MECHANISMS OF NON-ENVELOPED VIRAL EXIT FROM THE HOST

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ABSTRACT OF THE THESIS

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Viral exit from the host cell is a critical step of the viral lifecycle. Enveloped viruses have employed numerous mechanisms to exit their host including direct budding out of the plasma membrane, budding into the secretory pathway to be trafficked out, or budding into the endosomal membrane system to be exocytosed. Comparatively little is known about how non-enveloped RNA viruses such as Poliovirus (PV), Coxsackievirus B3 (CVB3), Rotavirus, Reovirus exit the host cell. Here I showed PV hijacks and diverts the host autophagy pathway to capture numerous virions in autophagosomes which are then trafficked by actin machinery to filopodial extensions and fuse with the plasma membrane to release infectious large vesicles containing mature polio virions. I also demonstrated the infectivity of the virus when inside a vesicle is higher then when free likely due to the vesicles containing many virions as the “clustered bombs”. Finally, I found the exported viral vesicles are highly enriched in phosphatidylserine lipids and that the infection on subsequent host cells is dependent on not only the poliovirus receptor but also the phosphotidylserine lipids on the vesicles. My findings suggest that non-enveloped viruses also carry a piece of the host with them on their pathogenic journey. The lipid and protein components within these membranes potentially modulate the pathogenesis of

non-enveloped viruses within the host and provide a new paradigm for viral spread and tropism.

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CHAPTER 1

Introduction and hypothesis for the thesis

POSITIVE STRANDED RNA VIRUSES

Viruses are small infectious agents responsible for many important human diseases such as Poliomyelitis, common cold, Yellow Fever, Hepatitis, AIDS among others. There are seven genome types encompassing all known families of viruses, including double-stranded DNA viruses, single-stranded DNA viruses, double-stranded RNA viruses, positive-stranded RNA viruses, negative-stranded RNA viruses, positive-stranded RNA viruses with a DNA replication intermediate stage (i.e. retroviruses) and double-stranded DNA viruses with a single-stranded RNA intermediate stage.

Positive stranded RNA viruses, which are the viruses that are being investigated in this thesis, carry their genetic materials in a single strand of RNA that is of “positive sense” and thus can be translated by the host translation machinery much like host mRNA molecules. The RNA nucleic acid is encapsidated by a protein shell which can then be further enveloped by a membrane typically obtained from the host cell. The positive stranded RNA viruses are further classified into families such as the:

Picornaviradae: examples include Poliovirus (PV), Coxsackievirus (CVB), Rhinovirus and enterovirus 71;

Coronaviridae: examples include Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and Middle East Respiratory Syndrome Coronavirus (MERS-CoV);

Flaviviridae: examples include Hepatitis C virus (HCV), Dengue virus and West Nile virus (WNV);

Tombusviridae: example Tombusvirus.

POSITIVE STRANDED RNA VIRUSES AND DISEASE

Positive stranded RNA viruses infect a variety of host cells including human, animal, plant and insect. Diseases caused by positive stranded RNA virus infection are not only widely spread throughout the human population but also have resulted in significant economic damage across the planet. Numerous human diseases such as Poliomyelitis, foot and mouth disease, hepatitis A and C Dengue fever, SARS and West Nile fever are caused by positive strand RNA viruses. Poliovirus alone caused more than 21,000 paralytic cases in the United States in 1952. More recently, the SARS virus led to 774 deaths during 114 days in 2003 (Stadler, Masignani et al. 2003) and a West Nile virus outbreak in 2012 resulted in 41 deaths in the US alone. While vaccines are available to a few members of the Positive strand RNA viruses (e.g. Poliovirus, Hepatitis A), there is little treatment by way of vaccine or antiviral available against the majority of these viruses. However knowing the mechanisms of infection, replication and pathogenesis will potentially provide new therapeutic targets.

THE PLUS STRAND RNA VIRUS LIFECYCLE

All positive stranded RNA viruses, whether they may be enveloped or non-enveloped, all share a common life cycle which consists of the following stages:

- attachment to the host cell surface;
- entry of the viral particle;
- uncoating of the viral particle;
- translation of the viral RNA by host ribosomal machinery;
- replication of the viral genome;
- packaging of the viral genome;
- release of the viral particles.

Specific receptors on the plasma membrane of the host cell allow viral particles to attach and enter. The cellular receptor is one of the determinants of the host range for infection. For example in the case of HIV, the CD4 receptor enables the virus to infect only helper T-cells and dendritic cells. Expression of a particular receptor however does not guarantee viral replication. For reasons yet unknown, the poliovirus receptor CD155 is ubiquitously expressed but poliovirus only replicates in gut epithelial and neuronal cells (Zhang and Racaniello 1997).

Viral particles often get internalized via endocytosis after binding to their receptors. For poliovirus, a clathrin-, caveolin- and microtubule-independent, but actin-dependent endocytosis pathway has been demonstrated to be required for internalization (Brandenburg, Lee et al. 2007; Brandenburg, Lee et al. 2007). Once internalized there is conformational change in the Poliovirus capsid which allows for release and transport of the viral RNA through a plasma membrane RNA channel into the cytosol. For Hepatitis C virus, an enveloped virus, after endocytosis, the envelope of the HCV fuses with the endocytic membrane thus releasing the core viral capsid particle into the cytosol (Ciesek and Manns 2011).

After entry, viral particles are destabilized and genomic RNAs are released into the cytosol (Strauss, Levy et al. 2013). The viral RNA is then translated into a polyprotein. The polyprotein subsequently undergoes a series of proteolytic cleavages to produce a variety of structural and nonstructural proteins. The structural proteins go on to form the new capsids and envelope components of progeny virions. The nonstructural proteins are the replication machinery which synthesize the viral RNA genome. The newly synthesized positive strand RNA molecules are then utilized for either translation (to make more structural and non-structural proteins), replication (as a template) or are packed into new virions. Finally the new progeny virions leave the host cells. For enveloped viruses this always involves taking membranes from the host, such as budding from the plasma membrane or budding into the secretory pathway and being trafficked out. For non-enveloped viruses, the dogma has been that the cells have to lyse in order for the viruses to get out. This, however, as you the reader will see in this thesis, turns out not to be the case.

POSITIVE STRANDED RNA VIRUSES GENERATE REPLICATION ORGANELLES

One of the most exclusive features of positive strand RNA viruses is that their genomes are replicated on the host intracellular membranes. These viruses all reorganize intracellular membranes to generate specialized membrane-based platforms, so-called replication organelles, on which they assemble their replication machinery (Denison 2008; Miller and Krijnse-Locker 2008).

Picornaviruses, coronaviruses and flaviviruses utilize membranes of the endoplasmic reticulum (ER) and Golgi apparatus/ trans-Golgi network (TGN) to generate their replication organelles (Schlegel, Giddings et al. 1996). In addition, endosomes, lysosomes and mitochondria have also been shown to be membrane sources for replication organelles for togaviruses and nodaviruses (Magliano, Marshall et al. 1998).

Why replicate on membranes? It is widely believed that membranes may facilitate replication by limiting the diffusion of replication machinery to a 2-D plane and concentrate viral and host proteins as well as viral RNA. In particular, at the earliest stages of infection when viral proteins are a minority in the vast sea of host proteins, binding and assembling on a membrane may provide a kinetic leg up in getting replication started, as well as protecting viral RNA from being degraded by host innate immune responses (Lyle, Bullitt et al. 2002).

Moreover, we have shown that membranes provide specific lipids that modulate replication reactions. In particular, our published studies have shown that many positive strand RNA viruses, including poliovirus, coxsackievirus, echovirus, rhinovirus, hepatitis C virus and aichi virus all rely on phosphatidylinositol 4-phosphate (PI4P) and cholesterol lipids for replication. We have shown that these viruses have evolved specific mechanisms to enrich for these two lipids at the ER/Golgi/TGN membranes: they recruit host phosphotidylinositol 4 kinase III β (PI4KIIIβ) to the membranes in order to generate high levels of PI4P lipids; and they drain the plasma membrane cholesterol pools by internalizing these lipids via endocytosis and subsequently

targeting these endosomes to the replication organelles (Hsu, Ilnytska et al. 2010; Ilnytska, Santiana et al. 2013). Furthermore we have shown that PI4P acts as a docking lipid for soluble viral RNA dependent RNA polymerases such as the poliovirus 3Dpol protein, as well as facilitating the regulated proteolytic processing of the viral polyprotein precursors. (Hsu et al., 2010; Ilnytska et al., 2013)

We have shown that inhibiting PI4K IIIβ from producing PI4P lipids blocks the replication of multiple different viruses while allowing the host cells to stay viable. One explanation for the latter is that PI4K IIIβ is a member of a family of PI4Ks. While the viruses have all evolved to hijack only one family member (Altan-Bonnet and Balla 2012), the host potentially utilizes the kinase activity of other members of the family and thus meet its PI4P needs. Our discovery of PI4K IIIβ as being required for the replication of multiple different positive strand RNA viruses has generated much excitement and created the possibility of one day developing panviral therapeutics. This is an important area since often hosts are infected with multiple different viruses at any given time, thus finding a single treatment that can target multiple different viruses is a holy grail in the fields of virology and infectious diseases.

