UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of Phosphoinositides in the Secretion of Hepatitis C Virus

A dissertation submitted in partial satisfaction of the requirements for the degree
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in

Biology

by

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2013
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Chair

University of California, San Diego

2013
DEDICATION

To my parents, Kenneth and Dorice Bishé, and my sister Kerry, who have always believed that I could do whatever I set my mind to, and whose belief has helped convince me.
The Earth is just too small and fragile a basket for the human race to keep all its eggs in.

—Robert A. Heinlein
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ABSTRACT OF THE DISSERTATION

The Role of Phosphoinositides in the Secretion of Hepatitis C Virus

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Hepatitis C virus (HCV) RNA replicates within the ribonucleoprotein complex, assembled on the endoplasmic reticulum (ER)-derived membranous structures closely juxtaposed to the lipid droplets that facilitate the post-replicative events of virion assembly and maturation. It is widely believed that the assembled virions piggy-back onto the very low density lipoprotein particles for secretion. Lipid phosphoinositides are important modulators of intracellular trafficking. HCV-infected cells display a fragmented and dispersed Golgi pattern, implicating involvement in virion morphogenesis. Golgi-localized phosphatidylinositol 4-phosphate (PI4P) recruits proteins involved in Golgi trafficking to the Golgi membrane and promotes anterograde transport of secre-
tory proteins. Here, we sought to investigate the role of Golgi-localized PI4P in the HCV secretion process. Depletion of the Golgi-specific PI4P pools by Golgi-targeted PI4P phosphatase hSac1 K2A led to significant reduction in HCV secretion without affecting replication. Reducing PI4P levels via knockdown of PI4 kinase IIβ led to the same effect. We then examined the role of PI4P binding proteins GOLPH3, FAPP1 and FAPP2 in the viral secretion process. GOLPH3 is shown to maintain a tensile force on the Golgi, required for vesicle budding via its interaction with an unconventional myosin, MYO18A. Silencing GOLPH3 led to a dramatic reduction in HCV virion secretion, as did the silencing of MYO18A. The reduction in virion secretion was accompanied by a concomitant accumulation of intracellular virions, suggesting a stall in virion egress. These studies establish the role of PI4P and its interacting protein GOLPH3 in HCV secretion and strengthen the significance of the Golgi secretory pathway in this process.
Chapter 1

Introduction

1.1 History and Background of Hepatitis C

Hepatitis C virus (HCV) was first documented in the 1970s, identified as a causative agent of non-A, non-B hepatitis [1]. However, the precise nature of this newly discovered viral pathogen was not fully explained until 1989, when the “non-A, non-B viral hepatitis” later to be termed Hepatitis C virus, was first cloned and sequenced [2]. HCV is acquired almost exclusively via blood-to-blood contact, primarily through intravenous drug use [3, 4]. Blood transfusions performed prior to the ability to screen for HCV may also account for a significant fraction of infected individuals. Sexual transmission is rare [5].

Upon transmission, HCV RNA is usually detectable in the blood within seven to 21 days [6-9]. Hepatitis C is considered acute during the first six months of infection, after which it progresses to chronic HCV [10]. Some infected patients spontaneously clear the viral infection on their own, never progressing to chronic hepatitis, but this occurs in only a small percentage (~14-46%) of cases (Figure 1.1) [9]. Chronic Hepatitis C is often asymptomatic, and so most patients are unaware of their infection, which can
Figure 1.1: Natural Progression of Hepatitis C
The progression of Hepatitis C following infection with HCV, if untreated. Chronic Hepatitis C is often asymptomatic and can go undiagnosed for 30 years or more.
remain silent for over decades without the development of any overt symptoms. This is one of the main reasons that the U.S. Centers for Disease Control and Prevention (CDC) has recommended that all members of the “Baby Boom” generation get tested for HCV [11].

HCV infection is a looming silent pandemic with an estimated 160 to 170 million individuals, or 2-3% of the global population afflicted [12]. Approximately 70% of these infected individuals develop chronic hepatitis C which often progresses to fibrosis, cirrhosis and hepatocellular carcinoma [12]. HCV infection is the leading indication for liver transplantation in the United States [13]. Currently, seven distinct genotypes of HCV have been characterized throughout the world, often with their own geographic distribution, and are further categorized by subtypes [14, 15]. Genotype 1, for instance, has two primary subtypes, 1a and 1b. Subtype 1a is found primarily in North America and Europe, while 1b is distributed throughout the world [15]. The genotype of the virus is an important factor in determining viral progression and outcome, and also response to treatment [9]. Treatment options for Hepatitis C are limited. The current therapeutic regimen of pegylated interferon and ribavirin is often poorly tolerated, and less than fully effective against the HCV genotype 1 that is predominant in Northern America and Europe. Two inhibitors of viral protease NS3, telaprevir and boceprevir, have been recently approved and many more direct-acting antivirals are in various phases of development. Although direct-acting antivirals are very effective, the rapid emergence of resistant mutants can lead to recurrence of infection, underscoring the need for the development of treatment strategies that target host factors critical for viral lifecycle [16-19].
1.2 Molecular Biology and Lifecycle of HCV

HCV is a member of the Flaviviridae family of viruses, whose members possess a positive-stranded RNA genome. This genome, like those of other plus-stranded RNA viruses, is directly translated into the complete set of viral proteins [20]. The HCV genome is roughly 9.6 kb in length and encodes a ~3000 amino acid polyprotein (Figure 1.2, A). The entire viral genome is translated into a large polyprotein that is cleaved co- and post-translationally by cellular and viral proteases to form the viral proteome of three structural and seven non-structural proteins (Figure 1.2, B) [21-23]. The structural proteins are translated first and consist of the core and surface glycoproteins E1 and E2. The six major non-structural proteins, NS2, NS3, NS4A, NS4B, NS5A and NS5B perform a variety of cellular functions to ensure successful viral replication. NS2 and NS3 are two distinct viral proteases that co-translationally cleave the rest of the NS proteins [20-22]. NS2 is an autoprotease that cleaves the NS2/NS3 junction, while NS3 has a broad range of viral targets, including cleavage of the remaining NS proteins [20, 24]. A small membrane protein, p7, which is thought to act as an ion channel is encoded between the structural and non-structural proteins (Figure 1.2, B) [25].

The HCV lifecycle begins upon entry of an infectious HCV particle into the cell. This particle is likely associated with a large very low-density lipoprotein (VLDL), in the form of a lipoviral particle (LVP) [26]. The exact method of HCV entry is not fully known, but several cell surface proteins have been identified as important players in the process. The HCV E2 envelope glycoprotein is crucial for recognition and attachment at the cell surface. Potential binding partners of E2 include the low-density lipoprotein
Figure 1.2: Diagram of HCV Proteins.
Diagram of the HCV genome (A) and proteome (B). Structural proteins core, E1 and E2 are in green. Non-structural proteins NS2, NS3, NS4A, NS4B, NS5A, NS5B are in green. p7 is a small membrane protein located between the other structural and the non-structural proteins.
receptor (LDLR), glycosaminoglycans, or CD81, a tetraspanin [16, 27-30]. Also possibly playing a role are scavenger receptor B1, claudin-1 and occludin [31-37]. After attaching to the cell, the virus is thought to enter via clathrin-mediated endocytosis [38]. The viral capsid uncoats via a pH-mediated transition inside the cell, and the exposed RNA travels to ribosomes on the ER, where the viral RNA acts as a functional mRNA and is translated through its internal ribosome entry site (IRES), located in the 5' non-translated region (NTR), upstream of the viral coding sequence [39-42]. The HCV 5' NTR has been extensively studied both at the structural and functional level. An RNA pseudoknot structure regulates initiation of translation within the IRES element and miR-122 microRNA binds specifically within the IRES element [39, 43]. miR-122 maintains the stability of HCV RNA and enhances replication with a modest effect on translation, an unusual property of miRNAs [43, 44]. miR-122 antagonists bring down the viral load in chimpanzees infected with HCV, demonstrating its promise as a therapeutic agent [45].

The 3000 amino acid residue polyprotein, targeted to the ER, undergoes cleavage via viral proteins NS2 and NS3, resulting in the full suite of viral proteins. The translated non-structural proteins then perform a variety of functions to ensure the efficient establishment of a cellular environment fit to reproduce more HCV virions. HCV replication, like that of several other positive-stranded RNA viruses, occurs on endoplasmic reticulum (ER)-derived modified membranous structures termed “membranous webs” (Figure 1.3) [22]. HCV replication is accomplished by the large viral NS5B protein, which acts as a RNA-dependent RNA polymerase (RdRP). These membranous webs support the continued replication via the action of the HCV RdRP NS5B, which generates large amounts of viral RNA. Some of this RNA is translated into
proteins at the ER, while a larger portion is used to create more RNA and eventually packaged into the viral particle. The virus uses its positive-stranded RNA as a template for negative stranded intermediary RNAs, which in turn are used as templates for new positive-stranded viral RNA. The viral replication complexes are often closely juxtaposed to lipid droplets (LDs), which are thought to play a key role in the assembly of the viral particle. HCV core protein travels to LDs, which are also associated with viral NS5A protein [26, 46]. The specific steps of the viral assembly process are unknown. The steps of viral secretion also remain to be characterized, though the fact that the virus is associated with low-density lipoprotein (VLDL) components such as apolipoproteins B and E (ApoB, ApoE) have garnered interest in the pathways of VLDL synthesis and secretion.

In addition, HCV modulates host lipid metabolism, leading to the enrichment of intracellular lipids to facilitate membrane fluidity and availability, as well as the accumulation of LDs critical for assembly of viral replication complexes and morphogenesis respectively [23, 46, 47]. Viral replication complexes are often localized adjacent to LDs, which subsequently serve as platforms for post-replicative events of viral assembly and maturation [46]. Although not completely understood, the prevailing view is that viral particles exit the cell by co-opting the very low-density lipoprotein (VLDL) secretion pathway, which is quite distinct from typical cellular protein secretion (Figure 1.3) [48-52].
Figure 1.3: Diagram of HCV Lifecycle.
The lifecycle of the Hepatitis C virus in seven steps. (i) HCV entry. (ii) pH-dependent fusion of envelope and uncoating of viral RNA takes place in endosomes. (iii) Translation begins via the viral IRES, on rough ER. (iv) Assembly of HCV ribonucleoprotein complexes (RNP). (v) RNP complexes begin RNA synthesis from negative-stranded intermediaries to produce plus-stranded viral RNA in membranous web-like structures derived from the ER. (vi) Plus-stranded RNA is packaged and encapsidated. (vii) HCV maturation and secretion process occur, possibly through the Golgi. Major elements of both vi and vii remain to be characterized. Figure courtesy of G. Syed and A. Siddiqui [47].
1.3 Development of an HCV Cell Culture Model

For a long time after the discovery and sequencing of HCV, the options available for studying the virus were very limited. Without a virus that could replicate in cultured cells, and lacking a small animal model, researchers were restricted to the use of primary human hepatocytes and the inoculation of chimpanzees, the only non-human animal capable of supporting an HCV infection [53, 54]. Infection of primary human hepatocytes is extremely inefficient, difficult and cumbersome which necessitated the development of a cell culture system to further study the virus. As the HCV genome consists of a plus-stranded RNA that essentially functions as mRNA, merely transfecting small amounts of virus RNA into the cell can result in the translation of the viral proteome, establishing an infection. Despite this ameliorating trait of plus-stranded RNA viruses that often makes them easier to work with, a number of obstacles needed to be surmounted in order to generate a viable cell culture replication system. Viral RNA levels in HCV infected cells are often quite low, and the amplification of viral RNA via polymerase chain reaction (PCR) is often error-prone. This is compounded by the lack of error-correcting functionality inherent in the NS5B RNA-dependent RNA polymerase, which results in highly mutable viral RNA genome [20]. The HCV genome sequences from even a single infected individual can vary up to 10%, creating a highly variable population of quasispecies [14]. Finally, these viruses can often only replicate in particular cells or cell types within an individual, which are often difficult to grow in cell culture. The difficulties imposed by the above constraints explain why it took over ten years after the virus was sequenced before an efficient cell culture system for generating infectious HCV
was finally developed.

One of the most challenging hurdles to overcome was the difficulty in successfully amplifying the often low levels of viral RNA present in patients without the incorporation of significant errors. These errors can arise both from the amplification technique used to generate the RNA sequence, as well as the mutations inherent in the viral quasispecies. To resolve the issue, consensus HCV genomes were developed by sequencing multiple copies of the HCV genome from a single infected individual. Mutations deleterious to viral replication or infection efficiency should show up in only a minority of the sequenced genomes, so that a master consensus sequence should consist primarily of the base genome. Sequencing was performed, and the sequences aligned to create a genotype 1a H77 consensus genome [53, 54]. The synthesized genome was then injected directly into the livers of chimpanzees, and was shown to both replicate efficiently and to produce infectious HCV particles in vivo [53, 54].

These genomes, however, were not able to successfully replicate in any cultured cells, possibly due to the inability at the time to efficiently detect low levels of viral RNA within cultured cells [20]. Using a similar consensus genome technique, another group of researchers was able to generate an HCV consensus genome with a built-in selectable marker. The HCV structural proteins core, E1, E2 and p7 were replaced with a neo resistance gene, and an additional IRES element added, allowing for the selection of cells able to replicate HCV RNA. This HCV genome was termed the subgenomic replicon, and under selection was able to actively replicate HCV RNA [55]. The generation of replication-competent HCV genomes also resulted in the discovery of a number of adaptive mutations in several HCV non-structural proteins which allowed for greater
replication efficiency in infected cultured cells [56, 57]. In addition, it was also
discovered that variations within the pool of cultured cells themselves can cause HCV to
replicate more efficiently [58, 59]. These discoveries led to the generation of the Huh 7.5
and Huh 7.5.1 clones, where specific cell clones of Huh 7 cells were screened for their
ability to replicate HCV very efficiently in culture. These extra permissive cells were then
cured of HCV infection and cultured. These Huh 7 derivitive cell lines can natively
support much higher levels of viral replication [59, 60]. Among the reasons that these
cells are more permissive of viral replication is the fact that the cell's double-stranded
RNA sensor, retinoic acid inducible gene I (RIG-I) is mutationally inactivated [61].
Despite these successes, however, none of the viral genomic replicons generated using
this technique were able to form infectious viral particles in culture. This is most likely
due to the fact that the cell culture adaptive mutations, while supporting the replication of
HCV RNA, actually hindered virus production [20, 62, 63].

