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BUILDING PLUS STRAND VIRAL RNA REPLICATION

PLATFORMS

by

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ABSTRACT OF THE THESIS

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Many RNA viruses remodel intracellular membranes of their hosts to generate specialized sites for RNA replication. How membranes are remodeled and what properties make them conducive for replication are unknown. Here we show how RNA viruses can manipulate multiple components of the cellular secretory pathway to generate organelles specialized for replication that are distinct in protein and lipid composition from the host cell. Specific viral proteins modulate effector recruitment by Arf1 GTPase and its guanine nucleotide exchange factor GBF1, promoting preferential recruitment of phosphatidylinositol-4-kinase III β (PI4KIII β) to membranes over coat proteins, yielding uncoated phosphatidylinositol-4-phosphate (PI4P) lipid-enriched organelles. The PI4P-rich lipid microenvironment is essential for both enteroviral and flaviviral RNA

replication; PI4KIII β inhibition interferes with this process. The potential mechanism by which PI4P mediated viral RNA replication are either tethering RdRP protein to replication membranes or regulating the RdRP and its precursor protein enzymatic activity. Our findings reveal how RNA viruses can selectively exploit specific elements of the host to form specialized organelles where cellular phosphoinositide lipids are key to regulating viral RNA replication.

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List of Abbreviations

Arf1	=ADP-ribosylation factor 1
ATCC	=American Type Culture Collection
ATP	=Adenosine triphosphate
BFA	=Brefeldin A
CAR	=Coxsackievirus and Adenovirus receptor
CERT	= Ceramide transfer protein
CVB3	= Coxsackievirus B3
DMEM	=Dulbecco's modified Eagle's medium
ER	=Endoplasmic reticulum
ERGIC	=ER-Golgi intermediate compartment
FAPP	=Four-phosphate-adaptor-protein
GAP	=GTPase-activating protein
GBF1	=Golgi brefeldin A resistant guanine nucleotide exchange factor 1
GFP	=Green fluorescent protein
GEF	=Guanidine exchange factor
HCV	=Hepatitis C virus
IPTG	= Isopropyl-beta-D-thiogalactopyranoside
MHC class I	= Major histocompatibility complex class I molecule
OSBP	= Oxysterol-binding protein
PCBP2	= Poly (rC) binding protein 2
PH	= Pleckstrin homology
PI	= Phosphatidylinositol-1-phosphate
PI3P	= Phosphatidylinositol-3-phosphate
PI4P	= Phosphatidylinositol-4-phosphate
PI5P	= Phosphatidylinositol-5-phosphate
PIP ₂	= Phosphatidylinositol-4, 5-phosphate
PIP ₃	= Phosphatidylinositol-3, 4,5-phosphate
PI4K	= Phosphatidylinositol 4-Kinase
PI4KIII β	= Phosphatidylinositol 4-Kinase type III β
PI4KIII α	= Phosphatidylinositol 4-Kinase type III α
RdRP	= RNA dependent RNA polymerase
RI	= Replicative intermediate
RF	= Replicative form

RT	= Room temperature
SDS-PAGE	= Sodium dodecyl sulfate polyacrylamide gel
TGN	=Trans-Golgi network
VSVG	=Vesicular stomatitis virus G
Vpg	=Viral protein of the genome
UMP	=Uridine 5'-monophosphate
UTR	=Untranslated region

CHAPTER 1

Introduction and Literature Review

PLUS STRAND RNA VIRUS CLASSIFICATION

Viruses infect and replicate themselves within living host cells. Based on the genetic materials they contain and their mode of replication, viruses can be divided into 7 groups. These are double-stranded DNA viruses, single-stranded DNA viruses, double-stranded RNA viruses, positive-stranded RNA viruses (also called plus strand RNA viruses), negative-stranded RNA viruses, positive-stranded RNA viruses with a DNA replication intermediate stage and double-stranded DNA viruses with a single-stranded RNA intermediate stage (Baltimore, 1971). In addition to different groups, the International Committee on Taxonomy of Viruses has established a universal system to classify viruses and arrange them in a hierarchical rank using corresponding italic suffixes (with the exception of the species level): order (- *virales*), family (- *viridae*), subfamily (- *virinae*), genus (- *virus*) and species.

Plus strand RNA viruses carry their genetic material in a single strand of positive sense RNA that can be directly translated into proteins by host translational machinery. The viral particles of plus strand RNA viruses

include a positive sense RNA and a protein coat (also called the capsid). Some have an additional membrane-based envelope surrounding the protein coat. Examples of plus strand RNA virus families are Picornaviridae: poliovirus, coxsackievirus and rhinovirus; Coronaviridae: severe acute respiratory syndrome coronavirus (SARS); Flaviviridae: hepatitis C virus (HCV) and dengue virus; and Tombusviridae: tombusvirus.

PLUS STRAND RNA VIRUSES AND DISEASES

Plus strand RNA viruses infect a wide range of host cells that include human, plant, insect and bovine. Diseases resulting from plus strand RNA virus infection have not only created widespread pandemics, they have also had a great economic impact in the world. Well known plus strand RNA viruses that result in human diseases include: poliovirus (Poliomyelitis), coxsackievirus (Hand, foot and mouth disease), hepatitis A and C virus (Hepatitis), dengue virus (Hemorrhagic fever and shock syndrome) and SARS virus (Severe acute respiratory syndrome). For example SARS virus affected 8098 people and led to 774 deaths during the 114 day SARS epidemic in 2003 (Stadler et al., 2003). In this thesis, we focus on plus strand RNA virus

replication. Studying plus strand RNA virus replication will provide insights to identifying new therapeutic targets for the infectious diseases that are caused by these viruses.

PLUS STRAND RNA VIRUS LIFECYCLE

All plus strand RNA viruses have a common life cycle composed of several stages including entry, uncoating, translation, replication, packaging and egress. Viral particles bind to specific receptors on the plasma membranes of host cells. The cellular receptor determines whether a specific virus can infect a particular host cell. For example, CD155, the poliovirus specific receptor, is expressed in epithelial and neuronal cells. Thus poliovirus is capable of infecting epithelial and neuronal cells. Upon binding, viral particles often get internalized through endocytosis. In the case of poliovirus, a clathrin-, caveolin-, flotillin-, and microtubule-independent endocytosis pathway is exploited (Brandenburg et al., 2007). After entering the cells, viral particles are uncoated and they release genomic RNA into the cytoplasm of infected host cells. The released RNA is translated into a polyprotein. Subsequently, the polyprotein undergoes proteolytic processing

to generate a repertoire of structural and nonstructural proteins. Viral RNA is replicated in the cytoplasmic membranes by means of the nonstructural proteins. The plus strand RNA is then packaged with structural proteins, which make up the protein coats to form a mature virion. Finally, the mature virions egress out of the host cells. In the case of enveloped viruses, virions also hijack some of the host secretory or endocytic membranes to form an envelope on the way out of cells.

THE GENOMIC STRUCTURE OF POLIOVIRUS AND COXSACKIEVIRUS

Poliovirus and coxsackievirus have identical genomic organization. Their genomic material is a plus sense polarity single-stranded RNA (Holland et al., 1960). With a genomic size of 7500 nucleotides, their genomic RNA is separated into three regions: a 5' untranslated region (5'UTR), a coding region and a 3' untranslated region (3'UTR). Similar to message RNA, the genomic RNA has a polyA tail which is located after the 3'UTR. The most unique feature of the genomic RNA is that its 5' end is covalently linked to a viral protein named Viral Protein of the genome (VPg) (Flanegan et al., 1977;

Lee et al., 1977). The tyrosine residue of Vpg forms a phosphodiester bond with the 5' terminal uridylic acid of viral RNA (Paul et al., 1998). The Vpg linked to 5' RNA initiates the transcription of the viral genome (Paul et al., 1998). The coding region, 5' and 3' UTR of the RNA contain secondary structures, which are essential for viral translation and replication. The RNA at the 2C coding region has a secondary structure called cre (2C). The cre (2C) is involved in the generation of primer for RNA polymerization reaction. The 5' UTR has two areas that carry secondary structures, a cloverleaf and an internal ribosome entry site, while the 3' noncoding region has one secondary structure. The cloverleaf and 3' UTR each play a critical role in replication (Brown et al., 2005; Herold and Andino, 2001). The internal ribosome entry site is involved in the initiation of translation (Pelletier et al., 1988; Pelletier and Sonenberg, 1988). The coding region contains 11 genes which are as follows (in order from 5' to 3' direction): VP4, VP2, VP3, VP1, 2A, 2B, 2C, 3A, 3B, 3C and 3D (Kitamura et al., 1981; Racaniello and Baltimore, 1981). The genes encode two sets of proteins: structural and nonstructural. The structural proteins include mature VP4, VP2, VP3, VP1 and their precursors, VP4-VP2 (VP0), VP4-VP2-VP3-VP1 (P1). The nonstructural

proteins are 2A, 2B, 2C, 3A, 3B, 3C 3D as well as intermediate proteins such as 2A-2B-2C (P2), 3A-3B-3C-3D (P3), 2BC, 3AB and 3CD(Wimmer et al., 1993).

The functions of individual viral proteins during the viral life cycle have mostly been worked out. As mentioned earlier, structural proteins are involved in the formation of the viral capsid. The poliovirus capsid is an icosahedral with an external and an internal surface. VP1, VP2 and VP3 form the external surface whereas VP4, VP1 and VP2 are on the internal surface of icosahedral shell(Hogle, 2002). The nonstructural proteins are directly or indirectly involved in viral replication. The 3D protein is the key enzyme that catalyzes the RNA synthesis reaction. It is an RNA dependent RNA polymerase (RdRP). Structurally, 3D contains three subdomains: finger, palm and thumb. The RNA synthesis reaction mainly takes place in the palm subdomain. In addition, 3D synthesizes its protein primer by means of the 3AB and 3CD proteins.

Viral proteolytic processing and host membranous structure formation indirectly regulates viral RNA replication. With an efficient proteolytic process, sufficient nonstructural proteins are generated to support replication.

2A, 3C and 3CD are proteases. 3C is the protease responsible for majority of viral protein processing (Malcolm, 1995; Palmenberg, 1987). 3CD, the precursor cleaves itself to generate 3C and 3D. 2A cuts between VP1 and 2A (Cameron et al., 2010; Palmenberg, 1987, 1990). In addition, 2B, 2C, 2BC and 3A (Cho et al., 1994; Choe and Kirkegaard, 2004; Suhy et al., 2000) induce the formation of membranous structure that has been proposed to serve as a platform for the assembly of replication complexes (see the detailed description in plus strand RNA virus generate a replication organelle).

THE REPLICATION MECHANISM OF POLIOVIRUS AND COXSACKIEVIRUS

As stated earlier, the replication of coxsackievirus and poliovirus begins by making the viral proteins essential for replication. These replication proteins are 2A, 2B, 2C, 2BC, 3A, 3B (also called Vpg), 3AB, 3CD, 3C and 3D. Deletion of any of these viral proteins RNA from the viral genome results in complete loss of viral replication (Sean and Semler, 2008). The viral RNA synthesis reaction is catalyzed by 3D using viral RNA as a template and a Vpg covalently-linked short chain of RNA oligonucleotides as a primer. There

are two steps for RNA synthesis: minus strand and plus strand synthesis. 3D first converts the genomic plus strand RNA into its complementary strand called the minus strand. Then the synthesized minus strand RNA is used as a template to generate more progeny of the plus strand. The following is the currently proposed model for negative and positive strand synthesis (Sean and Semler, 2008).

1) Negative strand synthesis

To initiate negative strand synthesis, a non-translating plus strand RNA template forms a ternary complex with 3CD and Poly(rC) binding protein 2 (PCBP2) at the 5' end of the RNA template (Parsley et al., 1997). In addition, 3CD recognizes and binds to the cre (2C) on the RNA template (Paul et al., 2000; Rieder et al., 2000). Since 3CD has a protease activity, it cleaves itself and 3AB to liberate 3C, 3D, 3A and 3B (Vpg) near the cre (2C) region. Subsequently, the 3D protein near cre (2C) uses cre (2C) as a template to generate Vpg-pU-pU, which acts as a primer for the RNA polymerization reaction (Paul et al., 2000; Paul et al., 1998). Since 3D catalyzes the RNA dependent RNA polymerase reaction in a 5' to 3' direction, the initiation step of the RdRP reaction has to take place at the 3' end of the plus stranded RNA.

To resolve this issue, the plus-stranded RNA template circularizes through an interaction between the ternary complex and polyA binding protein, which binds to the polyA tail at the 3' end of the plus strand (Herold and Andino, 2001). By circularization, the 3D-primer complex gets transferred to the 3' end. The primer binds to the polyA tail through the formation of hydrogen bonds between uracil on the primer and adenine on the polyA tail. Once bound, 3D begins to synthesize and elongate negative strand RNA based on the plus-stranded RNA template. As a result, a double strand RNA intermediate, called replicative form (RF) RNA, is produced.

2) Positive strand synthesis

Positive strand synthesis begins by synthesizing the primer Vpg-pUpU. Presumably, it uses the same manner as in negative strand synthesis (Goodfellow et al., 2003). Next, RF RNA is unwound to release the negative strand RNA from RF followed by the transfer of the 3D-primer complex to the 3' end of the negative strand. The unwinding of RF is hypothesized to be by binding of viral and cellular proteins to the positive and negative strands of RNA. PCBP2 and 3CD bind to the positive strand of RNA; 3AB, 3CD and 2C bind to the negative strand. Then Vpg-pUpU anneals to

adenines on the polyA tail of the 3' end of the released negative strand, and 3D starts to catalyze the polymerization reaction using a single negative strand as a template. During viral infection, the ratio of positive to negative strands is approximately 40 to 1 (Giachetti and Semler, 1991; Novak and Kirkegaard, 1991). Since there are more positive strands being generated, it is thought that a single negative strand can act as a template to simultaneously synthesize up to eight positive strands (Richards et al., 1984). As a result, a replicative intermediate (RI), which has a double-stranded RNA core and up to eight different lengths of plus strand RNA, is formed. Using the RI, an abundance of plus strand RNA is generated from one minus strand template.

PLUS STRAND RNA VIRUSES GENERATE A REPLICATION

ORGANELLE

Among stages of its life cycle, viral replication is the most critical step. One of the most unique features of plus strand RNA replication is that its replication relies on host intracellular membranes compartments termed replication organelles. Viruses assemble their replication machinery and

replicate their RNA on the cytosolic leaflet at replication organelle membranes (Denison, 2008; Miller and Krijnse-Locker, 2008). Replication organelle membranes for picornaviruses, flaviviruses, and coronaviruses originate from the endoplasmic reticulum (ER) and Golgi apparatus/ trans-Golgi network (TGN) (Schlegel et al., 1996), whereas endosomes, lysosomes and mitochondria are thought to be the membrane source for togaviruses and nodaviruses (Magliano et al., 1998).

The advantage of replicating RNA on the surface of intracellular membranes is not clear. The intracellular membranes may (1) concentrate viral/host proteins and viral RNA by limiting diffusion, (2) provide specific lipids that participate in or modulate the replication reactions, (3) provide a scaffold to support viral replication complex, (4) and/or even potentially protect viral replication complexes from the attack of host innate immune components (Lyle et al., 2002).

POLIOVIRUS AND COXSACKIEVIRUS

The presence of a poliovirus replication organelle was first observed in poliovirus infected HEP-2 cells, revealed by electron microscope study (Dales

et al., 1965). These replication organelles first appeared 3 hrs post infection. By 7 hrs. post infection, they had propagated extensively and filled almost the entire cytoplasm. Later, this same structure was found in coxsackievirus-infected cells(Jezequel and Steiner, 1966) as well.

Using a sucrose gradient, these replication organelles could be isolated(Caligiuri and Tamm, 1969) (Bienz et al., 1992). The size of the replication organelles is approximately 50 to 400nm in diameter (den Boon et al., 2010). Isolated replication organelles have compact and rosette-like structures. The formation of the rosette-like structures is reversible. At a low salt and temperature condition, the rosette-like replication organelles dissociate resulting in the generation of vesicle-like structures with extended tubules. When the temperature increased to 30°C, the rosette structure reforms(Egger et al., 1996).

Immunoelectron microscopy, immunofluorescence and biochemical analysis have begun to reveal some of the composition and function of replication organelles. The replication organelles contain viral proteins (2C, 2B, 2BC, 2ABC, 3C, 3D, 3CD) and RNA (plus strand, minus strand RF and RI) that are involved in the viral replication process (Bienz et al., 1987; Bienz et

al., 1994; Bienz et al., 1992; Bolten et al., 1998). Furthermore, the isolated replication organelles are capable of actively transcribing viral RNA in vitro. (Bienz et al., 1994; Caligiuri and Tamm, 1969). When replication organelle membranes are disrupted by detergent, the in vitro viral RNA transcription is completely abolished (Bienz et al., 1992) (Fogg et al., 2003). Collectively, all evidences demonstrate that isolated replication organelles indeed involved in viral replication.

HOST SECRETORY PATHWAY PROTEINS AND LIPIDS

Replication organelles of plus strand RNA virus are composed of host membranes. Host secretory organelles (e.g. ER, Golgi apparatus) are specialized intracellular compartments that govern protein/lipid synthesis, modification, degradation and secretion. They provide the largest source of intracellular membranes. Proteins involved in the process of trafficking between these compartments are called secretory pathway proteins. Among them, small GTPase proteins and their effectors are the most critical proteins in regulating membrane and protein trafficking.

ADP RIBOSYLATION FACTOR1 (Arf1)

ADP-ribosylation factor 1 (Arf1) is a master regulator of trafficking between the ER and Golgi. Like all small GTPase proteins, Arf1 cycles between the inactive (GDP bound form) and active (GTP bound form) state. Two protein families regulate the switch between the inactive and active states: Guanidine exchange factors (GEF), which activate Arf1 and GTPase activating proteins (GAP), which inactivate it. Once Arf1 is active, it becomes membrane associated and recruits its effectors to membranes. Arf1's known major effectors include coat proteins that regulate membrane curvature and budding such as COPI complex and clathrin and lipid modifying enzymes such as phosphatidylinositol 4 kinase type III β (PI4KIII β) and phospholipase D.

GOLGI BREFELDIN A RESISTANT GUANINE NUCLEOTIDE EXCHANGE

FACTOR 1(GBF1)

GBF1 is a high-molecular-weight GEF for Arf1. Like all Arf1 GEFs, GBF1 contains a Sec7 domain, a protein module of approximately 200 amino acids initially identified in *Saccharomyces cerevisiae* (Achstetter et al., 1988) and later found in mammalian proteins. GBF1's Sec7 domain is responsible

for stimulating the exchange of GDP for GTP on Arf1, thus it is called the catalytic domain (Jackson and Casanova, 2000). To activate Arf1, GBF1 forms a complex with Arf1-GDP. The Sec7 domain of GBF1 promotes the exchange of GDP to GTP on Arf1 to generate GBF1-Arf1-GTP. Next, GBF1 is released from membranes whereas Arf1-GTP stays on the membrane. Consequently, Arf1-GTP recruits its downstream effector to locally modulate the membrane properties. Therefore, GBF1 determines when and where Arf1 is activated (Niu et al., 2005). In mammalian cells, GBF1 and Arf1 are both localized to the ER, ER-Golgi intermediate compartment (ERGIC), and the Golgi apparatus (Garcia-Mata and Sztul, 2003; Kawamoto et al., 2002; Zhao et al., 2002). As a result, GBF1 regulates membrane and protein trafficking in the secretory pathway through activation of Arf1.

PHOSPHATIDYLINOSITOL 4-KINASE TYPE III β (PI4KIII β)

One of the Arf1 effectors, which can modulate membrane lipid composition, is PI4KIII β , an enzyme that catalyzes the production of phosphatidylinositide 4-phosphate (PI4P) from PI by the addition of a phosphate group to the 4th position of the inositol ring. PI4KIII β was first cloned and characterized in *Saccharomyces cerevisiae* (Flanagan et al., 1993),

followed by cloning and characterization in rat (Nakagawa et al., 1996), human (Meyers and Cantley, 1997), bovine (Balla et al., 1997) and drosophila (Brill et al., 2000). This enzyme is structurally similar to phosphoinositide-3-kinases. Unlike the structurally unrelated type II PI4K kinases, PI4KIII β has a low affinity for adenosine triphosphate (ATP) and its activity is relatively insensitive to adenosine. In mammalian tissue, PI4KIII β is highly expressed in the heart, skeletal muscle and the pancreas (Meyers and Cantley, 1997). In mammalian cells, at steady state, the majority of PI4KIII β is localized to the Golgi apparatus though it is present in the nucleus as well (Bruns et al., 2002; de Graaf et al., 2002; Wong et al., 1997). This Golgi localization of PI4KIII β is due to its recruitment by Arf1 since it is one of Arf1's effectors. Several proteins interact with PI4KIII β , and thus regulate its activity. Arf1, neuronal calcium sensor 1, Protein kinase D and Eukaryotic protein translation elongation factor 1 α 2 stimulate PI4KIII β activity (D'Angelo et al., 2008). The function of PI4KIII β is diverse. It regulates TGN to plasma membrane transport, exocytosis (mast cell, pancreatic cell and PC12), cytokinesis, spermatogenesis and yeast survival (Brill et al., 2000; Brill et al., 2011; Flanagan et al., 1993; Kapp-Barnea et al., 2003).

PHOSPHATIDYLINOSITOL-4-PHOSPHATE (PI4P)

PI4P is a monophosphorylated phosphoinositide that constitutes only 0.05% of the total lipid in cells (Lemmon, 2008). Although PI4P is a much less abundant lipid in cells, it regulates several important cellular functions. For example, it regulates trans Golgi compartment to plasma membrane trafficking and secretion. The role of PI4P in regulating cellular function is through its downstream effectors. All of its effectors have a PI4P binding domain such as pleckstrin homology (PH) domain, and are thus capable of binding to PI4P. To date, lipid transfer proteins and coat complex adaptor proteins are the two groups of well-documented effectors (D'Angelo et al., 2008). For example, four-phosphate-adaptor-protein (FAPP), ceramide transfer protein (CERT), oxysterol-binding protein (OSBP) are lipid transfer proteins. Adaptor protein complex 1 (AP-1), Golgi-localized, γ -ear-containing Arf-binding proteins (GGA) and EpsinR are coat complex and adaptor proteins.

In mammalian cells, PI4P production is governed by several types of phosphatidylinositol 4 kinase (PI4K) proteins: II α , II β , III α , III β . The

subcellular localization of these PI4K isoforms determines where synthesis of the PI4P lipid takes place. All PI4Ks except III α are localized to the Golgi. III α is found in ER and plasma membranes. Besides the Golgi, II α and II β also reside in endocytic organelles.

PLUS STRAND RNA VIRUS MODEL SYSTEM: POLIOVIRUS AND COXSACKIEVIRUS

The majority of studies in this thesis utilize poliovirus and coxsackievirus B3 (CVB3). CVB3 and poliovirus belong to the family of picornaviridae and the genus of enterovirus and are acute infection viruses. With an overall infection duration of 8 to 10 hours, and well-characterized replication components, CVB3 and poliovirus make a perfect model system to investigate the biogenesis and role of replication organelles.