ASSEMBLY AND RELEASE OF VIRAL PARTICLES

The newly synthesized positive strand viral RNA molecules undergo an encapsidation process to generate infectious particles. This is a crucial step in the viral life cycle. The protein shell provides a protective role for the positive

stranded RNA to survive in the extracellular environment. The assembly process includes the formation of the structural units from the structural proteins, assembly of the individual structural units into a coat, incorporation of the viral genome (which can happen simultaneously along with the assembly of the subunits). The positive stranded RNA viruses use a variety of assembly mechanisms to encapsidate the viral genomes. For example for HCV, the core protein which makes the capsid is first localized at the lipid droplets of liver cells where it recruits and assembles around the newly synthesized viral RNA. This core particle than buds into the lumen of the endoplasmic reticulum, thus picking up its envelope (Jones and McLauchlan 2010). For coronaviruses like SARS, their genomic RNA is encapsidated in the cytoplasm by multiple copies of the N protein (de Haan and Rottier 2005), thus forming the helical ribonucleic protein (RNP). Whether the RNP complex is generated at the replicative structures is presently unknown. For others such as poliovirus, direct interaction between the non-structural polio protein 2C and structural polio protein VP3, has been reported to be required for encapsidation of the poliovirus RNA molecules (Liu, Wang et al. 2010), although where and when in the cell this interaction takes place is unknown and thus one of the areas of investigation of this thesis.

RELEASE OF VIRAL PARTICLES FROM THE CELLS

During the process of exiting the host cell, the lipid membrane surrounding the capsid of enveloped viruses can be acquired from either intracellular membrane sources (such as endosomes, secretory organelles, multivesicular

bodies) or the plasma membrane. For example in the case of West Nile virus, the virions all assemble and bud out from the plasma membrane (Ng, Howe et al. 1994). HCV, on the other hand buds into the ER and is carried out of the host cell via constitutive secretory trafficking (Tamai, Shiina et al. 2012) whereas coronaviruses such as SARS and MERS bud into the ER-to-Golgi intermediate compartments (ERGIC) (Stertz, Reichelt et al. 2007).

In contrast, non-enveloped viruses are thought to be released from cells without having any host membranes around them and widely thought to be via a cellular lytic mechanism. While cell lysis could certainly be a mechanism by which non-enveloped viruses can exit, some reports in the literature over the past 50 years challenge this idea. First, it has been reported that echoviruses establish persistent infections with the cells continuously secreting infectious particles (Zhang and Racaniello 1997). Second Hepatitis A virus (HAV) has been recently shown to be released from cells without lysis of the infected cells (Feng, Hensley et al. 2013) possibly through a mechanism that involves the ESCRT machinery and multivesicular bodies. Third when polarized monolayers of human epithelial cultures are infected with poliovirus, the monolayer integrity of Caco-2 cell culture remains intact and poliovirus is preferentially released from the apical surface (Tucker, Thornton et al. 1993). Fourth, cells persistently infected with poliovirus continuously secrete infectious virus (Colbere-Garapin, Christodoulou et al. 1989; Lloyd and Bovee 1993). Finally in primary neuron cultures, the death of poliovirus-infected neuron is delayed and virus appears to spread from cell to cell by trans-synaptic transmission (Daley, Gechman et al. 2005). **Indeed**

Kirkegaard et al., have recently proposed that autophagosomes may play a role in the non-lytic export of poliovirus although to date there has been no direct evidence showing autophagosomes carrying viral particles being released from cells (Jackson, Giddings et al. 2005).

POLIOVIRUS AND AUTOPHAGY MACHINERY

Autophagic/lysosomal system is a degradation process that engulfs and breaks down cellular proteins and organelles within double membrane vesicles (Cuervo, Bergamini et al. 2005). Autophagy is general induced to maintain cell homeostasis under stress such as nutrient deprivation, as well as development and tumorigenesis. Double membrane structures are the features of autophagosomes; recently, it was proposed that the isolation membranes are from the mitochondria-associated endoplasmic reticulum (ER) membrane (MAM) (Hamasaki, Furuta et al. 2013). Autophagy-related (ATG) protein machinery is the main way to form the autophagosome. Many kinds of proteins are involved in the formation and elongation of isolation membrane, such as ATG5, ATG12, Beclin1, etc. In addition, a cytosolic form of LC3 (LC3-I) protein is lipidated by phosphatidylethanolamine (PE) to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is specifically recruited to autophagosome membranes (Tanida, Ueno et al. 2008). Autophagosomes fuse with endosomes and lysosomes that contain acid hydrolases to become a degradative autolysosome (Lamb, Yoshimori et al. 2013).

Autophagy is also an important cellular defense mechanism against

intracellular pathogens (Deretic, Saitoh et al. 2013). During infection, many DNA and RNA viruses induce accumination of autophagosomes or autophagy-related vesicles. These increased autophagosomes or autophagy-related vesicles are caused by either production of newly formed autophagosomes or inhibition of their maturation (Dreux and Chisari 2010).

Double-membraned cytoplasmic vesicles have been observed during poliovirus infection. Co-expression of the poliovirus non-structural proteins 2BC and 3A induce the formation of double-membrane vesicles which contain modified LC3 (Cho, Teterina et al. 1994; Suhy, Giddings et al. 2000). Moreover, poliovirus 3A protein inhibits autophagy-related vesicles movement along the microtubules by binding and blocking a member of the dynein complex (Kondratova, Neznanov et al. 2005; Taylor, Burgon et al. 2009). In addition, autophagy proteins are involved in progeny poliovirus yields (Jackson, Giddings et al. 2005).

HYPOTHESIS

Collectively the findings above all hint at non-lytic mechanisms at work to release non-enveloped viruses from cells. **Here in this thesis we hypothesized that non-enveloped RNA viruses hijack the cellular autophagy pathway to exit the host without lysis.** To test this hypothesis we utilized the poliovirus as a model virus because of its relatively short lifecycle (~10 hours from start to finish); amenability to live cell/infection imaging; wealth of reagents (antibodies, mutants etc) available; high multiplicity of infection; and finally its genetic

similarity to many other important human viruses causing diseases such as coxsackievirus, rhinovirus, echovirus and enterovirus to which no current treatments are available. To test this hypothesis we:

1) Characterized the translation and encapsidation sites using high resolution confocal imaging and super resolution imaging to establish the spatio-temporal connection among viral RNA translation, replication and ecapsidation sites and we demonstrated that mature poliovirions containing viral RNA are formed and remain on replication organelles until viral RNA synthesis is plateaued.

2) Demonstrated the spatio-temporal dynamics of poliovirions during infection and showed that the autophagosomal machinery is hijacked by poliovirus to capture multiple poliovirions and is subverted away from its typical degradative fate of fusing with lysosomes and instead is targeted to the plasma membrane where it is then subsequently released from, without disrupting the permeability barrier, into the extracellular medium as a vesicular packet containing multiple virions.

3) Characterized the protein and lipid composition of the released membrane vesicles containing non-enveloped virions.

4) Demonstrated the infection efficiency of viruses within vesicles relative to free (outside vesicles) viruses.

5) Demonstrated an increase in phosphotidylserine enriched microvesicle release from cells infected with non-enveloped RNA viruses, including both positive and double stranded RNA viruses.

6) Demonstrated that phospotidylserine is critical for poliovirus infection and thus providing a new paradigm for how poliovirus can enter host cells.

CHAPTER 2

Materials and Methods

Materials

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.

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.

Poliovirus type 1 (Mahoney) was provided by Dr. Georgiy Belov (University of Maryland, MD). To generate viral stock, confluent HeLa cells were infected by PV for 24hrs. The infected cells underwent freeze-and-thaw cycle three times, and supernatant was collected and stored at -80°C.

DNA construct:

Plasmid and replicon constructs were kindly provided by following laboratories. Arf1-GFP, Arf1-RFP (Jennifer Lippincott-Schwartz; National Institutes of Health, MD), LC3-RFP (Radek Dobrowolski; Rutgers University, NJ), LactC2-GFP (Sergio Grinstein; The Hospital for Sick Children, Canada).

Antibodies:

Antibodies were purchased or provided by the indicated vendor or laboratory. Anti-CVB3 3A (Lindsay Whitton; The Scripps Research Institute, CA), anti-3AB (Geroge Belov; University of Maryland, MD), anti-A12 (Robert Purcell; National Institutes of Health, MD). All secondary antibodies were from Invitrogen and Jackson Immunochemical.

Chemicals:

Brefeldin A, paraformaldehyde, and saponin (Sigma; St. Louis, MI), PIK93 (Kevin Shokat, UC Berkeley, CA), 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)

Microscope:

All imaging was performed on a Zeiss LSM780 confocal laser scanning

confocal microscope (Carl Zeiss, USA) equipped with lasers emitting 405nm, 488nm, 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.

Methods

Integrity of Plasma Membrane Assay (Cell Viability Assay)

Cells were infected with poliovirus and then harvested at each time point. The percentage of trypan blue positive cells was counted using trypan blue with Countless Automated Cell Counter (Life Technologies, CO).

Immunofluorescence and Confocal Microscopy

Cells were plated on glass coverslips and fixed with 3.7% PFA for 15 minutes at RT. Cells were permeabilized with 0.2% Saponin or 0.1% Triton X-100 and sequentially incubated with primary and fluorophore-tagged secondary antibodies. Coverslips then were mounted and imaged with Zeiss LSM 510. Confocal images were analyzed with Zeiss LSM or Image J software.

Live-Cell Imaging

All imaging was performed on a Zeiss LSM780 confocal laser scanning confocal microscope (Carl Zeiss, USA) using a Plan-Apochromat 40x/1.40 oil immersion objective and GaAsP detectors. Live cells were maintained on the microscope stage in a temperature, CO_2, and humidity-controlled environmental

chamber. Time-lapse images were acquired every 10 seconds for the duration of infection.

Plaque Assay

Monolayers of HeLa cells were infected with virus. After 30 minutes absorption, virus was removed and cells were overlaid with 0.5% agar in DMEM. Plaques were formed at 48 hours incubation; agar overlay were removed and cells were stained with 0.1% crystal violet.

Phosphatidylserine-Enriched Vesicle Isolation

Cells were infected with poliovirus for 30 minutes and replaced with fresh serum free media for another 8 hours. Extracellular media then was collected from virus-infected cells. Media was spun down at 500xg for 5 minutes (4°C). Supernatant was collected and spun down again at 5000xg for 10 minutes (4°C). The pellet was resuspended in 1X binding buffer and the following procedure was performed as the manual from the annexin V microbead kit (Milteny Biotec, CA).