The final step towards generating an infectious cell culture HCV genome required
a rare case, where a consensus genome was isolated from a Japanese patient displaying
fulminant hepatitis, an especially severe case of hepatitis with very high viral loads. This
was designated as Japanese Fulminant Hepatitis, or JFH-1 [64]. The JFH-1 consensus
genome, when transfected into Huh-7 cells, is able to reproduce viral particles that are
infectious both to naive primary and cultured cells as well as in chimpanzees [65-67].
Shortly after the completion of cloning the JFH1 clone of HCV, a chimeric virus was
developed using the JFH-1 non-structural proteins combined with the structural proteins
from a separate virus, the J6(CF) isolate, to create the chimeric JC-1 construct [68]. JC-1
proved to be even more infective than the successful JFH-1 genome, and generates
consistently high titers of virus. Several additional constructs of HCV genomes have been synthesized with the goal of more easily detecting changes in HCV RNA replication levels, such as the insertion of a *Renilla* luciferase gene inserted between the p7 and NS2 regions of JC1, termed JC1-RLuc 2A [69]. The viral RNA can be quantified by measuring the luciferase activity of the cells rather than the time- and labor-intensive quantitative (q)RT-PCR methods.

Despite the increasing success of HCV cell culture systems, there are still a number of limitations to our use of the virus in the laboratory. Currently, we have only a small range of genotypes available to study. Genotype 1a and 1b have been successfully cloned into replicon systems, but genotype 2a (JFH-1 and its derivatives) remains the only genotype able to productively infect the human hepatoma cell line Huh-7 and its derivatives. A cell culture system for the five remaining genotypes has not yet been developed, and despite the cell culture successes of the JFH1 and derived systems, it is not clear exactly why the JFH-1 consensus genome is inherently much more successful than others at reproducing in cell culture. Huh-7, and another very similar hepatocellular cell line, HuH6, remain the only cell lines permissive for HCV replication [59, 70]. There are many obstacles to studying HCV outside of infected patients that still need to be overcome before we fully understand the causes of and significant differences between genotypes and their ability to reproduce outside of an infected human being.
1.4 PI4P and Phosphoinositides

Phosphoinositides (PIPs) are phosphorylated derivatives of phosphatidylinositol (PI), an essential phospholipid component of the eukaryotic cell membrane (Figure 1.4) [71]. PI can be mono-, di-, or tri-phosphorylated at the D-3, D-4 and D-5 positions of the inositol ring in various combinations to generate seven different phosphoinositides, which have distinct biological activity (Figure 1.5) [71]. A cohort of cellular phosphoinositide kinases and phosphatases control the interconversion of phosphoinositide species. So far 18 phosphoinositide interconversion reactions mediated by 19 phosphoinositide kinases and 28 phosphoinositide phosphatases have been identified [72]. PIPs are predominantly localized on the cytosolic side of membranes and are fundamental constituents of the cytosol-membrane interface [73]. The distinct make up of PIPs within a membrane constitutes a lipid code or membrane signature that defines the identity of the membrane and associated organelles. These PIPs interact with various effector and adaptor proteins, which localize to specific organelles via their PIP interacting domains [74]. A number of recent studies have implicated PI4P and several interacting partners in HCV infection. In uninfected cells PI4P is primarily localized to the Golgi, with smaller distinct pools existent in the ER [75, 76]. HCV infection robustly stimulates ER-specific PI4P pools by activating phosphatidylinositol(4)phosphate kinase IIIα (PI4KIIIα), responsible for generating ER-specific PI4P [77, 78]. Phosphoinositides serve two main functions in the cell; establishing membrane identity for various cellular organelles by acting as mediators of protein-protein interaction, and signaling [79]. Spatio-temporal modulation of a cohort of phosphoinositide kinases and phosphatases
Figure 1.4: Phosphatidylinositol.
Diagram of a standard phosphatidylinositol (PI) molecule containing two fatty acid chains bound to a glycerol molecule. The inositol ring is bound via a phosphate group.
Figure 1.5: Phosphoinositides (PIPs), PI-Kinases and Phosphatases.
Schematic representation of phosphatidylinositol, the seven PIP varieties, kinases and phosphatases involved. Kinases are represented in black, phosphatases in red.
maintains a distinct population of PIPs that help define the membrane characteristics of
different organelles. For instance, PI(4,5)P2 is located primarily at the plasma membrane
(PM), whereas PI3P is predominantly an endosomal marker, and PI4P is present most
frequently at the Golgi (Figure 1.6) [75]. These PIP populations in turn interact with
proteins possessing binding domains specific for each phosphorylated derivative and thus
serve as primary modulators of membrane-cytosol interactions and organelle identity [80,
81]. For example specific PI4P binding pleckstrin homology (PH) domains shared by the
lipid transfer proteins such as oxysterol binding protein (OSBP), the ceramide transporter
protein (CERT) or four phosphate adaptator proteins 1 and 2 (FAPP1 and FAPP2) allow
their localization to the Golgi (Figure 1.7) [82]. Most interactions between PIPs and their
binding domains are weak, and require interactions with membrane proteins or membrane
bound GTPases that often function as co-receptors with PIPs for the recruitment of
cytosolic proteins to specific membrane compartments [83]. As mammalian cells express
over 100 small GTPases, a wide variety of unique membrane domains can be established
through dual interactions with specific GTPase and PIP configurations [80, 81]. Apart
from the regulation of signal transduction at the membrane surface, the myriad functions
of phosphoinositides include cytoskeleton and membrane reorganization, regulation of
intracellular vesicular trafficking and Golgi secretory functions [80, 81]. In addition, PIPs
and their byproducts can function as members of signaling pathways [79]. The well-
known secondary messengers, inositol 1,4,5-trisphosphate (IP3) and diacyl glycerol
(DAG) are produced by phospholipase C (PLC) from PI(4,5)P2, where both components
of the phosphoinositide function downstream to initiate a plethora of cellular responses.
PI4P, long considered merely an intermediary in the synthesis of PI(4,5)P2 for signaling
Figure 1.6: Cellular Localizations of Various Phosphoinositides. Diagram of a typical cell, highlighting the locations of various PIPs in different cellular membranes. EE: Early endosome; LE/MVB: Late endosome/multi-vesicular body. Some PI kinases are also given in their cellular locations. PI4Ks are in yellow ovals, PI3K in cyan.
Figure 1.7: PI and PI4P Binding Proteins at the Golgi.
Close-up of Golgi panel from Figure 1.6. PI4P binding proteins at the Golgi/TGN are localized, as well as the lipids or other proteins to which they bind. Lipid transfer proteins (LTPs) Nir2, CERT, and OSBP are shown shuttling their preferred lipid cargo from the ER; PI (pink), ceramide(green), and cholesterol (orange), respectively.
at the plasma membrane, has recently garnered more attention as a number of studies have characterized its role in the maintenance of Golgi structure and function. PI4P is primarily produced by the monophosphorylation of PI at the D-4 position of the inositol ring by one of four different phosphatidylinositol 4 kinases (PI4Ks) [84]. PI4P can also be produced via the dephosphorylation of PI(4,5)P2 by inositol polyphosphate-5-phosphatases [85]. PI4P is the most abundant monophosphorylated PIP in the cell, and the majority of PI4P localizes to the Golgi [86]. The Golgi has also been recognized for harboring highest levels of PI4K activity in the cell. PI4Ks have been categorized into two types: type II, which displays sensitivity to adenosine, and type III, which are sensitive to Wortmannin [76]. Based on the domain structure there are two isoforms of each type of PI4K; PI4KIIα and PI4KIIβ; PI4KIIIα and PI4KIIIβ (also known in shorthand as PI4Kβ or PI4KB). The Golgi is home to two types of PI4Ks; PI4KIIIβ, considered the kinase primarily responsible for establishing Golgi PI4P; and PI4KIIα, which has recently received attention due its possible role in trans-Golgi network (TGN) to plasma membrane (PM) transport [75, 87]. These PI4Ks locate primarily at the Golgi, but the kinases can also be found in other membrane compartments in smaller amounts, namely in the endosomes, plasma membrane, and nucleus [75]. Of the non Golgi-localized PI4 kinases, PI4KIIβ is mostly cytosolic, and can be recruited to the PM through activation by Rac1 [74]. PI4KIIIα is primarily located at the ER, and maintains a distinct, though smaller, population of PI4P, which apparently contributes to the formation endoplasmic reticulum exit sites that regulate export from ER during acute and chronic increase in ER cargo load [88, 89]. PI4KIIIα has also been shown to play a role in regulating PI4P levels at the PM [90, 91]. There are a number of phosphatases for
various PIPs, and the primary PI4P phosphatase is called hSac1. hSac1 phosphatase is responsible for de-phosphorylation of PI4P with notable specificity [92]. Under normal conditions, hSac1 primarily localizes to the ER, though a small amount can be found in the ER-Golgi and Golgi compartments [92]. hSac1 localizes to the ER via its C-terminal K(X)KXX (KEKID) domain, and mutations to this structure cause hSac1 to accumulate at the Golgi rather than the ER [92, 93].

A number of Golgi-specific cellular proteins localize to that compartment via their interactions with PI4P. Lipid transfer proteins (LTPs) OSBP and CERT are important for lipid transport between the ER and Golgi for cholesterol and ceramide, respectively [94, 95]. These two LTPs, along with a phosphatidylinositol and a phosphatidylcholine LTP, Nir2, associate with the vesicle-associated membrane protein-associated proteins (VAPs) at the ER, and likely coordinate the majority of the lipid composition of the Golgi membranes by facilitating the formation of hypothetical ER-Golgi membrane contact sites by simultaneous interaction with PI4P in Golgi and VAP in ER [96]. Each of the proteins contains a PH binding domain, accounting for their association with PI4P. This binding is crucial for their localization to the Golgi compartment [97]. FAPP1 and FAPP2 localize to the TGN, also via a PH domain [74, 98]. FAPP2 also plays a role in lipid transport, and is additionally important for apical Golgi-to-PM transport in polarized cells. A recently characterized PI4P-binding protein, GOLPH3 also binds PI4P through a binding pocket similar to a PH domain [93]. GOLPH3 interacts with an unconventional myosin, MYO18A, which in turn joins the TGN to the cellular actin cytoskeleton. The tensile force exerted on the Golgi by this interaction is thought to give the Golgi its flattened stack morphology, and is also important for vesicle budding from the TGN [93].
In the quest for identifying novel host factors critical for the HCV lifecycle, studies applying genome wide or targeted siRNA screens have identified the phosphatidylinositol-4-phosphate (PI4P) pathway as pivotal element in HCV replication [99-104].

1.5 Significance of PI4P and PI4 Kinases in HCV Replication

Viruses are obligatory intracellular parasites and rely on host cell infrastructure for viability and proliferation. Plus-stranded RNA viruses are critically dependent on intracellular membrane rearrangement for RNA replication [105, 106]. They modulate lipid metabolism to influence lipid composition of membranes to enhance fluidity and membrane curvature to ease membrane remodeling. RNA viruses are known to utilize membranes of various organelles including ER, Golgi, trans-Golgi, endosomes and mitochondrial outer membrane to assemble replication platforms [107]. Many RNA viruses, such as poliovirus, coxsackievirus, Aichi virus and enterovirus71 require enrichment of PI4P in replication membranes for active replication which they achieve by recruitment of host PI4Ks for synthesis of PI4P from PI by various mechanisms [78, 108-110]. In the case of HCV, studies utilizing siRNA-based screens have established the requirement of PI4Ks for HCV replication [77, 111-114]. Liver biopsies from HCV patients also displayed high levels of PI4P indicating that induction of PI4P is a clinical hallmark of HCV infection [77]. However, some discrepancies exist with regards to the role of specific PI4K isoforms, due to variations in experimental set up, analysis, and
HCV genotypes involved. Hsu et al, were the first to observe robust stimulation of ER-localized PI4P pools during HCV genotype 1b infection [78]. The typical Golgi/TGN localization of PI4P in uninfected cells was altered upon HCV infection to a discrete punctate distribution of PI4P in the cytoplasm [47, 78]. Both PI4KIIIα and β were implicated in the induction of PI4P levels, and expression of ER-localized hSac1 phosphatase in HCV infected cells both reduced PI4P levels and led to a decline in viral RNA synthesis [78]. Subsequent studies established the role of PI4KIIIα in membrane reorganization into replication competent membranous web-like structures [77, 115]. These studies further strengthen the involvement of PI4KIIIα in HCV replication by revealing that HCV NS5A protein physically interacts and activates PI4KIIIα. NS5A domain 1 was shown to be required for recruitment and activation of PI4KIIIα. Deletion mutants of PI4KIIIα and NS5A map the interaction domain of PI4KIIIα to amino acids 401-600 and domain I of NS5A [116]. Abrogation of PI4KIIIα activity by chemical inhibitor PIK93 or siRNA-mediated gene silencing resulted in aberrant clustering phenotype of NS5A in contrast to the normal reticular staining pattern [77]. Likewise, NS3 and NS5B immunostaining also showed aberrant clustering into large masses that entirely colocalized with NS5A, suggesting that silencing PI4KIIIα results in aggregation of viral replicase proteins [77, 114, 115]. These studies suggest that NS5A recruits PI4KIIIα to the membranous replication compartment and stimulates PI4KIIIα activity resulting in robust induction of PI4P pools that are required to maintain the integrity of the membranous web structure (Figure 1.3) [115]. It has also been suggested that HCV alters cellular distribution of PI4P causing enrichment in the HCV-membranous web with a concomitant depletion in plasma membrane and Golgi [117]. Despite the dramatic
increase in cellular PI4P levels by HCV infection, the overall expression level of the PI4KIIIα is not significantly altered, suggesting that increased PI4P levels are the result of an increased kinase activity of PI4KIIIα [77, 115]. Berger et. al also showed that the viral NS5A protein both recruits and activates PI4KIIIα and that its activation is required for membranous web integrity as well as viral replication. The significance of another PI4K isoform in HCV replication, PI4KIIIβ, normally localized to the trans-Golgi, is less clear [71]. PI4KIIIβ was never a top hit in several siRNA screens performed for cellular factors of HCV replication. PI4KIIIβ does not colocalize or interact with NS5A, and its overexpression does not rescue HCV replication hampered by PI4KIIIα silencing [77, 114]. Interestingly, Hsu et al reported that knockdown of PI4KIIIβ had stronger effect on reduction of HCV genotype 1b replication than the knockdown of PI4KIIIα. This discrepancy could have been due to the use of only genotype 1b replicon in their assay. Tai and Salloum also implicated PI4KIIIβ in HCV replication but suggested the involvement of an alternative replication mechanism, since PI4KIIIβ knockdown did not affect membranous web formation or the accumulation of PI4P pools at these sites [114]. However, it should be noted that Tai et al collected and assayed culture supernatants for Gaussia luciferase acitivity to assess the effect of PI4KIIIβ knockdown on HCV replication. PI4KIIIβ silencing is likely to affect Golgi-secretory functions and hence may perturb secretion of the Gaussia luciferase reporter [118]. The changes they observed may have been in HCV secretion rather than in replication, as they failed to isolate the pathways sufficiently. Zhang et al also confirmed the requirement of the PI4KIIIβ for HCV genotype 2a replication and also noted the role of the small GTPase ARF1 and its guanine nucleotide exchange factor GBF1 [119]. ARF1 is known to recruit PI4KIIIβ to
the TGN to stimulate PI4P production [120]. Taken together, PI4KIIIβ may play a role in HCV genotype 1b and 2a replication, but is not necessary for the formation of membranous webs.