OVERVIEW OF THE RESEARCH PROJECT

Plus strand RNA viruses hijack and tailor host to facilitate their own RNA replication, including the generation of replication organelles. How replication organelle membranes are formed and what are their common properties, if any, which make them conducive for viral replication, are unknown. Host secretory pathway, in particular the ER, provides the largest source of intracellular membranes. Therefore, we hypothesize that plus strand RNA viruses hijack the host secretory pathway to generate a membrane environment, which facilitates viral replication. To initially test our hypothesis, we characterized the enteroviral replication organelles using imaging and molecular genetic tools to establish the connection between host secretory membranes and viral replication organelles (chapter 3). We showed that replication organelle membranes were enriched in host PI4KIII β enzymes. We then demonstrated the critical role of PI4KIII β by showing that it produces a PI4P microenvironment that is essential for RNA replication (chapter 4). We next investigated the prevalence of a PI4P microenvironment among plus-strand RNA viruses, by examining the replication organelles formed during Hepatitis C virus replication (chapter 5). Finally we investigated the

mechanistic role of PI4P lipids in viral replication (chapter 6). Our work in this thesis not only provides insight into how plus strand RNA viruses exploit the host secretory system to produce a unique lipid/protein platform for viral replication but also identifies a new panviral therapeutic target for combating viral infections.

CHAPTER 2

Materials and Methods

Materials

A) CELL LINES:

HeLa Cells

HeLa cells are human epithelial cell lines. They were obtained from American Type Culture Collection (ATCC; Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplement with 10% heat-inactivated fetal bovine serum, 25mM HEPES buffer, 4mM L-glutamine, 100 units/ml of penicillin and 100 µg/ml of streptomycin.

Huh-7 and NNeo/3-5B (RG) Cells

Huh-7 and NNeo/3-5B (RG) cells were provided by Dr. Stanley Lemon (University of North Carolina at Chapel Hill, NC). Huh-7 cells are human hepatic cell lines. Huh-7 cells were maintained in DMEM supplemented with 2 mM L-glutamine, 5% heat-inactivated fetal bovine serum, 100 units/mL of penicillin and 100 µg/mL streptomycin. NNeo/3-5B (RG) Cells are Huh-7 derived cell line contains autonomously replicating, subgenomic, dicistronic,

selectable HCV RNAs from an infectious molecular clone HCV-N of genotype 1b virus and expresses the HCV non-structural proteins NS3-NS5B. This replicon-bearing cell line was cultured in the same media described above in the presence of 500 µg/mL G418.

B) VIRUSES:

CVB3 was provided by Dr. Frank van Kuppeveld (Utrecht University, Utrecht). To generate viral stock, confluent HeLa cells were infected by CVB3 for 24hrs. The infected cells underwent freeze-and-thaw cycle three times, and supernatant was collected and stored at -80°C.

C) DNA CONSTRUCTS:

Plasmid and replicon constructs were kindly provided by following laboratories. Arf1-GFP, Arf1-RFP, εCOP-GFP, GalT-YFP (Jennifer Lippincott-Schwartz; National Institutes of Health, MD), ERGIC53-GFP (Theresa Ward, London School of Tropical Medicine, London), Kinase-dead PI4Kβ D656A (Tamas Balla, National Institutes of Health, MD), HCV replicon J6/JFH (p7-Rluc2A)/delta E1 E2 (Charles Rice, The Rockefeller University, NY), CVB3 replicon pRib-Rluc (Frank J.M. van Kuppeveld, Utrecht University, Utrecht), Poliovirus replicon pXpA-Ren R (Geroge Belov, University of Maryland, MD).

D) ANTIBODIES:

Antibodies were purchased or provided by the indicated vendor or laboratory. Anti-CVB3 3A (Lindsay Whitton; The Scripps Research Institute, CA), anti-GalT (EG Berger, University of Zurich), Arf1 (Julie Donaldson; National Institutes of Health, MD), GBF1 (Jennifer Lippincott-Schwartz, National Institutes of Health, MD), anti-PI4KIII β (Upstate, Billerica, MA), anti- β COP (Sigma, St. Louis, MO), anti-TGN46 (Cell Signaling, Danvers, MA), anti-ERGIC53 (Sigma, St. Louis, MO), anti-HCV NS5A (Charles Rice; The Rockefeller University, NY). All secondary antibodies were from Invitrogen and Jackson Immunochemical.

E) SMALL INTERFERING RNA (siRNA):

siRNAs targeting specific genes were obtained from the following vendors with the indicated target sequences.

Target Genes	Target Sequences (5' TO 3')	Vendor
Arf1	1. UGA CAG AGA GCG UGU GAA C	Dharmacon

	2. CGG CCG AGA UCA CAG ACA A 3. ACG AUC CUC UAC AAG CUU A 4. GAA CCA GAA GUG AAC GCG A	(Lafayette, CA)
GBF1	1. CAA CCA CAA UGU UCG UAA A 2. GAA CAG GGA UCG UGU GGG C 3. GAA CCU GCC CAG UGU AUU U 4. GCA AGG ACU UUG AGC AAG A	Dharmacon (Lafayette, CA)
Non-targeting	AA TTC TCC GGA CGT GTC ACG T	Qiagen (Germantown, MD)
PI4K β	AA CAC GAA GGA UCA UGU GGU A	Qiagen (Germantown, MD)

F) CHEMICALS:

Brefeldin A, paraformaldehyde, and saponin (Sigma; St. Louis, MI), PIK93 (Kevin Shokat, UC Berkeley, CA), Live cell Renilla substrate Endu-Ren (Promega; Madison, WI), Bovine serum albumin (Fisher Scientific; Pittsburgh, PA), formaldehyde (EMD; Gibbstown, NJ), phosphate buffer saline (G biosciences; Maryland Heights, MI), DMEM (Mediatech; Manassas, VA), Fetal bovine serum (ATLANTA biological; Lawrenceville, GA), HEPES (Fisher

Scientific; Pittsburgh, PA), glutamine, penicillin and streptomycin (GIBCO; Carlsbad, CA)

G) MICROSCOPE:

All imaging was performed on a Zeiss LSM510META confocal laser scanning

confocal microscope (Carl Zeiss, USA) equipped with lasers emitting 458nm, 488nm, 514nm, 565nm and 633nm laser lines. For high-resolution light-level imaging only 40X and 63X oil immersion objectives with 1.4 numerical apertures were utilized with pinhole set at 1.2 Airy units.

Filters and laser used for following fluorophores and fluorescent proteins

	Excitation	Emission
Alexa 488	488 nm	BP 500- 550
Alexa 561	561 nm	BP 575- 615
Green Fluorescent Protein	488 nm	BP 500- 550
Red Fluorescent Protein	565 nm	BP 575- 615
Yellow Fluorescent Protein	514 nm	BP 535- 590

Methods

Immunofluorescence and Analysis

Cells were plated on glass coverslips, fixed with 4% formaldehyde PBS solution (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) at room temperature (RT). Coverslips were permeabilized with either 0.2% Triton or 0.2% saponin and sequentially incubated with primary and fluorophore-tagged secondary antibodies. Confocal images were obtained and analyzed with Zeiss LSM or Image J software.

To quantify the relative fluorescence of PI4KIII β in 3A-myc labeled structures, images were analyzed using LSM-FCS software (Carl Zeiss, USA). The Golgi and cytosol regions of interest were selected for both 3A and neighboring non-3A expressing cells, and mean fluorescence intensity of PI4KIII β in those regions was obtained. The relative fluorescence was determined by following formula: $[(I_{\text{Golgi}} - I_{\text{Cytosol}})_{\text{3A expressing cell}} / (I_{\text{Golgi}} - I_{\text{Cytosol}})_{\text{non-3A expressing cell}}] * 100$, where I_{Golgi} is the mean fluorescence intensity per pixel at the Golgi and I_{Cytosol} is the mean fluorescence intensity in the cytosol.

Live-Cell Imaging

All imaging was performed on a Zeiss LSM510META confocal laser scanning confocal microscope (Carl Zeiss, USA) using high-magnification, high numerical aperture objectives. Live cells were maintained on the microscope stage in a temperature, CO₂, and humidity-controlled environmental chamber. Time-lapse images were acquired every 5 min for the duration of infection.

Fluorescence in Situ Hybridization

Alexa555-labeled CVB3 plus-strand RNA-specific probes were synthesized using FISH Tag RNA kit (Invitrogen Corp., CA). The infected cells were fixed with 4% formaldehyde followed by overnight permeabilization with 70% ethanol. Cells were rehydrated in SSC buffer and hybridized with RNA probes overnight in hybridization buffer. GBF1 or Arf1 was immunostained with primary and Alexa488- labeled secondary antibodies in the absence of detergents.

Replicon Assays

pRib-Rluc (CVB3), pXpA-Ren R(poliovirus) and J6/JFH(p7-Rluc2A) (Hepatitis C virus) plasmid with Renilla luciferase gene as reporters in place of structural genes was utilized to measure viral RNA replication. Plasmids were in vitro-transcribed and RNAs transfected into HeLa cells grown in 96-well plates. Cells were incubated with 60 μ M live-cell Renilla substrate and light units were recorded with multiwell plate reader at 15 min intervals up to 16 hr. at 37°C. The kinetic of viral replication was generated in order to identify the time point when the light signal is maximal. The r.l.u% is calculated by dividing the light units of individual samples by the average of the maximal light units of the control group samples.

Cell viability quantification

Plasmid-transfected, siRNA- or PIK93- treated cells were assessed by Cell Titer-Glo cell viability assays (Promega, Madison, WI) as described in manufacturer's protocol.

RNA Polymerase Lipid-Binding Assay

Recombinant PV polymerase (3D) in pET26Ub-3D was purified as

described (Gohara et al., 1999). Lipid dot-blot strips were purchased (Echelon Biosciences, UT). The strips were incubated in blocking buffer for 1 hr. at RT and then incubated in the same buffer with purified 3D overnight at 4°C. The blots were then washed in TBST-50 buffer (50mM Tris, 150mM NaCl, 0.1% Tween20 at pH 7.4). To detect lipid-protein interactions, strips were incubated with anti-3D antibody for 1 hr. at RT. Blots were washed as before and incubated with anti-rabbit horseradish peroxidase for 1 hr. at RT. 3Dpol bound to the lipids immobilized on the membrane was visualized by incubating with chemiluminescent substrate.

PI4P Lipid Extraction and Quantification

PI4P lipid was extracted according to the manufacturer's protocol (Echelon Biosciences, UT) described by Gray (Gray et al., 2003) with slight modification. Briefly, HeLa cells were harvested, and PI4P lipids were extracted in cold 0.5M Trichloroacetic acid (TCA) for 5 min. Cell pellets were washed twice with 3 ml of 5% TCA/1 mM EDTA solution and pellets were suspended in 3 ml of a methanol: chloroform (2:1) solution, incubated at RT for ten minutes and subjected to centrifugation. To obtain phosphatidylinositol

lipids, 2.25 ml of methanol: chloroform: 12N hydrochloric acid solution (80:40:1) was added to the pellets followed by fifteen minutes incubation at RT. Supernatant was mixed with 0.75 ml of chloroform before adding 1.35 ml of 0.1N hydrochloric acid to generate organic and aqueous phases. The organic phase containing the phosphatidylinositide was dried in a vacuum dryer, and subjected to PI4P quantification by protein lipid overlay assay (Echelon Biosciences, UT). PI4P samples from different post-infection time points were spotted on nitrocellulose membrane strips and blocked with 3% BSA, 0.1% Tween-20, PBS solution for 1 hr. to remove non-specific binding. PI4P lipids were detected by PI4P detectors derived from FAPP1 proteins (Dowler et al., 2002) followed by secondary and tertiary antibody incubation. The signal was visualized by SuperSignal chemiluminescent substrate (Pierce, IL). The concentration of PI4P was determined by correlating the intensity of samples to a standard curve generated from known concentrations of PI4P.

siRNA Transfection

Cells were seeded in 96-well plates (for the replicon assay) or 12-well plates (for western blot analysis) 1 day before siRNA transfection. Typically 50

nM of each siRNA was transfected using Dharmafect1 (Dharmacon, CO) and incubated for 48 hr. To verify the knockdown efficiency of proteins, 10 to 20 μ g of cell lysate were used in western blots.

Statistical Analysis

Data were expressed and plotted as means \pm standard error of the mean (SEM). Unpaired student's t tests were used to compare the mean of control and experimental groups. The p value and sample size of each experimental group is provided in the respective figure legends.

K126A mutant generation

The K126A RdRP mutant was generated using QuickChange site-directed mutagenesis kit (Agilent Technologies) according to manufacturer's protocol. Briefly, a pair of mutagenesis primers was synthesized (Sigma). The sequence of mutagenesis primers is listed as follows and the mutation sites are underlined. Forward primer: 5' TAC GTA GCA ATG GGA AAG GCT AAG AGA GAC ATC TTG AAC AAA 3'; Reverse primer: 5' CAA GAT GTC TCT CTT AGC CTT TCC CAT TGC TAC GTA CGG GT3'. To amplify

the K126A RdRP plasmid, a PCR reaction was performed followed by Dpn1 digestion at 37°C for 2 hours. The Dpn1 digested solution was transformed into DH5α cells. Positive clones were identified based on DNA sequencing result (Genewiz, South Plainfield, NJ).

Poliovirus RdRP Protein Expression

Recombinant Poliovirus RdRP (3D) in pET26Ub-3D was expressed according to the protocol previously described (Gohara et al., 1999) with slight modification. Briefly, the pET26Ub-3D plasmid was transformed into BL21 (DE3) pCG1 *E.coli*, and a transformed *E.coli* colony was grown overnight at 37°C in 10 ml of NZCYM broth supplemented with 25 µg/ml of Kanamycin, 20 µg/ml of chloramphenicol and 0.4% dextrose. To prepare the starter culture for protein induction, the overnight culture was used to inoculate 250 ml of fresh media to reach a final OD=0.05. The *E.coli* starter culture was then grown at 37°C until the OD reached 0.6 to 0.8. Subsequently, the starter culture was cooled to 25°C, and isopropyl-beta-D-thiogalactopyranoside(IPTG) was added to a final concentration of 500 µM to induce Poliovirus RdRP expression and incubated overnight at 25°C. On the next day, the RdRP-

expressing *E.coli* were harvested by centrifugation at 6000 xg for 10 minutes at 4°C, and the *E.coli* pellet was washed once with T50E2 buffer(50 mM Tris, 2mM EDTA, pH=8). The pellet was stored at -80 °C.

Poliovirus RdRP Protein Purification

Recombinant Poliovirus RdRP (3D) in pET26Ub-3D was purified according to the protocol previously described (Gohara et al., 1999) with slight modification. The frozen pellet was thawed and suspended in T50E2 solution at a ratio of 1gram pellet in 21 ml of T50E2 solution. The suspended solution was frozen (liquid nitrogen) and thawed (30C water bath) 3 times followed by the addition of lysozyme to a final concentration of 0.1 mg/ ml and protease inhibitor cocktail to 1X (Thermal Scientific). Then the suspended solution was sonicated for 10 seconds on 20 second off cycle for 8 times at a intensity of 4.5 (Sonifier Cell Disruptor; Heat Systems-Ultrasonics Inc.) followed by the addition of additives to these final concentrations (10% glycerol, 1% NP40, 10mM β -mercaptoethanol, 0.3M NaCl, 60 μ M ZnCl₂). Cell debris was removed by centrifugation at 237000 rcf for 30 minutes at 4°C. Next, 0.25% (v/v) of polyethylenimine was added to cleared lysate in order to precipitate

nucleic acid. The lysate was stirred slowly at 4°C for 15 minutes and then centrifuged at 48384 xg for 30 minutes at 4°C. The PEI supernatant was decanted and solid pulverized ammonium sulfate particle was slowly added to 40% saturation. The ammonium sulfate-precipitated material was then pelleted by centrifugation at 75600 xg for 30 minutes at 4°C. The ammonium sulfate pellet was resuspended in 1.5 ml dialysis buffer (50mM HEPES, 20% glycerol, 10mM β -mercaptoethanol, 0.5M NaCl), and dialyzed in 600 ml of dialysis buffer for overnight. The dialyzed sample was aliquotted and stored at -80C.

RNA dependent RNA polymerase activity assay

The enzymatic activity of RdRP was determined by the UMP incorporation method(Flanegan and Baltimore, 1977). The UMP incorporation assay was performed as previously described(Gohara et al., 1999) with minor modification. Specifically, the partially purified RdRP proteins were incubated with primer (2 μ M of dT₁₅), template (100 μ M of poly(rA)₄₀₀), 500 μ MUTP and 0.2 μ Ci/ μ L [α -³²P]UTP in the presence of reaction buffer. The reaction buffer contained 50 mM of HEPES, pH 7.5, 10 mM of 2-mercaptoethanol, 5mM of MgCl₂ and 60 μ M of ZnCl₂. The enzymatic reactions were carried out in a

total volume of 25 μ L using 500 ng of RdRP at 30°C for 0, 5, 10 and 30 minutes. At the end of the incubation time points, the reactions were quenched by the addition of 0.5 M EDTA to a final concentration of 50 mM. Then, 10 μ L of the quenched reaction was spotted onto DE81 filter papers and dried completely. The papers were washed three times for 10 min in 5% dibasic sodium phosphate and rinsed in absolute ethanol. Bound radioactivity was quantitated by liquid scintillation counting.

CHAPTER 3

The dynamics of replication organelle biogenesis and associated host secretory machinery

Replication organelles are the membranous compartments that are formed in the cytosol of plus strand RNA viruses infected cells. Viral replication proteins assemble on the cytosolic outer membrane leaflet surface of these membranes and viral RNA replication takes place on them. It is not clear what the characteristics of these membranes are and where these membranes originate from. There are some indications that they originate from secretory membranes. For example in virally infected cells, it was shown that host secretion was inhibited (Choe et al., 2005; Deitz et al., 2000; Doedens and Kirkegaard, 1995; Moffat et al., 2007). The trafficking between the ER-Golgi intermediate compartment (ERGIC) and Golgi was also blocked (Beske et al., 2007). For CVB3, the viral proteins (3A, 2B and 2BC) when ectopically expressed alone inhibit protein secretion (Cornell et al., 2006). Moreover, Brefeldin A (BFA), a toxin isolated from *Penicillium brefeldianum*, has been reported to inhibit poliovirus RNA synthesis (Maynell et al., 1992).

Notably, BFA binds to the catalytic domain of GBF1, an Arf1 GEF, stabilizing the Arf1-GDP- GBF1 complex and thus preventing GDP/GTP exchange by Arf1 (Peyroche et al., 1999). Collectively the above evidence suggests that the host secretory pathway organelles and in particular Arf1 and GBF1 proteins are likely involved in regulating picornaviral replication processes.

In this chapter, we used CVB3 as a model viral system to examine the dynamics of plus strand RNA replication organelle biogenesis and established the connection between viral replication organelles and host secretory pathway membranes. Using high-resolution confocal microscopy, we systemically analyzed the properties of replication organelles.

RESULTS

Enteroviral replication sites and host secretory pathway organelles

We first characterized of RNA virus replication sites during enteroviral infection. Two hours after infection with CVB3, we were able to detect newly synthesized viral plus strand RNA molecules (by fluorescence in situ hybridization) localized to the host secretory Golgi/ TGN compartments by colabeling with Arf1-GFP (Figure 3-1), a green fluorescent protein (GFP)-

tagged version of the host small GTPase Arf1, which functionally mimics the native Arf1 GTPase in both uninfected and virally infected cells(Niu et al., 2005; Presley et al., 2002). Viral replication protein 3A, a tail-anchored membrane protein(Towner et al., 1996), and 3D, RdRP, both components of the viral replication enzyme complex (Richards and Ehrenfeld, 1990), were also localized to the Golgi apparatus and TGN compartments (Figure 3-1) signifying that these compartments were sites of initial viral RNA synthesis.

By 4 hr. post-infection, at the peak of enteroviral RNA replication, both viral RNA and viral replication enzyme levels rapidly increased. At this stage, Arf1 GTPase, viral RNA and 3Dpol (Figure 3-2, 4 hr. p.i.) were redistributed to discrete cytoplasmic structures, which we term "RNA replication organelles". These organelles persisted until the death of the cell, ~10 hr. after start of infection, during which time both viral RNA and viral protein levels continued to increase at these structures (Figure 3-2, 8 hr. p.i.). Cellular GBF1, the GEF for Arf1, which have previously shown to be required for enteroviral RNA replication(Belov et al., 2007), was colocalized with Arf1 (Figure 3-3A) and viral RNA throughout infection (Figure 3-3B). The replication organelles at 4 hr. post-infection were formed adjacent to ER exit sites as determined by

antibody labeling of sec31, a component of COPII coats, which are recruited by activated Sar1 GTPases to the ER (Figure 3-4A).

We tested whether Arf1, GBF1 and ER exit sites were required for the biogenesis of the replication organelles. We inhibited Arf1 or GBF1 by targeting their expression with siRNAs (Figure 3-3C), and we inhibited ER exit site biogenesis by ectopically expressing in cells a dominant-negative, GTPase-inactive Sar1 protein (Sar1 [T39N]) (Figure 3-4B), which is known to block the formation of ER exit sites (Kuge et al., 1994). Cells were then transfected with CVB3 replicons, which contain the full length of viral RNA genome except for the genes encoding the structural proteins, these are instead replaced by renilla luciferase gene as a readout reporter for replication. Depleting Arf1, GBF1 or ectopically expressing Sar1 mutants inhibited CVB3 replicon by 70%, 80% and 50% respectively (Figures 3-3C and 3-4B). These results indicated that the secretory pathway machinery GBF1, Arf1, and Sar1 proteins were all required for viral RNA replication.

Enteroviral replication organelles and host secretory pathway proteins

When we examined by high-resolution confocal imaging the replication membranes formed at four hours post infection, we found that even though Arf1 was present on the organelle membranes, many of Arf1's effectors including COP1, clathrin and clathrin adaptor γ -adaptin, which are all required for sorting/budding of cargo such as Golgi enzymes, were not colocalized with Arf1 at these organelles (Figure 3-5A to D). The lack of localization of these proteins to replication membranes was surprising given that Arf1 is able to bind and hydrolyze GTP at these sites (Belov et al., 2007) and hence *a priori* capable of recruiting these effectors. Consistent with the absence of these coats, these organelles were also not containing Golgi enzymes (Figure 3-5D), which typically sort into GBF1/Arf1-GTP/COP1 membranes at the ERGIC and Golgi membranes of uninfected cells (Lanoix et al., 1999).

COP1-dependent membrane budding mediates anterograde transport from the ERGIC and therefore is required for the maintenance of the Golgi apparatus (Lee et al., 2004). The absence of Golgi enzyme localization to these organelles suggested a disruption of anterograde transport. In addition ERGIC/Golgi matrix protein, GM130 (Figure 3-5E) were also absent from

these membranes. Nevertheless, these organelles did contain a combination of other TGN, Golgi and ERGIC components including TGN46, GGA1, Rab1b proteins as well as some ERGIC 53 (Figure 3-6A to D). Notably, recent reports have found that depletion of COPI proteins from cells can result in a loss of secretory pathway compartmentalization and the formation instead of membrane-bound structures that contain components of TGN, Golgi and ERGIC proteins (Styers et al., 2008). Similarly, our findings here suggest that decoupling GBF1/Arf1 activity from COPI recruitment to membranes also results in a complete reorganization of the secretory pathway away from distinct separate conventional organelles.