Ribopuromycylation

HeLa cells on the coverslips were infected with viruses when the cells reached 80% confluence. Then cells were incubated with DMEM (7.5% FBS supplemented with 91 μM PMY and 208 μM emetine) for 5 min at 37°C before different post infection time. Cells were washed with cold PBS twice and then

incubated with permeabilization buffer for 2 min on ice. After the extraction step, cells were washed with polysome buffer and fixed with 3% PFA for 15 min at RT. After being washed with PBS, cells were maintained at 4°C before immunofluorescence staining. The method was developed and described in David et al. (2012).

siRNA Transfection

Cells were seeded in 24-well plates one day before siRNA transfection. 100 nM of each siRNA was transfected with Dharmafect1 (Dharmacon, CO) and incubated for 48 hours. Supernatant was collected for examining virus titers via plaque assay and cells were lysed for western blot analysis.

Statistical Analysis

Data were expressed and plotted as means ± standard error of the mean (SEM). Unpaired student's t tests were used to compare the mean of control and experimental groups.

Super-Resolution 3D-SIM Imaging

Super-resolution 3D-SIM imaging was performed on a Zeiss ELYRA S.1 system (Carl Zeiss, USA). Images were acquired with a Plan-Apochromat 63x/1.40 oil immersion objective and an Andor iXon 885 EMCCD camera. Fifteen images per plane (five phases, three rotations) and 0.125 mm z section of 3 mm height were required for generating superresolution images. Raw images were

reconstructed and processed to demonstrate structure with greater resolution by the ZEN 2011 microscope software (Carl Zeiss, USA).

Vesicle Isolation

Cells were infected with poliovirus for 30 minutes and replaced with fresh serum free media for another 8 hours. Extracellular media then was collected from virus-infected cells. Media was spun down at 500xg for 5 minutes (4°C). Supernatant was collected and spun down again at 5000xg for 10 minutes (4°C). Supernatant and pellet (vesicles) were collected for infection.

Vesicle Infection

Collected vesicles were resuspended in serum free DMEM and then added onto fresh confluent HeLa cells. After four hours incubation, the cells were harvested and lysed for western blot analysis.

CHAPTER 3

The spatio-temporal dynamics of viral RNA translation, synthesis and encapsidation

Genomic RNAs of picornaviruses contain a poly(A) tract at the 3' terminus (Yogo and Wimmer 1972), but do not own 7-methylguanosine cap like cellular mRNAs. Since the genome lacks a 5' cap, the translation of picornaviruses is not cap-dependent. Instead, ribosomes bind through an internal ribosome entry site (IRES) located at the 5' non-coding region of the viral genome. IRES contains RNA secondary structure elements and is required for translation initiation (Jang, Krausslich et al. 1988). The secondary structures are able to interact with cellular canonical or noncanonical factors to recruit the ribosomes to viral RNA (Semler and Waterman 2008). In addition, virus shuts off host translation by cleaving the eukaryotic initiation factor 4F (eIF4F), which is crucial for the cap-dependent translation (Krausslich, Nicklin et al. 1987). By shutting down the host translation, the cellular translation factors are free to be utilized for viral translation. The newly synthesized viral proteins and cellular factors are required for replication organelles. Once replication complexes are formed, more viral RNAs will be synthesized.

As previous studies showed, newly synthesized viral RNAs are made at the replication organelles. These RNAs can then be utilized as templates for translation, replication or being packed into mature virions, yet how these processes can proceed without interference from each other remains to be fully

understood. The direction of protein synthesis and viral RNA polymerization is opposite to each other, thus a template cannot be used for both reactions at once. It has been shown the viral protein 3CD represses translation and promotes negative-strand RNA synthesis in vitro (Gamarnik and Andino 1998). It might suggest there is a switch between translation and replication. However, whether a single RNA template is used for both translation and replication is still unclear. In addition, viral RNAs have to be packed at the later stages of infection. Picornaviruses do not have an RNA encapsidation signal for the specificity of encapsidation (Porter, Ansardi et al. 1995). Therefore, the localization of viral RNAs for packaging is important as well.

In this chapter, we used PV and CVB3 as the model viral systems to investigate the spatio-temporal dynamics of viral RNA translation, synthesis, and encapsidation. Using super-resolution microscopy (3D Structural Illumination microscopy; 3D-SIM) we established the spatio-temporal connections between viral replication organelles translation and encapsidation sites and systematically dissected the distribution of viral and host protein machinery on the replication organelles.

RESULTS

Enteroviral RNA translation sites are spatially segregated from replication organelles

We first characterized of RNA translation sites during enteroviral infection. Active translation sites are able to be detected by Ribopuromycylation method (Figure 3-1) (David, Dolan et al. 2012). Puromycin (PMY) is a Tyr-tRNA analogue that enters ribosome A site and incorporates into the nascent chain C terminus (Pestka 1971). By blocking with the chain elongation inhibitors cycloheximide (CHX) and emetine while translating, immobilized ribosomes are capable of being detected by anti-puromycin antibodies via immunostaining. The cells without infection showed the ER-pattern staining which demonstrated that most of translating ribosomes were ER-bound in the normal situation. After two hours infection with viruses, fewer translation sites were seen than in mock cells (Figure 3-2A). The decrease in translation signal was even more significant at three hours post infection. The change in translation levels was due to shutting off the host cell translation: viral 2A protease is known to induce cleavage of the eukaryotic initiation factor 4F resulting in shut down of all cap-dependent translation (Krausslich, Nicklin et al. 1987). Additionally, cleavage of Poly(A)-binding protein by 3C protease is also known to inhibit host cell translation (Kuyumcu-Martinez et al., 2004).

Significantly an increase in translation signal was observed at four hours post infection, potentially because the cap-independent viral RNA translation now dominated and fluorescence intensity of the cells was quantified (Figure 3-2B). Since host cell translation is almost entirely shut down at three hours post infection, the fluorescence signal localization we detected at four hours post infection is showing active enteroviral RNA translation sites.

The spatio-temporal localization of viral RNA translation sites relative to RNA synthesis sites was demonstrated by costaining with antibodies against puromycin and 3AB protein (Figure 3-3). 3AB is localized on the membranes of the replication platforms (Hsu et al., 2010). By two hours post infection, not many replication platforms had formed and translation sites likely contained both host and viral RNA translation activities (Figure 3-3, 2hrs p.i.). However at 4 hours post infection, viral RNA translation sites were found to be adjacent to the now discrete replication sites (Figure 3-3, 4hrs p.i.). Furthermore, SIM imaging revealed that viral RNA translation sites were closely surrounding but still spatially segregated from replication organelles with the typical distance between them ~ 100 nm (Figure 3-4A and 3-4B).

Viral RNA encapsidation is localized to the replication organelles

VP1 is a subunit of capsids. When capsid structures are formed, there are 60 copies of VP1 incorporated into the virion. We used VP1 as an indicator to localize the location of virions. VP1 is a cytosolic protein, thus the majority of VP1 proteins were dispersed in the cytoplasm (Figure 3-5A). However, some of VP1 were concentrated at the replication organelles. As previous studies showed, new viral RNAs are synthesized on the replication organelles. Therefore, non-structural proteins being recruited to the replication organelles might be morphogenesis- related. We used 3A antibody to immunoprecipitate replication complexes and we found VP1 proteins were in physical interaction with 3A proteins on the replication complexes (Figure 3-5B). In addition, since direct

interaction between VP3 and 2C is known to be required for enterovirus morphogenesis (Liu, Wang et al. 2010). My data suggested that the sites of viral RNAs packaging are likely on the replication organelles themselves.

However, the subunit VP1 of capsids does not represent the mature virions, thus the encapsidation sites still cannot be indicated. A poliovirus neutralizing monoclonal antibody, A12, was produced from Purcell lab (Chen, Chumakov et al. 2011). The antibody is able to recognize antigenic sites (D-antigen) only in the mature virion. We took advantage of the specificity of this antibody to determine the localization of virions by immunostaining. The mature virions were seen by 3.5 hours poliovirus post infection, and appeared exclusively on the replication organelles (Figure 3-6B). Nevertheless, VP1 proteins were broadly dispersed in the cytoplasm, although some of them were colocalized with mature virions. The locations of virions were also confirmed by D-antigen antibody with A12 antibody (Figure 3-6A).

Furthermore, SIM was utilized to obtain spatial organization on replication organelles and encapsidation sites. 3D-SIM imaging demonstrated that VP1 proteins were together with mature virions colocalized to 3AB protein containing replication sites (Figure 3-7A). The mature Virions were formed on top of the PI4P enriched lipid domains where newly viral RNAs are synthesized (Figure 3-7B and 3-7C) as determined by dsRNA staining (Hsu et al., 2010).

CONCLUSIONS

In this chapter, we demonstrated that active viral RNA translation sites are

closely juxtaposed but spatially segregated from viral RNA synthesis sites. We also show that capsids containing viral RNA are formed at the viral RNA synthesis sites. Taken together our findings indicate that translation RNA templates and replication RNA templates are spatially segregated at the peak of viral replication (4 hrs p.i.). However since our signal to noise is low for the first 3 hours and some host translation is still active, viral RNA translation sites at early stages are difficult to make a distinction.

Figure 3-1: Ribopuromycylation method (RPM)

HeLa cells are infected with CVB3 or PV for 4 hours. Puromycin is added into living cells with elongation inhibitors (Emitine and CHX). Cells then are fixed and immunostained with anti-puromycin mAbs. Puromylated nascent chains are able to be detected via indirect immunofluorescence.