Although the requirement of PI4P for HCV replication and integrity of the membranous web has been strongly established, the precise nature of this requirement still needs to be unraveled. In principle, HCV NS4B protein is capable of inducing membrane rearrangement [121, 122]. Recent studies, however, implicate a formation of a more heterogeneous population of membrane structures involving double membrane vesicles and monomembrane vesicles, suggesting a more complex underlying mechanism [77, 105, 123]. The protein-lipid and protein-protein interactions might facilitate the membrane recruitment and confined enrichment and local concentration of viral proteins and critical host factors for establishment of functional replication complexes. For instance, the RNA dependent RNA polymerase of poliovirus, a positive-sense RNA virus of Picornaviridae family shows high affinity towards PI4Ps despite lacking canonical PI4P binding domains [78]. The potential for HCV RNA-dependent RNA polymerase (RdRp) or NS5B to interact with PI4P still needs to be ascertained. PI4P could also help induce conformational alterations of its binding partners, such as viral replicase proteins, modulating their activity. PI4P localized at viral replication sites may facilitate the recruitment of cellular proteins that harbor PI4P binding domains, such as PH domain-containing lipid transfer proteins like OSBP and CERT which may facilitate the enrichment of cholesterol, sphingomyelin, and/or ceramide in replication compartments [96, 124, 125]. The dual interaction exhibited by such LTPs, with PI4P via their PH domains and with the ER by virtue of FFAT motifs, may lead to formation of hypothetical
membrane contact sites between replication membranes and the ER to sustain a supply of PI and lipids to the replication compartments. Indeed the role of OSBP and CERT in HCV replication has been already established and cholesterol-depleting agents such as β-cyclodextrins have been shown to disrupt the membranous web [121, 126-128]. Incorporation of cholesterol in HCV replication membranes may promote membrane fluidity, elasticity, and curvature, generating domains required to sequester proteins for effective interaction. PI is synthesized in the ER and delivered to other cellular locations via vesicular transport or by cytosolic PI transfer proteins such as Nir2 [73]. Exploring the role of Nir2 in HCV-mediated induction of PI4P in replication sites may prove useful. Since HCV replication complexes are closely associated with LDs that serve as platforms for viral assembly, the lipid alterations occurring at replication sties may also be evident in the viral envelope [26, 46]. A recent report characterizing the lipidome of the viral particle shows characteristic enrichment of phosphatidylcholine, sphingomyelin, cholesterylester and cholesterol in HCV virus particle [129]. In addition the local accumulation of anionic PI4P can also induce membrane curvature. Some viruses have been shown to induce highly ordered complex structures dependent on cholesterol termed “cubic membranes” that help in sequestration of viral proteins and RNA for optimal replication and protection of the viral components from innate immunity [130]. A study with metabolically stabilized derivatives of PI4P that can substitute the natural lipid in protein recruitment and membrane deformation suggests that recruitment of proteins by PI4P is essential for inducing membrane tubulation [131]). This suggest that mere enrichment of PI4P pools is not sufficient to induce assembly of active HCV replication complexes but the recruitment of proteins by the PI4Ps is essential for inducing necessary
membrane modifications. Identification of the precise function of PI4P and interacting viral or host proteins in assembling replication complexes could prove to be fundamentally important in designing novel anti-HCV therapeutics.

1.6 Role of Phosphoinositides in HCV Entry

The role of PI4K's on HCV entry highlights the importance of PI4 kinases in multiple stages of HCV infection. Trotard et al have reported that PI4KIIIα and β are also essential for HCV entry [111]. However they observed differential sensitivity of various HCV genotypes to the two PI4KIII isoforms suggesting that the two kinases regulate distinct events during HCV entry [111]. Silencing PI4KIIIα expression protected cells from HCVpp infection of genotype 1a whereas silencing PI4KIIIβ inhibited entry of HCVpp of genotype 1a and 1b [111]. The direct link of PI4Ks with the endocytic pathway is via PI4P that serves as precursor for PI(4,5)P2 synthesis at plasma membrane and is required for recruitment of adaptor proteins involved in clathrin-mediated endocytosis [132]. Interestingly the PI4KIIIα and β knockdown only had a subtle effect on clathrin-mediated endocytosis suggesting that the effect of HCV entry is not due to inhibition of endocytosis [111]. Similar to other viruses such as VSV, internalization of HCV also requires the endocytic pathway however the specific dependence of HCV on PI4Ks for entry suggests the effect of PI4Ks on HCV binding or fusion partners. Silencing of PI4KIIIα or β did not disrupt the cell surface expression of HCV receptors CD-81 and SR-BI and further investigations are required for analyzing the effect on other HCV receptors [111]. It should be noted that PI4KIIIα and β differ in their subcellular
localization and associated functions, for example PI4KIIIβ modulates Golgi-associated intracellular traffic but PI4KIIIα does not, suggesting that the two PI4KIII isoforms may differentially regulate the cell surface expression of membrane proteins [84]. This might contribute towards observed differences of HCV genotypes on requirement of specific PI4K isoforms for HCV entry. Several other groups who analyzed the effect of PI4Ks on HCV replication have not found any effect of PI4K knockdown on HCV entry. HCV entry is a multi-step process involving complex interplay between various receptors and subsequent downstream signaling which may involve phosphoinositides other than PI4P, hence further investigations are needed to unravel the mechanistic details of the precise role of PI4Ks in HCV entry.

1.7 HCV and PI3K activation

PI4P is not the only PIP of significance in HCV infection. Several reports have indicated the activation of PI3K/Akt pathway in HCV infection and several downstream kinases like mTOR and S6K1 [133-136]. PI3K is the kinase responsible for the production of PI(3,4)P2 as well as PI(3,4,5)P3 [137]. These PIPs function as second messengers for a variety of signaling pathways related to cellular growth, survival and differentiation. PI3K consists of a regulatory subunit, p85, and a catalytic subunit, p110 which phosphorylates PI4P and PI(4,5)P2. The molecular mechanism(s) of HCV-mediated PI3K and Akt activation are still not clear, though oxidative stress has been implicated in their activation [138]. HCV NS5A protein has been shown to interact with the PI3K regulatory subunit p85 and release inhibition of catalytic p110 subunit [133,
The activation of p110 leads to the formation of PI(3,4)P2 and PI(3,4,5)P3 at the plasma membrane followed by subsequent recruitment of Akt that binds PI(3,4,5)P3 or PI(3,4)P2 via its PH domain. Upon recruitment to the plasma membrane Akt is activated by Ser 473 phosphorylation by mTORC2 and Thr 308 by phosphoinositide dependent kinase 1 [140, 141]. In addition to its role in multiple cellular processes such as glucose metabolism, cell proliferation, transcription, translation and cell migration, Akt also functions as anti-apoptotic protein by inhibiting the pro-apoptotic protein Bad and Caspase 9 [134, 142]. Non-lytic chronic viruses such as Epstein-Barr virus and Polyomavirus that activate PI3K by inhibiting p85 seem to exploit the anti-apoptotic potential of Akt to promote cell survival and maintain a persistent infection. Due to its ability to promote cell survival and block apoptosis, Akt has been implicated in many cancers. Increased oncogenic capacity of Akt in HCV infected cells could play a role in the development of hepatocellular carcinoma. The PI3K/Akt pathway is also implicated in promoting de novo lipogenesis by activating SREBPs in response to insulin [143]. HCV also stimulates de novo lipogenesis by promoting SREBPs activation [144]. The PI3K/Akt pathway could help mediate SREBPs activation in HCV infection. Interestingly, NS4B expression has been shown to promote SREBPs activation and de novo lipogenesis via the PI3K/Akt pathway [145]. PI3K dependent Akt activation is also regulated by lipid phosphatase and tensin homolog (PTEN), which negates the effect of PI3K and dephosphorylates PI(3,4,5)P3 back to PI(4,5)P2 thereby disrupting the membrane localization and subsequent activation of Akt [146]. Recently it has been shown that expression of HCV genotype 3a core protein down-regulated PTEN expression by microRNA-dependent blockade of PTEN mRNA translation leading to the
appearance of large lipid droplets [147]. HCV positive hepatocellular carcinomas also display reduced PTEN expression [148].

1.8 HCV and Lipid Metabolism

Alteration of host lipid metabolism is a well-known hallmark of HCV infection in patients. Chimpanzees infected with HCV upregulate lipid metabolism genes, and about 30% of cellular proteins associated with the HCV RNP complexes are related to lipid metabolism [149, 150]. Hepatic steatosis, where lipid droplets accumulate excessively in the liver, is one of the most prominent signs of HCV infection, occurring in 50-73% of patients, depending on genotype [151, 152]. Analysis of lipid levels in patients infected with HCV shows heavy modulation of specific lipids such as sphingomyelin and some phospholipids [153].

In addition, HCV has been shown to affect lipid homeostasis on the cellular level in specific ways [47, 154]. HCV increases lipogenesis in infected cells by activating the sterol regulatory element-binding protein (SREBP) [47, 144]. SREBPs are ER-localized transcription factors which can activate genes for cholesterol and fatty acid biosynthesis [155]. SREBP inhibition has been shown to block HCV replication [149]. Fatty acid synthase (FAS) is another protein implicated in HCV infection. FAS levels increase during HCV infection, assisting in the formation of replication complexes [149]. When FAS is inhibited, HCV replication is limited as well as viral release. The unfolded protein response (UPR), which has been shown to play a role in lipid metabolism is also upregulated by HCV, helping to promote lipogenesis, and steatosis [156-159]. HCV also
impairs lipid export. Domitrovich et al. noted that VLDL secretion and synthesis is downregulated upon HCV infection [160]. The reduction in VLDL synthesis was a result of downregulation of MTP at the transcriptional level. Mirandola et al. showed that lower levels of apolipoproteinB-100 in the sera of chronically infected HCV patients [161]. Stimulated lipogenesis, impaired β-oxidation, and lipid export all contribute to the steatosis observed in HCV infected patients [47, 151, 152, 154].

One of the crucial reasons for HCV's promotion of these lipid pathways is the promotion of lipid droplets (LDs) for HCV replication and possibly assembly [47, 154, 162]. HCV undergoes replication in the ER-derived membranous web structures closely juxtaposed to LDs. In order to facilitate replication, HCV causes LDs to accumulate around the perinuclear region in close proximity to the ER [163]. Treatment of HCV infected cells with NDGA, a negative regulator of lipid synthesis, resulted in reduced SREBP activation, and reduced the number of lipid droplets observed within the cell [154]. This reduction had a negative effect on HCV replication [154]. NDGA also reduces the levels of VLDL secretion by inhibiting its synthesis, and subsequently NDGA also reduced the levels of HCV secretion in infected cells [154].

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Chapter 2

Materials and Methods

2.1 Reagents and Plasmids

The plasmid pJFH1 was a gift of Dr. T. Wakita (Tokyo, Japan). The plasmid JC1 RLuc 2A and mouse monoclonal antibody 9E10/A3 for NS5A were gifts of Dr. C. Rice (Rockefeller University, NY). The plasmid pEGFP hSac1 K2A was a gift of Dr. P. Mayinger (Oregon Health and Science University, Portland, OR). Anti-ApoB was purchased from Chemicon International (Temecula, CA). Antibodies against Calnexin, MYO18A, and TGN38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PI4P antibody was purchased from Echelon Bioscience (Logan, UT). Mouse monoclonal anti-core antibody was purchased from Affinity Bioreagents (Golden, CO). Rabbit anti-GOLPH3 antibody was purchased from Abcam (Cambridge, MA). Rabbit polyclonal anti-TGN46 was purchased from Sigma-Aldrich (Saint Louis, MO). Fluorescence-conjugated donkey secondary antibodies for immunofluorescence against mouse, rabbit, goat, and human primary antibodies were purchased from Invitrogen (Carlsbad, CA). Primers and probes for qPCR of PI4KIIIβ and FAPP1 and FAPP2 were purchased from Integrated DNA Technologies (Coralville, IA). Stealth siRNAs were purchased from Invitrogen, with the following sequences:
GOLPH3: 5' UCUGGAAUACGUGGCUGUAUGUUAA 3'
   UUAACAUACAGCCACGUAAUCCAGA
MYO18A: 5' AUUCUCAUCACUCUCCAUCUCAUCC 3'
   GGAUGAGAUGGAGAGUGAGAAGAAU
PI4KIIIβ1: 5' ACUUGAUGAAUUCUCUCUCAGGAGC 3'
   GCUCCUGAGAGAGAAUUCAUCAAGU
PI4KIIIβ2: 5' AAAGGCUGACGUCUCAAAGGCCAGA 3'
   UCUGGGCUUUGAGACGUCAGCUCUUU
PI4KIIα: 5' CAGCGGUCUUCUAGCGAGUCCUACA 3'
   UGUAGGACUCGUAGAAGACCGCGUG

ON-TARGET plus siRNAs for FAPP1 and FAPP2 were purchased from Dharmacon (Thermo Fisher Scientific).

