white fluorescence. Bars = 10 micrometers.
Figure 3.7 Rab5 and 7 co-localizes in C5B cells. C. Quantitation of Rab-Rab co-localization in C5B cells using the PC method. Significant co-localization is indicated by PC values of 0.5 or greater. Quantitations are from at least 5 cells that are positive for both Rabs and NS4B expression. Notice the PC values indicating strong co-localization of Rab5 with Rab7, seen as white fluorescence (B, xiii-xvi, d). There is weak merging of Rab5 and Rab1, 2 or 6 fluorescence.
Figure 3.8 Rab5 effectors are differentially associated with the HCV RC. A. Fifty micrograms of protein was separated by SDS-PAGE followed by immunoblotting with various antibodies specific to various Rab5 effectors. Notice that the various EE proteins display differential association with the isolated Bound fraction.
Figure 3.8 Rab5 effectors are differentially associated with the HCV RC. B. Huh7 and C5B cells were dual stained with NS4B and Rabex5 (i-vi) or NS5A and Clathrin (vii-xii) specific antibodies and processed for fluorescence microscopy. Notice that Rabex5 co-localizes with NS4B foci while it was not detected in the Bound fraction by immunoblot. Clathrin does not co-localize with the HCV RC and is also absent in the Bound fraction by immunoblot. Bars = 10 micrometers.
Figure 3.9 Dominant Negative Rab6 disrupts Rab5-NS4B association. C5B cells were co-transfected with WT YFP-Rab5 and DN CFP-Rab1 (i-iv, a), DN CFP-Rab2 (v-viii, b) or DN CFP-Rab6 (ix-xvi, c and d). At 24h post transfection the cells were immunostained with NS4B specific antibody and processed for confocal microscopy. WT YFP-Rab5 is pseudo-colored green for imaging purposes and magnified areas (a-d) are indicated by white boxes. Notice that there is less Rab5-NS4B merging with co-expression of DN Rab6, as indicated by the increased red NS4B fluorescence showing in the merged image. Bars = 10 micrometers.
Figure 3.10 Some Dominant Negative Rabs disrupt NS4B foci. A. C5B cells were transfected with WT or DN YFP-Rab5 (i-vi, a and b), WT or DN CFP-Rab6 (vii-xii, c and d) and WT or DN CFP-Rab7 (xiii-xviii, e and f). At 24h post transfection the cells were immunostained with NS4B specific antibody and processed for confocal microscopy. Rab fluorescence was pseudo-colored green. Notice that expression of DN Rab5, 7 and to a lesser extent 6 results in a disruption of typical NS4B foci staining pattern (b, d and f). Bars = 10 micrometers.
Figure 3.10 Some Dominant Negative Rabs disrupt NS4B foci. B. C5B cells were transfected with WT or DN CFP-Rab11 (i-vi, a and b), WT or DN CFP-Rab1 (vii-xii, c and d), and WT or DN CFP-Rab2 (xiii-xviii, e and f). At 24h post transfection the cells were immunostained with NS4B specific antibody and processed for confocal microscopy. Rab fluorescence was pseudo-colored green. Notice that expression of DN Rab 11, 1 or 2 does not affect the typical NS4B foci staining pattern (b, d and f). Bars = 10 micrometers.
Figure 3.11 Rab2, 5 or 7 siRNA silencing reduces HCV replication levels. A. C5B replicon cells were transected with siCONTROL, Rab2, 5 or 7 siRNA. At 48h post transfection total cellular RNA was extracted and used to detect the level of HCV RNA by qRT-PCR. HCV RNA levels were normalized to cellular GAPDH mRNA levels. Note that siRNA directed to Rab2, 5 or 7 results in a decrease in HCV RNA synthesis of about 50%. B. Immunoblot analysis with Rab2, 5 or 7 specific antibodies to verify the knockdown efficiency after siRNA transfection. Each siRNA greatly decreases its target Rab protein levels. C. Immunoblot analysis with Rab2, 5 or 7 specific antibodies to verify that the siRNAs did not cause off target effects. Note that the non-targeted Rab levels remain constant after siRNA transfection. GAPDH specific immunoblot was also performed to check for cytotoxic effects after siRNA transfection.
Figure 3.12 Model of endocytic Rab recruitment into NS4B foci. Model 1 depicts NS4B-bound vesicles being transported to the plasma membrane, which are then retrieved back into the cytosol via the endocytic pathway, where Rab5 and Rab7 are recruited into NS4B foci. In model 2, NS4B recruits Rab5 and Rab7 into NS4B foci when they are all present on the ER membrane during protein synthesis. The Rabs identified in the bound fraction by western blot and mass spectrometry, or co-localizing with NS4B foci in fluorescence microscopy are indicated.
Chapter 4

The NS4B C-Terminal Domain Participates in Virus Production

Recent evidence suggests that the CTD of NS4B protein participates in post-replication steps of the HCV life cycle. Mutation of the JFH1 NS4B amino acid N216A had no impact on replication while increasing virus production (62). Two basic residues, H217 and H250, have also been implicated in virus assembly and release (101). Our lab has also shown that swapping sequences of the NS4B CTD between genotype 2a (gt 2a) strain JFH1 and gt 1b strain Con1 (J/C1-C) led to a roughly 5-fold decrease in virus production (51). The mechanism by which NS4B participates in virus production has not been identified. Therefore, we hypothesized that chimeric NS4B CTD viruses could be employed to identify suppressor mutations that rescue virus production, and consequently highlight new potential NS4B interactions important for virus production. The genotype 1a strain H77 was used to make another NS4B CTD domain swap (J/H-C) in order to test the effect of a more drastic sequence change. The NS4B CTD amino acid sequences are highly conserved between genotype 1a, 1b and 2a viruses (Figure 4.1A). The few non-conserved amino acid residues between the three NS4B CTD sequences should then contribute to any differences in virus production. The chimeric NS4B CTDs caused no defect in RNA replication while decreasing infectious virus production. The defect in virus production seems to be at the step of virus assembly. Chimeric NS4B CTD viruses also displayed a marked decrease in NS5A stability and phosphorylation without affecting levels of another HCV RC protein, NS3. Serial passaging in cell culture led to the generation of suppressor mutations in the chimeric viruses that increased infectivity and NS5A protein levels. Interestingly, two of the suppressor mutations were found in
both the J/C1-C and J/H-C virus, highlighting their importance for infectious virus production.