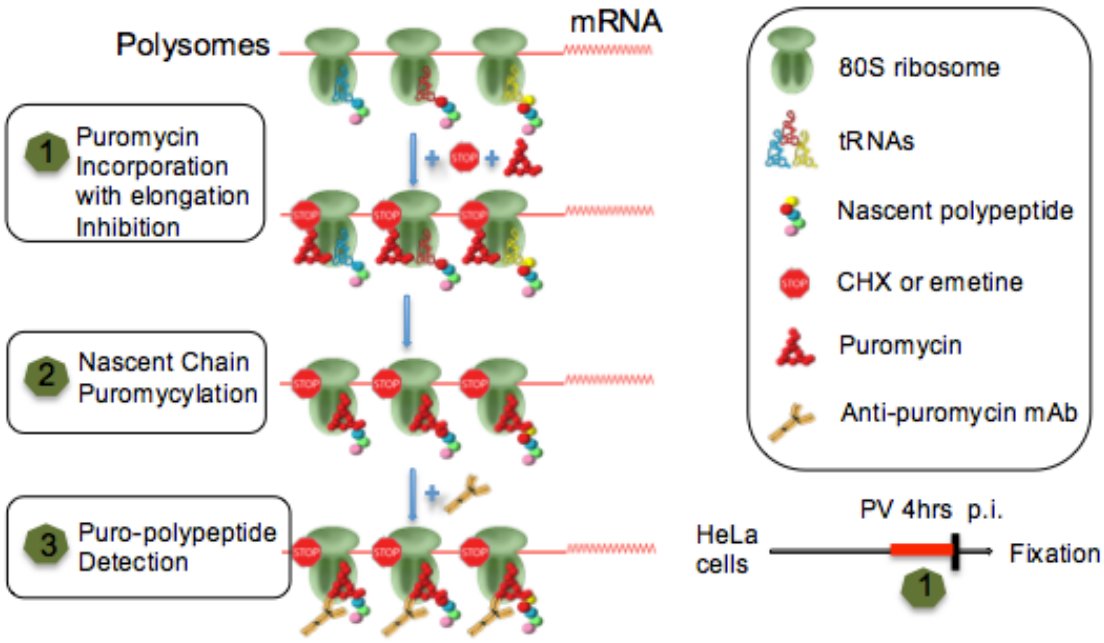
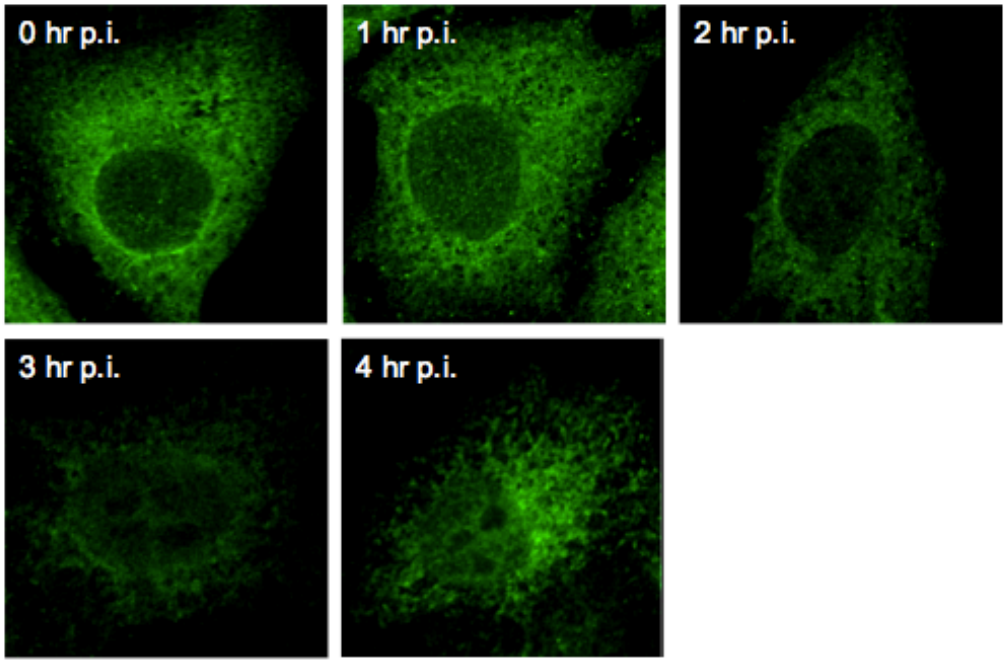

Polysomes
mRNA
1 Puromycin Incorporation with elongation Inhibition
2 Nascent Chain Puromycylation
3 Puro-polypeptide Detection
80S ribosome
tRNAs
Nascent polypeptide
CHX or emetine
Puromycin
Anti-puromycin mAb
PV 4hrs p.i.
HeLa cells
Fixation
1

Figure 3-2: RPM detects translating ribosomes.

A) HeLa cells were infected with poliovirus at different time points and incubated with PMY (puro) and inhibitors for 5 min at 37°C before digitonin extraction and fixation. Samples then were immunostained with antibodies (12D10) to detect PMY, which indicated the active translation sites.

B) Cellular translation levels of poliovirus infection over time were quantified by intensity of fluorescence from puromycin immunostaining images. Polioviral RNA translation is increased at 4 hours post infection. Less translation was seen at 3 hours post infection due to shutting off host cell translation.

A.


0 hr p.i.
1 hr p.i.
2 hr p.i.
3 hr p.i.
4 hr p.i.

B.

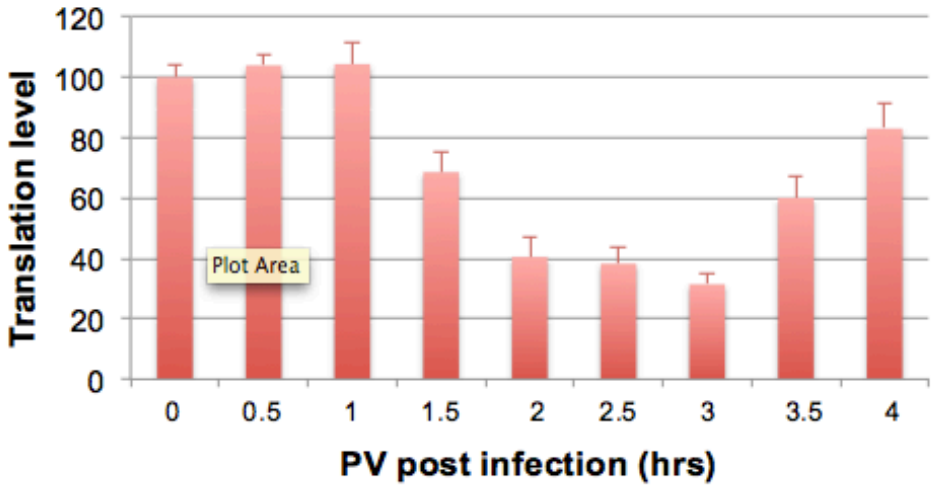

Translation level
120
100
80
60
40
20
0
Plot Area
0
0.5
1
1.5
2
2.5
3
3.5
4
PV post infection (hrs)

Figure 3-3: Active viral RNA translation sites are approximate on the replication organelles.

HeLa cells were infected with poliovirus and treated by ribopuromycylation method. Samples were stained with anti-puromycin and anti-3AB antibodies and analyzed by confocal microscopy. Localization of 3AB indicates the replication organelles. Active polioviral RNA translation sites are juxtaposed to replication organelles at 4 hours post infection.