FAPP1 Pooled siRNAs:
1: 5' AAAGCAUGUUUGACUGAUA 3'
   UAUCAGUCAAACAUGCUUU
2: 5' GCAUAAAGAUGGCGAGUUUG 3'
   CAAACUGGCAUCUUUAAUGC
3: 5' UCACAACGCUUGAGGAAUG 3'
   CAUUCCUCAAGCGUUGUGA
4: 5' GGUGUAUUGGUAGCUUAAUA 3'
   UUAUAAGCUACAAUACACC

FAPP2 Pooled siRNAs:
1: 5' GGUAAGAACUCAGAACAUA 3'
UAUGUUCUGAGUUCUUACC
2: 5' CGUCAAUCUAUGUCAAG 3'
CUUGACAUUAGAUUUGACG
3: 5' GGAGGAUAAGAGCACUA 3'
UAGUGCUCUUUAUAUCCUCC
4: 5' AAGAUUCUGUGACCGGAAA 3'
UUUCCCGUCACAGAAUCUU

2.2 Tissue Culture

Human hepatoma cell lines Huh7 and Huh7.5.1 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 g/ml streptomycin (Gibco), and 1 mM minimal essential medium with nonessential amino acids (Gibco). The Huh 7.5.1 cell line was a kind gift of F. Chisari (Scripps Institute, La Jolla, CA). Huh7 and Huh 7.5.1 cells were incubated at 37°C, 5% CO₂ in water-jacketed incubators (Thermo Forma).

2.3 HCV Infection of Cultured Cells

HCV infectious viral particles were produced from the JFH1 strain performed at a multiplicity of infection (MOI) of 1. Chimeric JC1 virus was also used at MOIs ranging from 0.01 to 1 to infect Huh 7.5.1 cells. These JFH1- or JC1-infected Huh7.5.1 cells were transfected with either DNA plasmids or siRNAs at 6 days post-infection. Media was changed 16 hours post-transfection and replaced with fresh media. Cultured supernatants
and cell lysates were collected at 9 days post-infection. Cell lysates were repeated to 3 cycles of freeze-thaw, using liquid nitrogen for 5 minutes, followed by 37 degree incubation to release infectious virus intact. Lysates were resuspended in 0.2 ml of 10% FBS DMEM.

2.4 FFU Assay

The infectious virion titers of collected supernatants and lysates were determined by a focus-forming-unit assay as described by Gastaminza et al [164]. 100 µl of previously uninfected Huh7.5.1 cells were plated on a 96-well plate at a concentration of 1x10^5 cells/ml. After 24 hours growth, the media was removed and replaced with HCV-infected supernatants or lysates. Serial dilutions used to infect cells ranged from 1:1 to 1:100,000. After 72 hours exposure, plates were removed and washed with phosphate buffered saline (PBS), followed by fixing with 4% paraformaldehyde (PFA) in PBS. Fixed cells were then washed twice with PBS, and blocked for one hour at room temperature with a FFU blocking buffer consisting of 3% bovine serum albumin (BSA), 10% fetal bovine serum (FBS), 0.5% Triton X-100 in PBS. Blocking buffer was removed and cells were then incubated for two hours at room temperature with the primary antibody human anti-HCV E2 protein, in FFU binding buffer, 3% BSA, 0.5% Triton X-100 in PBS. Cells were then washed three times with PBS and incubated for one hour at room temperature with secondary antibody goat anti-human Alexa-555 (Life Technologies). Cells were again washed three times with PBS and then all but 50 µl PBS removed. Plates were imaged with an Olympus IX71 fluorescence microscope, and
'focus-forming-units,' (FFU), clusters of 2-50 HCV-infected fluorescing cells, were counted (Figure 2.1).

2.5 DNA and RNA Transfection

DNA transfection was performed using the TransIT LT1 Reagent (Mirus Bio, Madison, WI). Huh7 or Huh7.5.1 cells were plated on 35mm dishes to reach 70% confluency on day of tranfection. 5 μg of plasmid DNA was mixed with 300 μl Opti-MEM minimal-serum media. This was mixed with an equal amount of Opti-MEM containing 7.5 TransIT LT1, shaken gently, and incubated at room temperature for 30 minutes. A combined volume of 600 μl was added dropwise to each well. Media was removed after 16 hours and replaced with fresh 10% FBS DMEM with Pen/Strep. For RNA transfection, cells were prepared as above, but prior to transfection, media was changed to 10% FBS in DMEM, with no antibiotics. siRNAs were transfected using the Lipofectamine RNAi Max reagent (Invitrogen). siRNAs, to a final concentration of 20 μM, were incubated for 30 minutes at room temperature with 9 μl RNAi Max reagent, and added dropwise to cells, as described [93]. Media was removed after 16 hours and replaced with fresh 10% FBS DMEM with Pen/Strep. Cell lysates were collected 48 hours post-transfection to analyze for protein and RNA knockdown.
Figure 2.1: Foci-Forming Units
Sample foci observed after immunostaining for HCV E2 protein. Three foci of 2-50 infected cells are visible in the image.
2.6 Quantitative Reverse-Transcription Polymerase Chain

Reaction (RTPCR)

Total cellular RNAs were purified by the AGPC method [165]. Cell lysates or supernatants were incubated at -80° C with 1 ml Trizol reagent (Ambion). In order to precipitate the small amounts of viral RNA extracted from culture supernatants, ten micrograms of Saccharomyces cerevisiae tRNA was added to each supernatant as a carrier (Sigma-Aldrich). Trizol-treated samples were thawed at room temperature, 200 μl chloroform was added, and samples were vortexed for 15 seconds, then constantly agitated for 3 minutes. Samples were then placed on ice for 10 minutes, and centrifuged at 12k RPM for 15 minutes at 4° C. 300 μl supernatant was removed from the top of all samples derived from cell lysates, and transferred to a fresh tube. 500 μl supernatant was removed from the top of all samples derived from culture supernatants (to account for the extra volume) and transferred to fresh tubes. Equal volumes of 2-propanol were added to each sample, and mixed by inversion. After one hour incubation at -20° C, samples were centrifuged at 12k RPM for 15 minutes at 4° C. Supernatant was removed, leaving precipitated RNA in a small pellet. The pellet was washed twice with 1 ml of 70% ethanol followed by additional centrifugation at 12k RPM, 4° C. RNA was resuspended in DEPC-treated water containing 0.2 units/μl of RNAse Out, a RNAse inhibitor, and either used immediately for RTPCR or stored at -80° C. HCV RNA was quantified on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) as described previously [166]. For the quantification of PI4KIIβ RNA, 200 ng cellular RNA was subjected to PCR using the Euroscript Reverse Transcription kit (Euroscript).
JFH1 or JC1 RNA was detected as previously described [166]. Primers and probes for RTPCR for HCV, PI4KIIIβ and FAPP1 and FAPP2 were purchased from Integrated DNA technologies, with the following sequences:

HCV Probe: 5'/56-FAM/CTGCAGAAACCGGCTGAGTACAC/36-TAMSp/ 3'
HCV Primer 1: 5' CGGGAGAGCCATAGTG 3'
HCV Primer 2: 5' AGTACCACAAGGCCTTTCG 3'
PI4KIIIβ Probe:
5'/56-FAM/CCGCCCATC/ZEN/ACCATCCACAAACTCT/3IABkFQ/ 3'
PI4KIIIβ Primer 1: 5' TGAACATGTCGCCATCCAG 3'
PI4KIIIβ Primer 2: 5' TCATCCTCTCCAGCTCACC 3'
FAPP1 Probe:
5'/56-FAM/TCTCTTTGA/ZEN/AGACCCAGATAGACCTGTTC/3IABkFQ/ 3'
FAPP1 Primer1: 5' GCTTTTCTTCAGGAATGGTTGC 3'
FAPP1 Primer 2: 5' GCCGAAGAACCTACTCATCACAGATTAC 3'
FAPP2 Probe:
5'/56-FAM/ACAGAAGAG/ZEN/GAACCATCGAGGCTC/3IABkFQ/ 3'
FAPP2 Primer1: 5' CCAGGCATCTTCAAGGAGAATC 3'
FAPP2 Primer 2: 5' TGTACAAGTGGAACCAACTATCTG 3'

2.7 Western Blotting

Cells grown in 35-mm dishes were transiently transfected (as above) and incubated for 48 hours, washed once with PBS, and lysed on ice with 500 μl of lysis
buffer (0.2% [wt/vol] deoxycholic acid, 20 mM Tris-HCl, 150 mM NaCl, 0.1 mM EDTA, 250 mM sucrose), supplemented with 1X Halt protease inhibitor single-use cocktail (Thermo Scientific, Rockford, IL). The cellular lysates were frozen at -80°C, followed by centrifugation at 13,400 g for 10 min. 20 μg of the clarified lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Lysates were mixed with 4X sodium dodecyl sulfate (SDS) loading buffer (30% SDS, 40% glycerol, bromophenol blue dye, 20% fresh β-mercaptoethanol in 2M Tris) and boiled at 95°C for five minutes to denature the proteins. After boiling, cells were loaded onto cast SDS-gels ranging from 6-12% polyacrylamide, depending on protein size and separation requirements. Gels were run in SDS running buffer (200 mM glycine, 2.5 mM Tris, 350 μM SDS) at a constant 30 mA for 2-4 hours, depending on gel concentration and separation requirements. Gels were then wet-transferred overnight to methanol-treated polyvinylidene fluoride (PVDF) membrane (Millipore) at 15V.

Post-transfer PVDF membranes were blocked in either a solution of 0.1% Tween-20 in PBS (PBS-T) supplemented with 5% milk, or PBS-T supplemented with 3% BSA depending on specific antibody performance. Incubation with primary antibodies was performed in the same blocking solution, with antibody concentration varying depending on the antibody, for 2-12 hours at room temperature or 4°C, depending on antibody characteristics. Membranes were then washed five times in PBS-T for six minutes each. Secondary antibody incubation was performed in blocking buffer, using either mouse, rabbit, or goat secondary antibodies conjugated to HRP enzyme (Thermo Fisher). After 1-3 hours incubation at room temperature, bands were detected using the Immobilon Western HRP reagent (Millipore), and imaged using a Kodak Image Station 440 gel
camera.

2.8 Immunofluorescence Microscopy

Infected and/or transfected cells were grown on glass coverslips, washed with PBS, and fixed in 4% paraformaldehyde in PBS at room temperature. Fixed cells were permeabilized and blocked in antibody-binding buffer (PBS, 20 μM digitonin, 1% [wt/vol] bovine serum albumin) for 2 hours at room temperature. Fixed cells were incubated in the presence of primary antibodies overnight at 4º C. Coverslips were washed three times with PBS, followed by the incubation with corresponding secondary antibodies for the detection of primary antibodies for 2 hours at room temperature. After incubation with secondary antibodies, the coverslips were again washed 3 times with PBS, then treated with ProLong gold antifade reagent (Invitrogen) containing DAPI (4,6-diamidino-2-phenylindole) for nuclear staining, and sealed to slides. The slides were analyzed by confocal laser scanning microscopy (Olympus FV1000).

2.9 In vitro RNA Transcription and Transfection

The plasmid encoding HCV (JFH1) was linearized by digestion with the restriction endonuclease XbaI (New England Biolabs), and subsequently treated with mung bean nuclease (New England Biolabs) to create blunt ends. This treated product was used as a template for RNA synthesis using a RiboMAX Large Scale RNA production system-T7 (Promega, Madison, WI). RNA was extracted by the acid guanidium thiocyanate phenol-chloroform (AGPC) method using Trizol (Ambion). 10
micrograms of extracted JFH1 RNA was electroporated into Huh7 or Huh7.5.1 cells. Huh7 or Huh7.5.1 cells were trypsinized and washed with ice-cold PBS and resuspended at 1x10⁷ cells/ml in Cytomix buffer. Synthesized RNAs were mixed in 0.4 ml of cell suspension in a 4-mm gap electroporation cuvette (Genesee Scientific, San Diego, CA), pulsed at 260 V for 25 ms in a square wave mode using a Gene Pulser Xcell electroporation system (Bio-Rad, Hercules, CA).

2.10 Luciferase Assay

Huh 7 or Huh 7.5.1 cells infected with chimeric JC1-RLuc 2A were lysed using luciferase lysis buffer (.1M potassium phosphate, 0.5% NP-40, 1mM DTT). Lysates were then measured for luciferase activity using the Oicomp I luminometer (Gem). Lysates were mixed with luciferase assay buffer (0.1M potassium phosphate, 5mM ATP, 0.1mM DTT, 15mM MgSO₄) and the renilla luciferase substrate (Promega). Luminosity was measured for one minute.

2.11 ssHRP Assay

Huh7 or Huh7.5.1 cells were transfected with the ssHRP plasmid, a kind gift of Dr. Vivek Malhotra (Barcelona, Spain) following the DNA transfection protocol as above. Media was changed 18 hours post-transfection. In the case of DNA co-transfection, culture supernatants were collected at 32, 40, and 48 hours post-transfection for assay of HRP activity. For RNA transfection, cells were seeded to result in half
normal density, and transfected with ssHRP. 18 hours post-DNA-transfection, media was changed to 10% FBS containing no antibiotics. Cells were then transfected with siRNAs according to the RNA transfection protocol as above. 48 hours post-RNA-transfection, culture supernatants were collected and assayed for HRP activity. 100 μl of supernatant was added to 50 μl 1X TMB substrate solution (eBiosciences). Supernatants were incubated for five minutes in the presence of substrate. Colorimetric reaction was halted by the addition of 50 μl of 0.5N sulfuric acid. Absorbance at 450 nm was measured using an ELx800 Universal Microplate Reader (Bio-Tek Instruments Inc).

**2.12 Cell Viability Assay**

Cell viability was measured using the *In Vitro* Toxicology Assay Kit, Resazurin based (Sigma). Resazurin reagent was added at 1:10 dilution to transfected cells at 48 hours post-transfection. Media was incubated in presence of cells for 4 hours, and color change from oxidized state (blue) to reduced state (red) measured. The color change is a measure of living cells' bioreduction ability. Absorbance was measured at 600 nm using an Ultrospec 1000 UV/visible spectrophotometer (Pharmacia Biotech).
Chapter 3

Results: Modification of Golgi-Localized PI4P Levels

3.1 Expression of hSac1 K2A Reduces PI4P Levels at the TGN

Since a number of PIPs and PIP-associated proteins such as kinases and binding proteins have been associated with various aspects of the HCV lifecycle, we were interested in looking at the role of PI4P in the process of viral secretion. In addition, morphological changes to the Golgi often occur in HCV infected cells, where the Golgi displays a fragmented phenotype (Figure 3.1). The bulk of total cellular PI4P localizes to the Golgi, and PI4P is by far the most abundant PIP in the Golgi itself. The PI4P pools localized at the Golgi play a critical role in standard Golgi secretory functions, and serve to anchor a number of proteins important for maintaining the lipid composition of the organelle [75, 126, 167]. Thus the targeted reduction of Golgi PI4P seemed like an ideal place to start to see if HCV secretion was dependent upon those PI4P pools. hSac1 is a phosphatase that normally localizes to both the ER and Golgi, and de-phosphorylates
Figure 3.1: Golgi Fragmentation Induced by HCV Infection
HCV infected Huh7 cells were immunostained with HCV envelope protein E2 and trans-Golgi marker TGN46. The nuclei were counter stained with DAPI. Courtesy of A. Siddiqui.
PI4P to PI [92]. The phosphatase activity is very specific, though it has some limited activity for the other PIPs phosphorylated at the 4-position, PI(3,4)P$_{2}$, PI(4,5)P$_{2}$, and PI(3,4,5)P$_{3}$. This activity is also less consequential due to the protein's normal localization, where PI4P is the predominant PIP.