**Chimeric NS4B C-Terminal Domain Viruses are Defective in Virus Assembly**

The chimeric NS4B CTD sequences (J/C1-C and J/H-C) were first expressed in a subgenomic luciferase (Luc) replicon system (Figure 4.1B) and JFH1 infectious virus system (Figure 4.1C) to test their effect on genome replication and virus production respectively. Efficiency of RNA replication was measured by Luc reporter gene expression in cell extracts at 4 hours (4 h), 2 days (D2), D4 and D6 post transfection (pt). At D2pt the chimera show a slight decrease in genome replication, but by D6pt have reached WT levels of replication (Figure 4.2A). To test virus production, chimeric viral genomes were transfected into Huh7.5 cells and infectious virus production (in FFU/ml) was determined at D3pt and D6pt. The genomes containing chimeric NS4B CTD sequences showed a 5- to 10-fold decrease in infectivity at both D3 and D6, with the J/H-C virus being the most defective (Figure 4.2B). This further suggests a role for the NS4B CTD in virus production while having only a slight kinetic effect on genome replication.

The defects in virus production for the chimeric viruses imply that either virus assembly or virus release could be altered relative to JFH1. If chimeric viruses were defective in assembly, a decrease in intracellular virus infectivity would be expected, whereas a defect in virus release would result in a buildup of intracellular virus relative to secreted virus. To address these two possibilities, chimeric genomes were transfected into Huh7.5 cells to measure titers of intracellular and extracellular virus at D3pt. Both the J/C1-C and J/H-C viruses showed much higher extracellular virus titers relative to intracellular, displaying that they are competent for virus release (Figure 4.3A). As
previously shown in Figure 4.2B, the chimeric virus extracellular titers were once again lower than JFH1. A large majority of the infectivity (roughly 95%) in all of the viruses was also found in the supernatant (Figure 4.3A). To test if the chimeric viruses display a visual defect in virus assembly, the association of Core, NS5A and lipid droplets (LD) was examined in JFH1 and mutant viruses. In normal HCV assembly, the NS5A and Core proteins can be found in a ring around the LD, which is believed to be the site of assembly (96). The J/H-C virus was compared to JFH1, as it had the most pronounced virus production defect. However, when visualized by fluorescence microscopy the J/H-C and JFH1 viruses showed no visible difference in NS5A-Core-LD staining pattern (Figure 4.3B, panels i-x). It is possible that the chimeric viruses are displaying a lower titer level than JFH1 if they are producing defective virus particles. In order to test this theory, secreted virus particles were pelleted on a sucrose cushion in order to determine the number of genomic RNA copies per focus forming unit (FFU). When comparing the specific infectivity (FFU/RNA copies) of JFH1 relative to J/C1-C and J/H-C virus, there is no significant decrease in specific infectivity for the chimeric viruses (Figure 4.3C). In fact, the J/H-C virus may be even more infectious relative to JFH1 once the virus particle is assembled, as there are fewer RNA copies found per FFU. This could suggest that the chimeric viruses are merely assembling viruses at a slower rate than JFH1 virus, or there is a defect in either particle release or particle infectivity.

**NS4B C-Terminal Domain Chimera Decrease Levels of NS5A Protein and Phosphorylation**

It is unclear how NS4B protein participates in HCV particle assembly. It is possible that NS4B directly chaperones HCV RNA from the replication complex to the
site of virus assembly. However, there is no clear evidence that NS4B is able to bind to positive sense HCV RNA. It is also possible that NS4B is involved in virus assembly by means of interactions with virus and host factors. NS4B does bind to NS5A protein, which is a HCV phosphoprotein involved in particle assembly (3, 32). The phosphorylation state of NS5A is linked to HCV production, with the hyperphosphorylated form (p58) of NS5A being required for HCV particle assembly (128). There is also evidence that the NS4B CTD regulates the formation of NS5A p58 (69). Therefore, we hypothesized that the HCV NS4B CTD chimeras have a deleterious effect on the ability of NS4B to mediate NS5A p58 formation. Huh7.5 cells were transfected with viral RNA from JFH1 and the CTD chimeras, and at D6pt cell extracts were collected for immunoblot analysis of NS5A protein. JFH1 transfection and virus spread results in high levels of both phosphorylated forms of NS5A (p56 and p58) in the cell extract (Figure 4.4A). Interestingly, the overall levels of NS5A protein and p58 form were lower in the CTD chimera constructs, especially in the J/H-C chimera (Figure 4.4A). Densitometry of the NS5A p56 and p58 band intensities suggests a decrease of around 3-fold in the NS5A p58 to p56 ratio in the chimeric viruses relative to JFH1 (Figure 4.4B). This could result from the NS4B CTD contributing to NS5A p58 formation, or possibly even NS5A stability. Attempts at immunoblotting NS4B proved difficult, with protein becoming detectable at late time points (D12pt) post electroporation. NS4B displays similar protein levels in the JFH1 and J/C1-C viruses, and lower levels in the J/H-C virus (Figure 4.4A). The NS4B antibody is polyclonal so it is possible that the chimeric NS4B sequences decreased antibody binding in the J/H-C mutant.
The differences in NS5A expression after viral genome electroporation could be due to the fact that JFH1 virus is growing to higher titers and spreading through the cell culture more rapidly than the CTD chimeric viruses (Figure 4.2B). This could cause higher HCV protein levels in general due to a larger percentage of the cells in the population being infected by HCV. However, NS3 levels at D6pt were similar between JFH1 and J/C1-C infected cells, and only slightly decreased in the J/H-C infected cells (Figure 4.4A). We then moved to testing the levels of NS5A and NS3 in cells expressing JFH1 and chimeric replicon RNA, as they display similar replication efficiency by D6pt (Figure 4.2A). In this manner, virus growth and spread through the cell culture can be removed from the interpretation, and we postulated that NS5A and NS3 levels would be similar to that of the viruses in all replicon constructs. This was the case, as shown by the similar levels of NS3 but decreased levels of NS5A in the same cells at D3 and D6pt (Figure 4.4C). Therefore, it seems that the NS4B CTD is able to mediate NS5A stability and phosphorylation state. Also, the very low NS5A levels at D3pt in the J/C1-C and J/H-C replicons (Figure 4.4C) suggest that HCV replication does not require high levels of NS5A protein.