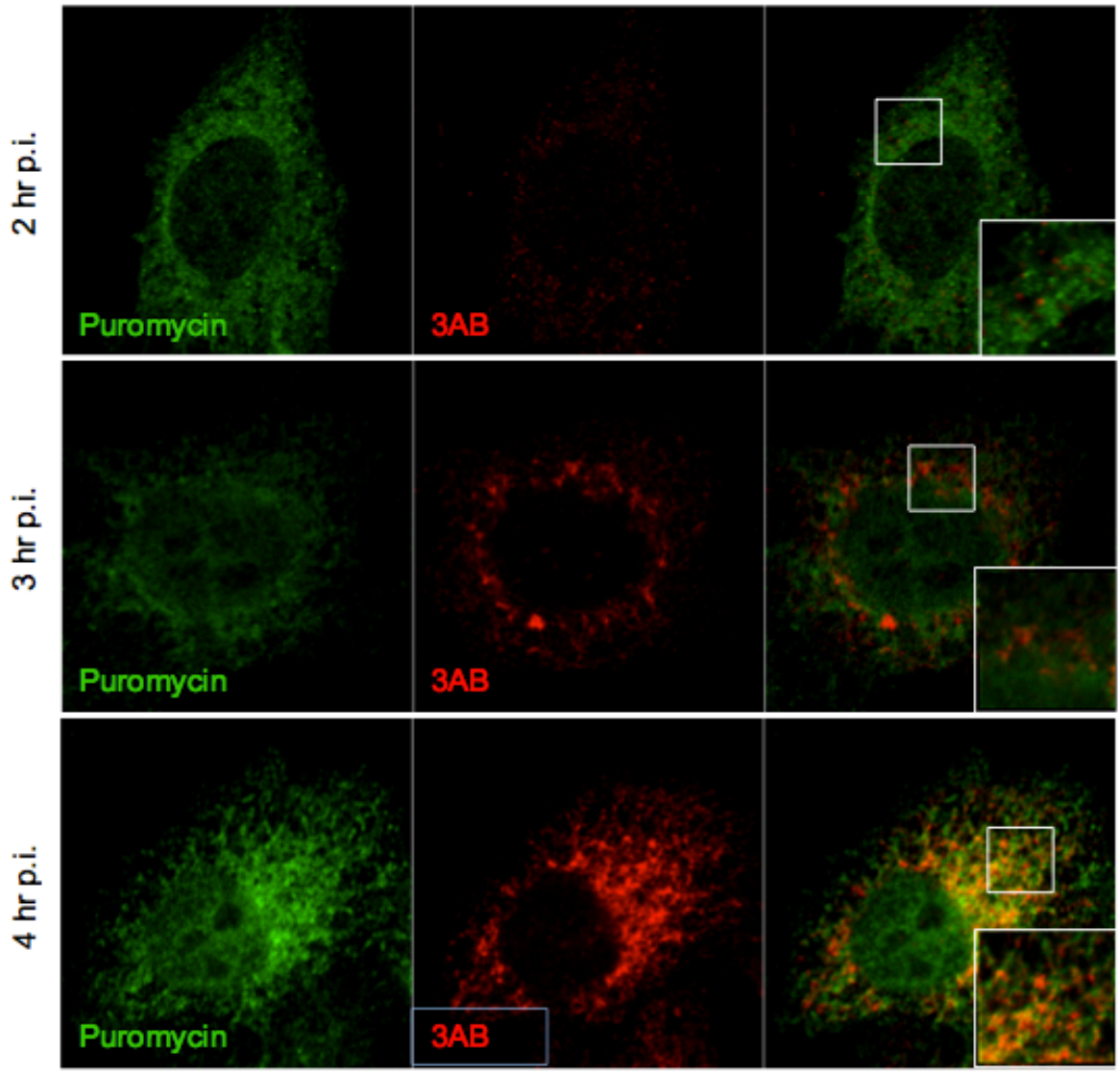

2 hr p.i.
Puromycin
3AB
3 hr p.i.
Puromycin
3AB
4 hr p.i.
Puromycin
3AB

Figure 3-4: Active viral RNA translation sites are juxtaposed to but spatially segregated from replication organelles.

A) SIM imaging of distribution of RNA translation sites on poliovirus replication organelles at 4 hours post infection.

B) 3D rendering of cropped region from SIM imaging. Scale bar indicates 200nm.

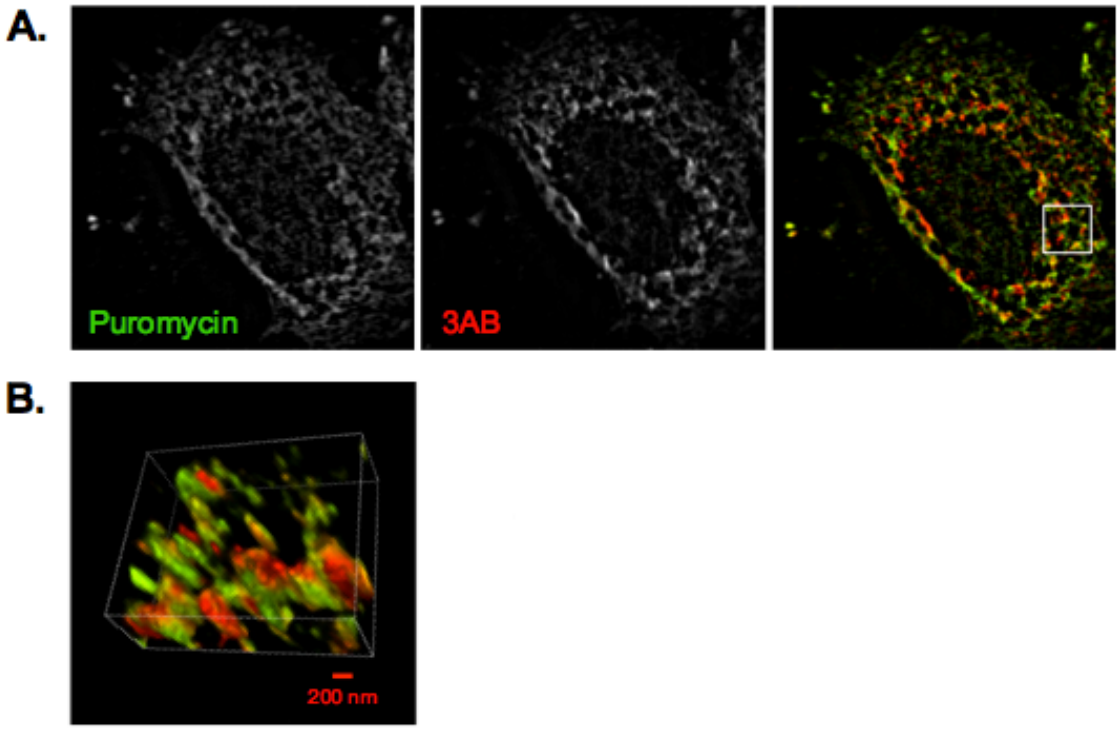
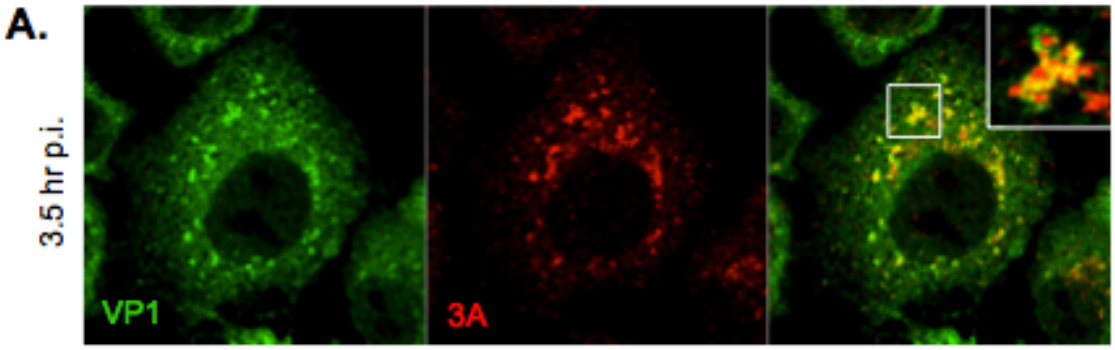

A.
Puromycin
3AB
B.
200 nm

Figure 3-5: Capsids are in physical interaction with viral replication organelles.

A) HeLa cells were infected with CVB3 for 3.5 hours and immunostained with anti-VP1 and anti-3A antibodies. Localization of 3A indicates the replication organelles.

B) HeLa cell lysates from cells infected with CVB3 or mock (-) for 4 hours were pulled down with anti-3A antibody. Coimmunoprecipitation of 3A with VP1 and VP3 was shown at the peak of replication.


A.
3.5 hr p.i.
VP1
3A

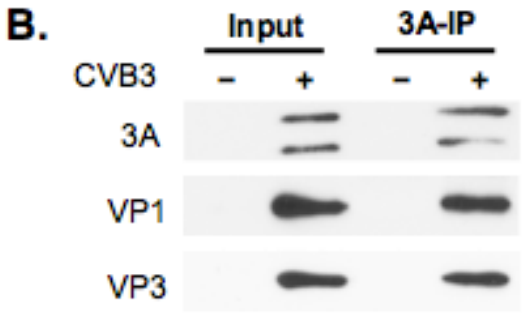

B.
Input
3A-IP
CVB3
–
+
–
+
3A
VP1
VP3

Figure 3-6: Capsids containing polioviral RNA are localized to discrete structures in the cytoplasm at 3.5 hours post infection.

A) Poliovirus-neutralizing MAb (A12) and D-antigen antibodies both recognize the capsids containing viral RNA. HeLa cells were infected with poliovirus for 3 hours.

B) Capsids containing viral RNA are more discrete rather than VP1, subunit of capsids. HeLa cells were infected with poliovirus for 3.5 hours.