Since we were interested in investigating the role of Golgi-specific pools of PI4P, the standard hSac1 protein would be not entirely useful for the study of viral secretion as under standard conditions a large fraction of the cellular hSac1 localizes to the ER [92]. PI4Ps in the ER, along with the kinase PI4KIII$\alpha$, have been found to be important for the establishment of proper HCV replication [77, 114]. To restrict hSac1 to the Golgi alone, we took advantage of a protein localization mutant called hSac1 K2A. Two lysine residues at the C-terminal KEKID domain of wild-type hSac1 were mutated to alanine to form the mutant protein designated hSac1 K2A [92]. The KEKID domain is normally responsible for localizing the protein to the ER, and the two lysine residues are crucial. Without the functional KEKID domain, hSac1 K2A localizes almost entirely to the Golgi compartment and retains its full phosphatase activity (Figure 3.2) [92]. Additionally, the hSac1 K2A was fused to an EGFP marker to allow for easier cellular localization. We first tested to ensure that hSac1 K2A was bringing down PI4P levels in the cell in a directed manner.

Huh7 cells were plated on glass coverslips to ensure roughly 50% confluence on the day of harvesting. After 24 hours to adhere to the coverslips, the cells were transfected with 5 $\mu$g of either hSac1 K2A EGFP, or an EGFP-expressing empty plasmid. Transfection was performed according to the procedure described in Materials and Methods. Two days post-transfection, media was removed, coverslips washed with PBS,
Figure 3.2: hSac1 K2A Expression Reduces PI4P Levels and Alters Golgi Morphology

Cells expressing hSac1 K2A EGFP (green) were plated on coverslips and stained for both PI4P and TGN marker TGN46 (red). Cells expressing hSac1 K2A EGFP are marked with arrows, while untransfected cells are noted with a star.
and the cells fixed with 4% PFA in PBS. The cells were stained for PI4P, as well as Golgi marker TGN46, in addition to the natural fluorescence of the hSac1 K2A EGFP. Expression of hSac1 led to reduction of the PI4P pools selectively in the Golgi, with little effect on the non-Golgi PI4P pools. (Figure 3.3, A, see the TGN46+PI4P panel). In the areas where hSac1 K2A is expressed (green), there is no evidence of PI4P (blue). Huh7 cells infected with HCV were also similarly transfected, to ensure that the hSac1 K2A activity remained constant in the presence of infection. In agreement with several previous reports, we first observed the robust stimulation of total cellular PI4P pools in HCV infected cells (Figure 3.3, B, see the uninfected and HCV infected cells in the PI4P panel) [77]. Transient expression of hSac1 K2A effectively reduced the levels of Golgi-localized PI4P in both uninfected and HCV-infected cells, without reducing the levels in areas where hSac1 K2A was not expressed (Figure 3.3, B, PI4P and hSac1 K2A-EGFP panel).

In addition, in concurrence with previous reports, the morphology of the Golgi is altered in hSac1 K2A expressing cells [92, 93]. Normally, the TGN has a fairly tight, perinuclear distribution in Huh7 or Huh7.5.1 cells (Figure 3.2, B, untransfected cells marked with stars). However, in the cells expressing hSac1 K2A, the Golgi distribution changes, becomes more ball-like, and tends to localize to one side of the nucleus more readily (Figure 3.2, B, transfected cells marked with arrows). This change in morphology has been associated with reduced ability of the cell to bud vesicles normally, and can interfere with cellular trafficking [92, 93].
Figure 3.3: Microscopy of hSac1 K2A in Huh 7.5.1 Cells
A) Huh 7.5.1 cells were transfected with hSac1 K2A EGFP (green) were plated on coverslips and stained with markers for PI4P (blue), TGN46 (red), and DAPI (cyan). B) Huh 7.5.1 cells infected with HCV JC1 virus were transfected with hSac1 K2A EGFP (green) and plated on coverslips. Cells were stained for HCV E2 protein (cyan), TGN46 (blue), and PI4P (red). Cell nuclei are outlined with white ovals, and hSac1 K2A expressing cells noted with arrows.
3.2 PI4P Reduction Alters Various Golgi Secretory Pathways

Since PI4P is a major modulator of Golgi secretory capacity, we wanted to investigate if the Golgi-specific depletion of PI4P by ectopic expression of hSac1 K2A affected the conventional secretory capacity of the cell. The ssHRP construct is a secreted form of horseradish peroxidase, in which the enzyme is fused to a signal sequence, which is responsible for the protein’s secretion. ssHRP is thought to follow a traditional, small protein secretion pathway through the TGN. Measuring the HRP activity of the culture supernatants provides a quick and easy measure of the secretory capacity of the transfected cells. Huh7 cells were co-transfected with ssHRP and either hSac1 K2A or a control, GFP-expressing plasmid. Culture supernatants were collected at 32, 40, and 48 hours post-transfection and subsequently analyzed for HRP activity. As expected we observed a dramatic reduction in the secretion of ssHRP in cells expressing hSac1 K2A compared to the untransfected control cells (Figure 3.4, A). This shows that standard secretion is heavily impaired by the removal of PI4P from the TGN.

Given the preponderance of evidence suggesting a link between HCV secretion and VLDL, we also sought to establish whether PI4P depletion in the TGN had any effect on VLDL secretion. VLDL represent a large cargo containing a heavily lipided apolipoprotein B, triglycerides, a triacylglycerol-rich lipid droplet, and often apolipoprotein E [154]. Its secretion pathway, though not fully elucidated, is thought to differ from traditional small protein secretion such as ssHRP. The VLDL secretion is also asdfad
Figure 3.4: hSac1 K2A ssHRP Assay. 
(A) ssHRP Assay. Cells expressing a secreted horseradish peroxidase (HRP) were transfected with a control vector or hSac1 K2A. HRP activity in the culture supernatants was measured at 32, 40, and 48 hours post-transfection. (B) Cell viability was assessed for hSac1 K2A transfected cells via a resazurin-based toxicology assay, as described in Materials and Methods.
likely to involve the Golgi, but the specific mechanisms are still unknown [46]. VLDL secretion is evaluated by Western blot analysis of apolipoproteinB-100 (ApoB), the major component of VLDL, in the cell culture supernatants. Serum-free culture supernatants of Huh7 cells transfected with hSac1 K2A were collected at 48 hours post-transfection and analyzed by Western blot. hSac1 K2A expression resulted in a modest decrease in the levels of secreted ApoB in the culture supernatants compared to untransfected control cells (Figure 3.5, A-D). Collectively, our results demonstrate that the depletion of Golgi-localized PI4P disrupts the secretion capacity of the Golgi apparatus resulting in reduced secretion of cellular secretory components traversing diverse Golgi secretory pathways.

3.3 hSac1 K2A Expression Inhibits HCV Secretion Without Affecting Replication

Given the inhibition of Golgi secretion by hSac1 K2A, we were interested in investigating the effect of Golgi-specific depletion of PI4P in HCV-infected cells. One of the primary concerns was attempting not to affect the replication of the virus, so as to isolate the cellular effects on viral secretion. Huh7.5.1 cells were infected with HCVcc (JC1 RLuc 2A) supernatants and passaged for five days. JC1 RLuc allows for the quick assessment of viral replication in HCV-infected cells. A RLuc reporter was added to the JC1 chimeric construct in between the p7 and NS2 domains. Containing its own promoter, the RLuc activity of the cell lysates provides an accurate measure of the level of viral replication ongoing in the cells. This reporter reduces the replication ability of the JC1 virus significantly due to the increased RNA load required for synthesizing a new
Figure 3.5: Effect of hSac1 K2A on ApoB Secretion. Secreted (A) and intracellular (B) apolipoprotein B was measured by Western blot, and the resulting images quantified by band intensity (C-D).
genome, but the main pathways and assembly/export of the virus are unaffected and infectious viral particles are produced. At day 6 post-infection, where viral production is beginning to approach its maximum, cells were transfected with either hSac1 K2A or a control vector. HCV replication was then measured by quantification of the RLuc activity of the cell lysates as described in Materials and Methods. hSac1 K2A expression had no significant effect on the luciferase activity of the cell lysates, suggesting that Golgi-specific depletion of PI4P pools did not affect HCV replication (Figure 3.6, A).

The most efficient and useful assay for determining viral infectivity is the foci-forming unit (FFU) assay. Since the JC1 system produces infectious viral particles in Huh 7.5.1 cells, the culture supernatants accumulate viral particles, and can subsequently be used in serial dilutions to infect naive cells. Huh 7.5.1 cells can be gently lysed using repeated freeze-thaw cycles in order to release fully infectious HCV viral particles that are resident within the cells. The analysis of both of these components serves to isolate all of the infectious virions produced by an infected cell culture population. After three days incubation, the newly-infected cells are stained for HCV E2 protein, which is broadly expressed in infected cells. The FFU assay is the most useful for studying viral secretion, because in order to produce infectious supernatants, the initial steps of viral replication, assembly, and maturation must all take place. We wanted to examine the effect of hSac1 K2A-mediated depletion of the Golgi-localized PI4P pool on HCV virion secretion using the FFU assay. Huh7.5.1 cells were infected with the highly infectious JC1 virus and subsequently transfected at day six with hSac1 K2A. Media was changed the day after transfection, and then allowed to accumulate virus for 48 hours. Both the culture supernatants and the cell lysates were collected and analyzed for viral infectivity by FFU
Figure 3.6: hSac1 K2A Affects HCV Secretion but not Replication.

(A) JC1-RLuc 2A-infected Huh 7.5.1 cells were transfected with hSac1 K2A or a control vector, and replication levels measured by renilla luciferase activity. (B-C) JC1-infected Huh 7.5.1 cells were transfected with a control vector or hSac1 K2A. Supernatants (B) and lysates (C) were collected and used to infect naive cells in an FFU assay.
assay. The culture supernatants from transfected cells were found to be significantly less infectious than those transfected with a control plasmid (Figure 3.6, B). However, the intracellular infectivity derived from the lysed cells remained high. In fact, the intracellular infectivity actually increased in similar magnitude to the observed decrease in the extracellular infectivity (Figure 3.6, C). Taken together, these data suggest that PI4P depletion from the Golgi inhibits HCV secretion, and subsequently that the assembled and infectious viral particles are retained within the cell.

3.4 Silencing of PI4 Kinase IIIβ Alters Cell Morphology and Secretion

As an alternative to the overexpression of the hSac1 K2A phosphatase to reduce the levels of Golgi PI4P in the cell, we also wanted to examine whether the Golgi-localized PI4 kinases (PI4Ks) had a specific role in the HCV secretion process. There are two kinases of two separate sub-types responsible for phosphorylating PI to PI4P at the Golgi complex, PI4KIIIβ and PI4KIIα. Of these, PI4KIIIβ is thought to be the one primarily responsible for maintaining the levels of PI4P throughout the TGN [76]. We examined the cellular distributions of PI4KIIIβ in infected cells, and found that PI4KIIIβ does indeed localize well to the Golgi in HCV infected cells, and co-localizes with PI4P markers as well (Figure 3.7, B and C). We chose to focus on silencing PI4KIIIβ in infected and uninfected cells via an siRNA. Huh 7.5.1 cells were transfected with either a control siRNA or an siRNA against PI4KIIIβ. Cells were harvested after 72 hours, and RNA extracted by the AGCP method using Trizol, as described in Materials and Methods.
Figure 3.7: Microscopy of HCV Infected Cells
HCV infected cells were plated on coverslips, fixed, and stained for (A) HCV NS5A (cyan), GOLPH3 (red), GM130 (green), and DAPI (blue). (B) HCV NS5A (cyan), PI4KIII β (red), TGN46 (green), and DAPI (blue). (C) HCV E2 (cyan), PI4P (red), TGN46 (green), and DAPI (blue). Infected cells are noted by arrows, while uninfected cells are marked with an ’x’.
mRNA levels were analyzed by RT-PCR and quantified to assess the efficacy of the PI4KIIIβ siRNA knockdown. Two different siRNAs and a pool of both siRNAs, were used and achieved variable rates of knockdown (Figure 3.8). As the second siRNA was essentially as effective as the pooled siRNAs, we used siRNA 2 for most of the rest of the experiments performed knocking out PI4KIIIβ.

Microscopy was performed on cells transfected with either a control siRNA or siRNA 2 against PI4KIIIβ. Cells were stained for TGN marker TGN46, as well as PI4KIIIβ, and PI4P (Figure 3.9, B and C). The transfection efficiency of the siRNAs was nearly 100%, which was quantified using a fluorescently-labeled siRNA additive (data not shown). In the untransfected cells, PI4KIIIβ (green) co-localizes very well with both the trans-Golgi marker TGN46 (red) as well as PI4P (blue) at the Golgi (Figure 3.9, A, TGN46+PI4KIIIβ and PI4KIIIβ+PI4P panels). The Golgi retains its characteristic perinuclear morphology. However, in the siRNA transfected cells, nearly all the PI4KIIIβ is gone from the cells, with just a small fraction of green fluorescence remaining in the cells (Figure 3.9, B, DAPI+PI4KIIIβ panel). In addition, the majority of PI4P (blue) is also gone from the cells where PI4KIIIβ has been knocked down (Figure 3.9, B, DAPI+PI4P and PI4P+PI4KIIIβ panels). The efficacy of the knockdown, and the subsequent reduction in PI4P, is also represented by the quantification of the total fluorescence intensity of the siRNA-treated cells for both PI4KIIIβ and PI4P (Figure 3.10, A and B). The small amounts of PI4P remaining in the cells does not appear to co-localize with TGN46, suggesting that the PI4P reduction is primarily Golgi-localized, with the remaining PI4P signal likely representing non-Golgi pools of PI4P (Figure 3.9, B, TGN46+PI4P panel). Finally, the Golgi morphology in the siRNA transfected cells is
Knockdown of PI4KIIIβ

Huh 7.5.1 cells were transfected with siRNAs against a control vector, or against PI4KIIIβ. PI4KIIIβ levels were measured by RTPCR.
Figure 3.9: Microscopy of siPI4KIIIβ-treated Huh 7.5.1 Cells
Huh 7.5.1 cells were transfected with either a control siRNA (A), or siRNA against PI4KIIIβ (B). Cells were subsequently plated, fixed, and stained for DAPI (cyan), PI4KIIIβ (green), PI4P (blue), and TGN46 (red). Merges are as marked.
Figure 3.10: Quantification of PI4KIIIβ and PI4P Levels in PI4KIIIβ-Silenced Cells
Quantification of silencing efficiency from Figure 3.9. (A) PI4KIIIβ fluorescence intensity at the Golgi. (B) PI4P fluorescence intensity at the Golgi. Intensity was quantified using ImageJ software.
distinctly different than those of the control transfected cells. From a tight, perinuclear morphology, the Golgi condenses towards one side of the cell, and loses some of its tight distribution (Figure 3.9, B, TGN46 panels). This altered morphology is similar to that seen in the hSac1 K2A-expressing cells where the PI4P levels are also similarly reduced (Figure 3.3, A).