**Cell Culture Adaptive Viruses Express High Levels of NS5A Protein**

In order to clarify the mechanism by which the chimeric viruses decrease virus production, cells electroporated with JFH1 or chimeric viral RNAs were serially passaged every three days in the hopes of generating viruses with adaptive mutations. Cells transfected with JFH1 RNA stop dividing by D12pt and die shortly thereafter, but the cells transfected with the chimeric CTD RNAs were passaged until at least D30pt (Figure 4.5A and B). The titer of the supernatants were tested for infectivity and the J/H-C virus
titer rose to JFH1 levels by D54pt (Figure 4.5A), while a second electroporation and passaging of the J/C1-C virus yielded titer levels similar to JFH1 by D30pt (Figure 4.5B). To confirm that these higher titer viruses were in fact revertant viruses, naïve Huh7.5 cells were infected at a multiplicity of infection (MOI) of 0.01 with J/C1-C D30pt (J/C1-C Ad1) and J/H-C D54pt (J/H-C Ad1) virus supernatants. As a control, Huh7.5 cells were infected with earlier timepoint parental virus supernatants that had not yet reverted. The JFH1 infected cells displayed virus titers of roughly 10- to 100-fold higher than the parental chimeric viruses at D9 post infection (pi) (Figure 4.5C). Infection of the cells with the Ad1 virus supernatants resulted in virus titers that were at least 10-fold higher than JFH1 at all timepoints tested post infection (Figure 4.5C). This confirms the Ad1 viruses’ phenotype of faster growth relative to JFH1 virus.

Since the parental chimeric viruses displayed lower levels of NS5A expression, we hypothesized that the Ad1 viruses were capable of producing higher NS5A levels in infected cells. Huh7.5 cells were infected at a MOI of 0.01, and at D9pi cell extracts were collected for NS5A specific immunoblotting. The extracts of cells infected with Ad1 viruses have much higher NS5A levels compared to JFH1-infected cell extracts (Figure 4.5D). In order to better visualize NS5A, twice the amount of total protein was loaded in the parental virus-infected cell extract lanes as compared to the Ad1-infected cell extracts. Therefore, the Ad1 viruses are revertant viruses that grow much more rapidly than JFH1 and are able to more stably express NS5A protein. Interestingly, the Ad1 virus infected cell extracts did not show a similar p58 to p56 NS5A ratio as seen in JFH1 infected cells (Figure 4.5D), implying that overall increased NS5A levels, not NS5A hyperphosphorylation, may be the cause of increased virus production.
Because the Ad1 viruses grew faster than JFH1, it is possible that they are more efficient in either virus assembly or virus release compared to JFH1. To test this, naïve Huh7.5 cells were infected at a MOI of 0.01 followed by titering of intracellular and extracellular virus at D9pi. Figure 4.5E shows that intracellular virus titers for both J/C1-C Ad1 and J/H-C Ad1 were roughly 1000-fold higher than JFH1. The ratio of secreted to intracellular virus was also similar to that seen in transfected cells (roughly 95% present in supernatant), implying that there is no change in the rate of virus release. This suggests that the Ad1 viruses are able to assemble virus particles more rapidly than JFH1.

**Suppressor Mutations Differentially Complement Virus Production**

In order to identify the mutations that were responsible for the increased growth kinetics of the Ad1 viruses, a cDNA pool was obtained from the total cellular RNA of Ad1 infected cells and used for sequencing. J/C1-C Ad1 contained four mutations, while J/H-C Ad1 contained two mutations. The two mutations identified in J/H-C Ad1 virus (NS4B N216S and NS5A C465S) were also found in J/C1-C Ad1 (Figure 4.6A). The remaining two mutations in J/C1-C Ad1 (p7 H31L and NS4B I236T) were specific to the J/C1-C Ad1 virus (Figure 4.6A). In order to determine if these mutations increase virus production by virtue of increasing RNA replication levels, the mutations were introduced singly or in combination into the subgenomic Luciferase J/C1-C replicon (Luc-J/C1-C). The single NS4B and NS5A mutations had little to no impact on HCV replication efficiency (Figure 4.6B), and the combined NS4B/NS5A mutations also had the same negligible effect (Figure 4.6C). The mutations resulted in replication levels very similar to that of the parental CTD chimera replicons, with an initial lag followed by levels reaching JFH1 levels by D6pt. The p7 mutation H31L was not included in this
investigation as the Luc replicons only contain the subgenomic NS3-NS5B HCV sequences, and p7 has not previously been implicated to participate in RNA replication.

Because the pseudoreversions had little impact on J/C1-C replicon RNA replication, we hypothesized that they would enhance virus production at a post-replication stage. The mutations were inserted singly or in combination into the parental chimeric viruses. The mutations had varying effects on J/C1-C virus production after transfection of Huh7.5 cells with the viral RNA. NS4B I236T had no effect on virus titer, but p7 H31L, NS5A C465S, and NS4B N216S single mutations all increased virus titer relative to J/C1-C by roughly 3-, 4- and 5-fold, respectively (Figure 4.7A). This implies that three of the mutations (H31L, N216S and C465S) are acting synergistically to increase virus production in the J/C1-C Ad1 virus. Figure 4.7B shows that a mutation-induced increase of J/C1-C virus titer correlates well to higher levels of NS5A protein and p58 hyperphosphorylation ratio in cell extracts at D6pt as detected by immunoblotting. The pseudoreversions had a similar effect in the J/H-C virus after testing viral titer with NS4B N216S and NS5A C465S present singly or in combination (Figure 4.7C and D), and the NS4B N216A mutation was included due to its previously reported effect in increasing virus production (62). The effects of the two common Ad1 mutations (N216S and C465S) were also tested by inserting them into the JFH1 genome. The NS4B mutations N216S/A both increase JFH1 titer by about 3-fold, but in the context of the J/H-C virus they show a much larger increase in titer, roughly 15-fold (Figure 4.7C). The single NS5A mutation C465S increased titers to a similar level in both JFH1 and J/H-C, being increased by about 6-fold and 8-fold respectively (Figure 4.7D). When the NS4B and NS5A mutations were combined in the J/H-C virus, titers
approached JFH1 levels (Figure 4.7D). As seen in the J/C1-C mutant viruses, the increase in virus titer seen in the J/H-C mutants correlates to higher levels of NS5A protein in cell extracts at D6pt (Figure 4.7E). This suggests that the NS4B N216S and NS5A C465S mutations are responsible for the increased J/H-C Ad1 virus production and NS5A protein levels.