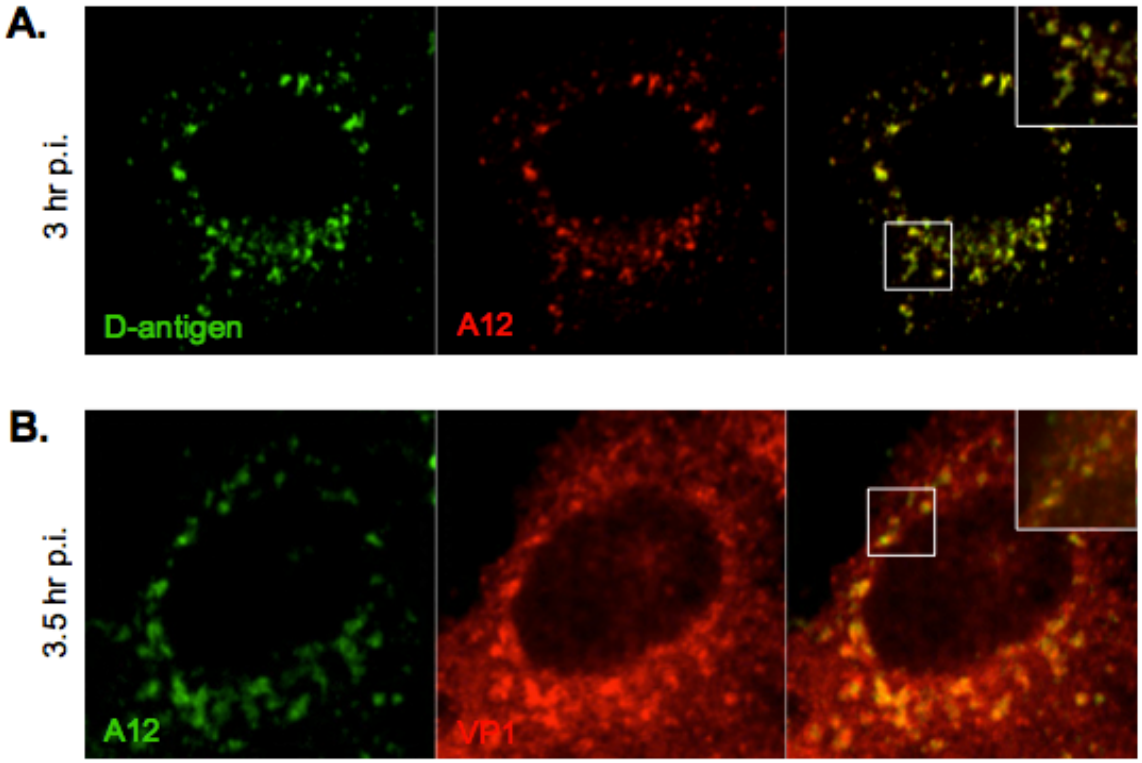

A.
3 hr p.i.
D-antigen
A12
B.
3.5 hr p.i.
A12
VP1

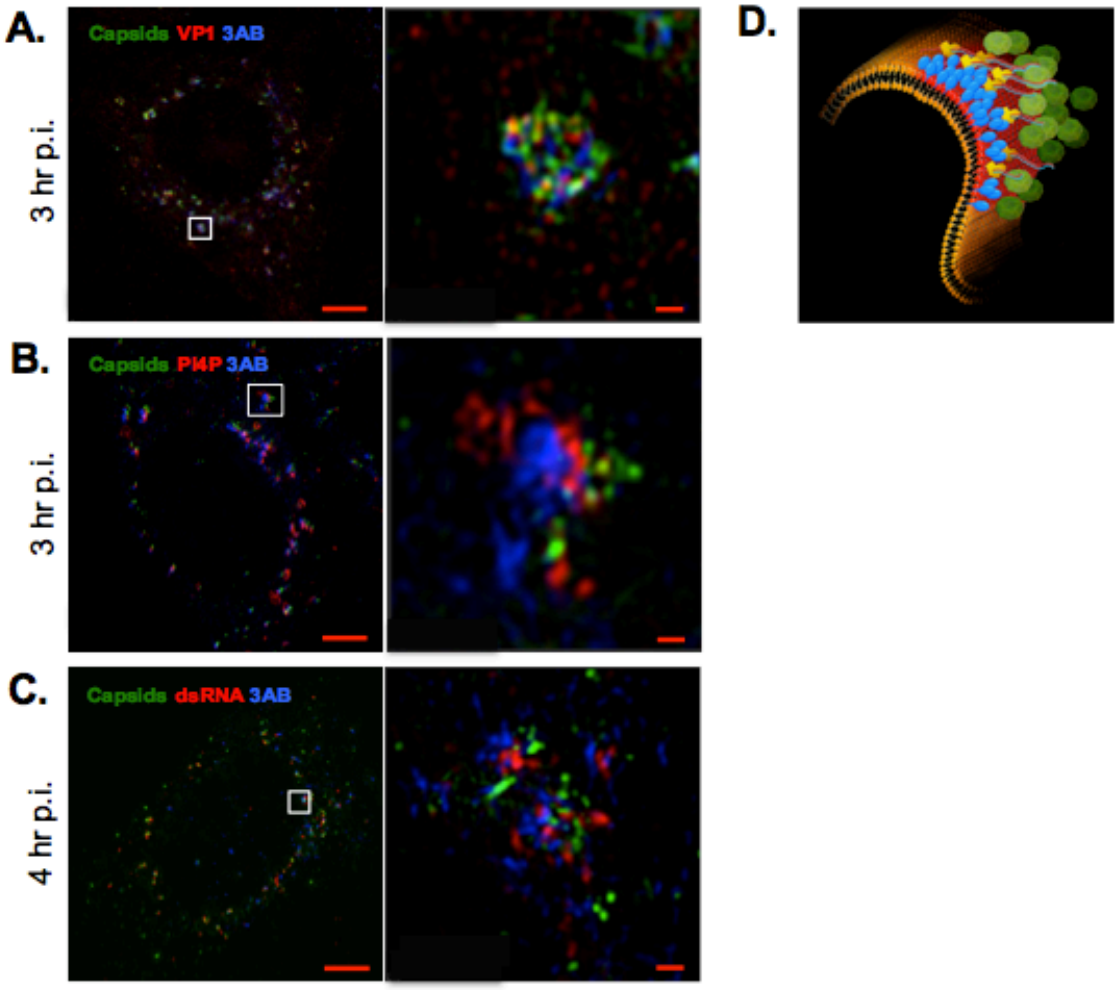
Figure 3-7: Dissection of replication organelle structures by super-resolution imaging.

A) Capsids are localized to the replication organelles. (Insets) Higher-detail information of replication organelle substructures when recorded with 3D-SIM. Scale bars indicate 5μm and 300nm respectively.

B) Capsids are localized to the PI4P-enriched membrane. Domains. (Insets) Higher-detail information of replication organelle substructures when recorded with 3D-SIM. Scale bars indicate 5μm and 200nm respectively.

C) Capsids are enriched at viral RNA synthesis sites on replication organelles. (Insets) Higher-detail information of replication organelle substructures when recorded with 3D-SIM. Scale bars indicate 5μm and 400nm respectively.

D) Model of the replication organelles. Membranes are enriched with PI4P lipids (red). 3AB proteins (blue) and replicating viral RNAs (yellow) are docking on the membranes. Mature virions (green) then are formed next to the replication sites.


A.
Capsids VP1 3AB
3 hr p.i.
B.
Capsids PI4P 3AB
3 hr p.i.
C.
Capsids dsRNA 3AB
4 hr p.i.
D.

CHAPTER 4

The mechanisms of Polioviral exit from the host

Where viral RNAs are encapsidated (i.e. packaged) is unknown. Previously, in chapter 3, we described viral RNAs were synthesized at the replication organelles and virions were also found to be localized on the membranes of replication organelles. Thus direct recruiting the capsid proteins to the replication organelles is the way for enteroviral packaging.

Once the viral RNAs are encapsidated, virions exit the cell. It has been shown that a large number of particles of progeny poliovirus are present in the cytoplasmic matrix at 5 to 7 hours post infection by electron microscopy (Dales, Eggers et al. 1965). However, how Poliovirus and indeed non-enveloped viruses in general are released from cells is still debated. The general dogma is that cells have to lyse in order to release the viruses. However virus-containing vesicles are frequently found near the cell surface within infected cells giving rise to the idea that vesicles may mediate virus release (Dunnebacke, Levinthal et al. 1969).

In this chapter, we characterized the spatio-temporal distribution of Poliovirions during the course of infection to better understand the dynamics of virus release.

RESULTS

Poliovirions undergo dynamic spatial transitions during infection

Given the localization of virions to the replication organelle membranes at

peak infection (Figure 3-7), we next investigated the fate of these virions as they exited the cells. The cells were infected with poliovirus for given time periods of 0, 3, 4, 6, 7, 8 hours. The location of the poliovirions, the subunits of the poliovirion capsids, and the replication organelles themselves were determined by immunostaining with anti-A12, anti-VP1, and anti-3AB antibodies respectively and confocal microscopy. No viral protein was seen at the beginning of infection (Figure 4-1, 0hr). At 3 hours post infection, replication organelles were formed as seen with 3Ab proteins clustered on perinuclear membrane bound organelles and some of subunits of capsids were also seen to localize to these viral RNA replication sites as well as being diffusely distributed across the cytoplasm (Figure 4-1, 3 hours pi). However, mature virions were only present on the replication organelles. As the infection progressed, accompanying the increasing RNA replication and translation cycles, more and more replication organelles were found to be formed (Figure 4-1 4 hrs pi). By this time, capsid subunits were dispersed across the cell, but the large majority of mature virions were still localized and concentrated at the replication organelles. Surprisingly, we observed a significant transition in the localization of mature viruses at 6 hours post infection, with the poliovirions dispersed from the replication organelles into the cytoplasm. Finally by 8 hours post infection, the levels of poliovirions within the cytoplasm were significant decreased suggesting release into the extracellular medium.

Given that the transition of poliovirions from the replication organelles to the cytoplasm (Figure 4-2A) correlated with the time when viral RNA synthesis is

plateaued (Yakobson, Mikhejeva et al. 1973), we tested whether this transition could be pre-empted by inhibiting viral RNA synthesis. Cells were treated with Guanidine HCl to block RNA synthesis (Figure 4-2B). Guanidine HCl is a reverse inhibitor of viral protein 2C that is responsible for negative strand RNA synthesis (Barton and Flanegan 1997). We added the inhibitor at 3.5 hours post infection. Poliovirions were now observed to transition into the cytoplasm at an earlier time point in the infection (Figure 4-2B). This suggested that inhibiting viral RNA synthesis accelerated virion release from replication organelles.

Poliovirus release via a non-lytic pathway

From our previous results, we had seen a significant decrease in the number of poliovirions by 8 hours post infection (Figure 4-1). Whether the decrease of intensity of staining is because the virions were released from the cells, we determined the virus titers from the supernatant of each time point of infection. Poliovirus release was seen from 5 hours post infection (Figure 4-3). Virus titers were increased dramatically at 7 to 8 hours post infection. However, whether the poliovirus release is because of the lysis of the cells, we investigated the integrity of the plasma membrane. Trypan blue, a small and cell impermeable dye, was used to test the intactness of the plasma membrane. The dye is able to stain the cells when the plasma membrane is damaged. We examined the plasma membrane integrity and compare it to the poliovirus release at each given time points. Amazingly, the plasma membrane remained intact during the course of infection (Figure 4-3). Since polio virions are too large to cross the

plasma membrane by diffusion through the bilayer even with a channel, the virus must use some other way to exit without damaging the plasma membranes.