In addition to observing similar morphological changes to the Golgi, we also sought to observe any effects that the knockout of PI4KIIβ had on standard secretion pathways from the cell. Huh 7.5.1 cells were DNA transfected with the ssHRP plasmid, then treated the following day with control siRNA or siRNA against PI4KIIβ. 48 hours post-RNA-transfection, culture supernatants were collected and assayed for HRP activity. The silencing of PI4KIIβ resulted in a decreased supernatant HRP activity, showing that small protein secretion is affected by the knockout (Figure 4.4, A). The magnitude of the result is somewhat smaller than that observed in the hSac1 K2A expressing cells. The reasons for the smaller effect size may be twofold. First of all, the DNA co-transfection of ssHRP and hSac1 K2A is likely more efficient than the sequential and separate DNA and RNA transfections using siRNA. Every single cell that received an ssHRP plasmid was also highly over-expressing hSac1 K2A, a highly effective phosphatase, which likely severely affected the HRP accumulation in the culture supernatants. Secondly, the knockdown of PI4KIIβ may not have resulted in the total abrogation of PI4P levels at the Golgi. PI4KIIα may play a substitute role for maintaining some low levels of PI4P in the absence of PI4KIIβ at the Golgi. In addition, the regulation of PI4P levels is in the cell is dynamic, so that in response to the loss of PI4KIIβ activity, PI4P phosphatases may have been somewhat downregulated to retain some presence of PI4P in the Golgi that was not
seen with the overexpression of hSac1 K2A. siRNA-treated cells were also assayed for the levels of VLDL (via ApoB expression) in the culture supernatants, but the levels were found to be similar to those of the control cells, reflecting the fact that VLDL particles and small proteins may follow different secretion pathways through the cell. VLDL secretion may not utilize the same PI4P-mediated secretory pathway through Golgi network that HCV maturation and vesicular trafficking does.

3.5 PI4P Reduction by Silencing of PI4KIIIβ Negatively Affects HCV Secretion

In order to assess the effect of PI4KIIIβ on the HCV secretion pathway, HCV JC1-infected Huh 7.5.1 cells were transfected with a control siRNA or siRNA against PI4KIIIβ. Cell lysates were collected 72 hours post-transfection and assayed via RTPCR to assess any effect of PI4KIIIβ knockdown on viral replication. Viral RNA levels were unaltered by PI4KIIIβ knockdown, showing that PI4KIIIβ does not appear to affect HCV replication (Figure 3.11, A). This lack of effect on replication was also confirmed in an experiment where PI4KIIIβ knockdown was effected prior to infection (Figure 3.12). Uninfected Huh 7.5.1 cells were treated via siRNA against PI4KIIIβ or a control siRNA, and subsequently infected with HCV JC1 supernatants 24 hours later. After 48 hours of exposure to the infectious viral particles, cell lysates were collected and again analyzed via RTPCR for viral replication. This experiment was designed to test the effect of PI4KIIIβ knockdown on the early stages of viral replication, before a full-fledged infection had occurred and cells were already producing and secreting infectious virus.
Figure 3.11: PI4KIIIβ Silencing Affects HCV Secretion Without Affecting Replication

(A) JC1 HCV infected Huh 7.5.1 cells were transfected with control siRNAs or siRNA against PI4KIIIβ, and cell lysates analyzed by RTPCR to assess HCV replication. (B-C) JC1-infected Huh 7.5.1 cells were transfected with a control siRNA or siRNA against PI4KIIIβ. Supernatants (B) and lysates (C) were collected and used to infect naive cells in an FFU assay.
Figure 3.12: Pre-Silencing of PI4KIIIβ Prior to HCV Infection Does Not Affect Replication

Uninfected Huh 7.5.1 cells were pre-transfected with either control siRNA or PI4KIIIβ-targeted siRNA and subsequently infected with HCV JC1 virus. Replication levels were measured after 72 hours via RTPCR.
Silencing of PI4KIIIβ in naive cells prior to infection also had no significant effect on viral replication (Figure 3.12).

In addition to the RNA quantification, viral supernatants and lysates were collected and used to infect naive cells via FFU assay. Reduction in Golgi PI4P levels by knockout of PI4KIIIβ lowered the amount of secreted HCV (Figure 3.11, B). This effect on viral secretion occurred concomitantly with an increase in the intracellular viral infectivity assessed on the freeze-thaw treated cell lysates (Figure 3.11, C). This effect is similar to the one seen with the hSac1 K2A expressing cells, though the magnitude of the effect is somewhat larger, likely reflecting the increased transfection efficiency of the small siRNAs as opposed to the larger DNA plasmid. Taken together, these data suggest that when the PI4P levels in the Golgi are reduced, HCV is unable to efficiently exit the cell. It appears as though the preliminary steps of viral assembly are mostly unaffected, however the intracellular viral titer remains high, and in fact increases. This points to a specific block in the secretion pathway of HCV where viral particles prevented from exiting the cell are retained within the cell, thereby increasing the intracellular viral titer.

3.6 Silencing of Both PI4 Kinases Does Not Augment Suppression of Secretion

In order to assess any effect that different PI4 kinases may have on the PI4P populations of the Golgi, we were also interested in PI4KIIα. PI4KIIα also localizes to the Golgi, though it is less abundant and generally thought to play a less important role in the regulation of PI4P levels in the Golgi [76]. We were curious as to the effect that
knockdown of PI4KIIα would have in concert with the knockdown of PI4KIIIβ and whether any synergistic effect might be achieved by knocking down the levels of both Golgi resident kinases. Correspondingly, Huh 7.5.1 cells were transfected with either a control siRNA, an siRNA against PI4KIIα, siRNA against PI4KIIIβ, or a dual knockdown pool of siRNAs against both PI4KIIα and PI4KIIIβ. PI4KIIα levels were quantified by Western blot (Figure 3.13). PI4KIIα was knocked down effectively by the siRNA.

Huh 7.5.1 cells infected with JC1 were then silenced for each of the kinases separately and for the combined double silencing, and cell lysates and supernatants collected. Lysates and supernatants were subjected to RTPCR for analysis of viral RNA levels. Viral replication was unaffected by PI4KIIα silencing, or the dual knockdown (Figure 3.14, A). Culture supernatants, however, showed a reduction in viral RNA in both the single knockdowns as well as the dual knockdown, though there did not appear to be any additive effect on secretion suppression when both kinases were knocked down (Figure 3.14, B). Lysates and supernatants from transfected cells were then used to infect naive cells in an FFU assay. When looking at the extracellular viral titer, the silencing of PI4KIIα appeared to affect viral secretion, as did the dual silencing of both PI4KIIα and PI4KIIIβ, though there did not appear to be any synergistic difference between the double knockdown and that of the single kinase knockdown (Figure 3.15, A and B). However, taking the intracellular viral infectivity into account, the knockdown appears to have had more of an effect on viral assembly or maturation than on secretion. Knockdown of PI4KIIα, or the double knockdown, both lowered the intracellular infectivity significantly. This is in contrast to the effect of the knockdown of PI4KIIIβ, which caused
Figure 3.13: Silencing of PI4KIIα Via siRNA
Huh 7.5.1 cells were transfected with either a control siRNA, siRNA against PI4KIIβ, PI4KIIα, or both siRNAs. PI4KIIα levels were measured via Western blot, as well as a loading control, Calnexin.
Figure 3.14: Silencing of PI4KIIα Affects Secretion But Not Replication
HCV JC1 infected Huh 7.5.1 cells were transfected with either a control siRNA or siRNAs against PI4KIIβ, PI4KIIα, or both siRNAs. RNA was extracted from cell lysates (A) or culture supernatants (B) and analyzed for HCV RNA via RTPCR.
Figure 3.15: PI4KI\(\alpha\) Silencing Reduces Both Intracellular and Secreted HCV Infectivity

HCV JC1 infected Huh 7.5.1 cells were transfected with either a control siRNA or siRNAs against PI4KIII\(\beta\), PI4KII\(\alpha\), or both siRNAs. Culture supernatants (A) and cell lysates (B) were used to infect naive cells in an FFU assay.
the intracellular infectivity to increase (Figure 3.11, C, Figure 3.15, B). This data, taken together, suggests that PI4KIIIβ plays an important role in viral secretion, and its abrogation can cause the retention of infectious virus within the cell. PI4KIIα, on the other hand, seems to have some effect on the processes occurring prior to viral secretion, possibly at some stages of maturation of the virus, and only a limited effect on viral secretion.

### 3.7 Summary

The results of our studies discussed above suggest that the Golgi-specific pools of PI4P play a crucial role in the secretion of HCV. Overexpression of hSac1 K2A phosphatase, along with the siRNA-mediated knockdown of the primary Golgi PI4 kinase, PI4KIIIβ, appears to have very similar effects on both the Golgi morphology, secretion ability, and the ability of HCV to exit the cell in a normal fashion. Reducing the levels of PI4P at the Golgi, through multiple methods, results in the retention of viral particles within the cell. This points to the general import of the virions through Golgi, and raises the question of potential other facilitators of the secretion process, which utilize PI4P, in the overall schemes of HCV exit.

Chapter 4

Results: Examination of PI4P Binding Proteins Affecting HCV Secretion

4.1 PI4P Binding Proteins at the Golgi

With the observation that PI4P levels at the Golgi have a strong effect on HCV secretion, we were interested in determining whether Golgi-localized PI4P binding proteins played any role in the process of HCV exit. There are only a few proteins known to bind to PI4P at the Golgi, most of them utilizing a pleckstrin homology (PH) domain to bind PI4P [71, 75]. The known Golgi-localized PH-domain containing proteins are oxysterol binding protein (OSBP), ceramide transporter (CERT), and four phosphate adapter proteins 1 and 2 (FAPP1 and FAPP2) [71, 75]. The functions of two of these proteins, OSBP and CERT, in the HCV lifecycle have already been explored by Yutaka Amako in our laboratory. In addition, a novel PI4P binding protein called GOLPH3 was recently characterized by the Field lab and colleagues, which also uses PI4P to localize to the Golgi [93]. MYO18A is an interacting partner of GOLPH3 which, though not binding PI4P directly, plays an important role in the structure and function of the Golgi. Here, we
investigated the roles of GOLPH3, its binding partner MYO18A, and the possible roles of FAPP1 and FAPP2 in HCV secretion.

4.2 The Role of OSBP and CERT in the HCV Lifecycle

OSBP is a sterol sensor and helps transport cholesterol and hydroxycholesterol from their sites of synthesis in the ER into the Golgi [168, 169]. OSBP can bind to both the ER and Golgi through its FFAT and PH domains, respectively, and is able to help form membrane contact sites between the two organelles [96]. When it binds cholesterol, it can translocate from the ER to the Golgi to facilitate the transfer of lipids. CERT has a high degree of homology with OSBP, and also functions as a lipid transporter, transferring ceramide to the Golgi from the ER. OSBP and CERT also function together to help with the synthesis and transport of sphingomyelin into the Golgi [170]. OSBP was identified during a screen to identify proteins associated with HCV replication at the viral RNP complexes and was found to play an important role in HCV replication, but also in HCV secretion.

OSBP was knocked down via shRNA in HCV-infected cells by two separate shRNAs (Figure 4.1, A). shRNA1 resulted in the nearly complete knock down of the OSBP protein, while the shRNA2 displayed only a partial knockdown (Figure 4.1, A). The complete removal of the OSBP protein from the infected cells had a strong effect on both HCV replication and HCV secretion. Replication was reduced substantially, and the secretion of HCV was entirely prevented (Figure 4.1, B and C). The complete abrogation of HCV secretion is likely due to the profound effect of OSBP knockdown on viral
Figure 4.1: OSBP Reduction Via shRNA Interferes with HCV Replication and Secretion

(A) OSBP was knocked down in Huh 7.5.1 cells, and mRNA levels assessed by RT-PCR. HCV RNA levels in culture supernatants (B) and cell lysates (C) were assessed by RTPCR. (D) Supernatant infectivity was measured by FFU assay. From Amako et. al, 2009, used with permission [126].
replication: with hardly any viral RNA being effectively produced, there was essentially none available to be secreted from the cell. The second shRNA however, displayed much less of an effect on HCV replication, as the knockdown of OSBP was only partial. The effect of OSBP knockdown on secretion, however, was more observable in the partial knockdown. shRNA two reduced the replication levels only about 14%, while the reduction in secreted virus, measured by both RTPCR and FFU assay, was around 87% (Figure 4.1, B-D). This shows that OSBP plays roles in both the viral replication and viral secretion process.

CERT has also been implicated in the process of HCV secretion by studies in our laboratory. Protein kinase D (PKD) is a Golgi-localized kinase which phosphorylates both OSBP and CERT. This phosphorylation disrupts the Golgi localization of these two proteins, and that disruption inhibits efficient HCV secretion [167]. HCV negatively regulates PKD activation in infected cells, and the overexpression of a constitutively active PKD reduces HCV secretion [167]. In addition, the inhibition of CERT also impaired HCV secretion [127]. These studies establish the importance of several PI4P binding proteins in the HCV lifecycle. In light of these results, we focused our inquiries on characterizing the effect the other PI4P binding proteins on HCV infection.