Because the single mutant viruses display similar titer levels by D6pt, we hypothesized that if a particular mutation was more active in increasing virus production we would be able to detect a stronger effect on virus production after a low MOI infection, similar to that seen with the Ad1 viruses after infection (Figure 4.5C). This hypothesis is based on the fact that after electroporation of viral RNA the Huh7.5 cells have high amounts of genomic RNA copies inserted directly into the cytosol, which could mask more subtle differences in virus production. Indeed, after MOI 0.01 infection, the NS4B N216S and NS5A C465S mutant J/C1-C viruses spread more quickly through the Huh7.5 cell culture than the p7 H31L mutant J/C1-C virus (Figure 4.8A). A similar situation was seen with the NS4B and NS5A mutations increasing the growth of J/H-C virus (Figure 4.8B). Interestingly, the NS5A C465S mutation increased virus titer slightly more than in the NS4B N216S mutant viruses (Figure 4.8A and B). However, all of the chimeric viruses with single mutations did not display titer levels as high as JFH1. The lower titers of J/C1-C H31L did indicate that the p7 adaptive mutation is playing a lesser role in virus production compared to the NS4B N216S and NS5A C465S mutations.
**Some NS4B N216 Amino Acid Substitutions Result in Non-Viable Virus**

The above data regarding the NS4B N216 mutations confirm published reports that N216 is important in JFH virus production. Interestingly, both the J/C1-C Ad1 and J/H-C Ad1 viruses mutated this residue to N216S while producing higher levels of virus (Figure 4.5A and B). Also, N216A mutation in JFH1 has been shown to increase virus production in JFH1 and J/H-C virus (Figure 4.7C) (62). Therefore, we hypothesized that amino acid residues with similar characteristics would be able to replace N216 and result in infectious virus. Of the other N216 substitutions tested, only N216T resulted in viable virus at D3 and D6pt (Figure 4.9A). N216T could be able to accommodate a water molecule between the threonine side chain and any possible interacting protein, similar to how N216S/A would also be able to accommodate a water molecule to facilitate hydrogen bonding. N216E, F or R changes would exclude possible hydrogen bonding interactions mediated by a water molecule if their side chains, which extend farther from the alpha carbon position, protrude too far and block a potential interaction. As expected, the N216T substitution in J/H-C increases levels of NS5A protein relative to the increase in virus titer, but was lower than NS5A levels in the higher titer J/H-C N216S and N216A mutants (Figure 4.9B).

**Possible Roles for the NS4B C-terminal Domain in Virus Assembly, NS5A**

**Phosphorylation and NS5A Stability**

Previously, the functions of the NS4B CTD were confined to RC formation and RNA replication (3, 45). New data in the literature has suggested that the NS4B CTD also plays a role in virus production. Three amino acid substitutions in the JFH1 NS4B CTD (N216A, H217V and H250A) have been shown to increase virus production (62,
Preliminary data from our lab has also shown that swapping amino acid sequences of the genotype 2a JFH1 NS4B CTD with that from genotype 1b Con1 strain (J/C1-C) results in a decrease in virus production (51). A more drastic change in sequence was also considered when creating the J/H-C chimeric NS4B CTD construct, as there is less sequence conservation in the NS4B CTD between JFH1 (gt 2a) and H77 (gt 1a) than between JFH1 and Con1 (gt 1b).

The subgenomic luciferase replicon system showed that the CTD chimeras caused a slight decrease in genome replication at early time points (D2pt), but by D6pt they have reached levels comparable to that of JFH1. However, the CTD domain swaps resulted in a 5- to 10-fold decrease in virus infectivity at D3pt and D6pt. Therefore, the decrease in virus production was not due to a disruption in HCV genome replication. The ratio of intracellular infectivity relative to secreted supernatant infectivity was unaffected in the chimeric viruses, suggesting that virus release was not altered by the CTD domain swap. The mechanism by which NS4B participates in virus assembly remains unclear. One possibility would be through interaction of the NS4B CTD with viral and host factors that participate in virus assembly. One such protein could be NS5A, which is found in the HCV RC and also present at lipid droplets (LDs). This suggests a role for NS5A in connecting the HCV RNA RC to virus production, and interaction with NS4B could be how NS5A is anchored to the replication complex. The phosphorylation state of NS5A has also been linked to virus production, with the hyperphosphorylated p58 state being required for HCV particle assembly (128). NS4B could be binding to NS5A in order to position it properly for phosphorylation by the cellular kinases CKIα and CKII. On D6pt, the chimeric viruses displayed both a decrease in NS5A protein levels and p58
phosphorylation state relative to the JFH1 virus. However, levels of NS3 protein were similar between JFH1 and the chimeric viruses, suggesting that NS5A is displaying decreased stability. It is possible that the lower NS5A levels are representative of the lower virus production levels of the chimeric CTD viruses, which would spread more slowly through the cell culture and result in fewer infected cells in the population. However, NS5A levels and p58 phosphorylation levels in chimeric SGR-Luc replicons were also reduced relative to JFH1, yet again displayed similar levels of NS3 protein. Since RNA replication levels are similar in the replicons and virus spread is not involved, the assumption is that NS5A stability is reduced. This hypothesis must still be directly tested, as the preliminary evidence for decreased NS5A stability is only from immunoblot results. Metabolic labeling can directly assay for NS5A stability in the context of chimeric NS4B CTD sequences.