Host PI4KIII β and Enteroviral replication organelles

Phospholipid-modifying enzyme PI4KIII β , which catalyzes the production of PI4P lipids from PI (D'Angelo et al., 2008), is one of the critical downstream effectors of Arf1, recruited to and activated by Arf1 at the TGN and Golgi apparatus membranes and required for membrane trafficking (Balla et al. 2006; Godi et al., 1999). Given the absence of COPI or clathrin effectors at GBF1/Arf1 labeled replication organelles, we tracked the fate of

PI4KIII β during enteroviral infection. Before infection, Arf1 and PI4KIII β were colocalized at the Golgi apparatus (Figure 3-7, 0hr) but at 4 hours post infection, in striking contrast to COPI and clathrin, PI4KIII β remained colocalized with Arf1 (Figure 3- 7, 4hr). This localization remained for the rest of the infection (Figure 3-7, 6hrs) and was correlated with GBF1/Arf1 localization (Figure 3-3A). Furthermore, PI4KIII β was not just spatiotemporally correlated with replication organelles but was in a physical association with the viral replication enzyme complex. Upon immunoprecipitation of PI4KIII β from infected cells at 4 hr. post-infection, the enteroviral 3A, 3AB, 3CD, and 3D proteins, which are all components of the replication complex, coprecipitated with it(Hsu et al., 2010).

CONCLUSIONS

In this chapter, we found that replication organelles were unconventional intracellular organelles with a distinct combination of a subset of Golgi/TGN/ERGIC proteins. In particular, the replication organelles were enriched in Arf1 and GBF1. Most notably, among Arf1 effectors only, PI4KIII β was localized to replication organelles.

Figure 3-1: Viral RNA and replication protein (3A, 3D) subcellular distribution in early stages of CVB3 infection.

Top panel: HeLa cells expressing Arf1-GFP were infected with CVB3 for 2hr. Cells were fixed and stained with Alexa 555 labeled plus strand RNA probe follow by anti-GFP antibody.

Middle panel: HeLa cells were infected with CVB3 for 2 hr. Then cells were fixed and coimmunostained with anti-3A and TGN46 antibody.

Bottom panel: HeLa cells expressing GalT-YFP were infected with CVB3 for 2hr. Cells were fixed and coimmunostained with anti-3D and anti-GFP antibody. All fluorescence images were confocal images of optical slice thickness~ 1 μm . Scale bar, 10 μm .

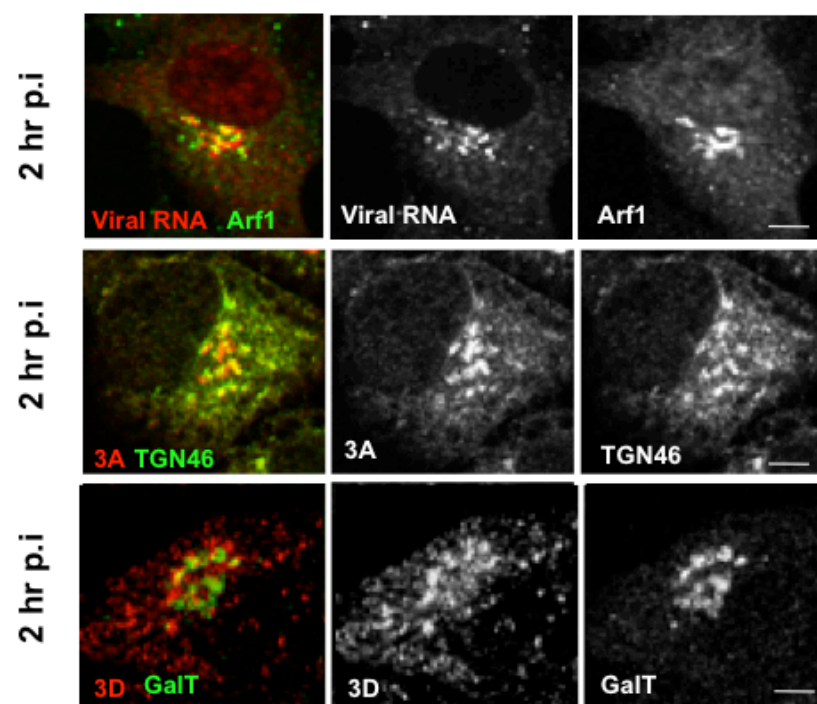


Figure 3-2: Viral RNA and replication protein (3A, 3D) subcellular localization at peak and late stages of CVB3 infection.

Top panel: HeLa cells expressing Arf1-GFP were infected with CVB3 for 4hr.

Cells were fixed and coimmunostained with anti-3D and anti-GFP antibody.

Middle / bottom panel: HeLa cells expressing Arf1-GFP were infected with CVB3 for 4hr(middle panel) or 8 hr. (bottom panel). Cells were fixed and stained with Alexa 555 labeled plus strand RNA probe follow by anti-GFP antibody.

All fluorescence images were confocal images of optical slice thickness~ 1 μ m.

Scale bar, 10 μ m.

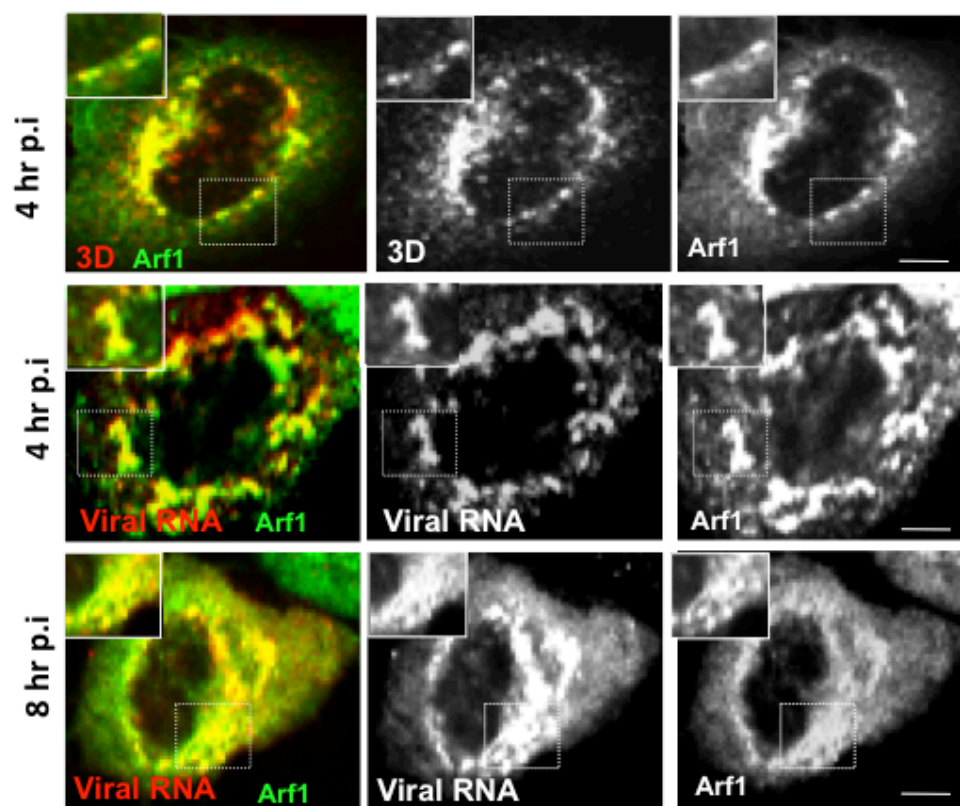


Figure 3-3: GBF1 is colocalized with viral RNA and Arf1 in CVB3 infected cells.

A) Arf1-RFP and GBF1-YFP dynamics in CVB3-infected HeLa cell. Confocal time-lapse images of single cell are presented.

B) GBF1 and viral RNA are colocalized in HeLa cells during CVB3 infection.

All fluorescence images were confocal images of optical slice thickness~ 1 μm .

Scale bar, 10 μm .

C) Arf1 and GBF1 facilitate viral RNA replication. CVB3 replicon assays in HeLa cells pretreated with siRNA against Arf1 or GBF1. Bar graph presents maximum replication values for each condition, normalized to control samples transfected with nontargeting siRNA. Error bars are SEM from eight replicates for each condition (** $p < 0.01$).

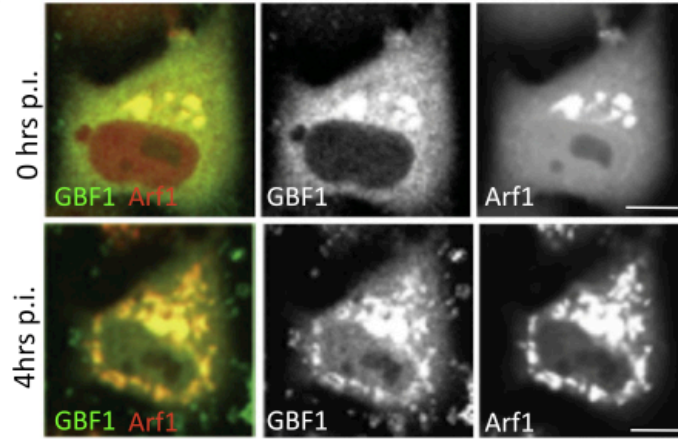
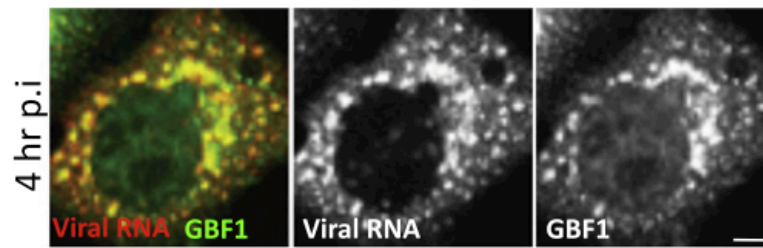
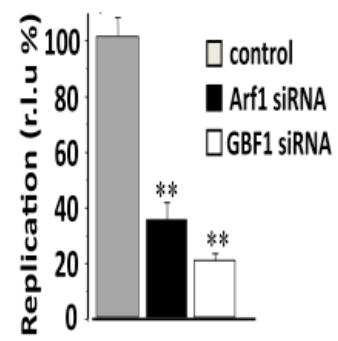
A**B****C**

Figure 3-4: Replication organelles forms adjacent to ER exit sites and functional ER exit sites facilitate viral RNA replication.

A) Replication organelles, at peak replication, emerge adjacent to ER exit sites.

Fluorescence images were confocal images of optical slice thickness~ 1 μm .

Scale bar, 10 μm .

B) PV replicon assays in HeLa cells transiently expressing Sar1[T39N] plasmid.

Bar graph presents maximum replication values, normalized to control samples transfected with GFP. Error bars are SEM from eight replicates for each condition (** $p < 0.01$). r.l.u% = relative light unit %.

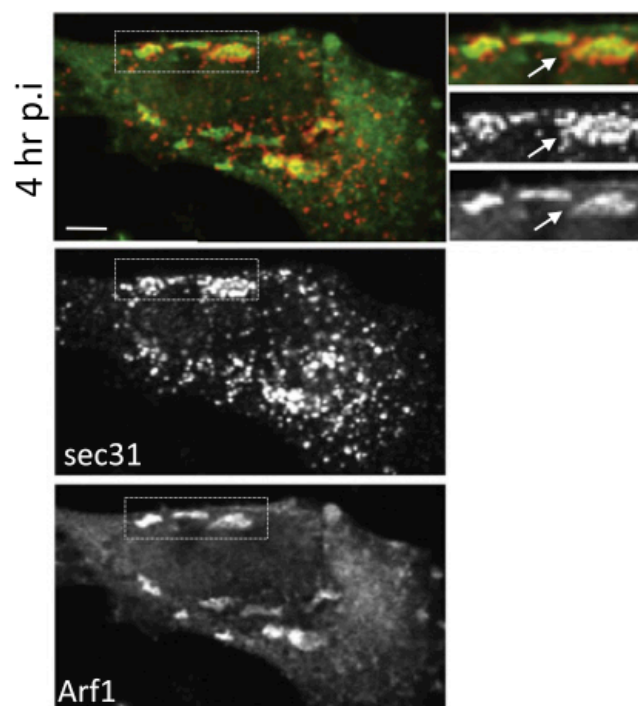
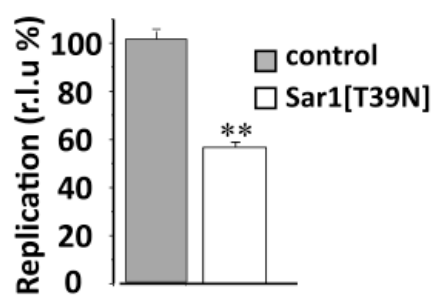
A**B**

Figure 3-5: The subset of host secretory pathway proteins that is absent from replication membranes.

HeLa cells **(A)** coexpressing Arf1-RFP/ ϵ COP-YFP; expressing Arf1-GFP **(B, C, E)**; or coexpressing Arf1-RFP/GaIT-YFP **(D)** were infected with CVB3 for 4 hr. Cells in (B), (C), (E) were fixed and coimmunostained with anti-GFP and (B) anti-clathrin heavy chain; (C) anti- γ -adaptin; (E) anti-GM130 antibodies. Arrows in (A), (B), (C), and (D) indicate Arf1-labeled membranes that do not label with ϵ COP-YFP, clathrin, γ -adaptin, and GaIT-YFP, respectively. All fluorescence images were confocal images of optical slice thickness~ 1 μ m. Scale bar, 10 μ m.

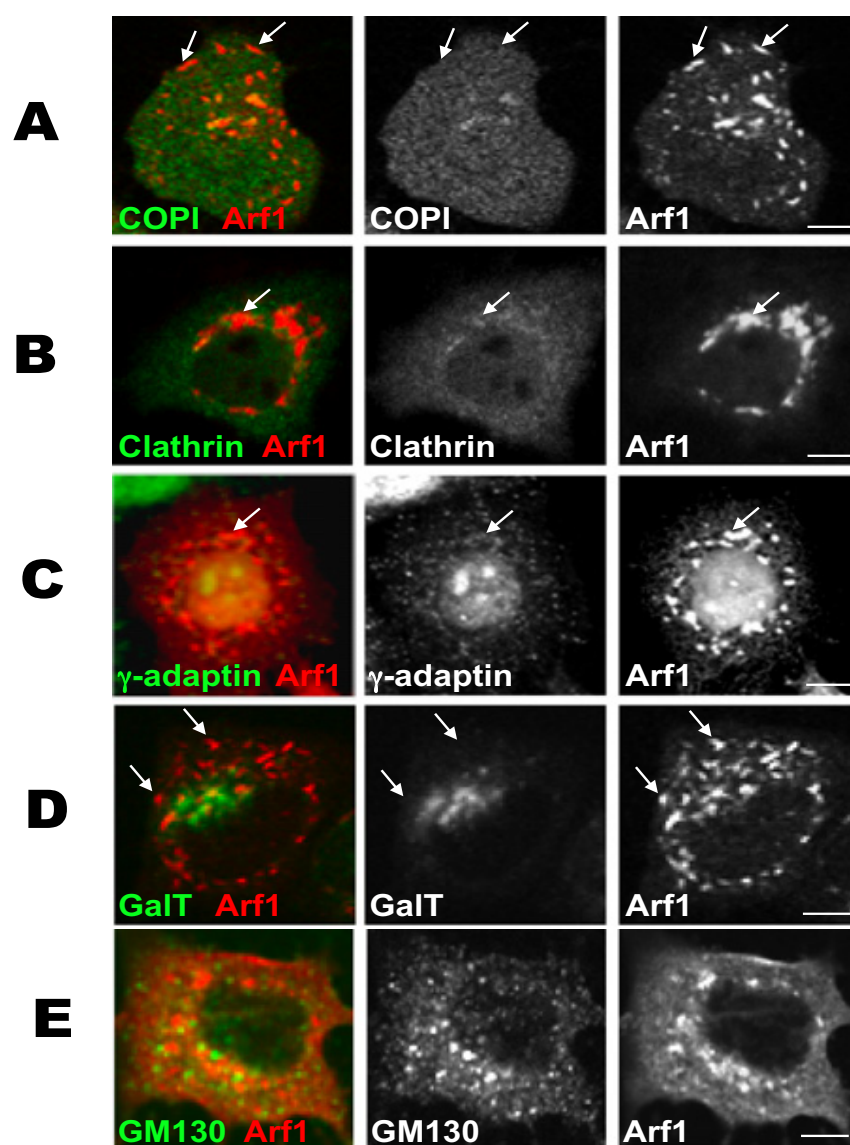


Figure 3-6: The subset of host secretory pathway proteins that is present at replication membranes.

HeLa cells expressing Arf1-GFP were infected with CVB3 for 4 hr. Cells were fixed and coimmunostained with anti-GFP and (A) anti-TGN46; (B) anti-GGA1; (C) anti-Rab1b; (D) anti-ERGIC53 antibodies. All fluorescence images were confocal images of optical slice thickness~ 1 μm . Scale bar, 10 μm

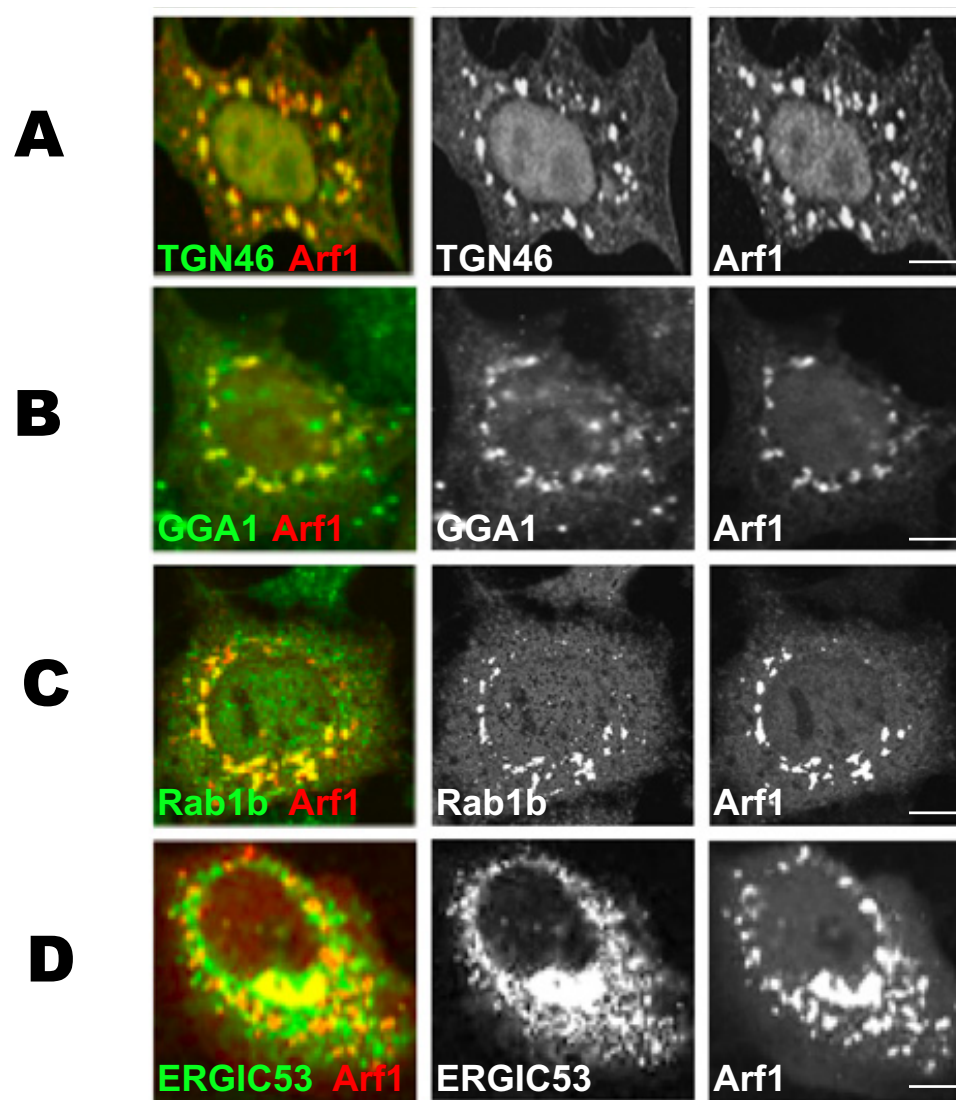
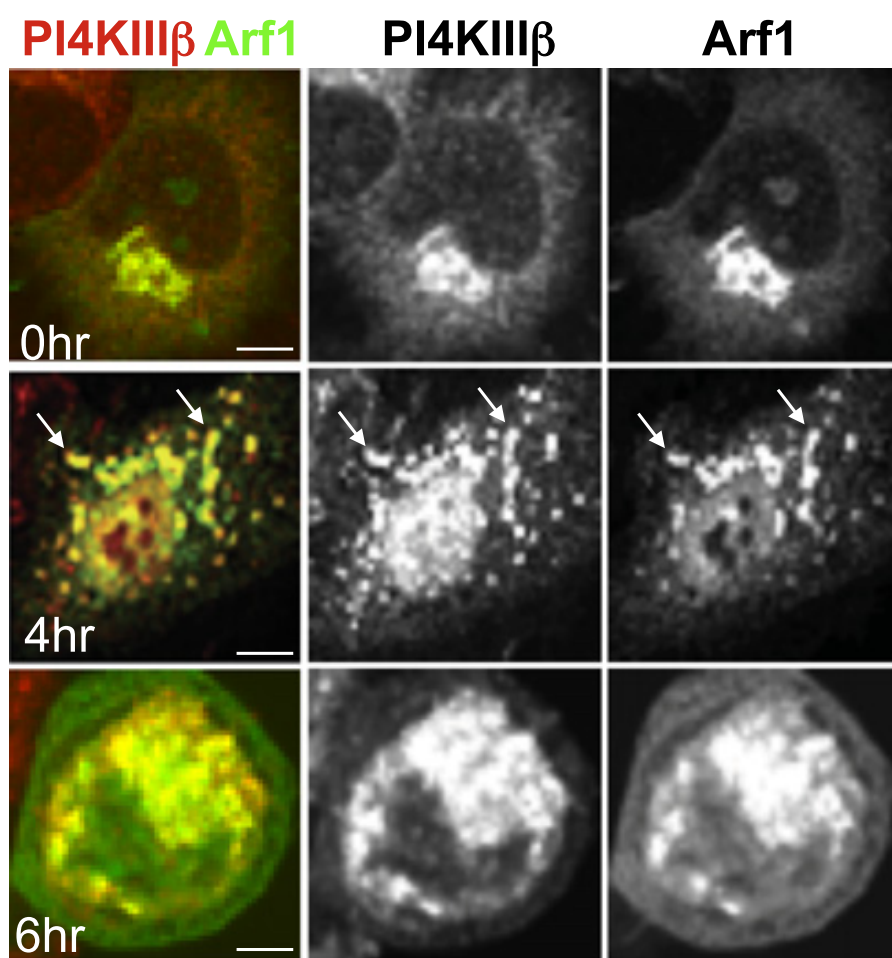


Figure 3-7:PI4KIII β Is Localized to Enteroviral RNA Replication**membranes.**

HeLa cells expressing Arf1-GFP infected with CVB3 were immunostained with anti-PI4KIII β and anti-GFP antibodies. All fluorescence images were confocal images of optical slice thickness~ 1 μ m. Scale bar, 10 μ m.



CHAPTER 4

The function of PI4KIII β during enteroviral replication

PI4KIII β is an evolutionarily conserved protein, and its homologs are found from yeast to human. Biochemical studies demonstrate that it is a lipid kinase that transfers a phosphate from ATP to the 4th position of the inositol ring of phosphatidylinositol 1-phosphate (PI) in order to generate PI4P (Meyers and Cantley, 1997). Structurally, PI4KIII β contains six domains: a proline-rich, a lipid kinase unique, an Fq, a serine-rich, a Hom2 and a catalytic domain (Balla and Balla, 2006). The enzymatic reaction is performed by the catalytic domain, which carries a critical aspartic acid residue (Asp 656) in its catalytic core. When aspartic acid 656 is mutated to alanine, PI4KIII β does not incorporate ³²P ATP into PI4P in an *in vitro* lipid kinase assay (Zhao et al., 2000). Thus this mutation is referred to as generating the kinase dead PI4KIII β -KD D656A protein.