4.3 GOLPH3 Is a Newly Discovered PI4P Binding Protein

Golgi phosphoprotein 3, or GOLPH3, is a protein that localizes to the Golgi via PI4P without the use of the PH domain found in OSBP, CERT, and FAPP1 & FAPP2 [93,
GOLPH3 was shown to be crucial for efficient vesicle budding from the TGN, and for TGN to plasma membrane (PM) trafficking [93]. When GOLPH3 is knocked down, cargo is unable to be properly sorted to the PM [93]. In addition, the Golgi tends to lose its characteristic ribbon shape, and condenses into a more ball-like structure, similar to the Golgi structures we observed upon PI4P removal from the Golgi. GOLPH3 was also found to interact with the actin cytoskeleton, linking to it through an unconventional myosin, MYO18A [93]. When the interactions between GOLPH3 and PI4P, GOLPH3 and MYO18A, or MYO18A and the actin network are removed, similar phenotypes are observed, namely the condensation of the Golgi, inefficient vesicle budding, and the inhibition of TGN trafficking [93]. Each of these components, and their successful interactions, are required for normal Golgi morphology and function. It has been hypothesized that the interaction between GOLPH3, MYO18A and the actin network is responsible for the characteristic 'flattened stack' morphology of the Golgi, where actin can exert a tensile force on the edges of the Golgi stacks, and contributing to the budding of vesicles from the trans face of the Golgi [93]. GOLPH3 then seemed like an ideal candidate for investigation in the process of HCV secretion through the Golgi.

4.4 GOLPH3 and MYO18A Silencing Affect HCV Secretion

HCV infected Huh 7.5.1 cells were transfected with siRNAs against either GOLPH3 or a non-targeted control. 72 hours post-transfection, cell lysates and
supernatants were collected. Lysates were analyzed by Western blot for GOLPH3 knockdown, as described in Materials and Methods. Over 95% knockdown in expression levels was achieved over the control siRNA (Figure 4.2, A). RNA was also extracted from the cell lysates and supernatants, and the RNA levels of HCV quantified by RTPCR. Intracellular RNA levels, representing HCV replication, were mostly unaltered by knockdown of GOLPH3 (Figure 4.2, B). However, the levels of RNA in the culture supernatants was reduced following the knockdown, suggesting that GOLPH3 effectively participates or facilitates the HCV secretion process (Figure 4.2, C).

We subsequently were interested in measuring the infectivity of the culture supernatants and the lysates, as well as any effect that the binding partner to GOLPH3, MYO18A, might have on viral secretion. HCV infected Huh 7.5.1 cells were transfected with control siRNA, or siRNAs against GOLPH3 or MYO18A. Western blot analysis showed that knockdowns for both GOLPH3 and MYO18A were effective, with ~95% and ~70% knockdowns achieved, respectively (Figure 4.3, A). These knockdowns were also mostly harmless to the cell, as shown by the cell viability assay described in Materials and Methods (Figure 4.4, B). Cell lysates subjected to freeze-thaw cycles to release intracellular viral particles, and culture supernatants were also collected and used to infect naive cells in an FFU assay. GOLPH3 and MYO18A knockdowns both had the similar effect of reducing the infectivity of the culture supernatants (Figure 4.3, B). In addition, the intracellular viral infectivity was increased by GOLPH3 or MYO18A knockdown (Figure 4.3, C). Taken together, this suggests that, similar to the effect seen by reduction of Golgi PI4P levels, infectious viral particles failed to be secreted properly from the infected cell, and are instead being retained somewhere within the cell. The
Figure 4.2: GOLPH3 Silencing Affects HCV Secretion But Not Replication
HCV JC1 infected Huh 7.5.1 cells were transfected with control siRNA or siRNA against GOLPH3. (A) Cell lysates were analyzed for GOLPH3 levels by Western blot. Cell lysates (B) and culture supernatants (C) were analyzed for HCV RNA content via RTPCR.
Figure 4.3: Silencing of GOLPH3 and MYO18A Decreases Secreted Viral Infectivity
HCV JC1 infected Huh 7.5.1 cells were transfected with control siRNA or siRNA against GOLPH3 or MYO18A. (A) Cell lysates were analyzed for GOLPH3 and MYO18A levels by Western blot. Cell lysates (B) and culture supernatants (C) were collected and used to infect naive cells via FFU assay.
Figure 4.4: ssHRP Secretion is Affected by GOLPH3, MYO18A, PI4KIIIβ Silencing

(A) Huh 7.5.1 cells were transfected with ssHRP and subsequently transfected with control siRNA, or siRNAs against GOLPH3, MYO18A, or PI4KIIIβ. Culture supernatants were collected and analyzed for HRP activity. (B) Cell Viability Assay. Transfected cells were assessed for cytotoxicity via a resazurin based toxicology assay.
intracellular accumulation of completely assembled infectious virions (intracellular infectivity) in similar conditions also suggests that Golgi-associated vesicle sorting and secretory function are not required for the virus assembly process. GOLPH3 and MYO18A were also tested for any effect on general protein secretion using the widely used ssHRP secretion assay. Huh 7.5.1 cells were transfected with the ssHRP plasmid, then subsequently transfected 18 hours later with siRNAs against GOLPH3 or MYO18A. The culture supernatants were collected 48 hours after RNA transfection, and the HRP activity measured. GOLPH3 and MYO18A knockdowns also showed a reduction in the supernatant HRP activity, showing that standard TGN to PM trafficking was impaired, as reported previously (Figure 4.4, A)[93].

### 4.5 FAPP1 and FAPP2 Silencing Have Little Effect on HCV

FAPP1 and FAPP2 are two additional PI4P binding proteins, which use their PH domain to localize to the TGN [94]. FAPP2 protein has roles in Golgi trafficking, coordinates with CERT to assist in sphingolipid synthesis helping to transfer glucosylceramide, is thought to play a role in anterograde trafficking through the Golgi, and may play a role in membrane tubulation [79, 84, 171]. FAPP2 has also been shown to be important for apical sorting of cargo in polarized cells [172]. FAPP1 is less well characterized, but may act with FAPP2 to induce membrane curvature and tubulation [79]. Since FAPP2 has been shown to play a role in cargo sorting in polarized cells, we were interested in seeing what effects FAPP1 and FAPP2 knockdown might have on HCV infected cells.
siRNAs against either a control, FAPP1, FAPP2, or both FAPP1 and FAPP2 were transfected into HCV-infected Huh 7.5.1 cells. These cells were incubated for 72 hours. Cell lysates and culture supernatants were collected for analysis. RNA was extracted from cell lysates, and FAPP1 and FAPP2 mRNA levels quantified by RT-PCR. Significant but not complete knockdowns were achieved for both FAPP1 and FAPP2, as well as the combined FAPP1 and FAPP2 knockdown (Figure 4.5). HCV RNA levels were also quantified by RT-PCR, for both cellular lysates and culture supernatants. The knockdown of FAPP1 and FAPP2 appeared to have little effect on viral replication, or secretion of viral RNA (Figure 4.6). Freeze-thaw treated cell lysates, and culture supernatants were also collected to infect naïve Huh 7.5.1 cells in an FFU assay. The FFU assay showed a slight reduction of both the intracellular and secreted viral infectivity (Figure 4.7). This decrease was modest and appeared insignificant but may point to a role for FAPP1 and/or FAPP2 in the maturation or assembly of HCV.
Figure 4.5: RTPCR of FAPP1 and FAPP2 mRNA Levels
Huh 7.5.1 cells were transfected with either a control siRNA or siRNAs against FAPP1, FAPP2, or both FAPP1 and FAPP2. Cell lysates were collected and mRNA levels for FAPP1 (A) and FAPP2 (B) assessed via RTPCR.
Figure 4.6: FAPP1 and FAPP2 Silencing Has Little Effect on HCV Replication or Secretion

HCV JC1 infected Huh 7.5.1 cells were transfected with either a control siRNA or siRNAs against FAPP1, FAPP2, or both FAPP1 and FAPP2. Cell lysates (A) and culture supernatants (B) were analyzed by RTPCR for HCV viral RNA.
Figure 4.7: FAPP1 and FAPP2 Silencing Reduces Intracellular and Secreted Infectivity Slightly

HCV JC1 infected Huh 7.5.1 cells were transfected with either a control siRNA or siRNAs against FAPP1, FAPP2, or both FAPP1 and FAPP2. Cell lysates (A) and culture supernatants (B) were collected and used to infect naive cells via FFU assay.
4.6 Summary

It appears as though multiple varieties of TGN localized PI4P binding proteins, some with PH domains, some without, have effects on the HCV secretion pathway. OSBP and CERT, key components of several lipid transport and synthesis pathways, both affect HCV secretion, and may play a role in HCV replication as well. Alteration of the Golgi structure and vesicle budding by knocking down GOLPH3 or its unconventional myosin partner, MYO18A also appear to affect HCV secretion. In the case of GOLPH3 and MYO18A, the effect seen is similar to the elimination of PI4P from the Golgi, wherein infectious HCV particles appear to be retained within the cell rather than properly secreted. FAPP1 and FAPP2, however, do not seem to play a significant role in the secretion process, showing only modest reductions in both HCV intracellular and secreted infectivity. Taken together, these studies highlight the roles that both PI4P, and PI4P binding proteins, play in the egress of HCV, and pinpoint the Golgi as the key site through which infectious virions travel to effect their release from the cell.

Portions of this chapter are reprints of the material as it appears in the Journal of Biological Chemistry, 2012. Bishé B, Syed GH, Field SJ, Siddiqui A. Role of phosphatidylinositol 4-phosphate (PI4P) and its binding protein GOLPH3 in hepatitis C virus secretion. Journal of Biological Chemistry, 2012. The dissertation author was the primary investigator and author of the paper.
Chapter 5

Discussion

Though the last ten years have seen an incredible explosion in our knowledge of the structure, function, and host-cell interactions of hepatitis C virus and the cells it infects, many parts of the viral life cycle remain to be fully characterized. One of the least studied aspects of this lifecycle is the viral secretion pathway. There are a number of reasons that we became interested in this topic, and why phosphoinositides, PI4P in particular, seemed fascinating targets for examination in the process of viral egress. One of the main indicators is the prevalence of HCV-induced alteration of cellular lipid synthesis and transport pathways. The modification of host cell lipid composition is key for successful viral replication and assembly, including a large increase in the number of cellular lipid droplets to assist in the establishment of the infectious process [154, 162]. Further, it has been well demonstrated that HCV has some dependence on the cellular VLDL biogenesis pathway for its secretion [46, 47, 150, 164, 173]. The occurrence of VLDL associated viral particles (lipoviroparticles) in patients’ sera further strengthens the common notion that HCV and VLDL secretion possesses some areas of overlap and that HCV may co-opt the VLDL secretory pathway for its secretion [48-52]. Although the intricate details of VLDL biogenesis are still unclear, it is well known that the VLDL
particles traffic through the Golgi compartments, possibly implicating a similar pathway for HCV virion egress [48, 49]. In digression to this assumption, some recent reports have implicated a role for the endosomal secretory components and ESCRT machinery for HCV virion secretion, similar in fashion to HIV virion secretion [174, 175]. However it should be noted that there is considerable amount of crosstalk between the endosomal and Golgi secretory pathway [176]. The TGN acts as an interface between the Golgi and the endocytic system and cargo vesicles emanating from the TGN sorting hub are also targeted to recycling, early and late endosomes [176]. Hence it is highly plausible that both pathways serve the purpose of HCV virion secretion in sequential or coordinated fashion. A recent study identified Rab11A, a small GTP binding protein that modulates trafficking from recycling endosomes and the Golgi as required for HCV virion secretion [104]. Coller et al. also showed a link, similar to that characterized by our laboratory, between PI4KIIIβ and HCV secretion. Silencing of PI4KIIIβ was found by Coller et al to increase the ratio of intracellular to extracellular virus in HCV infected cells [104]. Essentially, PI4KIIIβ silencing promotes the retention of fully infectious viral particles within the cell, rather than their proper secretion. Further study of the viral protein dynamics also implicated several members of the Golgi secretory pathway of the cell in HCV secretion, as well as providing images of HCV core protein in transit [104].

Nonvesicular lipid transport between the ER and Golgi affects the lipid composition of the Golgi membranes [96]. The alteration of lipid composition being a hallmark of HCV infection, proteins involved in the transport and maintenance of lipids in various cellular membranes obviously deserve scrutiny when attempting to characterize the processes of HCV assembly, maturation, and secretion. We and others
have shown that Golgi-associated OSBP and CERT are required for HCV virion secretion [126, 127]. OSBP is a sterol sensor and transporter and maintains cholesterol levels in the Golgi membrane, as well as playing a role in membrane contact sites between the ER and Golgi [126, 168]. CERT transfers ceramide, in concert with OSBP, from ER to the Golgi where it is used for synthesis of sphingomyelin [167, 177]. Both cholesterol and sphingomyelin are enriched in HCV virions, suggesting that the HCV virion envelope becomes enriched in these lipid molecules during their egress, or that the virion envelope may be derived from trans-Golgi membrane [129]. It is worth noting again that both OSBP and CERT localize to the TGN primarily through their PI4P-binding PH domains. The participation of both of these elements of the lipid transfer pathway highlighted our interest in PI4P as a possible component of the HCV secretion pathway.

Another interesting element to the puzzle of HCV secretion is protein kinase D. PKD, a Golgi kinase and a key component of PI4P regulated pathways at TGN, negatively regulates HCV secretion by triggering phosphorylation of OSBP and CERT, thereby inhibiting their function. In correlation with this hypothesis we observed that HCV downregulates PKD activity [167]. Since PKD is actually implicated in the activation of PI4KIIIβ by phosphorylation at the TGN, our observations suggest that although HCV reduces general secretion via the TGN, it likely modulates the local lipid composition of the TGN to specifically enhance HCV virion secretion [47, 167]. Interestingly, we also observed that in HCV infected cells the Golgi apparatus displays a dispersed and fragmented pattern (Fig. 3.1), which may contribute to a decrease in general secretion capacity. This ultrastructural change also hints towards plausible sequestration of Golgi compartments at the site of virion assembly for efficient secretion.
of the assembled virions. These observations strongly emphasize the major role of the Golgi network in HCV virion secretion. How HCV secretion benefits from Golgi fragmentation remains to be characterized. To further strengthen our hypothesis, we investigated the role of Golgi-localized PI4P, its major kinases, and several other proteins that utilize PI4P to situate themselves in their proper TGN location. We examined the recently identified PI4P interacting Golgi protein GOLPH3 and its binding partner MYO18A in HCV secretion process, as well as possible roles for the FAPP proteins.