Suppressor mutations were sought in the J/C1-C and J/H-C viruses in order to shed light on potential mechanisms by which the NS4B CTD is participating in virus production. After electroporation of viral genomes and serial passaging, each chimeric virus adapted to cell culture and showed JFH1-like virus production levels. The adapted (Ad1) viruses were confirmed to be more infectious than the parental viruses after a low MOI infection. After sequencing of the Ad1 genomes, 4 and 2 suppressor mutations were found in the J/C1-C-Ad1 and J/H-C-Ad1 viruses, respectively. Interestingly, the 2 mutations that were identified in J/H-C-Ad1 virus (NS4B N216S and NS5A C465S) were also present in the J/C1-C-Ad1 virus. The NS4B mutation N216S is located in the chimeric CTD of the protein, and is the same residue previously published to increase virus production when mutated to N216A (62). Combined with Figure 4.9A and B,
which display that some N216 substitutions will support virus replication, a role for the NS4B residue N216 in protein-protein interaction seems likely. If N216 is involved in hydrogen bonding with a NS4B binding partner, then the A/S/T substitutions could allow for a water molecule to bridge the altered N216 interaction (Figure 4.9C). The E/F/R mutations at N216, which result in no virus production, would not be able to substitute hydrogen bonds at the same location as N216 can (Figure 4.9C). The N216 mutations that increase virus titer relative to parental virus could be allowing for more productive NS4B interactions at residue 216, and therefore, higher virus production levels (Figure 4.9A). Such a potential interaction could be between NS5A kinases and the NS4B CTD, resulting in increased NS5A phosphorylation. The mutations of NS4B N216S or T can also change putative NS4B phosphorylation motifs in the CTD based on computer predictions, and direct phosphorylation of the NS4B CTD is an interesting theory to test. The online phosphorylation motif search engines NetPhos2.0 and PhosphoMotif Finder both predicted that changing NS4B N216 to S or T results in a new potential phosphorylation target residue at S/T216. These new phosphorylation motifs are potential substrates of Casein Kinase I and Protein Kinase A, both of which are kinases of the NS5A protein (106). The NS5A mutation C465S is located in the C-terminal domain III of the protein that is required for virus production, and phosphorylation in this domain is necessary for virus assembly (5, 128). The change of C465 to a serine residue also raises the interesting possibility that the adaptive mutation is introducing a novel phosphorylation site in domin III of NS5A. Indeed, the same phosphorylation motif search engines used above predict a novel Casein Kinase I and II target motif in the NS5A C465S mutant protein, both of which are known to be NS5A kinases (106). The
J/C1-C-Ad1 virus also contained the p7 mutation H31L, displaying a genetic interaction between p7 and NS4B. When inserted back into the parental viruses, the suppressor mutations cause similar increases in virus titer after electroporation. However, after a low MOI infection using the single mutant viruses, the largest fold increases are seen the NS4B N216S and NS5A C465S mutations. The increase in virus titer after infection with the p7 H31L mutant was smaller compared to the NS4B and NS5A mutations. The increases in virus titer were not due to increased replication, as the mutations did little to alter replication levels in the SGR-Luc replicon system.

**Potential NS4B C-Terminal Domain Interactions with p7 and NS5A to Promote Virus Production**

The NS4B CTD is oriented toward the host cell’s cytosol, which suggests that it could be involved in biochemical interactions with viral and host factors. In fact, the NS4B and NS5A suppressor mutations that were identified to increase J/C1-C or J/H-C virus production (NS4B N216S, NS5A C465S) are also located in the cytosol of the cell, while according to topology predictions, the p7 mutation H31L is the last amino acid in a transmembrane domain adjacent to the cytosol (Figure 4.6A) (23). Because of their common cytosolic localizations, the genetic interactions between NS4B and the other HCV proteins suggest two possible scenarios. First, the NS4B CTD could be directly binding to these cytosolic portions of p7 and NS5A in order to facilitate virus assembly. Secondly, the NS4B CTD could be binding to other viral or host factors that act as a bridge between NS4B and p7 or NS5A. These putative interactions could allow NS4B to link the HCV RC to the virus assembly machinery. This is possible because the HCV RC is known to be located in close proximity to the LD, the site of HCV assembly (96).
The effect of chimeric NS4B CTDs on NS5A phosphorylation state is also potentially important for virus production as the p58 form of NS5A is required for virus production (128). We have shown that the chimeric viruses with decreased virus production levels correspond to lower overall NS5A levels and lower relative levels of p58 hyperphosphorylation. Combining this with the facts that NS5A is a HCV RNA binding protein and NS5A acts as a bridge between the HCV RC and LD virus assembly sites an interesting possibility arises (54, 96). Figure 4.10 describes a possible model of how the NS4B CTD may be mediating NS5A phosphorylation and stability. Initially, NS4B and NS5A are associated in the context of the HCV RC where viral RNA is being amplified. At this stage, there may be few NS5A kinases present and could allow for NS5A to be susceptible for degradation by NS5A specific proteases, such as Caspases 3, 6 and Calpains (Figure 4.10A) (63, 64). The effect on HCV replication by these NS5A specific proteases remains unclear, but processing seems to cause a cytosolic localization of C-terminal NS5A cleavage products (63). The lower levels of HCV replication proteins at this early stage of replication could in turn recruit lower levels of NS5A kinases. This is based on the observation that only the basally phosphorylated p56 form of NS5A is required for RNA replication in the adaptive mutant subgenomic replicons (14). However, trans-acting low levels of p58 may be required for RNA replication in the JFH1 subgenomic replicon, and low levels of phosphorylation cannot be ruled out (41). As HCV RNA is amplified for packaging into virions, the NS4B CTD may then possibly recruit NS5A kinases into the RC, allowing for basal NS5A p56 phosphorylation (Figure 4.10B). This basal phosphorylation, especially of residues in domain III, is required for NS5A to associate with Core and LDs (Figure 4.10B). This change in NS4B
function could be due to increased levels of NS4B in the cell and in functioning replication complexes. NS4B levels could accumulate in the RC after translation of the viral polyprotein elsewhere in the cell, as NS4B is able to trans-complement HCV replication (62). Next, as NS5A becomes hyperphosphorylated it may reduce the affinity between HCV RNA and NS5A, allowing the HCV RNA to be more readily released from NS5A for encapsidation by Core (Figure 4.10C). NS5A hyperphosphorylation would increase the overall negative charge of the NS5A protein, which could in turn reduce its affinity for the negatively charged sugar-phosphate backbone of the HCV genomic RNA. In this manner, NS4B protein is putatively affecting both p56 and p58 NS5A formation, and regulates NS5A function as needed for replication or virus production. This is an intriguing hypothesis to test, and future studies could be aimed at uncovering more information on the mechanism of how the NS4B CTD mediates NS5A stability and phosphorylation.
**Figure 4.1 Chimeric NS4B CTD Constructs.** A. Amino acid sequence alignment of the NS4B CTD between genotype 2a JFH1 sequence with genotype 1b Con1 and genotype 1a H77 virus sequences. Conserved residues are highlighted by stars, and differences are highlighted by red text. The position of the two alpha helices located in the NS4B CTD are highlighted by lines above the sequences. B. Schematic of the chimeric viral genomes used in this study. The amino acids of the NS4B CTD from JFH1 was replaced by that of either Con1 or H77. C. Subgenomic luciferase replicon constructs used in this study.
Figure 4.2 J/C1-C and J/H-C chimeric viruses display decreased virus production. A. Ten micrograms of JFH1 or chimeric Luc replicon RNA was electroporated into Huh7.5 cells, and HCV replication was measured by Luc reporter activity in cell extracts at 4 h, 2d, 4d and 6dpt. Results are representative of at least 2 independent experiments with triplicate samples. The values are calculated as the fold increase in Luc activity normalized to 4 h input and the GND replication defective replicon. B. One microgram of JFH1 or chimeric viral RNA was electroporated into Huh7.5 cells and cell supernatant was collected at 3 and 6dpt. Virus titer was measured with the limiting dilution assay, and expressed as FFU/ml. Results are representative of at least 3 independent experiments with triplicate samples.
Figure 4.3 Chimeric CTD viruses have no visible defect in virus assembly. A. One microgram of JFH1 or chimeric viral RNA was electroporated into Huh7.5 cells, and at 3dpt cell supernatants and freeze/thaw prepared intracellular virus were collected. Infectious virus titers were expressed as total FFU due to the different volumes collected for the supernatant and intracellular viruses. Notice that roughly 95% of the total virus is found in the supernatant for all of the viruses. Results are representative of 5 independent experiments with triplicate samples. B. Huh7.5 cells were transfected with JFH1 or J/H-C viral RNA. At 2dpt the cells were immunostained with antibodies specific to Core (green) and NS5A (red), while lipid droplets (blue) were stained with LipidTox stain. Magnified areas are indicated with white boxes. Notice that core and NS5A strongly co-localize in both viruses (yellow color, iii and viii), and they form a ring around lipid droplets (v and x) that is a characteristic pattern of HCV assembly sites. Fluorescence image courtesy of Qingxia Han.
Figure 4.3 Chimeric CTD viruses are defective in virus assembly. C. One microgram of JFH1 or chimeric viral RNA was electroporated into Huh7.5 cells, and cell supernatants were collected at 3dpt. The virus titer was determined as total FFU using the limiting dilution assay. The virus in the remaining supernatant was then pelleted on a sucrose cushion, and the viral pellet was used to extract viral RNA. The total number of HCV RNA copies was then determined using qRT-PCR, and the specific infectivity was calculated as FFU/RNA copies. The specific infectivity of JFH1 was set as 1, and the chimeric virus specific infectivity was displayed compared to that of JFH1. Notice that the J/C1-C and J/H-C viruses do not show a large defect relative to JFH1.
Figure 4.4 Chimeric viruses display lower NS5A protein levels and phosphorylation.
A. One microgram of JFH1 or chimeric viral RNA was electroporated into Huh7.5 cells and cell extracts were collected at d6pt. 150 micrograms of protein was separated by SDS-PAGE followed by immunoblotting with NS5A, NS3, NS4B or GAPDH specific antibody. The NS4B immunoblot was performed with d12pt lysates due to difficulty in detection.
B. The ratio of hyperphosphorylated NS5A (p58) to basally phosphorylated NS5A (p56) was quantified from the average of 3 independent experiments using ImageQuant software.
Figure 4.4 Chimeric viruses display lower NS5A protein levels and phosphorylation.