The biological functions of PI4KIII β were revealed by genetic elimination of PI4KIII β and/or overexpression of PI4KIII β -KD D656A in cells. Genetic deletion of the PIK1 gene, the PI4KIII β yeast homolog, in *Saccharomyces cerevisiae* was lethal (Flanagan et al., 1993). In yeast with a

temperature sensitive mutation of the PIK1 gene, the secretion of invertase was reduced by 70% (Hama et al., 1999). Ectopically expressed PI4KIII β -KD, which decreases PI4P lipid levels in COS7 cells, also inhibited the membrane trafficking of VSVG proteins from the TGN to the plasma membrane (Godi et al., 2004a).

Besides protein secretion, PI4KIII β also plays an essential role in regulating actin organization. For example, spermatocytes of a PI4KIII β mutant fruit fly failed to form stable intercellular bridges and instead fused together to produce multinucleated spermatids. This phenotype suggests that cytokinesis failed, a process, which is regulated by actin (Brill et al., 2000).

In chapter 3, we had demonstrated that host PI4KIII β was preferentially recruited to replication organelle membranes of enterovirus infected cells. Here, we address the functional role of PI4KIII β in enteroviral replication. Given its localization to the replication organelle membranes, we hypothesized that PI4KIII β may be essential for the enteroviral RNA synthesis.

RESULTS

PI4KIII β Activity Is Required for Enteroviral RNA Replication

Given the localization of PI4KIII β at replication membranes (Figure 3-7), we tested whether PI4KIII β had any potential role in the viral replication process. First we depleted PI4KIII β mRNA, using siRNA, to reduce protein production. HeLa cells transfected with non-targeting or PI4KIII β siRNA-for 48 hours were subsequently transfected with poliovirus or CVB3 replicons, and bioluminescence was measured as an indicator of replication. We confirmed that PI4KIII β was indeed depleted by 99%, by western blotting the lysates from siRNA-treated cells with an antibody against native PI4KIII β (Figure 4-1 A). Especially, reducing PI4KIII β protein dramatically diminished RNA replication by ~80% (Figure 4-1B). Since in yeast PI4KIII β was shown to be an essential gene for survival, we asked if the inhibition of RNA replication was due to cell death. To assess cell viability, we measured ATP production. We found that there was no difference in cell viability between non-targeting and PI4KIII β siRNA- transfected cells (Figure 4-1C). Therefore, the reduction in viral replication we measured was not due to cell death but due to the absence of PI4KIII β . These findings demonstrate that PI4KIII β is critical for

enteroviral RNA replication.

In uninfected cells, the function of PI4KIII β is typically mediated through its enzymatic activity. So, we next asked whether PI4KIII β enzymatic activity was also required for enteroviral RNA replication. We used two methods to inhibit PI4KIII β enzymatic activity: using the small molecule inhibitor PIK93 or expressing the kinase dead PI4KIII β (D656A) in cells. PIK93 was added to cells immediately after viral replicon transfection. PIK93 selectively inhibits PI4KIII β activity by competitively binding to the ATP binding site of the enzyme (Knight et al., 2006). We found that in the presence of PIK93, enteroviral RNA replication was significantly reduced in a dose dependent manner (Figure 4-2 A). As for the PI4KIII β D656A-expressing cells, viral RNA replication was also reduced approximately by 70% when compared with GFP-expressing control cells (Figure 4-2 B). Hence, these experiments show that inhibition of PI4KIII β kinase activity also inhibits enteroviral RNA replication.

PI4P Lipids Regulate Enteroviral RNA Replication

Given the localization of PI4KIII β to replication organelles, and the inhibition of replication in the absence of PI4KIII β activity, we reasoned next that PI4P lipids may play a role in enteroviral replication. As a first step to test this possibility, we monitored the levels of cellular PI4P, the known lipid product of PI4KIII β activity, during infection with CVB3. Total PI4P lipid was extracted from uninfected or infected cells by removing the neutral lipids first, followed by extraction of the acidic lipids, which contain the PI4P lipid. The PI4P lipid level was determined by PI4P detectors and quantified by correlating the intensity of the samples to a standard curve generated from known concentrations of PI4P. We found a striking ~5-fold increase in PI4P levels within the first four hours of CVB3 infection (Figure 4-3A).

We conjectured that the increased levels of PI4P lipids during CVB3 infection were likely localized to the replication organelle membranes. To determine whether the PI4P lipids, along with PI4KIII β , were localized to these membranes, we utilized GFP-tagged FAPP1-PH proteins. These proteins are known to bind PI4P lipid containing membranes in the presence of Arf1 (Godi et al., 2004b; Toth et al., 2006). We co-expressed Arf1-RFP and FAPP1-PH-

GFP and acquired time-lapse confocal images of individual cells during infection to monitor both the distribution of Arf1 and PI4P lipids. At the start of infection the major PI4P pool in the cell was found at the Golgi/TGN compartment. But after four hours all the newly formed Arf1-labeled organelles contained PI4P lipids (Figure 4-3B). Taken together, this demonstrated that replication organelle membranes are highly enriched in PI4P lipids generated likely by the PI4KIII β enzymes localized at these organelles. To confirm that PI4KIII β indeed generated these PI4P lipids, we acutely treated cells 4 hrs. post infection with PIK93 and monitored the PI4P lipid levels on the replication organelles. Acute inhibition of PI4KIII β with PIK93 significantly reduced the PI4P lipids from the replication organelles thus strongly suggesting that the PI4KIII β at these organelles was responsible for the increased PI4P lipids (Figure 4-3 C). Abundant quantities of PI4P are generated during CVB3 infection implicating a probable role of the PI4P lipids themselves in viral replication. To test whether PI4P lipids, independent of PI4KIII β , have a role in regulating viral RNA replication, we ectopically expressed Sac1 phosphatase in cells and performed a replicon assay. Sac1 phosphatase has been shown to specifically convert PI4P lipids back to PIs

(Blagoveshchenskaya et al., 2008). Viral replication was also inhibited in Sac1-overexpressing cells, but the inhibition was ~40% (Figure 4-3 D), likely due to competition with endogenous PI4K activity which would have been producing PI4P. Taken together, our work suggests that the PI4P lipid environment is critical for viral replication.

Enteroviral 3A Protein Preferentially Recruits PI4KIII β to Membranes

Since we have shown that PI4KIII β is critical for the enteroviral life cycle, we conjectured that enteroviruses maybe able to selectively recruit PI4KIII β to the replication organelle membranes. Enteroviral 3A, a viral protein necessary for RNA replication, anchors into the membrane via its C terminal hydrophobic residues. It has been shown to activate Arf1 by binding to GBF1 during viral infection (Belov et al., 2007; Wessels et al., 2006). Given that 3A is able to activate Arf1 during enteroviral infection, we tested whether the CVB3 3A protein by itself could preferentially recruit PI4KIII β to membranes as we had previously observed during CVB3 infections. A myc-tagged version of the CVB3 3A protein was transiently expressed. Then the 3A,

PI4KIII β , PI4KIII α and/or COPI proteins were labeled by myc-, PI4KIII β -, PI4KIII α - or β COP- specific antibodies respectively. Results show that the 3A proteins were localized to perinuclear structures after expression (Figure 4-4, arrow). The levels of β COP, a component of the COPI coat complex, was decreased at these structures about 2-fold compared to neighboring cells not expressing 3A (Figure 4-4A). Significantly, despite decreased in β COP levels at 3A- labeled membrane structures, we found that PI4KIII β was redistributed with about a 3-fold increase to these membranes in the 3A-expressing cells relative to cells where 3A was absent (Figure 4-4B). Notably the recruitment to membranes of PI4K family member PI4KIII α was not enhanced by 3A (Figure 4-4C). Thus, 3A expression alone specifically enhances the membrane recruitment of one Arf1 effector, PI4KIII β , over another, COPI, and mimics the virally infected cell phenotype (Figure 3-5A; 3-7).

CONCLUSIONS

PI4KIII β , one of the few Arf1 effectors, was found to be localized to enteroviral replication organelle membranes (see Chapter 3). Our findings in this chapter indicate that a critical function for PI4KIII β during enteroviral infection is to produce high levels of PI4P lipids at the replication organelle membranes. When PI4P lipids are depleted from cells, we find that enteroviral replication is significantly inhibited, indicating the importance of the PI4P lipid microenvironment for the enteroviral life cycle. Lastly, we show that enteroviruses utilize 3A proteins to modulate Arf1 effector selection so as to preferentially recruit PI4KIII β enzymes to replication organelle membranes while suppressing the recruitment of COP1. The latter would potentially explain the disassembly of the Golgi apparatus during the course of infection (Figure 3-5 D and E) since coat proteins are required to maintain the secretory pathway.

Figure 4-1: PI4KIII β protein is required for enteroviral RNA synthesis

A) Western blots of HeLa cells treated with nontargeting (control) or PI4KIII β siRNAs (PI4KIII β).

B) Kinetics of inhibition by PI4KIII β siRNA presented for poliovirus replicon.

Poliovirus replicon assays in PI4KIII β (triangle) or nontargeting (square; control) siRNA treated HeLa cells. Error bars are SEM from six replicates for each condition (**p < 0.01). r.l.u% = relative light unit %.

C) Cell viability assay of HeLa cells treated with nontargeting (control) or PI4KIII β siRNAs (PI4KIII β siRNA).

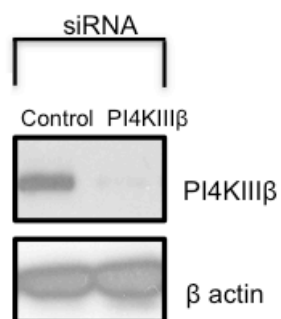
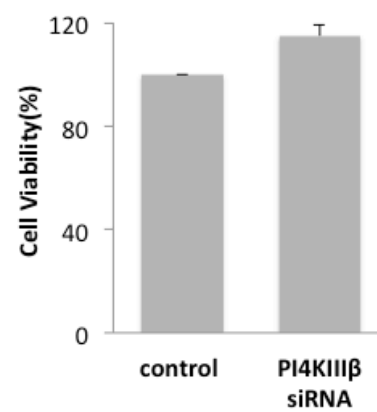
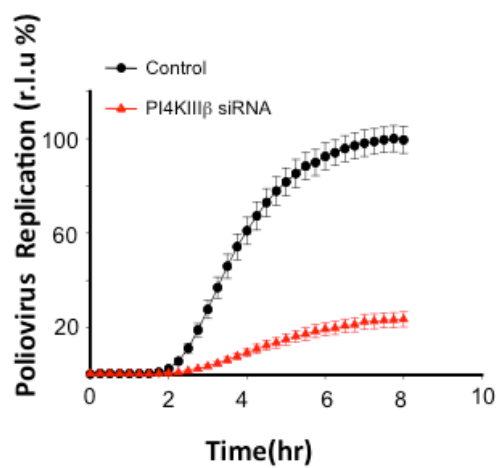
A**C****B**

Figure 4-2: PI4KIII β enzymatic activity is necessary for viral RNA replication

A) Poliovirus replicon assays in cells treated with 500 nM and 1 μ M PIK93 are shown. Bar graphs present maximum replication values for PV with PIK93 treatment, normalized to control (DMSO) treatment. Error bars are SEM from six replicates for each condition (**p < 0.01). r.l.u% = relative light unit %.

B) Poliovirus replicon assays in HeLa cells expressing kinase dead PI4KIII β (PI4KIII β -KD) or GFP (control) protein; bar graphs present maximum replication values for each condition, normalized to control (GFP) plasmid ectopic expression. Error bars are SEM from six replicates for each condition (**p < 0.01). r.l.u% = relative light unit %.

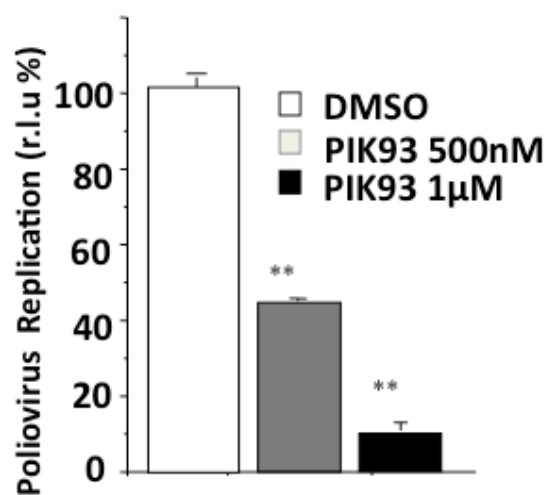
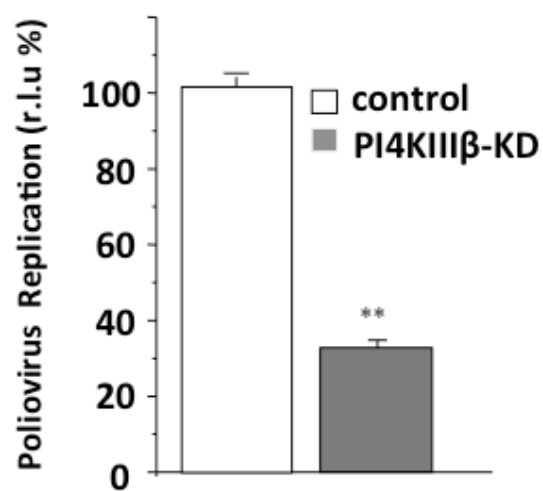
A**B**

Figure 4-3: PI4P Lipid Microenvironment within Replication Organelles**Regulates Enteroviral RNA Replication.**

- A)** Cellular PI4P lipid levels rise in CVB3 infection. Total cellular PI4P lipids were quantified over time. Error bars are SEM from duplicate samples (**p < 0.01).
- B)** PI4P lipids localize to enteroviral replication organelles. Time-lapse confocal images of single HeLa cell infected with CVB3, coexpressing FAPP1PH-GFP/ Arf1-RFP. The optical slice thickness of confocal images is ~ 1 μ m. Scale bar, 10 μ m.
- C)** PI4KIII β activity is responsible for PI4P lipids at enteroviral replication organelles. Time-lapse confocal image of single HeLa cell coexpressing FAPP1-PHGFP/ ARF1-RFP pre- and post-PIK93 treatment at 4 hr post-infection with CVB3.
- D)** Reduction in PI4P lipid levels inhibits enteroviral RNA replication. PV replicon assays in HeLa cells ectopically expressing Sac1 or GFP (control) are shown. Bar graph presents maximum replication values, normalized to control (GFP) plasmid ectopic expression. Error bars are SEM from eight replicates cells (**p < 0.01).

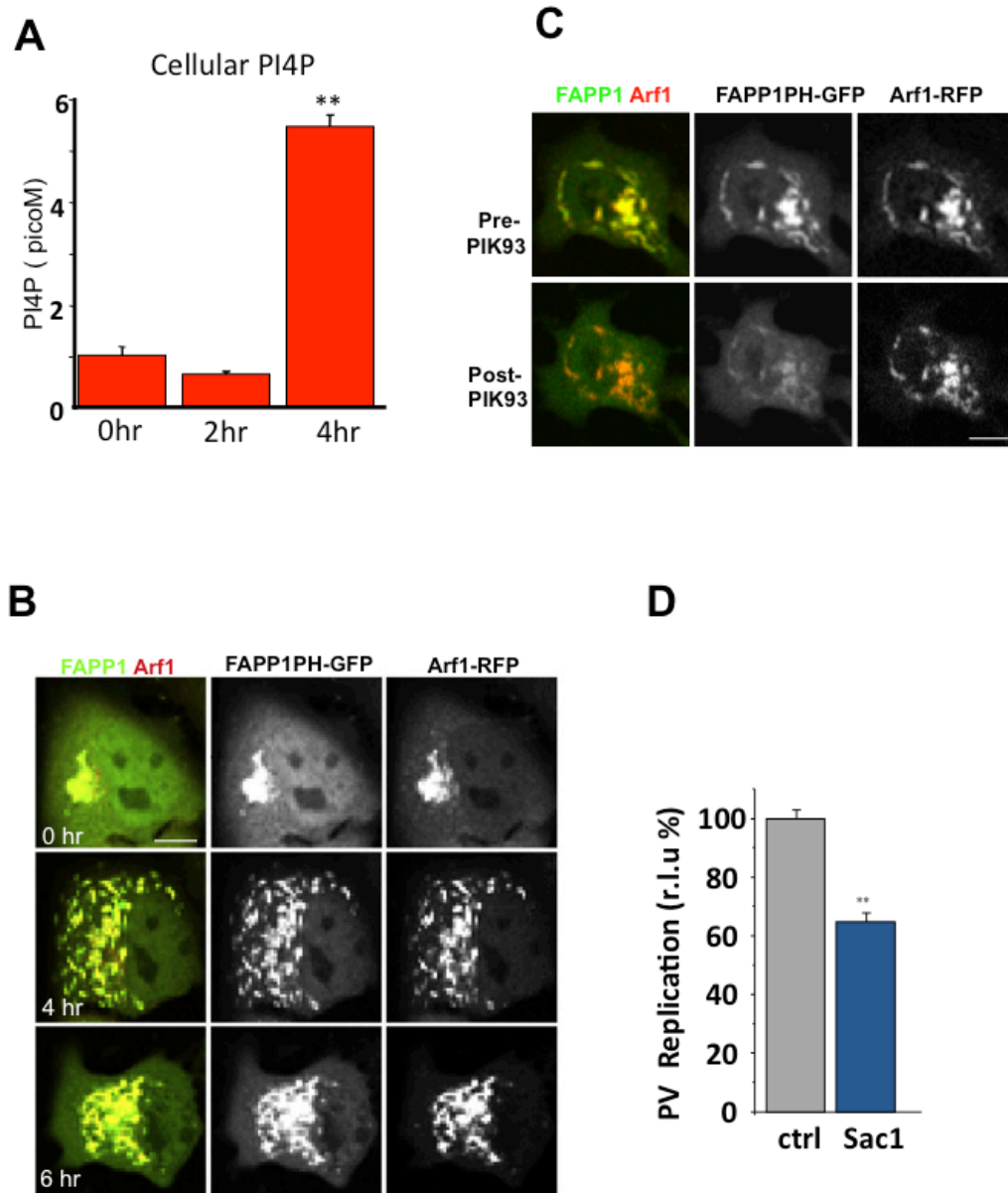
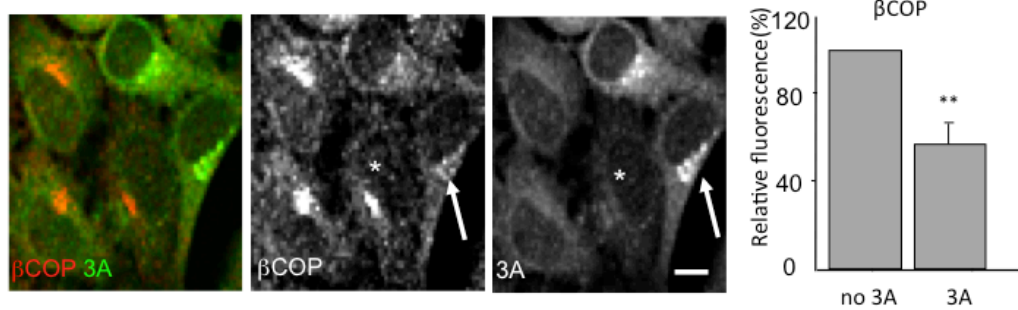
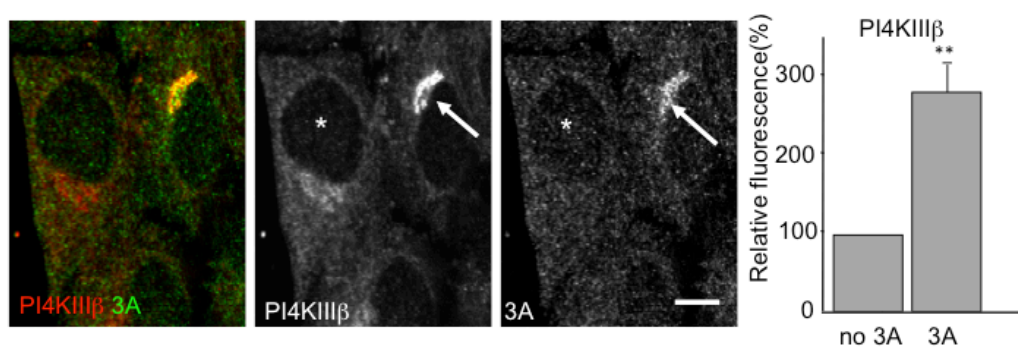
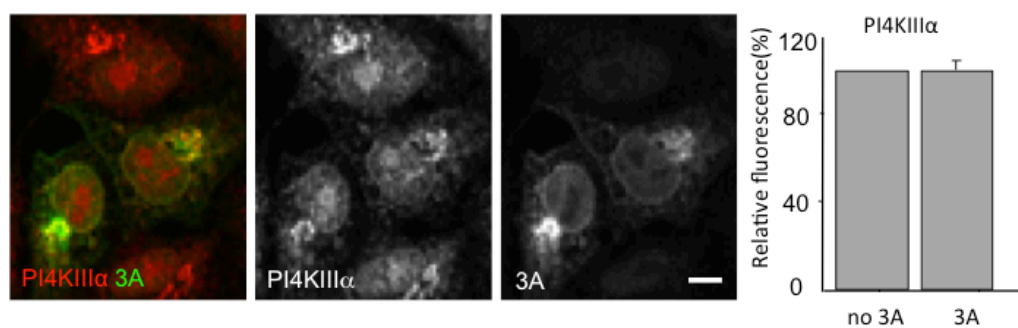


Figure 4-4: Enteroviral 3A Proteins Can Promote Selective PI4KIII β **Recruitment over Coat Proteins.**

HeLa cells were immunostained with antibodies to myc-tag and native: (A) β COP (B) PI4KIII β (C) PI4KIII α . Arrows and asterisk indicate cells where 3A is either expressed (arrow) or not (*). Bar graph represents quantification result of β COP (n = 10 cells), PI4KIII β (n = 13 cells) and PI4KIII α (n = 10 cells) antibody fluorescence associated with 3A-labeled membranes as (%) of their respective values at the Golgi apparatus of cells not expressing 3A. Error bars are SEM (**p < 0.001). All fluorescence images were confocal images of optical slice thickness~ 1 μ m. Scale bar, 10 μ m

A**B****C**

CHAPTER 5

The prevalence of PI4P lipids microenvironment in other plus strand RNA viruses

Previously, in chapter 3 and 4, we described how enteroviruses hijacked the Arf1/GBF1/PI4KIII β pathway to generate a PI4P enriched lipid microenvironment for their replication. The majority of plus strand RNA viruses rely on host membranes for replication (Salonen et al., 2005). Membranes are composed of different lipids, which may provide a suitable lipid environment to favor viral replication (Miller and Krijnse-Locker, 2008). Therefore, we hypothesized that there may be a common lipid environment that is created by plus strand RNA viruses to facilitate RNA synthesis process.