Poliovirus hijacks autophagosome-like structures for virus release

Previous work by Karla Kirkeggard et al., has suggested that Poliovirus may hijack the autophagosomal machinery to exit the cell. Given this by immunostaining for lipidated LC3-II as an autophagosome marker (Tanida, Ueno et al. 2008) we monitored the fate of the autophagy pathway in Poliovirus infected cells. We found that the amount of LC3-II was significantly increased over timecourse of infection (Figure 4-4A and 4-4B) and poliovrions were colocalized with autophagosomes by 7 hours post infection (Figure 4-4C).

Autophagy is a degradative process critical for maintaining protein and lipid homeostasis within cells (Cuervo, Bergamini et al. 2005). Many proteins are involved in the formation and elongation of autophagic membranes, such as autophagy-related (ATG) proteins, Beclin1, LC3, etc. Recently it was proposed that the isolation membranes are from the mitochondria-associated endoplasmic reticulum (ER) membrane (MAM) (Hamasaki, Furuta et al. 2013). Autophagosomes then fuse with endosomes and lysosomes that contain acid hydrolases to become a degradative autolysosome (Lamb, Yoshimori et al. 2013). Given this, we next investigated whether autophagosomes during Poliovirus infection fuse with lysosomes. First using antibodies against LAMP1, a lysosomal membrane protein we examined the distribution of lysosomes relative to virions. We found that lysosomes were not colocalized with virions (Figure 4-

5A). Secondly, we treated the cells with lysosomal protease inhibitors, leupeptin, pepstatin, and E64d, to inhibit autophagic degradation during infection. Lipidated LC3 (LC3-II) is incorporated into the autophagosome membrane and specifically degraded by the autolysosome; thus an increase of LC3-II level would be observed if the activity of the lysosomal proteases is blocked. However, the inhibitors did not rescue any LC3-II level. It is clear that, during infection, the autophagosomes do not fuse with the lysosomes. Collectively these data thus suggested that the typical degradative autophagy pathway was subverted during poliovirus infection.

We next investigated why autophagosome-lysosome fusion was blocked during viral infection. Syntaxin 17 (STX17) was shown as the autophagosomal SNARE protein required for fusion with endosome/lysosome (Itakura, Kishi-Itakura et al. 2012). Depletion of STX17 can result in accumulation of autophagosomes without degradation. We examined the localization of STX17 during viral infection using GFP-tagged STX17 expressed in HeLa cells along with FAPP1-RFP as a marker for both the replication organelles and as indicator of the progress of infection (Hsu et al., 2010). Remarkably we found that STX17 was recruited to replication organelles from 4 hours post infection until late stages (Figure 4-7) and was not colocalized with either virions (A12) or autophagosome structures (LC3-RFP) (Figure 4-6A and 4-6B). This recruitment of ATX17 away from autophagosomes may explain why the latter were not capable of fusion with lysosomes during infection.

Poliovirus release is increased or decreased by stimulating or disrupting the level of autophagy

We next tested whether virus release from cells could be modulated by modulating the autophagy pathway. First, we inhibited autophagy by depleting with siRNA Beclin1 and LC3, both of which are known to be required for this pathway. Notably while depletion of Beclin1 and LC3 did not affect viral replication (Figure 4-8A), poliovirus release was decreased by~ 7 fold (Figure 4-8B). Second we acutely stimulated autophagy by treating cells with Tat-Beclin1 peptide. Tat-Beclin1 is a cell-permeable peptide, which has recently been shown to stimulate autophagy within 4 hours of treatment (Shoji-Kawata, Sumpter et al. 2013). We saw ~ 7 fold increase in poliovirus release when the cells were treated with 50μM Tat-Beclin peptide for 2.5 hours (Figure 4-9).

CONCLUSIONS

Non-enveloped viruses were thought to be released because of cell lysing. In this chapter, our data support a non-lytic poliovirus release pathway. We have shown that by hijacking the autophagosomal machinery, poliovirions are capable of crossing the plasma membrane without disrupting or lysing the cell. In addition, we have found that poliovirus infection can block the fusion of autophagosomes with lysosomes through a not fully understood process that involves sequestration of the SNARE machinery involved in fusion away from the autophagosomes.

Figure 4-1: Capsids undergo dynamic spatial transitions during infection.

HeLa cells were infected with poliovirus for different time points and immunostained with anti-A12, VP1 and 3AB antibodies. Samples were imaged by confocal microscopy. Capsids containing viral RNA are appeared on the replication organelles at 4 hours post infection and released from replication sites at 6 hours post infection. Puncta structures of capsids are formed at 7 to 8 hours post infection.

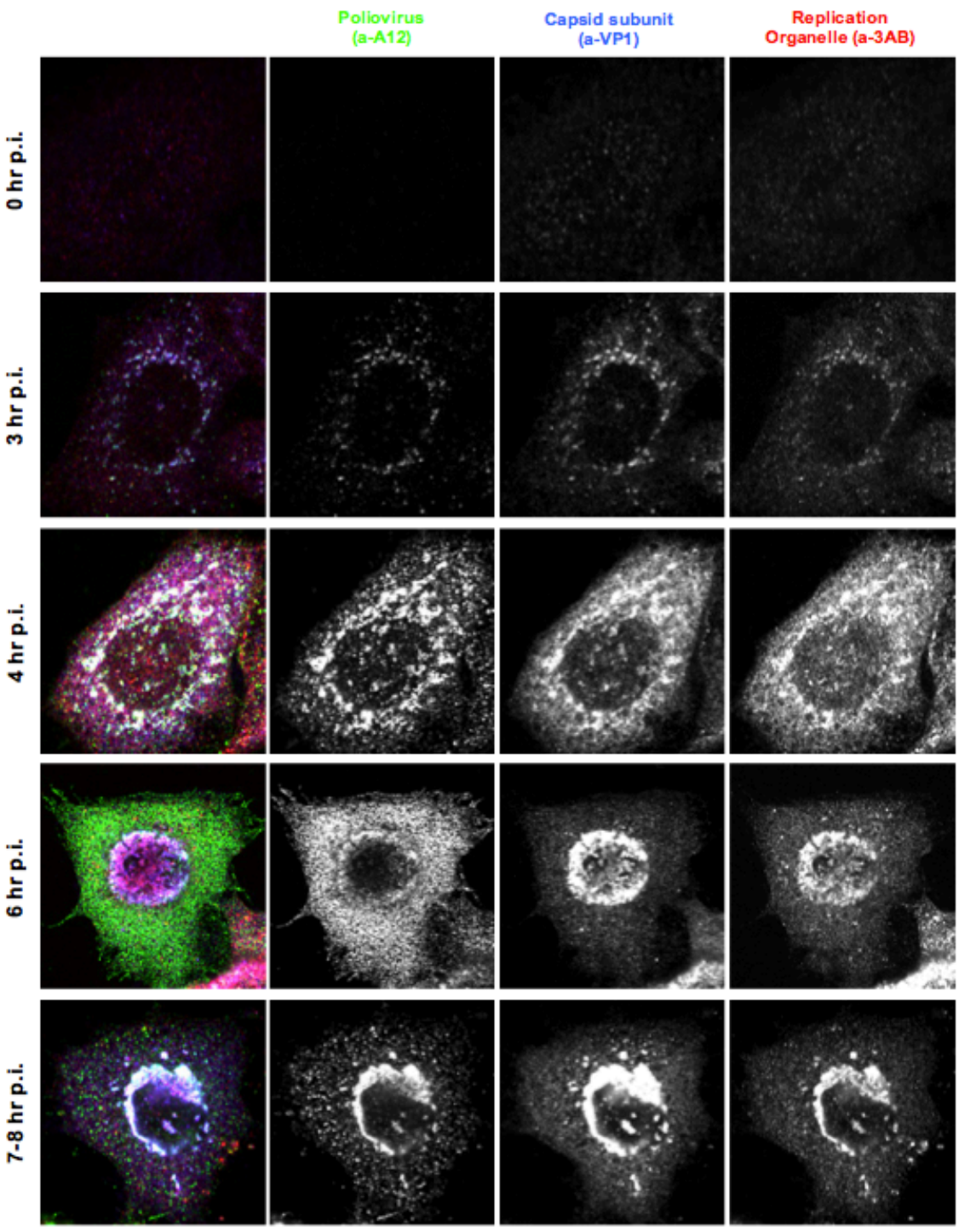

Poliovirus
(a-A12)
Capsid subunit
(a-VP1)
Replication
Organelle (a-3AB)
0 hr p.i.
3 hr p.i.
4 hr p.i.
6 hr p.i.
7-8 hr p.i.

Figure 4-2: Inhibiting viral RNA synthesis accelerates release of capsids from replication organelles.

HeLa cells were infected with poliovirus for 4.5 hours and imaged by 3D-SIM. Scale bar indicates 200nm.

A) Capsids are colocalized with 3AB at replication organelles at 4.5 hours post infection.

B) HeLa cells were infected and incubated with Guanidine HCl at 3.5 hours post infection for another one hour. GnHCl brings the transition of capsids at forward to early stages. Capsids are not colocalized with 3AB at replication organelles at 4.5 hours post infection.