C. Ten micrograms of JFH1 or chimeric Luc replicon RNA was electroporated into Huh7.5 cells. At d3 and d6pt cell extracts were collected and 150 micrograms of total protein was separated by SDS-PAGE followed by immunoblotting with NS5A, NS3 or GAPDH specific antibody. Notice that at d3pt the chimeric replicons show greatly reduced NS5A levels while having levels of NS3 that are comparable to that of JFH1.
Figure 4.5 Cell culture adapted chimeric viruses display increased NS5A levels. A. One microgram of JFH1 or chimeric viral RNA was electroporated into Huh7.5 cells followed by collection of cell supernatant and serial passage of infected cells every 3 days. Notice the J/H-C virus displays a large increase in titer level between days 30 and 36. B. One microgram of JFH1 or chimeric viral RNA was electroporated into Huh7.5 cells followed by collection of cell supernatant and serial passage of infected cells every 3 days. Notice the J/C1-C virus displays a large increase in titer level between days 21 and 24.
Figure 4.5 Cell culture adapted chimeric viruses display increased NS5A levels. C. Parental and cell culture adapted viruses were used to infect naïve Huh7.5 cells at a multiplicity of infection of 0.01 followed by titration of cell supernatants a 3d, 6d, 9d and 12dpi. Titers are displayed as FFU/ml, and results are representative of at least two independent experiments with triplicate samples. D. Cell extracts were collected at d9pi and 150 micrograms (parental viruses) or 75 micrograms (Ad1 viruses) of protein was separated by SDS-PAGE followed by immunoblotting with NS5A or GAPDH specific antibody. Notice that even though the adaptive virus infected cell extracts had half the amount of total protein loaded in the gel, they display much higher NS5A expression levels.
Figure 4.5 Cell culture adapted chimeric viruses display increased NS5A levels. E. Parental and cell culture adapted viruses were used to infect Huh7.5 cells at a multiplicity of infection of 0.01 followed by titration of cell supernatant and freeze/thaw prepared intracellular virus samples and d9pi. Titers are expressed as total FFU due to the different volumes of virus collected for the supernatant and intracellular viruses. Notice that the Ad1 viruses have roughly 95% of the total virus present in the supernatant.
Figure 4.6 Suppressor mutations have minimal effect on RNA replication. A. The suppressor mutations found in the cell culture adaptive viruses are located in the cytosol of the host cell (NS4B and NS5A mutations), or in the last residue of a transmembrane domain adjacent to the cytosol of the host cell (p7 mutation). Notice that the cytosolic localization for the suppressor mutations could indicate a potential biochemical interaction between the NS4B CTD and NS5A or p7.
Figure 4.6 Suppressor mutations have minimal effect on RNA replication. B. Ten micrograms of JFH1 or chimeric Luc replicon RNA with single mutations was electroporated into Huh7.5 cells, and HCV replication was measured by Luc reporter activity in cell extracts at 4 h, 2d, 4d and 6dpt. Results are representative of at least 2 independent experiments with triplicate samples. The values are calculated as the fold increase in Luc activity normalized to 4 h input and the GND replication defective replicon. C. Ten micrograms of JFH1 or chimeric Luc replicon RNA with combined NS4B and NS5A mutations was electroporated into Huh7.5 cells and Luc reporter activity was measured as in performed in B.
Figure 4.7 Suppressor mutations increase chimeric virus titer and NS5A levels. A. One microgram of JFH1 and mutant J/C1-C genomic RNA was electroporated into Huh7.5 cells, and the cell culture supernatant infectivity was titrated at 3d and 6dpt. B. Cell extracts were prepared at 6dpt and 150 micrograms of total protein was separated on SDS-PAGE followed by immunoblotting with NS5A and GAPDH-specific antibodies. Notice that mutant viruses with increased titer relative to parental J/C1-C virus also display increased NS5A levels.
Figure 4.7 Suppressor mutations increase chimeric virus titer and NS5A levels. C. One microgram of JFH1 and J/H-C genomic RNA containing NS4B N216 mutations was electroporated into Huh7.5 cells, and the cell culture supernatant infectivity was titrated at 3d and 6dpt. D. One microgram of JFH1 and J/H-C genomic RNA containing NS5A or NS4B + NS5A mutations was electroporated into Huh7.5 cells, and the cell culture supernatant infectivity was titrated at 3d and 6dpt. Notice that in both JFH1 and J/H-C the suppressor mutations increase virus titer. Titer data courtesy of Qingxia Han.
Figure 4.7 Suppressors mutations increase chimeric virus titer and NS5A levels. E. Cell extracts were prepared at 6dpt and 150 micrograms of total protein was separated on SDS-PAGE followed by immunoblotting with NS5A and GAPDH-specific antibodies. Notice that mutant viruses with increased titer relative to parental J/H-C virus also display increased NS5A levels.
Figure 4.8 Chimeric viruses containing single suppressor mutations are more infectious than parental viruses. A. Parental and J/C1-C single mutant viruses were used to infect naïve Huh7.5 cells at a multiplicity of infection of 0.01 followed by titration of cell supernatants a 3d, 6d, 9d and 12dpi. B. Parental and J/H-C single mutant viruses were used to infect naïve Huh7.5 cells at a multiplicity of infection of 0.01 followed by titration of cell supernatants a 3d, 6d, 9d and 12dpi. Titers are displayed as FFU/ml, and results are representative of at least two independent experiments with triplicate samples.
Figure 4.9 Some NS4B N216 mutations result in non-viable virus.
A. One microgram of JFH1 or NS4B N216 mutant J/H-C genomic RNA was electroporated into Huh7.5 cells, and the cell culture supernatant infectivity was titrated at 3d and 6dpt. B. Cell extracts were prepared at 6dpt and 150 micrograms of total protein was separated on SDS-PAGE followed by immunoblotting with NS5A and GAPDH-specific antibodies. Notice that mutant viruses with increased titer relative to parental J/H-C virus also display increased NS5A levels.
Figure 4.9 Some NS4B N216 mutations result in non-viable virus.
C. Model of accessibility of N216 mutation amino acid side chains to potential interacting factor X. Normally, N216 may be engaged in hydrogen bonding (dashed red line) with a NS4B interacting factor “X” (Yellow box). The various mutations at N216 that support virus replication (Top row: N216S, A or T) are of sufficient size to potentially accommodate water molecules between them and the binding partner of residue 216. The replication deficient mutants (N216E, F or R) provide steric obstacles to binding or are not capable of hydrogen binding.
Figure 4.10 Model of NS4B CTD mediating NS5A phosphorylation and stability. A. NS4B and NS5A interact in the HCV replication complex, however the lack of NS5A phosphorylation may make NS5A accessible to proteases. B. When sufficient RNA genomes are replicated the NS4B CTD may recruit NS5A kinases, allowing basal p56 phosphorylation to occur. This phosphorylation in NS5A domain III then allows interaction with Core and the Lipid Droplet (LD), which are required for virus assembly.
Figure 4.10 Model of NS4B CTD mediating NS5A phosphorylation and stability. C. The NS4B CTD continues to recruit and mediate NS5A hyperphosphorylation into the p58 form, which allows NS5A to perform other functions related to host cell modulation. The higher levels of negative charge on NS5A p58 due to hyperphosphorylation may also aid in the dissociation of HCV RNA from NS5A, facilitating virus encapsidation.
Chapter 5 - Conclusions and Discussion