HCV, an enveloped virus, is a member of the hepacivirus genus of viruses. It assembles replication complexes and replicates its RNA on host membranes that are derived from ER (Wolk et al., 2008). HCV is known to assemble its replication complexes on membranous webs which originate from host ER membranes (Wolk et al., 2008).. There are four lines of evidence that suggest that there may be a PI4P lipid environment present at the replication membranes of Hepatitis C virus. Trotard et al. showed that

knocking down PI4KIII α or III β genes significantly reduced HCV infection and the level of plus strand RNA (Trotard et al., 2009). Secondly, Berger et al. revealed that PI4KIII α was very closely associated with HCV replication complex protein NS5A and double stranded RNA, which was the intermediate stage of HCV RNA replication (Berger et al., 2009). Thirdly, when PI4KIII α mRNA was silenced, the formation of the HCV replication membranes was perturbed (Berger et al., 2009). Finally, Borawski et al. showed that HCV replication was sensitive to PIK93 (Borawski et al., 2009). Therefore in this chapter, we describe experiments to test whether HCV replication, similar to enteroviruses, is also dependent on a PI4P lipid microenvironment.

RESULTS

PI4P Lipid Is Enriched in HCV Replication Membranes

3-5B(HCV) is a Huh7 derived liver cell line and contains autonomously replicating, subgenomic, dicistronic, selectable HCV RNAs from infectious HCV-N1b strain as well as expressing HCV non-structural proteins (Ikeda et al., 2002). 3-5B(HCV) cells were coimmunostained with antibodies against

PI4P lipids and the HCV protein NS5A. NS5A is a membrane-associated protein that is part of the HCV replication complex and colocalizes with HCV RNA molecules (Wolk et al., 2008), thus allowing us to identify RNA replication membranes. In the parental Huh7 cells, the PI4P lipid pool was localized to the perinuclear Golgi apparatus/TGN region (Figure 5-1, bottom panel). However, in 3-5B(HCV) cells PI4P lipid levels were increased and they were localized to NS5A containing replication membranes (Figure 5-1, top panel). Since PI4KIII β generated PI4P at enteroviral replication membranes, we then examined if PI4KIII β was responsible for producing the PI4P pools in 3-5B(HCV) cells. We depleted PI4KIII β using siRNA and then investigated whether PI4P lipids were affected by performing immunofluorescence with antibodies against PI4P (Figure 5-2). Note that in these images, the intensity of PI4P image correlates to the total amount of PI4P within cell and is not saturated. Compared to the non-targeting siRNA treated control cells, the levels of PI4P lipids in PI4KIII β siRNA treated cells were significantly reduced (around 80% reduction) (Figure 5-2 middle panel), indicating that PI4KIII β was responsible for the bulk of the PI4P production at HCV replication membranes. In order to rule out the possibility that this phenotype was due to knocking

down PI4KIII α , we aligned human PI4KIII α mRNA(NM_058004) with PI4KIII β siRNA target sequence(AA CAC GAA GGA UCA UGU GGU A) using bl2seq program with blastn algorithm from National Center for Biotechnology Information. There was no significant similarity found between two nucleotide sequences suggesting that antisense strand of PI4KIII β siRNA can not complementary to any part of PI4KIII α mRNA resulting in PI4KIII α mRNA degradation. Therefore, it is unlikely that the effect of PI4P reduction in PI4KIII β siRNA treated cell is due to knocking down of PI4KIII α .

Given that, in sucrose gradient assays ER-localized PI4KIII α is co-fractionated with the HCV replication complex protein NS5A (Berger et al., 2009), we next determined whether PI4KIII α was also contributing to the PI4P pools. Therefore we performed immunofluorescence staining of PI4P lipids in cells depleted of PI4KIII α . Similar to PI4KIII β siRNA treated cells, the intensity of PI4P in PI4KIII α treated cells was also reduced but to a lesser extent (around 40% reduction) (Figure 5-2 right panel). This experiment indicated that both PI4KIII α and PI4KIII β enzymes contribute to the PI4P lipid pools at the HCV replication membranes. Notably, after our studies were published it was shown by Reiss et al., that PI4P lipid environment was also

present in the replication membranes from primary liver sections of HCV infected individuals thus indicating the clinical significance of this lipid for HCV pathogenesis (Reiss et al., 2011).

PI4P Lipid Regulates HCV RNA Synthesis

Next to investigate whether PI4P lipids were required for HCV RNA replication, we measured the replication of HCV J6/JFH (p7-Rluc2A) replicons (Jones et al., 2007) in PI4P lipid-depleted Huh7 cells (Figure 5-3). We applied three approaches to deplete PI4P lipid pools in Huh7 cells. First, we transiently transfected PI4KIII β -KD plasmid into Huh7 cells since PI4KIII β was the major enzyme producing PI4P lipids at HCV replication membranes. Next, we ectopically expressed Sac1 phosphatase to convert PI4P back to PI in Huh7 cells, thus resulting in decreased levels of PI4P. Finally, we combined first two approaches. Similar to our findings in enterovirus (Figure 4-2B), cells overexpressing PI4KIII β -KD exhibited HCV RNA replication that was decreased by 60%. In addition, HCV RNA replication was inhibited by more than 50% in Sac1-expressing cells. As predicted, when we co-expressed PI4KIII β -KD and Sac1 plasmid in Huh7 cells, HCV replication inhibition was

greater (~ 70%). Thus the PI4P lipid microenvironment is an important regulator of both enteroviral and hepaciviral RNA replication.

CONCLUSIONS

In this chapter, we characterized the lipid environment of HCV replication membranes. HCV replication membranes were enriched in PI4P lipids, which were produced by PI4KIII β and PI4KIII α . Depletion of PI4P lipids at the replication membranes significantly diminished viral RNA replication. Here, we provided the first evidence that PI4P lipid microenvironment was prevalent in HCV as well.

Figure 5-1: PI4P lipids localize to HCV replication membranes.

3-5(HCV) and Huh7 cells were fixed with 3.7% paraformaldehyde and co-immunostained with anti-PI4P and anti-NS5A antibodies. PI4P were labeled with secondary antibody conjugated with Alexa 555 (shown in Red), and NS5A were labeled with secondary antibody conjugated with Alexa 488 (shown in green). Scale bar, 10 μ m

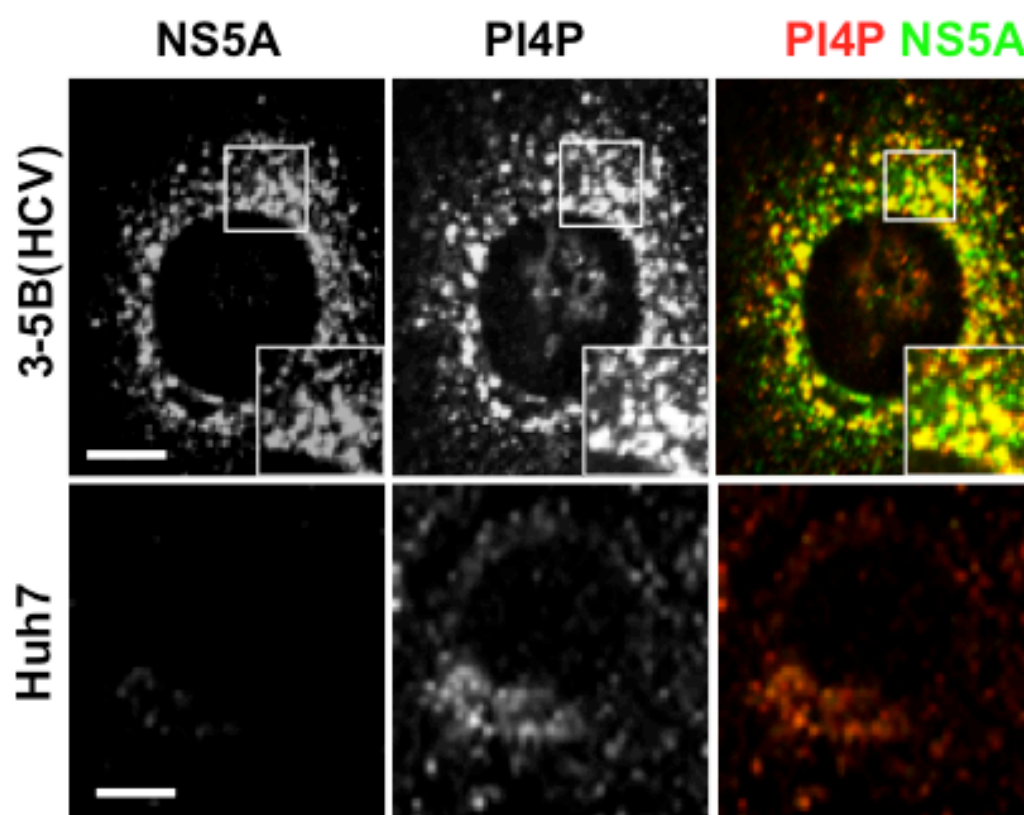


Figure 5-2: PI4KIII β is responsible for a significant fraction of PI4P lipids at HCV replication membranes.

3-5B(HCV) cells were treated with nontargeting, PI4KIII β , or PI4KIII α siRNA.

Cells were immunostained for PI4P lipids. Representative images of groups of siRNA-treated cells are shown. All fluorescence images were confocal images of optical slice thickness of $\sim 1 \mu\text{m}$. Scale bar, $10 \mu\text{m}$

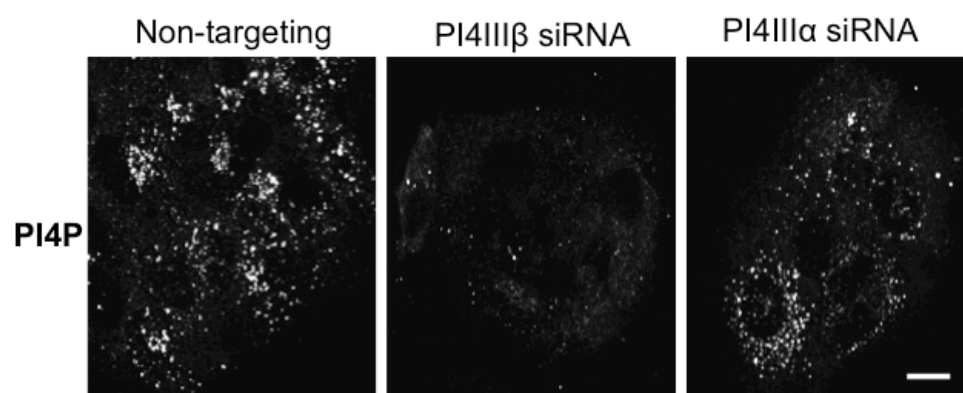
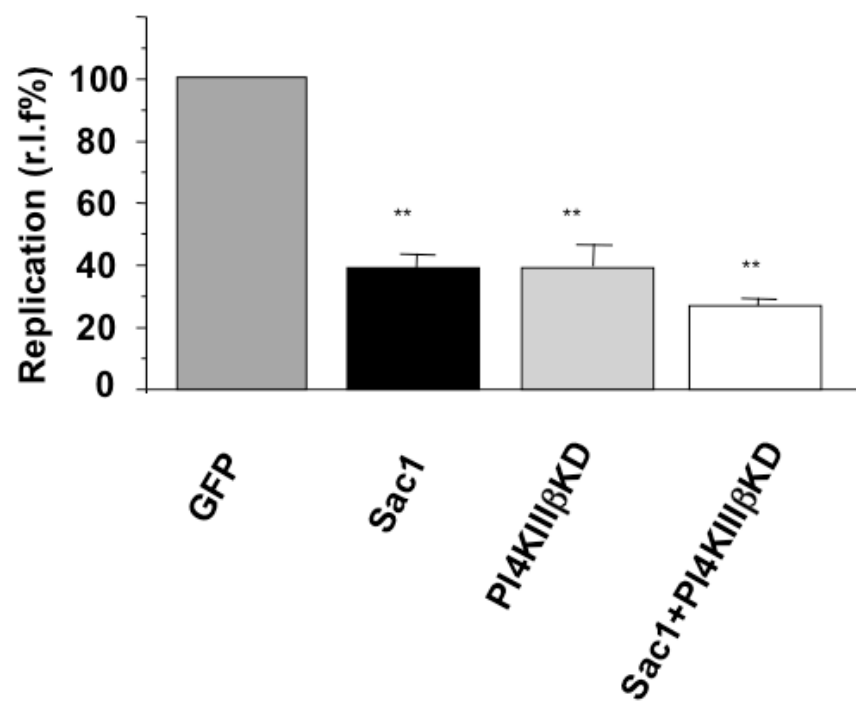


Figure 5-3: Reduction of PI4P lipids inhibits HCV replication.

Results of HCV replicon assays conducted with J6/JFH (p7-Rluc2A) replicons in Huh7 cells ectopically expressing Sac1, PI4KIII β -KD, or both plasmids are shown. Bar graph presents maximum replication value normalized to control (GFP) plasmid ectopic expression. Error bars are SEM from eight replicates of cells for each treatment condition (**p < 0.01).



CHAPTER 6

The mechanism of PI4P mediated viral RNA replication

Although phosphoinositides are a minority of membrane phospholipids, they conduct critical roles in regulating cellular physiological activities such as signaling, membrane trafficking and protein secretion. Cells can generate several species of phosphoinositides distinguished from each other by which position in the inositol ring of the phospholipid head group is phosphorylated. These are PI, phosphatidylinositol-3-phosphate (PI3P), PI4P, phosphatidylinositol-5-phosphate (PI5P), phosphatidylinositol-4, 5-phosphate (PIP₂) and phosphatidylinositol-3,4,5-phosphate (PIP₃).

Among monophosphorylated PI species, PI4P is the most abundant. Its amount is similar to PIP₂ within cells (Lemmon, 2008). PIP₂ is a plasma membrane-localized lipid known to generate secondary messengers using a G-protein coupled receptor signal transduction pathway, and thus it is considered a critical phosphoinositide within cells (Berridge et al., 1984; Berridge and Irvine, 1984). For a long time, PI4P was thought to function only as a PIP₂ precursor (D'Angelo et al., 2008). However, accumulating

evidence suggests that PI4P has its own role in regulating several cellular functions. At a steady state, the majority of PI4P is localized at the Golgi (Blagoveshchenskaya et al., 2008). Furthermore, it has been well established that PI4P plays a key role in trafficking at Golgi membranes in yeast (Walch-Solimena and Novick, 1999), mammalian cells (Godi et al., 1999) and plant cells (Preuss et al., 2006). The role of PI4P in regulating cellular function is through its downstream effectors. The effector proteins contain a PI4P binding domain, and thus are able to bind to PI4P. The known PI4P effectors include FAPP, CERT, AP-1 and GGA (see chapter 1 for detail description) which are involved in lipid metabolism and membrane trafficking.

Plasma membranes contain lipid microdomains which are made of cholesterol, glycolipid, sphingolipids and proteins (Simons and van Meer, 1988). Different signaling proteins with specific lipid binding domains can be recruited to these microdomains and execute signaling events (Simons and Toomre, 2000). Thus these domains serve as an important organizing center for the assembly of membrane associated signaling molecules (Simons and Ikonen, 1997). Phosphoinositides are also present in these domains. The head group of phosphoinositides is highly negatively charged. As a result, the

head group repels to each other. Having cholesterol in these domains help partition and organize phosphoinositides and proteins within membrane bilayers. Given our findings that replication organelle membranes of both entero and hepaciviruses are enriched in PI4P lipids and that these lipids are required for replication on these membranes, we now using computational, biochemical, molecular and imaging approaches, investigate the mechanisms by which PI4P lipids can regulate viral RNA replication.

RESULTS

RNA Dependent RNA Polymerase Binds PI4P Lipids

Enteroviruses utilize viral replication complex proteins to synthesize enteroviral RNA. To explore whether PI4P mediates the viral RNA synthesis reaction by serving as docking sites for replication complexes, we examined whether any of the replication complex proteins possess a specific affinity for PI4P lipids.

RdRP is a soluble enzyme without any known membrane association domains. The mechanism by which RdRP proteins can associate with replication membranes is unknown. Given our finding of in vivo colocalization

of RdRP with PI4P lipid enriched organelle membranes (Figure 3-1), we investigated whether RdRP protein had specific binding site(s) for PI4P lipids by incubating purified recombinant poliovirus RdRP proteins with nitrocellulose strips previously spotted with different cellular lipids including PI4P. As we expected, RdRP specifically and preferentially bound PI4P lipids over all other phosphatidylinositides and membrane lipids (Figure 6-1). Thus, RdRP by itself, independent of any of the other components of the viral replication complex has a specific affinity for PI4P lipids which may regulate RdRP binding to replication organelle membranes as well as modulating its enzymatic activity.

Identification of PI4P Binding Region on Enterovirus RdRP

We next determined the amino acid makeup of the PI4P binding site on RdRP (Figure 6-2). Sequence analysis of RdRP did not reveal any known canonical PI4P binding site such as a PH domain. Since the crystal structure of poliovirus RdRP has been elucidated, we used the AutoDock algorithm to predict possible binding residues on RdRP surface. AutoDock is simulation software that can predict any non-covalent interaction based on the formation

of the most energy-favorable chemical bonding between RdRP and PI4P. AutoDock randomly docks PI4P structures on the RdRP surface to see where energy favorable bonds can form.

Based on the software predictions, we identified six positively charged amino acid residues (Figure 6-3). They were lysines 24, 45, 126, 159, 276 and arginine 128. These residues might be responsible for PI4P head group binding since the head group is negatively charged. These residues were located in two distinct surface regions of the finger subdomain with a close proximity to thumb subdomain. Lysine 126 and arginine 128 were in the front of the finger domain. On the other hand, lysine 24, 45, 159 and 276 were in the back of the finger subdomain and were much closer to thumb subdomain.

Secondly, we created mutants that carried a single amino acid change for each of the six predicted residues. To do so, we changed positively charged amino acid to non-charged alanine. The point mutation was made by site-directed mutagenesis of a wild type RdRP protein-expressing plasmid. Simply, full-length mutant plasmids were synthesized by polymerase chain reaction using oligonucleotides that contained mutation sites as primers and wild type protein-expressing plasmids as templates. The high fidelity of DNA

polymerase was used to avoid generation of any nucleotide changes besides the desired mutation sites. Then, Dpn1 cleaved the methylated wild type template plasmid while it left the mutant plasmids intact due to their lack of methylation. The mutated plasmids were then subjected to DNA sequencing in order to confirm the mutated sites as well as to verify that no additional mutations were introduced into the rest of the coding regions.

Thirdly, the mutant proteins were expressed from the plasmids and purified (Figure 6-4) by utilizing a simplified version of an established protocol(Gohara et al., 1999). Briefly, each protein was expressed in BL21 (DE3) *Escherichia coli*. To evaluate whether the expression protocol worked successfully, wild type protein was expressed. The molecular weight of the poliovirus RdRP protein is fifty-two-kilodaltons. Data show that a 52-kilo Dalton band appeared on a SDS-PAGE protein gel (Figure 6-5A) which matches the size of poliovirus RdRP. In addition, this 52-kilo Dalton protein could be recognized by a poliovirus RdRP-specific antibody in western blot signifying that the protein expressed was indeed poliovirus RdRP (Figure 6-5B). The protein-expressing *Escherichia coli* were lysed by lysozyme in combination with sonication. Then the clear lysate was subjected to

polyethyleneimine precipitation followed by 0 to 40% ammonium sulfate precipitation. The polyethyleneimine precipitation removes nucleic acids, which interfere with the RdRP activity assay. Ammonium sulfate precipitation is a common way to purify protein by using salting out principle. Proteins precipitate out at a specific range of ammonium sulfate concentration depending on how many polar amino acids they have. As a first step in testing the purification protocol, we were able to get a partially purified wild type RdRP protein (Figure 6-6), with approximately 86 % purity. All of the RdRP mutants including K24A, K45A, K126A, R128A, K159A and K276A were successfully purified with similar purity percentages as found in wild type (data not show).

Finally, the PI4P lipid overlay assay was performed with both wild type and mutant proteins using the same total protein concentration. We directly compared the level of PI4P binding between wild type and mutant proteins. The level of PI4P binding is positively correlated with the intensity of PI4P signal in lipid overlay assay(Dowler et al., 2002). Thus the intensity of PI4P signal is a suitable measurement to quantify PI4P binding. After comparing the PI4P intensity of mutant to wild type proteins, we observed that

the K126A RdRP protein decreased PI4P binding by 70%(Figure 6-7) while there is no significant change in the rest of RdRP mutants (Figure 6-8). Our results indicate that K126 on RdRP is the key amino acid residue responsible for PI4P binding. The identification of the PI4P binding residue on RdRP may lead to the uncovering of mechanistic insights on how PI4P regulates enteroviral replication.

RdRP mutant with diminished PI4P binding ability reduces membrane association in Vivo

To determine whether PI4P facilitates the viral RNA replication process through tethering soluble RdRP protein to membranes, we made a K126A mutation on the RdRP precursor protein (3CD). When 3CD protein is expressed in HeLa cells, it cleaves itself to liberate mature RdRP. This 3CD expression system mimics the generation of RdRP during viral infection. Based on our findings, K126A RdRP significantly decreases PI4P binding (Figure 6-7). Hence the K126A mutation in 3CD is likely to yield RdRP protein with diminished PI4P binding, which may result in reduction of RdRP membrane association. To test this possibility, we expressed 3CD wild type

or K126A proteins in HeLa cells, and then measured membrane association of these two proteins. Membrane association was determined by digitonin assay (Lorenz et al., 2006). Digitonin binds to cholesterol (Nishikawa et al., 1984), and extracts cholesterol from any membranes. As a result, digitonin treatment is able to selectively permeabilize the plasma membrane, which contains the major pool of cholesterol within mammalian cells. If the protein tested is associated with any membrane structures, it stays inside the cells (Figure 6-9A). On the contrary, if the protein of interest freely moves in the cytosol such as the cytosolic GFP (Figure 6-9B), it is washed away after digitonin treatment. After digitonin treatment, the RdRP protein was visualized by immunofluorescence with RdRP specific antibody. We found that the immunofluorescent signal from the wild type RdRP protein remained after digitonin permeabilization indicating that the wild type RdRP was membrane bound (Figure 6-10 A top panel). Strikingly, the signal from the RdRP K126A mutant was completely gone in digitonin treated cells (Figure 6-10 A bottom panel) similar to what was observed in cytosolic GFP (Figure 6-9B) implicating that RdRP K126A was not associated with any membrane structures. Our finding suggests that RdRP may likely use PI4P lipids as

docking sites to tether itself to membranes. Therefore, it is possible that PI4P may regulate enteroviral RNA replication by tethering RdRP to replication organelle membranes.

PI4P regulate enzymatic activity of RdRP

Given that RdRP has an affinity to PI4P (Figure 6-1), we hypothesized that PI4P may act as a regulator for RdRP enzymatic activity. To test this hypothesis, we determined whether reduced PI4P binding of RdRP would result in decreased in RdRP enzymatic activity. We incubated partially purified wild type or k126A proteins with RdRP substrate and all other necessary components for the RdRP polymerization reaction. As we expected, the polymerase activity of k126A mutant decreases about 3-fold when compared to wild type (Figure 6-11A). In parallel, we loaded same amount of protein (1 μ g) on a SDS-page gel. There was no difference in protein loading between wild type and K126A (Figure 6-11B). Therefore, the reduction of polymerase activity in K126A was not due to less protein in enzymatic reaction. Our results suggest that PI4P binding may be necessary for RdRP enzymatic activity.