PI4P pools at the TGN regulate important functional and structural attributes of Golgi complex [100, 113]. siRNA screening for host factors revealed that PI4Ks are essential for HCV proliferation [99-102, 112]. Since ER-localized PI4 kinase, PI4KIIα has been shown to be important for viral induced membrane rearrangement and HCV replication, we primarily focused on investigating the role of Golgi-localized PI4P pools [77, 100]. We took advantage of Golgi-specific hSac1 K2A phosphatase to specifically deplete PI4P from the TGN. Reduction in Golgi PI4P levels reduced secretion from the cell generally, both in small protein secretion (ssHRP) as well as VLDL secretion. In the case of HCV infection, the depletion of the Golgi-localized PI4P pools reduced HCV secretion, but had no notable effect on viral replication. Although the extracellular infectivity (secreted virus titers) decreased, we observed a concomitant increase in the intracellular infectivity (intracellular virus titers) upon ectopic expression of hSac1 K2A suggesting that the viral replication and assembly processes are not affected but that there is stall in virus secretion leading to intracellular accumulation of assembled virus particles. The FFU assay, which revealed the levels of intracellular and extracellular infectivity, proved to be the most informative experiment for determining the effect of
disabling the Golgi structure and function on HCV secretion. The FFU assay allows us to exclusively examine the possible roles of host factors on viral secretion. RT-PCR analysis allowed us to confirm that replication in infected cells underwent little change through these modifications, while the increase in intracellular infectivity, which represents unsecreted virions, indicated that the assembly of the virus was occurring mostly unimpeded.

We did not achieve particularly high levels of expression of hSac1 K2A and the ectopic expression was slightly deleterious to the cells, contributing to widespread changes in Golgi morphology particularly as large amounts of hSac1 K2A were expressed in individual cells. This was likely a result of the typically highly over-expressed proteins. For this reason, as well as to increase the effect size on HCV secretion, we chose to examine the PI4P levels at the TGN by silencing PI4P kinases at the Golgi. We investigated the effect of depleting TGN-specific pool of PI4P by siRNA mediated knockdown of the predominantly active Golgi-resident PI4P kinase, PI4KIIIβ. Knockdown of PI4KIIIβ mimicked the effect seen with hSac1 K2A overexpression; a decrease in extracellular viral infectivity accompanied by an increase in intracellular infectivity. The accumulation of infectious virion in the cell under these conditions suggests that the Golgi-associated PI4P pools modulated by PI4KIIIβ, and Golgi-specific vesicle sorting and transport functions are not required for virion morphogenesis or assembly.

Analysis of the other Golgi-associated PI4 kinase, PI4KIIα, was also performed, both independently and in concert with PI4KIIIβ. We were interested in determining if there was a possible synergistic effect to be attained by knocking down both kinases to
attempt to further reduce Golgi PI4P levels, and hence HCV secretion. The knockdown of PI4KIIα, and the dual knockdowns of PI4KIIα and PI4KIIIβ again had no effect on viral RNA levels, showing that replication appears to be unaltered by the Golgi resident kinases, as expected. The secretion levels of HCV were both reduced by the knockdown of each kinase, as well as the dual knockdown. We did not observe any additive effect by knocking down the kinases together, rather than separately, as we might have expected. This is likely due to the fact that the PI4KIIIβ knockdown was quite effective at removing most PI4P from the TGN membranes (Figure 3.9, B). PI4KIIIβ is thought to contribute more to the maintenance of TGN PI4P pools than PI4KIIα, so this is not entirely unexpected. What was unexpected, however, was the effect that the single and dual knockdowns of PI4KIIα had on the intracellular viral titer. Knockdown of PI4KIIIβ decreased the infectivity of the culture supernatants, while increasing the infectivity of the cell lysates, pointing to a possible retention of viral particles inside the cell. The knockdown of PI4KIIα on the other hand, while similarly reducing secretion, also reduced the intracellular viral infectivity. This occurred in both the single and dual knockdowns. This might point to a role for PI4KIIα to affect a step of the viral lifecycle earlier than the secretion pathway, possibly in the assembly or maturation of the viral particle. PI4KIIα has not been studied extensively, and while it is known to localize to the Golgi, it may localize more to the cis- or medial Golgi, rather than the TGN where PI4KIIIβ seems to play a larger role. The localization of PI4KIIα seems to be primarily through palmitoylation of certain residues, but the exact mechanism is not well understood [76, 178]. The possible role for PI4KIIα in the viral lifecycle may merit further examination.
Given that the TGN-localized PI4P and several of its binding proteins have already been implicated in HCV secretion, we sought to further characterize the roles that additional PI4P binding proteins might play in the viral egress process [104, 126, 167]. GOLPH3 is a Golgi-associated protein and a functionally component of Golgi secretory pathway [93, 179]. GOLPH3 localizes to the Golgi via its interaction with PI4P and maintains a standard Golgi morphology by interacting with an unconventional myosin, MYO18A which in turn binds to F-actin. The interaction of GOLPH3-MYO18A-F actin triad, in addition to maintaining the stretched morphology of Golgi stacks, also provides a tensile force necessary for the budding of the cargo vesicles from TGN [93].

GOLPH3 and MYO18A knockdown likewise reduced the general secretion of proteins, represented by ssHRP, from the TGN to PM. VLDL secretion, on the other hand, was unaffected by GOLPH3 or MYO18A knockdown, showing that some secretion components may not be totally affected by these interactions (data not shown). In the case of HCV infected cells, we observed that knockdown of GOLPH3 or MYO18A affected HCV secretion similar to that of the TGN-specific depletion of PI4P. GOLPH3 or MYO18A knockdown did not affect HCV replication but did stall the secretion of viral particles, leading to their accumulation within the cell. This indicates that again, when Golgi secretory function is inhibited, viral particles replicate, are assembled, and mature into infectious virions as normal, but that the secretion of these particles is inhibited. This suggests that inhibiting vesicle budding from the TGN may be an important component of the method in which HCV exits the cell, and points to an extensive role for multiple Golgi components in the virus secretion process (Figure 5.1).

FAPP1 and FAPP2 were also interesting proteins to examine, particularly given
Figure 5.1: Diagram of HCV Secretion Pathway Model
Proposed model of late stages of HCV lifecycle, with the rough locations of proteins examined in this study marked.
their role in TGN to PM transport in polarized cells [176, 180]. These knockdowns in hepatocyte cells such as Huh 7.5.1 cells, however, did not appear to play as large a role in secretion as we had hoped. We observed only a small negative effect on HCV secretion, measured by both supernatant RNA levels, and supernatant infectivity by FFU assay. Additionally, the modest reduction in secretion was accompanied by a small reduction in the intracellular viral infectivity, not an increase as seen in the case of PI4KIIIβ knockdown. This effect does not seem especially significant, although it also might point to a role in the HCV life cycle further upstream than secretion. FAPP1 and FAPP2 proteins have not been functionally well examined, and most of the studies performed were in polarized cells, with distinct basal and apical cell surfaces [172, 176, 180]. The way these proteins might interact in non-polarized hepatocytes could differ, or the HCV secretion pathway might utilize a different set of proteins to ensure its successful budding from the TGN. The GOLPH3-MYO18A interaction may be more general in reducing TGN vesicle transport overall, which would explain the reduction previously observed. FAPP1/2 may represent a more specific role in the secretion process which may not be utilized by HCV. Another group studying HCV has recently had success in the generation of polarized hepatocytes capable of supporting HCV infection [181]. Studying the roles of the FAPP proteins in these cell types might prove to be more informative than in our non-polarized Huh 7 cells.

Some additional evidence supporting the importance of the Golgi apparatus in HCV virus secretion is derived from the effect of HCV infection on the Golgi morphology. HCV infected Huh7 cells displayed dispersed and fragmented Golgi uncharacteristic of its normal perinuclear distribution and stacked morphology (Figure
Markers for all Golgi compartments like GM130 (cis-Golgi marker) and TGN46 (trans-Golgi marker) displayed a dissembled Golgi morphology suggesting that the entire Golgi stack is fragmented in HCV infected cells. Although the Golgi apparatus showed a dispersed pattern in HCV infected cells, PI4KIIIβ and GOLPH3 proteins still localized to these fragmented Golgi stacks suggesting that these fragments are still a functional unit (Figure 3.7, A). The fragmentation of the Golgi in HCV correlates with the decrease in general secretory capacity of the infected cell but further implicates the organelle in important viral processes and hints towards sequestration of secretory components at sites of viral assembly. Golgi disruption and decrease in secretory capacity of host cells is a peculiar feature of many infectious agents such as Chlamydia, Poliovirus and Rhinovirus 1A, where the Golgi membranes serve an important role in viral replication and morphogenesis [182-184].

There are a number of interesting problems in the elucidation of the HCV secretion pathway, specifically as it relates to PI4P, still need to be explored. One of the primary, and in theory, simpler goals, is that of observing the trafficking of viral particles in living cells. This proves to be very difficult, however, as determining where a fully assembled and infectious viral particle is not a simple task. A fully assembled particle should contain core protein, as well as E1 and E2 envelope proteins, and a viral RNA molecule. These components however, are often widely distributed throughout the cell, as can be seen in the case of E2, which is often used as a marker for infected cells because of its ubiquitous expression (Figure 3.3, B and 3.7, C). Adding to the difficulty, detecting these cellular components via immunostaining may be difficult given the close association, possibly within the cell, to lipid molecules such as VLDL, which may make
it difficult for antibodies to penetrate and bind successfully to non-envelope protein components. Coller et. al recently demonstrated an interesting method for tracking a putative HCV molecule through the cell, showing where core, E2, and viral RNAs all appeared to co-localize [104]. These core puncta were able to migrate through the cell, and inhibition of microtubule polymerization slowed their trafficking [104]. This method of tracking might be further applied to study the silencing of proteins that cause the intracellular accumulation of HCV particles such as GOLPH3 or MYO18A, in order to pinpoint the cellular location where these particles might be retained.

In a similar vein, fractionation of infected cells treated to retain viral particles might be illuminating. Properly treated, cellular fractions of cell lysates could be separated and used independently to infect naïve cells to assess the infectivity of particular cellular organelles. When cells are treated to induce the intracellular accumulation of viral particles via PI4KIIIβ, GOLPH3, MYO18A, etc., any change in the infectivity of the various cellular fractions could be measured. This would highlight the location within the cell where the viral particles are being retained, and give a better idea as to the workings of the HCV secretion pathways. This kind of fractionation experiment will again likely prove quite difficult, however, due to the difficulties inherent in successfully fractionating membranous organelles like the TGN, and separating them sufficiently from other closely-juxtaposed membranes such as those of the ER. However, fractionation attempts may merit further study.

Targeting the cellular PI4P pathways for therapeutics may also prove useful in the future treatment of HCV in patients. The major problem with direct-acting antivirals such as the NS3 inhibitors telaprevir and boceprevir is the emergence of resistant mutants.
Antiviral therapies, which target host factors may lead to drugs with pan-genotype activity and a greater barrier to resistance. PI4Ks are critical for multiple events in the HCV life cycle, and several other viruses also require intracellular enrichment of PI4P for assembly of viral replication sites, making PI4Ks an excellent target for developing panviral therapeutics. Targeting of host factors is an attractive strategy, but can result in higher toxicity/intolerance, particularly in the case of targets like PI4Ks, which regulate multiple cellular processes. Successful future therapeutics may require selective and targeted inhibition of specific PI4K isoforms.

AL-9, a member of the 4-anilino quinazoline class of compounds previously considered to be an NS5A inhibitor has been recently shown to inhibit PI4KIIIα and prevent membranous web formation [117]. Pharmacological inhibition of PI4KIIIα with AL-9 resulted in altered subcellular distribution of NS5A reminiscent of that observed upon PI4KIIIα silencing, further confirming that the anti-HCV activity of these compounds involves inhibition of PI4KIIIα and depletion of PI4P from HCV replication sites [117]. New classes of NS5A-binding compounds show promise as extremely potent inhibitors of HCV replication, with EC50 values in the picomolar range [185]. While the molecular basis of their inhibition is not clear, it would be interesting to assess the ability of these compounds to interact with NS5A or deactivate PI4KIIIα. PIK93, a phenylthiazole has been used to inhibit HCV and Poliovirus replication [110, 186]. PIK93 inhibits both the PI4KIII isoforms α and β but has higher efficacy against the α isoform [187]. The lack of PIK93 specificity for PI4KIII isoforms and the fact that it inhibits PI3Ks at high concentrations raises concern about its potential as an anti-HCV agent [188]. Another potential inhibitor of PI4KIIIα, enviroxime, also displayed strong
inhibition of picornaviruses and HCV replication [110, 186, 189]. More specific PI4KIIIα inhibitors have been identified that potently inhibit replication of HCV genotype 1a and 1b replicons as well as genotype 2a virus [185, 190]. The PI4KIIIα inhibitors did not display cytotoxicity in multiple cell lines and primary cell types. However, their anti-proliferative activity in lymphocytes precluded further development, suggesting that intolerance/toxicity to PI4K inhibitors could prove to be an obstacle to their use in the treatment of HCV [190]. Mouse studies to assess the safety of PI4KIIIα inhibitors or knockdown show that homozygotic knockout of the kinase domain or knock-in of a kinase-defective PI4KIIIα displayed a lethal phenotype with widespread mucosal epithelial degeneration of the gastrointestinal tract [191]. Heterozygotes display a less severe phenotype [191]. These observations highlight the pivotal and sensitive role of PI4Ks in cellular physiology, signaling necessary caution in developing PI4K inhibitors for HCV treatment. Abrogation of PI4P signaling by using metabolically stabilized mimetics of PI4P may hold promise for future therapeutic treatments.

In summary, the work presented in this dissertation elaborates on the process of HCV secretion specifically as it is related to PI4P and its associated proteins. The studies strongly emphasize the role of Golgi components and Golgi secretory pathway in the HCV secretion process that may parallel protein secretion from the cell, and may pertain to the VLDL secretory pathway. Identification of the lipid messenger PI4P, the roles of PI4KIIIβ, PI4KIIα, and PI4P binding proteins in the process of HCV secretion may help provide newer avenues for therapeutic design of antiviral strategies, and further contributes to the general knowledge of the HCV lifecycle.
References


93. Dippold, H.C., M.M. Ng, S.E. Farber-Katz, S.K. Lee, M.L. Kerr, M.C.


113. **Borawski, J., P. Troke, X. Puyang, V. Gibaja, S. Zhao, C. Mickanin, J.**


179. **Wu, C.C., R.S. Taylor, D.R. Lane, M.S. Ladinsky, J.A. Weisz, and K.E.**