Significance to the Field

Despite the intensive research on NS4B during recent years, many of the exact roles of NS4B in the HCV lifecycle remain poorly understood. NS4B was previously shown to be required for membranous web formation and HCV genome replication, and only was very recently implicated in virus production. In the case of HCV RC formation, the early endosomal (EE) regulator Rab5 and late endosomal (LE) regulator Rab7 were both shown to be required for HCV genome replication by siRNA knockdowns (10, 121). We hypothesized that this requirement was due to the ability of Rab5 and Rab7 to mediate membrane fusion, which could allow NS4B to regulate membranous web formation by virtue of recruiting these factors to the site of the HCV RC. We tackled this question by using two main techniques, the first being NS4B specific immunoisolation of a subcellular fraction that was enriched in HCV replication machinery, and the second being immunofluorescence microscopy to visualize NS4B-Rab co-localization in the cell.

The theory that the membranous web contained EE factors important for formation of the HCV RC was controversial at the onset of this study. An initial publication from our lab demonstrated co-localization of Rab4 or Rab5 with NS4B foci in replicon cells, and that Rab5 binds to NS4B (121). Published data from this investigation regarding NS4B-Rab association, as well as other reports documenting the requirement of Rab5, Rab7 and the cellular autophagy machinery for HCV replication has strengthened this hypothesis (10, 34, 49, 88, 122).

The investigation of the role of the NS4B CTD in virus production had a different purpose. During this study, three publications documented a role for the NS4B CTD in
virus production (51, 62, 101). Therefore, our focus was on beginning to uncover mechanisms by which NS4B is participating in virus production. We hypothesized that creating chimeric NS4B CTD viruses and allowing them to accumulate suppressor mutations during serial passage in cell culture would give us clues as to the types of interactions that may be important for NS4B-mediated virus production. This study uncovered a genetic interaction between NS4B and p7 in virus production, which has not been previously documented. As p7 is known to have a role in virus production, this highlighted an interesting link between NS4B and virus assembly. Also, chimeric NS4B CTD sequences had an antagonistic effect on NS5A protein levels and NS5A p58 hyperphosphorylation. This is another potential mechanism of how NS4B may be mediating virus production through regulation of NS5A protein.