PI4P and cholesterol regulate viral 3CD polyprotein processing

Enteroviruses generate replication complex proteins by means of a series of proteolytic cleavages of their initial polyprotein. For example, RdRP is made from 3CD, a polyprotein which is the RdRP precursor. 3CD is a protease that cleaves itself to liberate RdRP and 3C protease where the RdRP subsequently catalyzes RNA synthesis and 3C proteolytically cleaves other viral replication proteins. Though it is a precursor of RdRP and 3C, 3CD has its own role in viral replication independent of RdRP or 3C enzymatic function. Notably 3CD itself also plays important roles during infection by priming RNA synthesis (Cornell and Semler, 2002) as well as being required to proteolytically cleave the capsid proteins to prepare for RNA packaging. Therefore, enteroviruses regulate 3CD proteolysis during infection in order to maintain 3CD levels. Indeed early in infection the levels of 3CD are low but gradually build as viral RNA synthesis reaches exponential rates (>3 hrs. pi)- thus requiring priming- and viral RNA is packaged (Figure 6-12).

Since 3CD contains the RdRP portion that has PI4P binding site, we wondered if PI4P has a role in modulating 3CD proteolytic activity as well. To test this, we treated poliovirus-infected HeLa cells with PIK93, which depletes

the PI4KIII β dependent PI4P pool in cells, and examined the level of 3CD and 3D as an indication of 3CD protease activity. We demonstrate that 3CD protease activity is stimulated in the absence of PI4P at 1.5hr post infection (Figure 6-13). Furthermore, when we ectopically expressed 3CD K126A protein in HeLa cells, 3CD K126A protein lost membrane association, probably due to reduced PI4P binding on RdRP. Next, 3CD proteolytic activity was measured by determining the 3CD and 3D level since 3CD cleaves itself to liberate 3C and 3D when 3CD is proteolytic active. Compared to wild type 3CD, we show that 3CD K126A has more 3D than 3CD suggesting that a loss of PI4P binding accelerates 3CD protease activity (Figure 6-14A). On the other hand, the ratio of 3CD and 3D change in K126A mutant may also be due to 3CD instability. To rule out this possibility, we probe for 3C protein, another product for 3CD protease. Similar to what we have found in 3D, 3C level also increase in K126A mutant (Figure 6-14 B). Hence, it is likely the increasing 3D level compared to 3CD in K126A mutant is a result of increasing 3CD protease activity. Consequently, PI4P may likely attenuate 3CD protease activity so as to maintain a sufficient level of 3CD, which executes its indispensable role in viral replication.

In addition to PI4P lipids, the replication organelles are also enriched in cholesterol. Notably, cholesterol acts as a negative regulator of poliovirus 3CD protease activity (Illytska et al., 2013). It is possible that PI4P and cholesterol cooperatively regulate 3CD protease activity. The head group of PI4P is negatively charged which repels to each other by electrostatic repulsion. The more negatively charged lipids membranes have, the higher the membranes fluidity. Thus, the PI4P enriched membranes can be highly fluid (Zhendre et al., 2011), which may prevent 3CD from binding on them. Cholesterol present at the replication organelle membrane can affect the packaging of PI4P, and thus, reduce the fluidity of PI4P enriched membranes (Zhendre et al., 2011). Therefore, PI4P and cholesterol may not only facilitate 3CD binding but also position 3CD in a specific conformation at the membrane such that its proteolytic activity will be attenuated.

CONCLUSIONS

PI4P lipids play a critical role in enteroviral (chapter 4) and hepaciviral (chapter 5) RNA replication through mechanisms yet to be investigated. Our findings in this chapter offer two prospective mechanisms by which PI4P mediates the viral RNA synthesis reaction. Viral replication complex proteins such as RdRP can bind to PI4P lipids on the replication membranes via a PI4P binding residue, which in RdRP is lysine 126. Potentially PI4P lipids may serve as docking sites to enable the assembly of replication complexes. Additionally, PI4P regulates the enzymatic activity of RdRP and the RdRP precursor protein, 3CD. The loss of PI4P binding on RdRP dramatically reduces the polymerization reaction. On the other hand, 3CD enzymatic activity was stimulated when PI4P binding site was mutated. It is plausible that PI4P lipids attenuate 3CD proteolytic activity to promote viral RNA replication.

Figure 6-1: Poliovirus RNA polymerase specifically and preferentially binds PI4P lipids.

A) Purified recombinant 3D enzyme was incubated with membrane strips that were previously spotted with different lipids. Antibodies detected 3D binding.

Two representative blots are shown.

B) Lipid binding was quantified and plotted in bar graph as a ratio over background non-lipid spotted membrane from three different experiments for each lipid type.

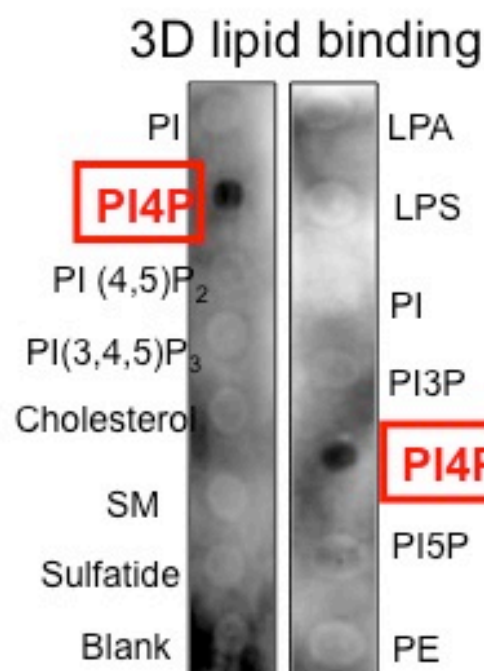
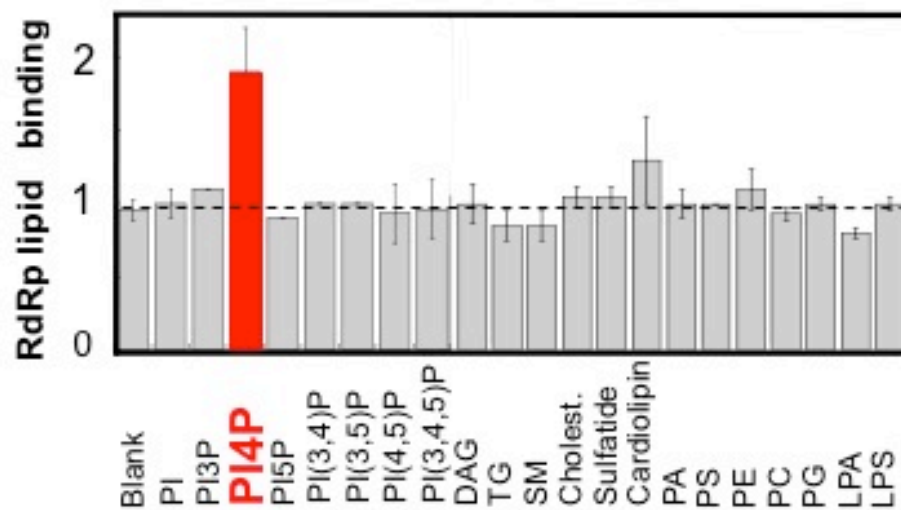
A**B**

Figure 6-2: Schematic flowchart depicting our approach to identify and verify possible PI4P binding residues in poliovirus RdRP.

The potential PI4P residues were predicted by computer simulation of docking experiment in AutoDock. All predicted residues were mutated, expressed and purified in *Escherichia coli* systems. Purified proteins were screened by lipid overlay assay to test the binding ability of mutant proteins to PI4P. Positive candidates were identified by reduction of PI4P binding ability.

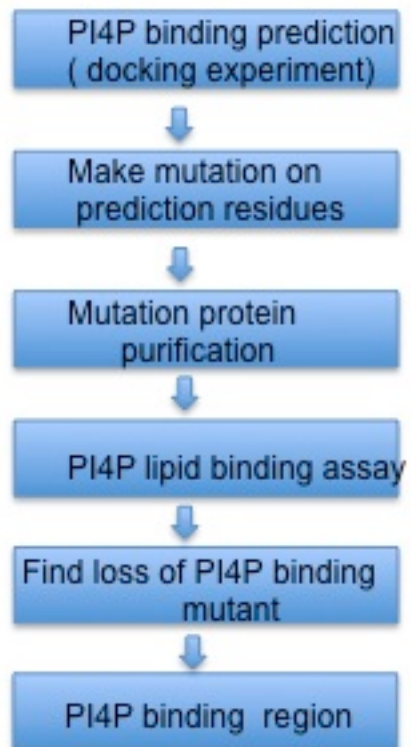
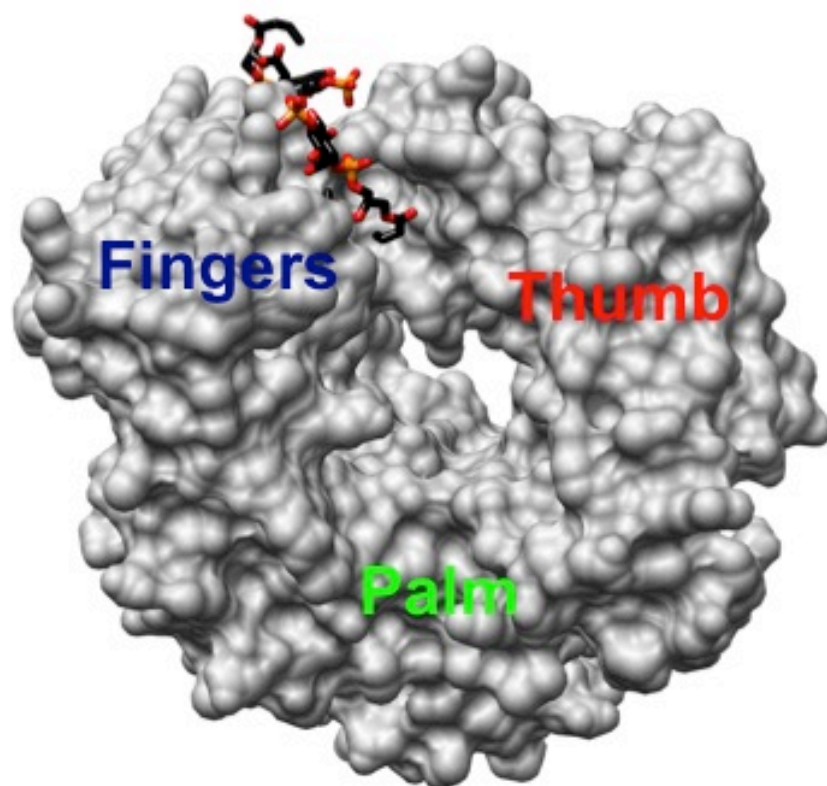


Figure6-3: Prediction of PI4P binding residues on Poliovirus RdRP.

The potential PI4P binding residues and their localization on the surface of poliovirus RdRP (front view). The PI4P binding residues include Lysine 24, 45, 126, 159, 276 and Arginine 128.



K24, K45, K126, R128, K159 K276

Figure6-4: The flowchart of poliovirus RdRP protein purification.

This flowchart depicts the procedure used for RdRP protein purification.

RdRP protein was expressed in *Escherichia coli*, and protein-expressing *Escherichia coli* were lysed and subjected to PEI and ammonium sulfate precipitation to produce a partially purified protein. Subscript denotes fraction:

S: supernatant; P: pellet; Pd: dialyzed pellet; M: mixture.

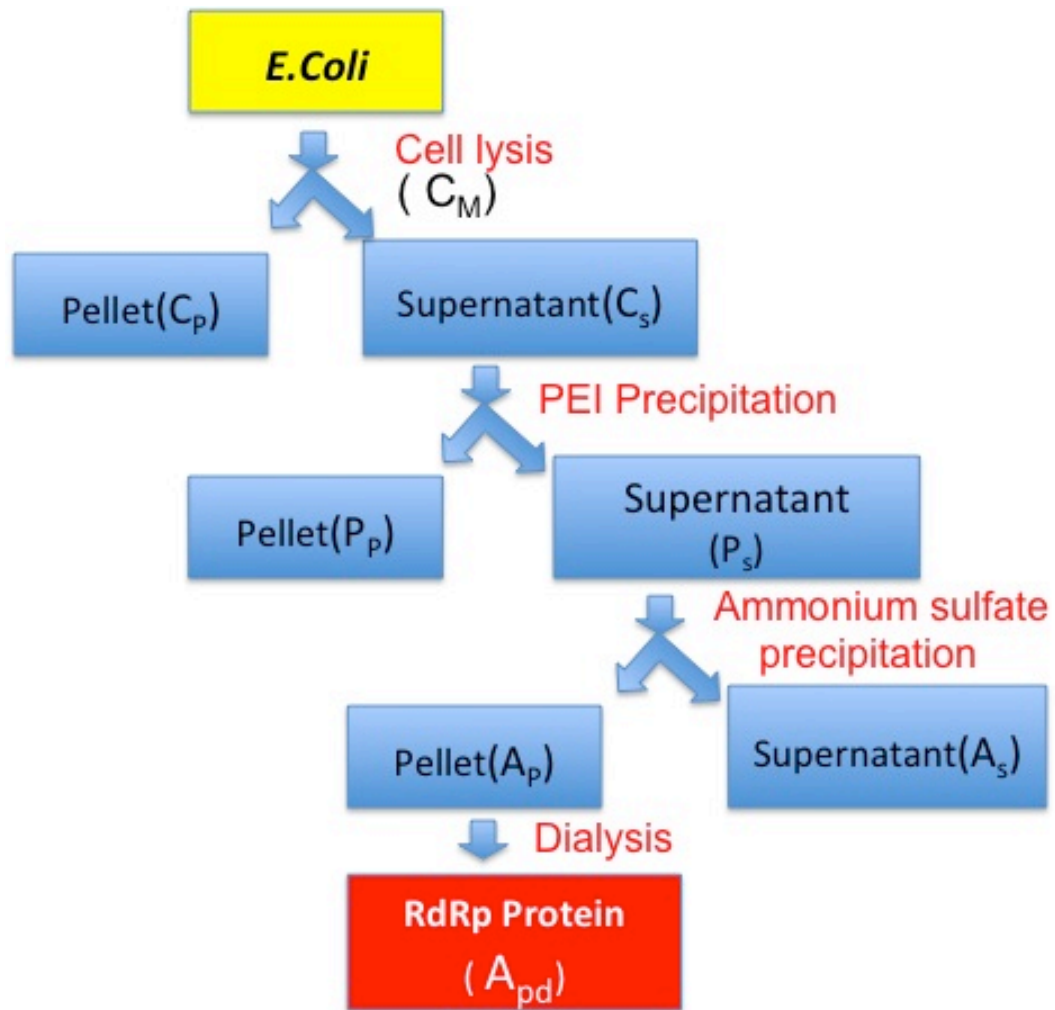


Figure 6-5: Wild type poliovirus RNA dependent RNA polymerase is expressed in *Escherichia coli*.

Escherichia coli were transformed with wild type poliovirus RdRP-expressing plasmids. Protein was induced by incubation with 500 μ M IPTG at 25°C overnight.

- A)** SDS-PAGE image of uninduced and induced samples. The same volume of uninduced and induced *Escherichia coli* lysate was loaded and run on an SDS-PAGE gel followed by coomassie blue staining to detect protein.
- B)** Poliovirus RdRP protein detection in a western blot of uninduced and induced *Escherichia coli*.

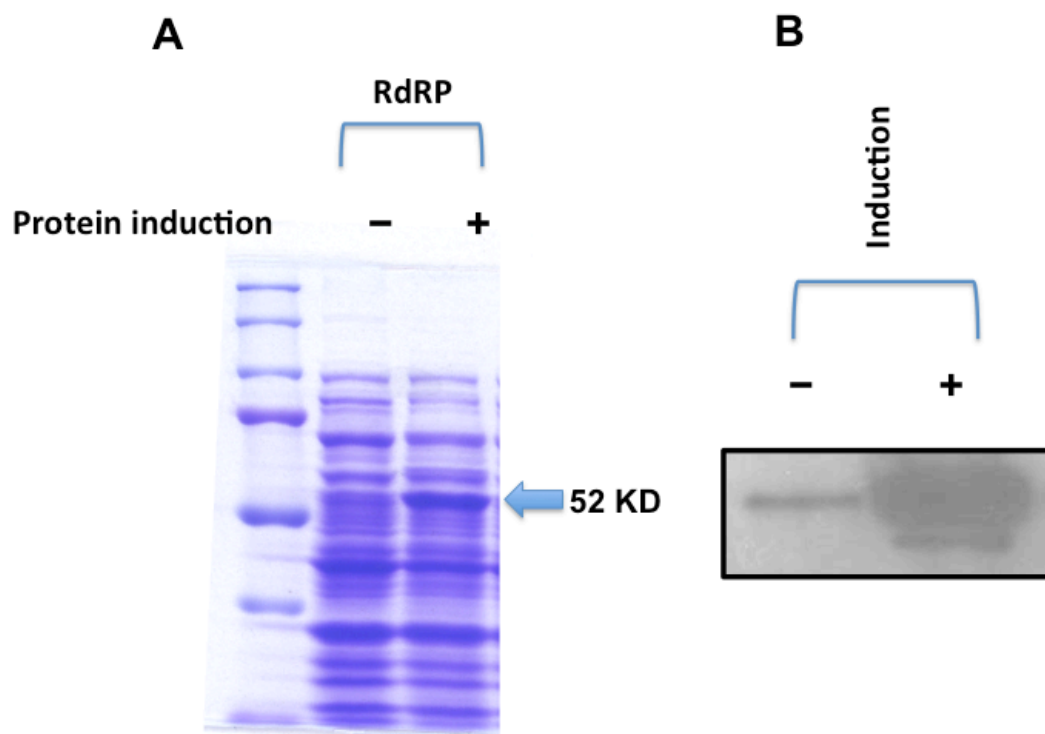


Figure 6-6: Wild type poliovirus RdRP is successfully purified.

Wild type poliovirus RdRP was purified according to the flowchart in Figure 6-

4. Fractions from the purification process were loaded on an SDS page gel to

monitor protein level during purification. The purified wild type RdRP protein

was found in the A_{pd} fraction. The fraction abbreviations are as follows.

Upper case letter represents the purification step: C: Cell lysis; P: PEI

precipitation; A: Ammonium sulfate precipitation. Subscript denotes the

fraction: S: supernatant; P: pellet; Pd: dialyzed pellet; M: mixture

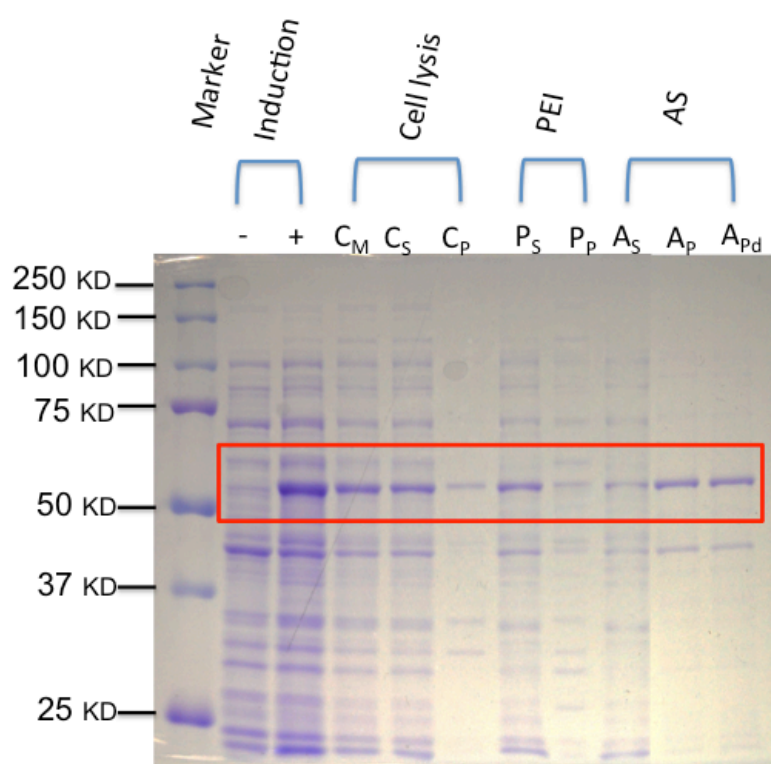


Figure 6-7: PI4P binding is significantly decreased in RdRP K126A protein.

Purified wild type poliovirus RdRP (WT) or RdRP K126A was incubated with membrane strips that were previously spotted with different lipids. RdRP binding was detected by RdRP specific antibody.

A) Lipid overlay assay of wild type and K126A RdRP protein

B)) Denatured protein gel loaded with wild type and K126A RdRP protein.

The image of SYPRO Ruby stained 10% of sodium dodecyl sulfate polyacrylamide gel is presented.

C) PI4P binding of wild type and K126A RdRP protein.

Bar graph show PI4P binding percentage normalized to amount of protein (Figure 6-7 B).

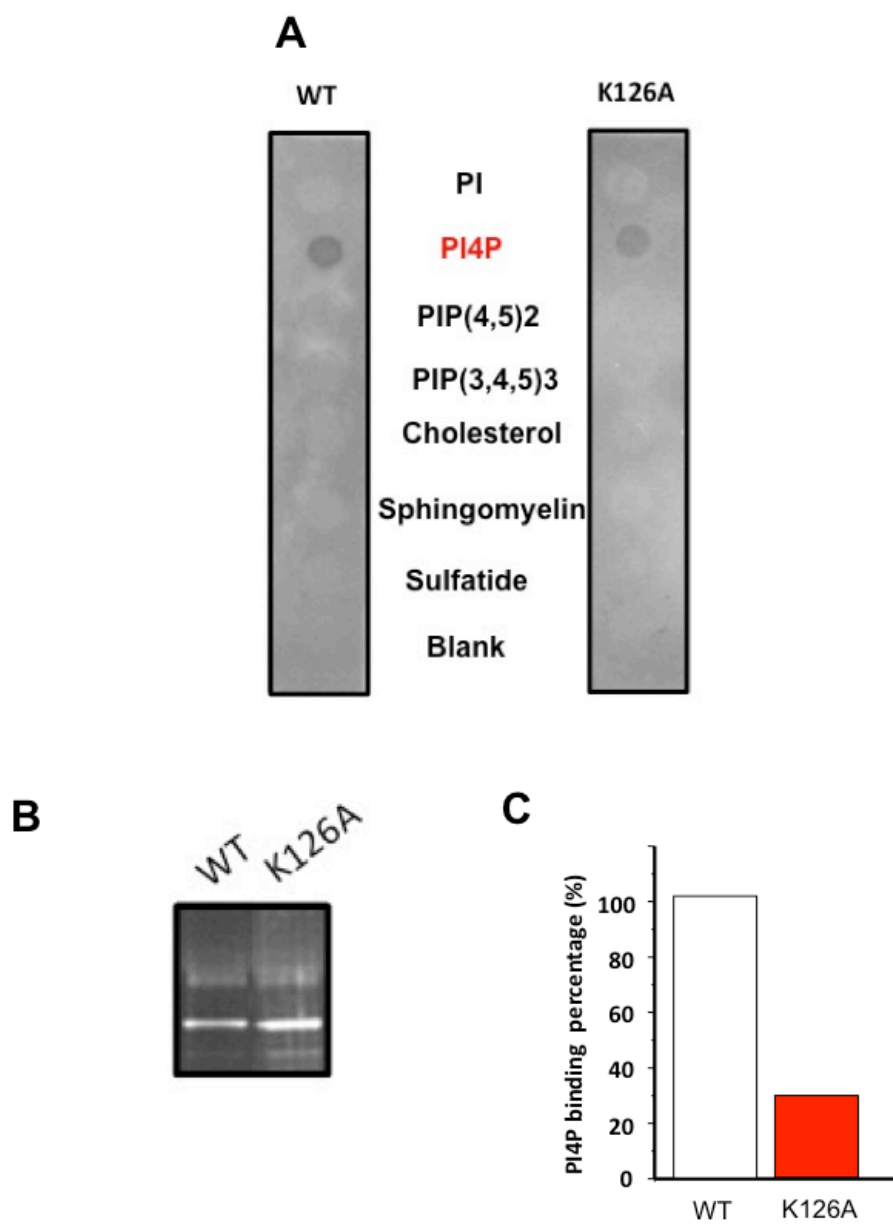


Figure 6-8: PI4P binding is not significantly decreased in RdRP K24A, K45A, R128A, K159A and K276A proteins.