**Future Directions- NS4B and Endocytic Rabs**

There are many interesting possibilities for future studies on the participation of endosomal Rabs on HCV RC formation and function. Our lab has previously identified binding between NS4B and the early endosomal (EE) Rab5 (121). It would be interesting to test if the late endosomal regulator (LE) Rab7 binds to NS4B. The similarities between Rab5 and Rab7 in NS4B foci co-localization, as well as their requirements for HCV genome replication would suggest that Rab7 can also bind to NS4B. Next, detailing the Rab domains required for association with NS4B foci or NS4B binding could be mapped. Rabs contain multiple structural motifs that participate in their targeting to membranes, including Rab Family (RabF) and Rab Subfamily (RabSF) domains (1, 2). These motifs are spread throughout the Rab protein sequences and act in concert to target Rabs to their cellular membranes. Chimeric Rab constructs
could be created in order to narrow down regions of Rab5 or Rab7 that are required for co-localization with NS4B foci. For instance, this would entail making a chimeric Rab protein that contained the N-terminal region of Rab5 and the C-terminal region of Rab2, and vice versa. This way, half of the chimeric Rab would originate from a Rab that co-localizes with NS4B foci and the other half would be from a Rab that does not co-localize with NS4B foci. Checking the NS4B foci co-localization pattern of the chimeric Rabs could indicate a possible region in Rab proteins to which NS4B is binding. Another avenue of investigation would be to test if NS4B can also bind to Rab5 effectors of vesicle fusion. Some Rab5 effectors that are important vesicle fusion machinery components are found in the Bound fraction, such as the vesicle tethering factor EEA1 and the t-SNARE syntaxin-13 (Figure 3.8A). Our lab has previously shown that EEA1 co-localizes with NS4B foci, and others have demonstrated that siRNA knockdown of EEA1 decreases HCV replication, highlighting its importance in HCV RC formation (10, 121). It would also be interesting to test if the Rab5 effector Rabex-5 binds to NS4B, since it does co-localize with NS4B foci but did not associate with the isolated HCV RC fraction by western blot. We also could test if expression of DN Rabs that disrupt NS4B foci pattern results in a decrease in HCV replication levels. We only used a siRNA knockdown approach to test HCV replication effects, but one would expect that a DN Rab that disrupts NS4B foci would also decrease RNA replication. Also, the single Rab siRNA treatment resulted in a roughly 50% decrease in HCV RNA levels. Combinations of the Rab siRNAs could be used to test for a more drastic reduction in HCV replication. Together, these potential assays could shed more light on the roles that endosomal Rabs are playing in HCV RC formation.
Future Directions- NS4B CTD and Virus Production

The main focus of future assays with the NS4B CTD and virus production will be to further characterize the phosphorylation and stability effects on NS5A. So far, the apparent decrease in NS5A phosphorylation and stability has only been shown by immunoblotting. NS5A stability in the context of J/C1-C or J/H-C virus genomes can be directly measured by pulse-chase analysis with $^{35}$S metabolic labeling. This can be performed with electroporation of the chimeric virus genomes or by plasmid transfection of an NS3-NS5A construct. This would be a direct measure of NS5A protein stability in the context of chimeric NS4B CTD sequences. Whether using the viral genomes or subgenomic sequences it is imperative that the NS3-NS5A sequences are present, as NS3-NS4B sequences have been previously shown to mediate NS5A phosphorylation (69). Testing the direct amount of NS5A phosphorylation can also be achieved with $^{32}$P orthophosphate metabolic labeling. After transfection of chimeric genomes and labeling, NS5A protein can be immunoprecipitated (IP) and used for scintillation counting for a measure of phosphate labeling. The orthophosphate labeled NS5A IP fraction can also be separated by SDS PAGE and potentially used to visualize p56 or p58 formation with a phosphoimager.
References

lines for subgenomic and genomic hepatitis C virus RNA replication. J Virol
76:13001-14.

requirements for hepatitis C virus (HCV) RNA synthesis in the HCV replicon cell

mediates membrane association of the hepatitis C virus nonstructural protein 5A.

19. **Bucci, C., A. Lutcke, O. Steele-Mortimer, V. M. Olkkonen, P. Dupree, M.

Govindarajan, M. Shapiro, M. St Claire, and R. Bartenschlager.** 2002.
Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells
prevent productive replication in chimpanzees. Proc Natl Acad Sci U S A

virus generated in cell culture is fully viable in vivo. Proc Natl Acad Sci U S A
103:3500-1.

22. **Cai, H., K. Reinisch, and S. Ferro-Novick.** 2007. Coats, tethers, Rabs, and
SNAREs work together to mediate the intracellular destination of a transport

23. **Carrere-Kremer, S., C. Montpellier-Pala, L. Cocquerel, C. Wochowski, F.
Penin, and J. Dubuisson.** 2002. Subcellular localization and topology of the p7

neuronal endocytosis and protease delivery to early endosomes in sporadic
Alzheimer's disease: neuropathologic evidence for a mechanism of increased

Membrane rearrangement and vesicle induction by recombinant poliovirus 2C

Bradley.** 1990. Hepatitis C virus: the major causative agent of viral non-A, non-B

Transmembrane domains of hepatitis C virus envelope glycoproteins: residues
involved in E1E2 heterodimerization and involvement of these domains in virus

28. **Clyde, K., J. L. Kyle, and E. Harris.** 2006. Recent advances in deciphering viral
and host determinants of dengue virus replication and pathogenesis. J Virol

29. **Cocquerel, L., C. Voisset, and J. Dubuisson.** 2006. Hepatitis C virus entry:
potential receptors and their biological functions. J Gen Virol 87:1075-84.


110


112. **Sakai, A., M. S. Claire, K. Faulk, S. Govindarajan, S. U. Emerson, R. H. Purcell, and J. Bukh.** 2003. The p7 polypeptide of hepatitis C virus is critical for
infectivity and contains functionally important genotype-specific sequences. Proc Natl Acad Sci U S A 100:11646-51.


VITA: David P. Manna

Education:

- **Ph.D., Biochemistry and Molecular Biology, Pennsylvania State University (2006-Present)**
  Research Advisor: Kouacou Konan
- **M.S., Biotechnology, University of Delaware (2004-2006)**
  Research Advisor: Daniel Simmons
  Thesis Title: “Mutational Analysis of the Central Channel in the Simian Virus 40 Large T Antigen Helicase”
- **B.S., Biotechnology (Cum Laude), University of Delaware (2000-2004)**
  Undergraduate Research Advisor: Daniel Simmons

Publications:


Conference Presentations

- American Society for Virology 2008 at Cornell University. “A Novel Hepatitis C Virus Replicase System With Robust RNA-Dependent RNA Polymerase Activity”. Workshop #45 RNA virus replication and gene expression” (15 min)
- American Society for Virology 2009 at the University of British Columbia. “Formation of a Competent Hepatitis C Virus Replication Complex Requires a Significant Contribution of Early Endosome-Associated Proteins”. Workshop #22 Positive strand RNA viruses and host protein interactions (15 min)
- American Society for Virology 2011 at the University of Minnesota. “Role of NS4B Protein in NS5A Hyperphosphorylation and Hepatitis C Virus Assembly”. Workshop #34 Virus Assembly Mechanisms (15 min)