Purified wild type poliovirus RdRP (WT), K24A, K45A, R128A, K159A, K276A or bacterial protein mixture from uninduced sample was incubated with membrane strips that were previously spotted with different lipids. RdRP binding was detected by RdRP specific antibody. The represented blot of lipid overlay assay from two-independent experiemnt was presented. The Bar graph shows PI4P binding percentage (%) normalized to that of its corresponding wild type protein (show in dashedline). The PI4P binding percentage (%) is calculated as following equation.

The PI4P binding percentage (%)= ((The PI4P intensity of RdRP mutant – background intensity) / (The PI4P intensity of RdRP WT-background intensity)) *100

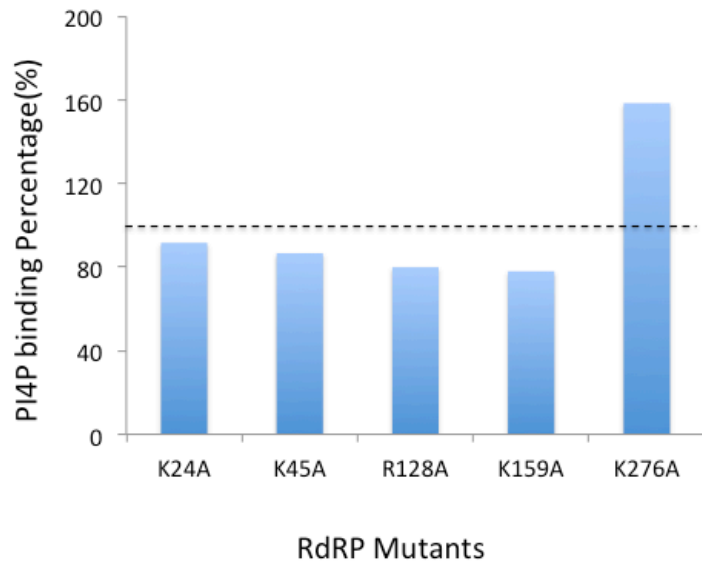
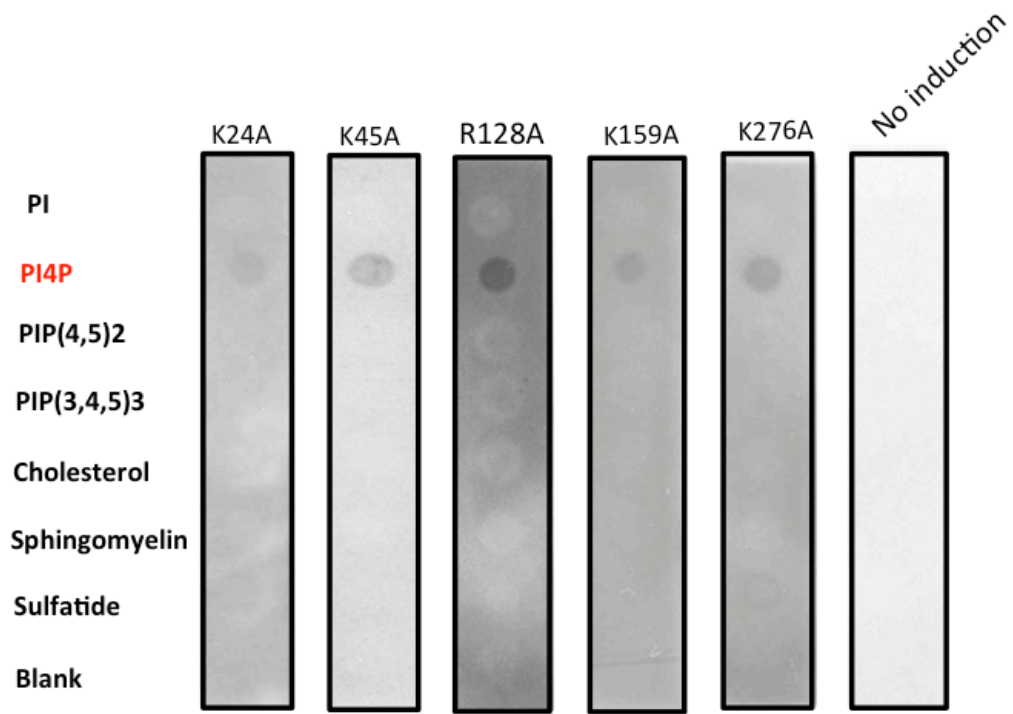


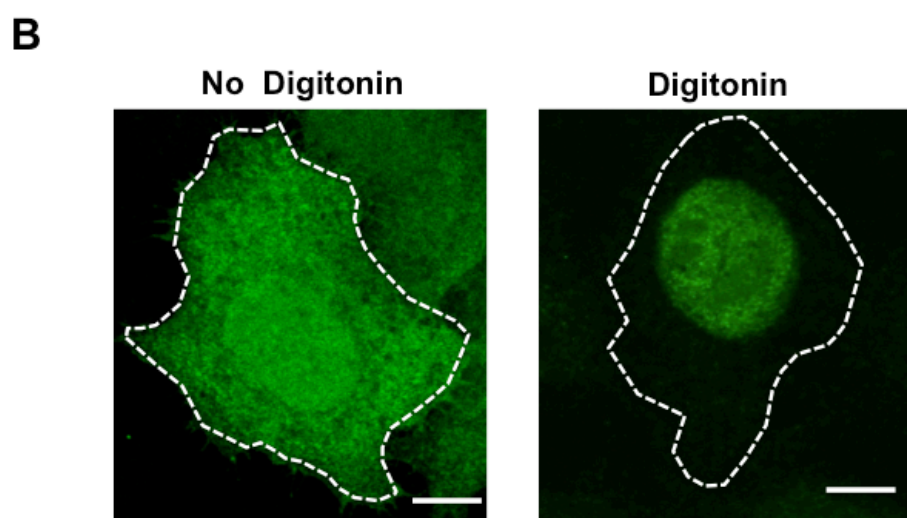
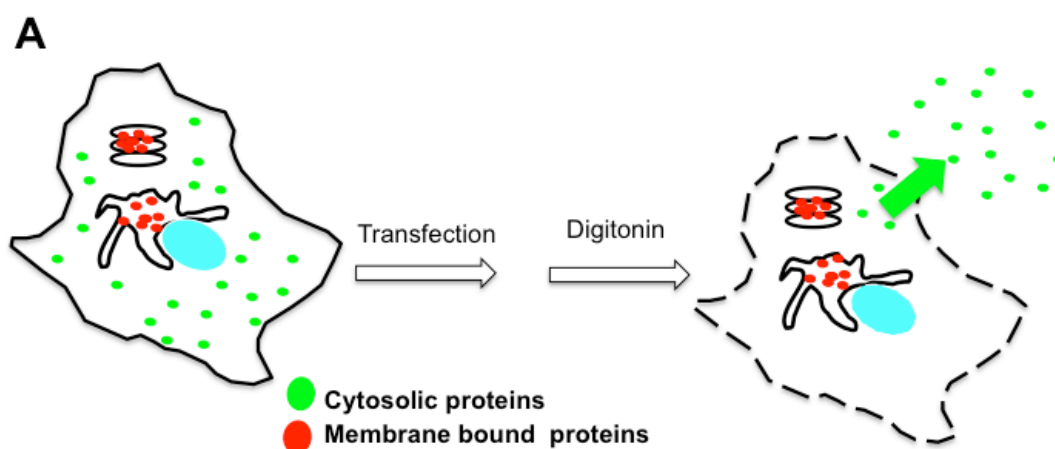
Figure 6-9: In vivo membrane association assay--- Digitonin

Permeabilization.

A) The schematic diagram depict the principle of digitonin assay

B) The confocal images of HeLa cells expressing cytosolic GFP pre and post

digitonin permeabilization. Scale bar, 10 μm



Cytosolic EGFP transfected cells

Figure6-10: RdRP K126A proteins lose membrane association in Vivo.

A) The represented confocal images of HeLa cells expressing wild type (WT) or K126A RdRP pre and post digitonin permeabilization are presented. HeLa cells were transiently transfected with wild type and K126A plasmid. Plasmid-expressing cells were treated with buffer (No Digitonin) or 40 µg/ml of Digitonin for 2 minutes followed by three washes. Cells were fixed with 3.7% formaldehyde, and then RdRP proteins were detected by immunofluorescence with RdRP specific antibody. Scale bar, 10 µm

B) The quantification result of HeLa cells expressing wild type WT or K126A RdRP pre and post digitonin permeabilization (n=10 cells for all groups).

The Bar graph represents RdRP staining fluorescence intensity as (%) with respective values to the average of fluorescence intensity of WT plasmid with no digitonin treatment. Error bars are SEM.

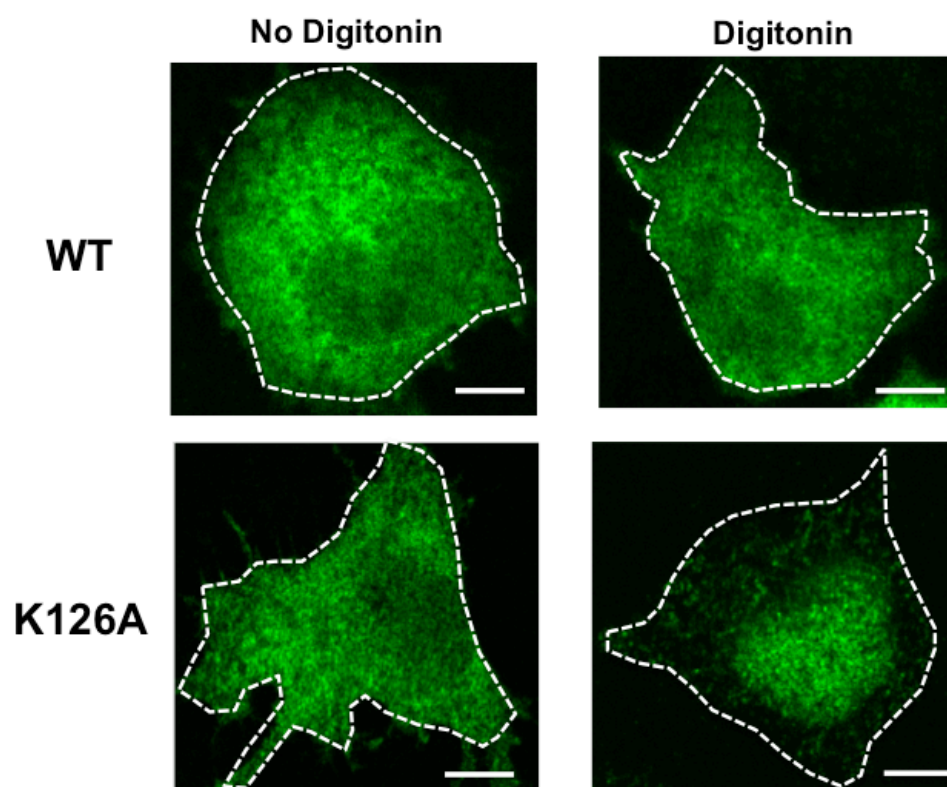
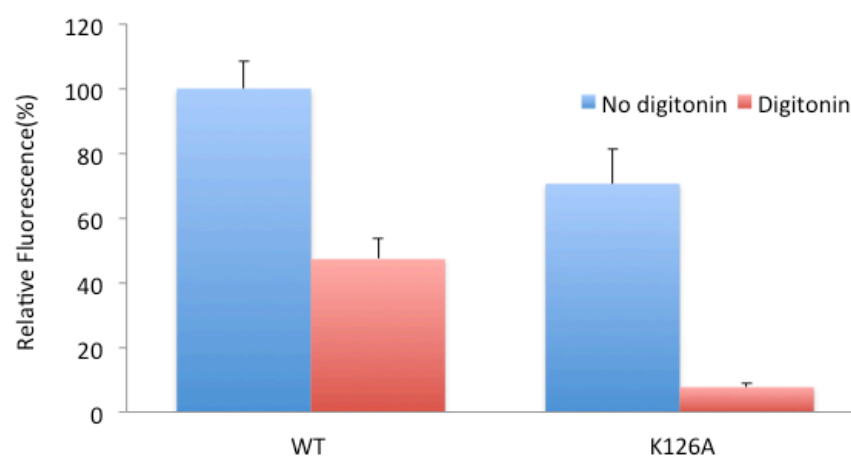
A**B**

Figure 6-11: RdRP K126A protein decreases in RdRP polymerization

assay. (Courtesy of Jamie Arnold from Craig Cameron Lab)

A) Kinetics of RdRP functional assay as measured by UMP incorporation.

UMP incorporation level of 500 ng of wild type or K126A RdRP at

varies of reaction time.

B) Denatured protein gel loaded with 1 µg of wild type and K126A RdRP

protein. The image of Coomassie Blue stained 8% of sodium dodecyl

sulfate polyacrylamide gel is presented.

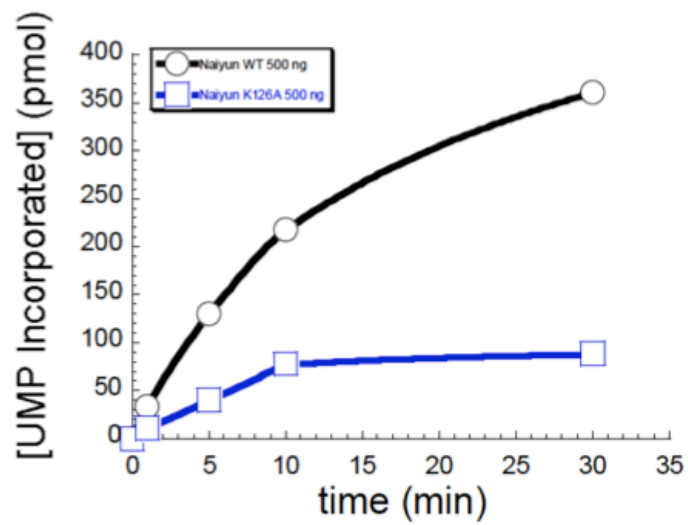
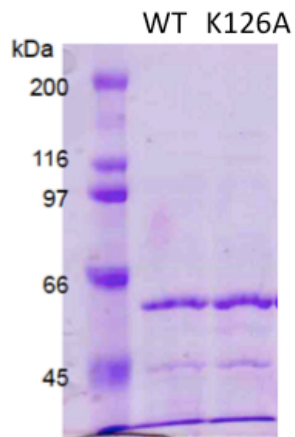
A**B**

Figure 6-12: Poliovirus generates 3CD and RdRP with different kinetics during infection.

Poliovirus 3CD and RdRP western blot image of poliovirus infected HeLa cell lysate. HeLa cells were infected with poliovirus for different duration of time. 50 µg of lysate was subjected to western blot with RdRP specific antibody, which recognizes both 3CD and RdRP protein. In order to visualize 3CD and RdRP over the course of infection at the same time, different exposure time was used.

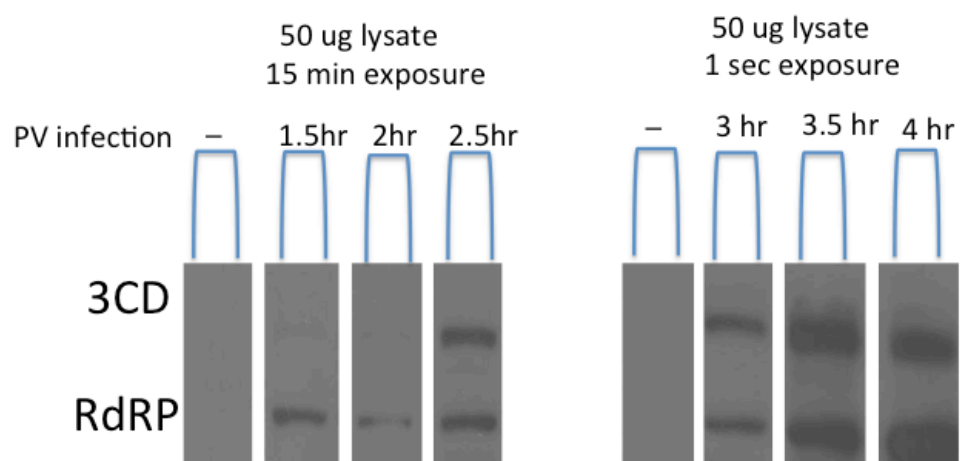


Figure 6-13: PI4P depletion leads to increased 3CD proteolytic processing.

A) Poliovirus 3CD and RdRP western blot image of poliovirus infected HeLa cell lysate with/without 0.5 μ M PIK93 treatment. The western blot image of one experiment is presented. HeLa cells were infected with poliovirus for 0.5, 1, 1.5, 2, 2.5, 3 hr. then treated with 1hr of 0.5 μ M PIK93. Cell lysates were harvested and subject to western blot with RdRP specific antibody, which recognizes both 3CD and RdRP protein.

B) The quantification result of western blot image in Figure 6-13A.

The intensity of each bands were quantified using ImageJ. The X-axis denotes different viral infection time in the presence or absence of PIK93; The Y-axis denotes the relative percentage of viral proteins. The relative percentage is calculated as following equation.

$$\left(\frac{\text{The intensity of 3CD or 3D}}{\text{the sum of the intensity of 3CD and 3D}} \right) \times 100 = \text{Viral protein relative percentage (\%)}$$

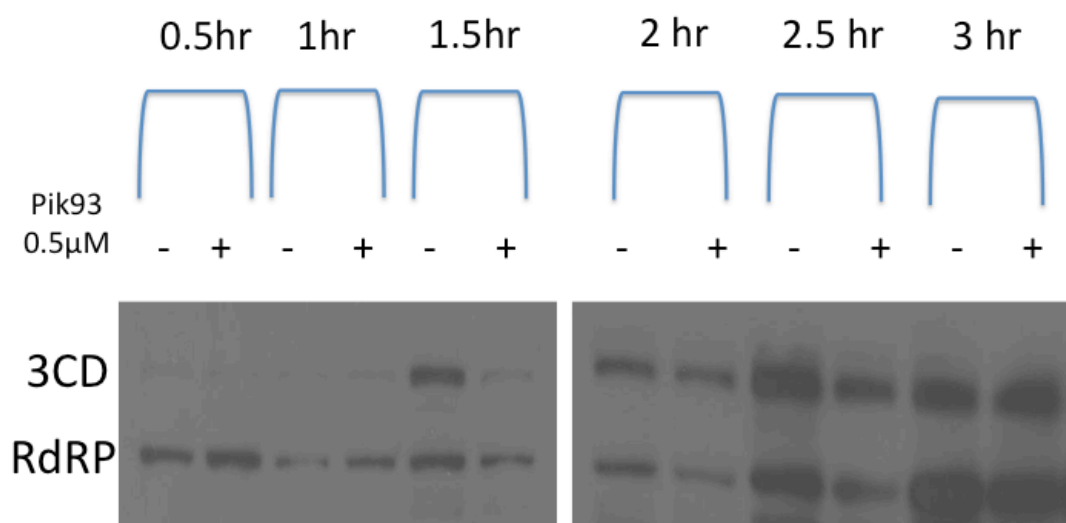
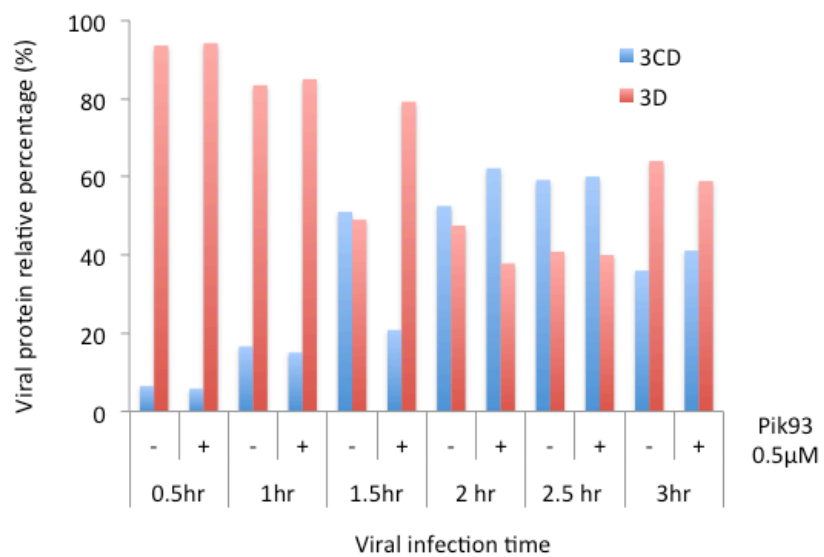
A**B**

Figure 6-14: K126A mutation results in accelerating 3CD proteolytic processing.

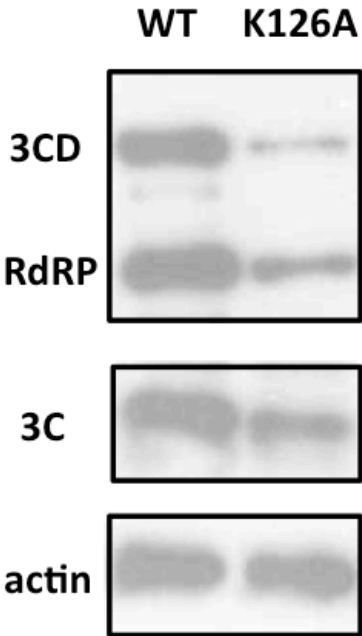
A) The western blot image of poliovirus 3CD, RdRP, 3C and actin from one experiment was presented. HeLa cells were transiently transfected with wild type and K126A plasmid. Cell lysates were harvested and subjected to western blot with RdRP specific antibody to monitor 3CD and RdRP level, 3C or actin antibody, which serves as loading control.

B) The quantification result of western blot images in Figure 6-14A. The intensity of each band was quantified using ImageJ. The X-axis denotes wild type or K126A plasmid expressing cell; The Y-axis denotes the relative percentage of viral proteins (%). The relative percentage is calculated as follows:

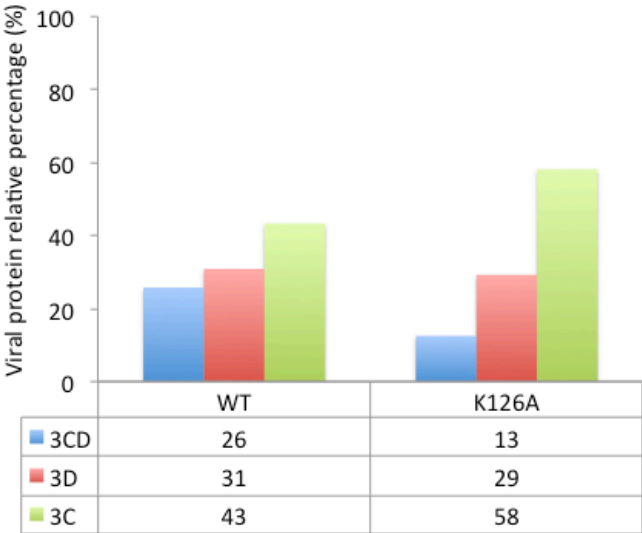
(The intensity of 3C, 3D or 3CD / the sum of the intensity of 3C, 3D and 3CD)

X100 = Viral protein relative percentage (%)

A



B



CHAPTER 7

Discussion and future directions

Model for building plus strand RNA virus replication platform

The work presented in this thesis describes how plus strand RNA viruses rewire the host secretory pathway proteins to build PI4P lipid enriched replication platforms. We demonstrate that enteroviruses (Poliovirus and CVB3) and Hepacivirus (hepatitis C virus) exploit host PI4KIII β enzymes and replicate their respective viral RNA on PI4P lipid-enriched membranes. Furthermore, we showed that the enteroviral membrane protein 3A can reorganize the host secretory trafficking pathway to enhance the recruitment of PI4KIII β to host membranes in order to generate the PI4P lipid-enriched membrane microenvironment. We also demonstrated that viral replication complex proteins, RdRP selectively can bind PI4P lipids, and that these lipids function as docking sites for the assembly of the viral replication machinery. PI4P binding may also regulate the enzymatic activity of 3D since in RNA polymerization assays, mutating a PI4P binding site inhibited the polymerase activity (Figure 6-11). Finally, we showed that PI4P along with cholesterol may regulates the proteolytic processing of 3CD in order to regulate 3CD and 3D

levels during viral RNA replication. Based on our findings we propose the following model for building the plus strand RNA virus replication platform:

Enteroviral RNA replication begins on existing PI4P lipid-containing organelles including the Golgi and TGN, which have the highest steady-state levels of this lipid in uninfected cells (Godi et al., 1999). Viral RNA replication involves a positive feedback loop, where newly synthesized viral RNA molecules are translated into increasing amounts of viral replication proteins including 3A, which then further replicate viral RNA. Since 3A modulates GBF1/Arf1 effector recruitment, its impact on GBF1/Arf1/coat/PI4KIII β interactions becomes inescapable as its levels rise. The selective recruitment of PI4KIII β to membranes over COPI eventually disrupts secretory membrane trafficking and leads to Golgi disassembly by decreasing anterograde transport from the ERGIC and intra-Golgi trafficking, both COPI-dependent processes (Lee et al., 2004). Furthermore 3A-bound membranes emerging de novo from ER exit sites will develop into PI4P lipid-enriched uncoated membranes (Figure 7-1A).

Membrane-bound enteroviral 3A proteins bind and modulate host GBF1/Arf1 to preferentially enhance recruitment of PI4KIII β to membranes

over COPI and other coat proteins. There PI4III β catalyzes the production of PI4P lipids, leading to the biogenesis of a PI4P lipid-enriched membrane microenvironment that is distinct from that in uninfected cells. This PI4P lipid-rich microenvironment will in turn promote the recruitment and stabilization on the membrane of the RdRP from the cytosolic pool. PI4P binding of RdRP increases its enzymatic activity to produce a more efficient RNA polymerization reaction. In addition, 3CD presumably binds to PI4P via its RdRP portion. The PI4P-bound 3CD attenuates its protease activity in order to keep a sufficient level of itself. 3CD regulates the priming of RNA replication. To initiate RNA replication, primer synthesis (Vpg-pU-pU) and viral RNA circularization are required. 3CD plays indispensable role in both processes (see chapter 1 for detail description). RdRP, 3CD, 3A and several other viral proteins then initiate RNA synthesis at these membranes. Membrane associated 3AB protein is likely the candidate that may be involved in this process (Figure 7-1 B). As part of the replication complex, purified 3AB has been shown to stimulate 3D polymerase and 3CD autoproteolytic activity in vitro (Lama et al., 1994; Molla et al., 1994). It is very possible that 3AB together with PI4P may cause the conformational changes of RdRP as to

optimize RdRP polymerization reaction at these membranes. Moreover, the presence of PI4P and cholesterol at these membranes may counteract 3AB mediated 3CD autoproteolytic activity stimulation. This way, sufficient 3CD protein can be generated in order to prime RNA polymerization reaction.

The host secretory pathway disruption and host defensive response

Host cells produce several lines of defense against viral infection. Specifically, viral infected cells produce soluble and membrane-bound proteins to stop viral replication, to kill them or to alert neighboring uninfected cells. The soluble proteins are cytokines such as the interferons, IL-6 and IL-8. Autocrine or paracrine interferons execute their antiviral response by inhibiting host translation and by inducing apoptosis (Goodbourn et al., 2000). IL-6 and IL-8 activate or attract immune cells to the viral infected cells' location. (Miller and Krangel, 1992). Subsequently, they facilitate the killing of viral infected cells. A well-known example of membrane-bound proteins is the major histocompatibility complex class I molecule (MHC class I). MHC class I is a heterodimer with 1 transmembrane domain, and it is widely expressed in all nucleated cells (Bjorkman et al., 1987). MHC class I performs its antiviral

action through the activation of CD8⁺ T cells. There are abundant viral proteins present in the cytosol of plus strand RNA-infected cells since their translation take place in the cytosol. Within infected cells, viral proteins go through the class I antigen presentation pathway to generate MHCI-peptide complexes on the plasma membrane. To initiate class I antigen presentation, viral proteins are degraded into peptide by proteasomes and then the degraded viral peptides are shuttled into the ER where they are loaded on MHC class I (Morrison et al., 1986). The viral peptide-loaded MHC class I trafficks to the plasma membrane where it is recognized by CD8⁺ T cells. As a result, CD8⁺ T cells are activated, and in turn they induce apoptosis of viral infected cells.

As stated earlier, enteroviruses utilize 3A to modulate Arf1 effector selection to the replication organelle membrane by the recruitment of PI4KIII β and exclusion of COPI. The elimination of COPI from membranes will lead to the disruption of the host secretory pathway. The disruption of secretory pathway integrity has been implicated as a cause of the suppression of IL-6, IL-8 and interferon secretion (Dodd et al., 2001) and of interfering with MHC class I antigen presentation (Deitz et al., 2000) during poliovirus infection. In

the case of coxsackievirus, inhibition of host anterograde trafficking likely causes a down regulation of MHC class I from the plasma membrane(Cornell et al., 2007). As a result, CVB3 infected mice fail to mount a functional MHC class I –dependent CD8+ T cell response(Kemball et al., 2009).

Therefore, viral-induced secretory pathway disruption via Arf1 effector selection--- COPI depletion offers an additional advantage to facilitate viral replication and propagation.

PI4P as docking site and much more during viral replication

In mammalian cells, PI4P lipids were previously viewed only as PIP2 precursors(D'Angelo et al., 2008). However independent functions have recently emerged. Several host proteins including CERT, OSBP, and FAPP1/2 specifically bind PI4P lipids (Lemmon, 2008) and PI4P lipids regulate selective autophagy and ER exit site biogenesis (Blumental-Perry et al., 2006; Yamashita et al., 2006). PI4P lipids can change the membrane curvature locally(Ishiyama et al., 2002; McMahon and Gallop, 2005). PI4P lipid-enriched membranes may generate high curvature membrane pockets during viral infection to shield viral components from host defense.

Furthermore, high curvature membranes may provide microdomains for the segregation of different viral activities that cannot take place at the same time. For example, it has been demonstrated that replication and translation cannot take place on the same strand of RNA as this creates a conflict between two sets of machineries (Gamarnik and Andino, 1998). At the same time, replication requires machinery that is generated from translation. To package mature virions, abundant viral RNA and capsid proteins are needed that are supplied by the replication and translation processes. Localizing replication, translation and packaging machineries to nearby specialized microdomains of PI4P-enriched membranes could provide a solution for the above problems. Viral translation can initially take place in one microdomain to generate replication and capsid proteins. Then the translated replication proteins diffuse to a nearby microdomain to assemble replication complexes thus promoting viral replication. Next, viral RNA produced by the replication and capsid proteins from translation are eventually packaged to produce mature virions at the third microdomain on the PI4P-enriched membranes.

Little is known about how soluble viral RNA polymerases are recruited to membranes. PI4P lipids may provide docking sites to concentrate viral

proteins for efficient RNA synthesis. Providing a docking site for RNA polymerase is especially important at the early stage of infection when RdRP concentration is low. Since RNA replication takes place in the cytosol, the low amount of RdRP has to be targeted to membranes where viral replication occurs. Poliovirus RdRP preferentially binds PI4P lipids over all other cellular lipid components. The lysine 126 residue of RdRP may recognize the head group of PI4P on the membrane, thus facilitating its binding to PI4P enriched membranes (chapter 6). Furthermore, PI4P depletion specifically perturbs viral RNA synthesis (Chapter 4). All these data provide evidence supporting the possibility that enteroviruses rewire host secretory machinery to generate PI4P lipid-enriched membranes in order to recruit and to concentrate RNA polymerases on membranes. In the future, it remains to be explored whether other viral replication complex proteins have affinities to PI4P or other lipid components on membranes. As an example, another possible lipid candidate in replication membranes is cholesterol. Ilnytska *et al.* demonstrated that enterovirus trafficks cholesterol to PI4P-enriched replication organelle membranes and that cholesterol is also required for enterovirus replication.

PI4P lipid binding may also induce conformational changes in RdRP

and modulate RdRP enzymatic activity. We have shown in this thesis that diminishing the PI4P binding of RdRP reduces its enzymatic activity significantly suggesting that PI4P binding may induce local “proper” conformational changes to stimulate RdRP activity. It will be interesting to see whether by addition of purified viral PI4P enriched membranes will result in increased RdRP enzymatic activity or not.

Poliovirus 3CD is a precursor to RdRP, an RNA polymerase and 3C, a viral protease. It has cysteine protease activity, and not only cleaves itself but also other viral proteins. Thus, 3CD regulates not only viral replication, but viral packaging as well. To have successful viral replication and packaging, 3CD, RdRP and 3C are indispensable. Therefore, during the course of infection, the 3CD protease activity has to be tightly regulated. Our findings indicate that PI4P and cholesterol are involved in the attenuation of 3CD protease activity. PI4P enriched membranes can be highly fluid (Zhendre et al., 2011) which may prevent viral proteins from assembling on them includes 3CD. Cholesterol can counteract this fluidity (Zhendre et al., 2011) and thereby position 3CD in a specific conformation at the membrane such that its autocatalytic processing will be attenuated.

Our findings in this thesis suggest a critical role of PI4P in providing docking sites for replication complex proteins such as RdRP and 3CD and also in modulating replication proteins' enzymatic activity. It will be worth pursuing how PI4P lipid binding regulates RdRP and 3CD enzymatic activity. It would probably occur by co-crystallization of 3CD or RdRP with PI4P.

PI4P in plus strand RNA viral packaging and egress

Poliovirus 3CD is a multi-functional protein which not only regulates viral replication but also viral packaging. In this thesis, we propose a potential role of PI4P in regulating 3CD enzymatic activity as a mechanism to regulate viral replication. Since 3CD also regulates viral packaging through its protease activity, we anticipate that PI4P executes a similar regulatory role in viral packaging. It will be interesting to investigate whether PI4P has any role in viral packaging, and if so, what mechanisms may be involved beyond this potential regulatory one.

HCV, an enveloped flavivirus whose replication enzymes are sequence- and structure-wise distinct from enteroviral enzymes (Dubuisson et

al., 2002), nevertheless depends on PI4P lipid-enriched membranes and PI4KIII β for replication (chapter5). Whereas most enteroviral infections disrupt secretory trafficking, flaviviruses utilize the secretory pathway to mature into virions and exit the cell(Mackenzie et al., 2007). HCV RNA is replicated on remodeled ER membranes whereas structural proteins are localized to lipid droplets(Miyanari et al., 2007). Through a complex assembly process not yet understood, virions bud out of the ER and are released from the cell through exocytosis. The presence of high levels of PI4P lipids at ER membranes, in addition to regulating HCV RNA replication, could impact the organization and kinetics of secretory trafficking and the budding/export of HCV. Indeed secretory trafficking is attenuated in HCV-infected cells (Konan et al., 2003).

Why do plus strand RNA viruses hijack PI4KIII β pathway?

Viruses rely on their host for viability and replication. During infection, the virus and host become engaged in a dynamic duet, lasting from several hours to potentially years (in persistent infections), in which the virus initiates a spatio-temporally ordered sequence of subcellular events, along the way dramatically altering cellular architecture and physiology. On the other hand,

viruses also adjust themselves to adapt to the environmental changes of host cells. It has also been shown that RNA viruses coevolve with their human host (Switzer et al., 2005). For example, the population of human immunodeficiency virus is selected by its host through the MHC molecule-CD8⁺ interface, which is the critical component for anti-viral response (Worobey M et al 2007).

Enterovirus replication is extremely dependent on the PI4KIII β pathway. Given that viruses coevolve with their host, we suspect that hijacking the PI4KIII β pathway might be a result of virus and host coevolution over a long period time. Coxsackievirus is a virulent heart virus in humans and mice. In the coxsackievirus-infected animal and human, coxsackieviruses selectively target and replicate their viral RNA in the heart and thus induce myocarditis. The heart tissue expresses Coxsackievirus and Adenovirus receptor (CAR), the coxsackievirus specific cellular receptor. Among the organs which express CAR including heart, kidney and liver, heart tissue has a level of CAR similar to the other tissues (Freimuth et al., 2008). It is not clear what makes the heart more susceptible to coxsackievirus infection given that the expression of viral receptor is the same. Interestingly, the human heart

tissue has a high level of PI4KIII β mRNA and presumably a high protein level as well. Since PI4KIII β is indispensable for enterovirus replication including coxsackievirus, PI4KIII β is likely the host factor that contributes to coxsackievirus heart tropism.

At the beginning of an infection, coxsackieviruses randomly infect tissues with CAR expression, including the heart. Heart tissue has high PI4KIII β expression. By an accumulation of genomic mutations, viruses begin to adapt to the heart environment. The PI4P lipid environment created by PI4KIII β is favorable to viral replication for the reasons we described in this thesis (chapter6). Thus, the PI4P lipid environment serves as a selection pressure for the virus. The viral populations that can take advantage PI4P environment are stabilized while populations, which don't fit, will inevitably die. Eventually, the viral populations, which can hijack the PI4KIII β pathway to generate a conducive PI4P lipid environment, become prominent.

Future directions: host lipid-modifying and metabolizing enzymes such as PI4Ks as potential targets for antiviral therapy

The replication step is one of the most critical processes in plus strand RNA viral life cycles since it determines the propagation of viral particles that contribute to the pathogenesis of infection. Thus, targeting proteins that are involved in the viral replication step as an antiviral therapy is considered one of the most effective approaches for halting viral infection. Since targeting viral proteins avoids disrupting host cells, the essential viral replication proteins are obvious targets for drug design. However, drug-resistant viruses are generated from the accumulation of viral genomic RNA mutations during the error-prone replication process of RdRP. Compared to viral replication machineries, host replication enzymes are thousand times more accurate due to their proofreading abilities. As a result, the host genome has much lower mutation rate than viral genomes. Following this logical progression, targeting host proteins that are required for viral RNA replication can potentially avoid the drug-resistant virus issue and prolong drug lifetime.

In this thesis we identified three host proteins; Arf1, GBF1 and PI4KIII β , that play critical roles in the generation of PI4P-enriched plus strand RNA

virus replication platforms, which support viral replication. Arf1 along with GBF1 are the master regulators for ER-Golgi vesicular trafficking through the recruitment of many Arf1 effectors such as COP, PI4KIII β etc. Perturbing the cellular function of Arf1 and GBF1 within the host will impact many downstream pathways including cell movement and COP- mediated protein secretion. Thus this would lead to inevitable side effects(Randazzo et al., 2000). This disadvantage of the side effect issues makes Arf1 and GBF1 implausible targets.

On the other hand, blockade of PI4KIII β , one of the Arf1 downstream effectors, will have much less impact on host cellular functions. There are other PI4K proteins present within host cells, and they can compensate the loss of PI4P lipid generation. Consistent with this point, when we inhibited the activity of PI4KIII β , we did not observe any cytotoxicity effect. As demonstrated in this thesis, poliovirus, coxsackievirus and hepatitis C virus are all dependent on PI4KIII β for replication. Therefore, PI4KIII β will be an attractive drug target for combating all these plus-strand viruses.

Based on our work, PI4KIII β is required for poliovirus, coxsackievirus (chapter4) and HCV (chapter 5) replication. Also, others have demonstrated

that PI4KIII β is essential for rhinovirus (Brown-Augsburger et al., 1999), SARS (Yang et al., 2012) Theiler's murine encephalomyelitis virus (Steurbaut et al., 2008) and Aichi virus (Greninger et al., 2012) replication. For HCV, both III α and III β are involved (Borawski et al., 2009; Hsu et al., 2010; Reiss et al., 2011). This raises the possibility that the PI4K family proteins are involved in plus strand RNA replication.

It remains to be explored- which other plus strand RNA viruses from the same families also utilize PI4K pathways to generate a suitable environment for their replication. In addition, mouse models of the above described viruses are available. One can use viral infected animal model to further validate the feasibility of PI4Ks as drug targets based anti-viral therapies in these animal models. Since PI4Ks are involved in building viral replication platforms, we envision that an inhibitor of PI4Ks will be more effective when used as preventive medicine. Unlike an antiviral vaccine that only prevents the infection of a specific serotype of viruses, an inhibitor of PI4Ks would work on all serotypes of viruses as they all utilize the same mechanism to replicate. Potentially, PI4K based medicine could apply to a broad spectrum of plus strand RNA viral infection.

Though targeting PI4Ks is less likely to cause side effects than is targeting Arf1 and GBF1, PI4K-based antiviral therapy still has to overcome the safety issue. One will have to identify compounds that only target one type of PI4Ks since targeting more than one PI4K may interfere with too many physiological responses and lead to undesirable side effects. In the case of PI4KIII β activity inhibition by PIK93, we did not find any differences in cell viability in vitro. While there is no cytotoxic effect in this cell culture system, one will have to perform toxicology studies in animals to further evaluate the safety of a PI4KIII β inhibitor such as PIK93. Recently, the groups cited below reported on the effects of PI4KIII β inhibition in animal models and their studies support the idea of the safety of PI4KIII β inhibitor. In preliminary data from GlaxoSmithKline, no toxicity effects were observed in mice or dog (personal communication with GlaxoSmithKline). Moreover, Frank van Kuppeveld's group reported that their PI4KIII β inhibition compound effectively reduces the viral load and viral-induced inflammation while there is no adverse effect in the CVB3-induced pancreatitis mouse model (Hilde van der Schaar et.al. 2013, Keystone Symposia on Positive Strand RNA viruses). The side effects of a PI4KIII β inhibitor were only noted under the condition of repeated doses

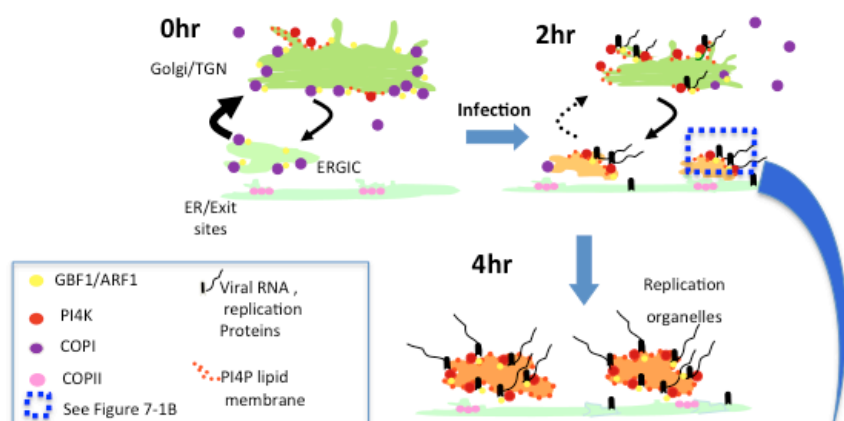
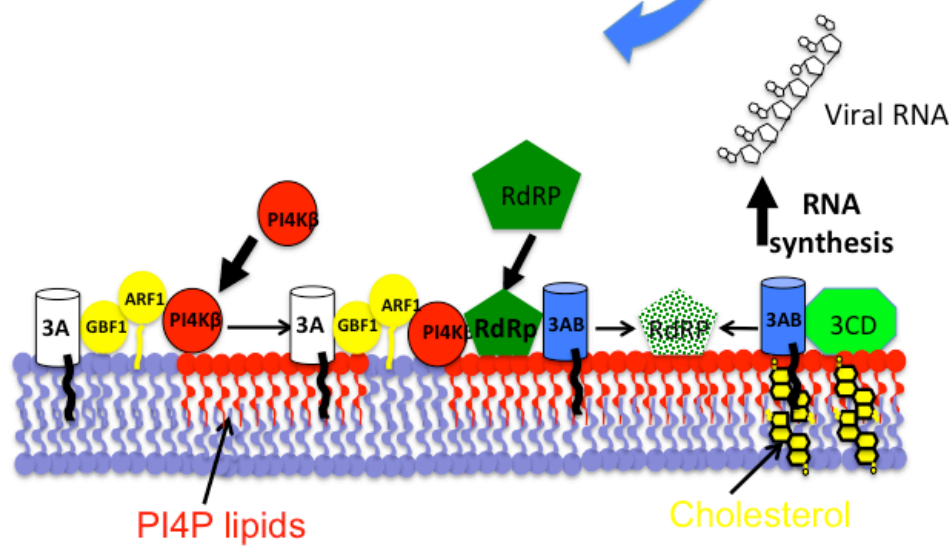
of a high concentration (Spickler et al., 2013). In the future, the safety margin will have to be carefully titrated. One will have to find a safe dosage concentration window that is also effective in blocking viral replication. If this issue can be solved, we believe a novel PI4K-based panviral therapeutic medicine will come soon.

Figure 7-1: The proposed model for building plus strand RNA virus replication platform.

A) In uninfected cells (0hr), there is a bidirectional trafficking (anterograde and retrograde) between Golgi /TGN and ERGIC. Upon infection (2hr), newly synthesis viral proteins such as 3A concentrate and initiate viral RNA synthesis on the Golgi/TGN membranes, where the pre-existing pool of PI4P lipids facilitates viral replication protein assembly and RNA replication.

Rising levels of 3A combined with its modulation of effector recruitment by GBF1/Arf1 will enhance the recruitment of PI4KIII β over COPI, leading to a decreased rate of anterograde transport out of the ERGIC and subsequent disassembled of the Golgi/TGN organelles. Blue intersect at 2hr is enlarged and described in Figure 7-1B. Enhanced recruitment of PI4KIII β over COPI results in the formation of uncoated PI4P lipid-enriched organelles adjacent to ER exit sites (4hr). The PI4P lipid enriched microenvironment of these organelles facilitates the viral RNA replication.

B) Tail-anchored membrane protein 3A by binding and modulating GBF1/Arf1 promotes PI4KIII β recruitment to the membrane bilayer. Recruited PI4KIII β will catalyze the production of a PI4P lipid microenvironment (red lipids). PI4P will in turn promote the synthesis of viral RNA through two possible mechanisms. First, PI4P may facilitate the recruitment of RdRP from the cytosolic pool to the membrane. Second, PI4P may regulate enzymatic activity of RdRP and 3CD. Potentially, viral 3AB and cholesterol at the membranes may assist PI4P mediated viral RNA synthesis reaction.

A**B**

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