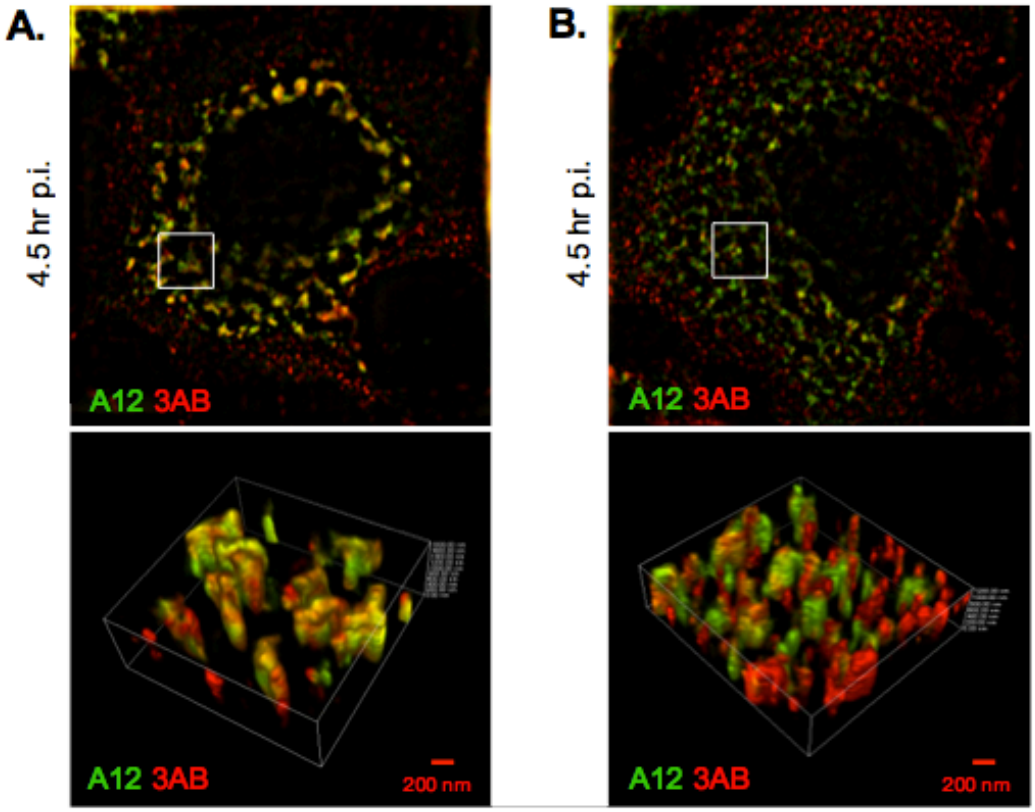

A.
4.5 hr p.i.
A12 3AB
A12 3AB
200 nm
B.
4.5 hr p.i.
A12 3AB
A12 3AB
200 nm

Figure 4-3: Plasma membrane integrity remains intact when poliovirus exits the cells.

Extracellular poliovirus was collected at each time point and virus titers were determined by plaque assay. Intactness of plasma membrane was verified with trypan blue staining. Percentage of trypan blue stained cells was counted.

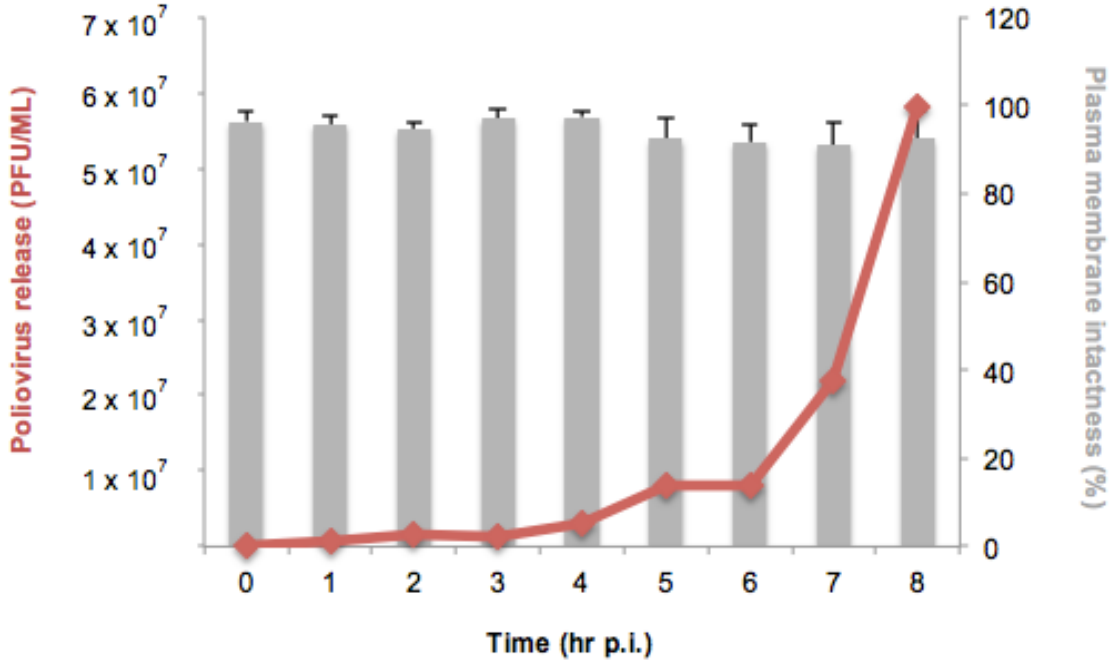

Poliovirus release (PFU/ML)
7 x 10^7
6 x 10^7
5 x 10^7
4 x 10^7
3 x 10^7
2 x 10^7
1 x 10^7
120
100
80
60
40
20
0
Plasma membrane intactness (%)
0
1
2
3
4
5
6
7
8
Time (hr p.i.)

Figure 4-4: Capsids are concentrated in induced autophagosome-like structures.

A) HeLa cells were infected with poliovirus for different time points and LC3 were detected by western blotting.

B) Quantification of LC3-II level. The protein levels were normalized with β-actin.

C) HeLa cells were infected with poliovirus for 7 hours and immunostained with anti-A12 and anti-LC3 antibodies. Capsids (A12) were localized with autophagosome-like structures (LC3).

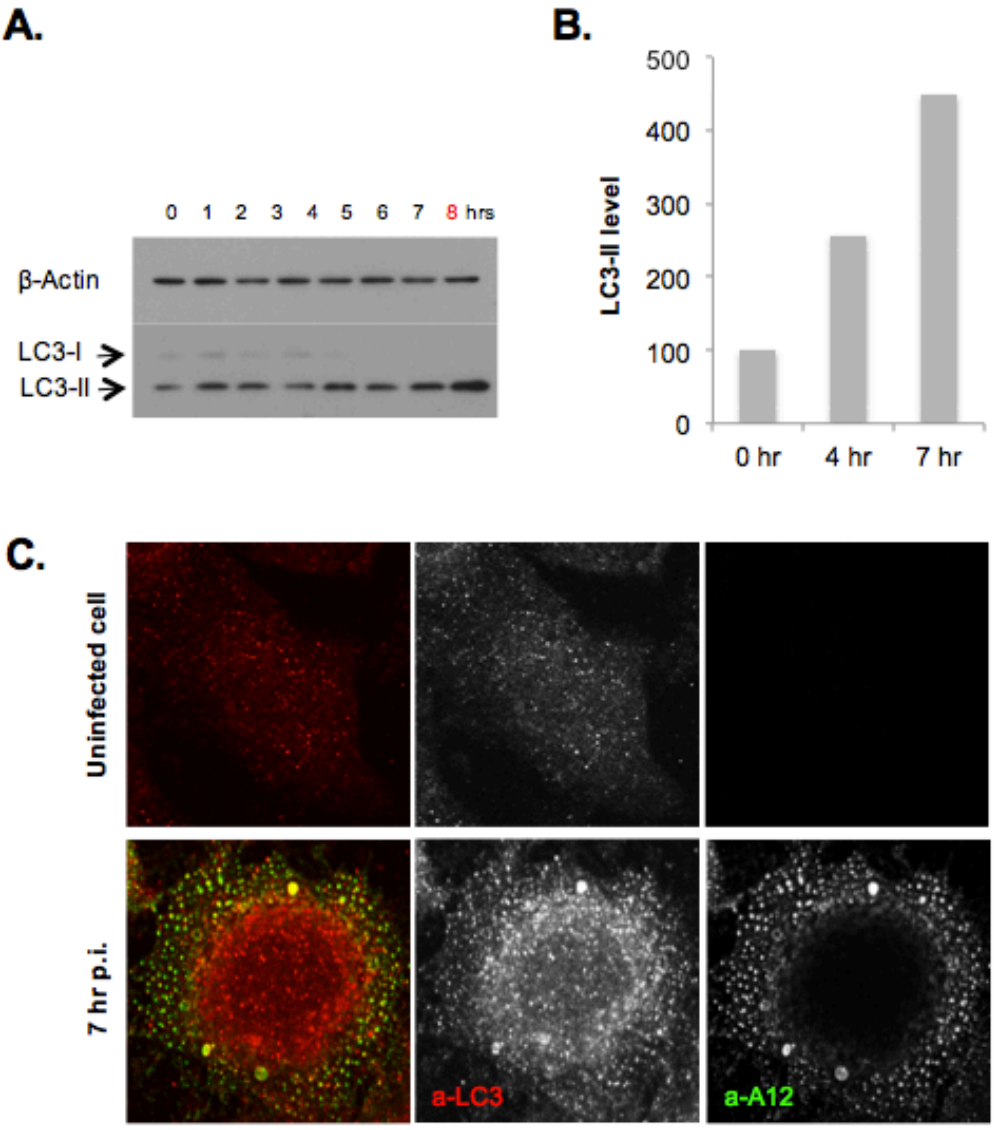

A.
0 1 2 3 4 5 6 7 8 hrs
β-Actin
LC3-I
LC3-II
B.
LC3-II level
500
400
300
200
100
0
0 hr
4 hr
7 hr
C.
Uninfected cell
7 hr p.i.
a-LC3
a-A12

Figure 4-5: Autophagosome-like structures do not fuse with lysosomes.

A) HeLa cells were infected with poliovirus for 7 hours and immunostained with anti-A12 and anti-LAMP1 antibodies. Autophagosome-like structures were not localized with lysosomes.

B) HeLa cells were infected with poliovirus for 7 hours. Leupeptin (20μM) or pepstatin (10μg/mL) together with E64d (10μg/mL) were added into the media at 1 hour post infection. LC3-II level was detected by western blotting.

C) Quantification of LC3-II level. The protein levels were normalized with β-actin.

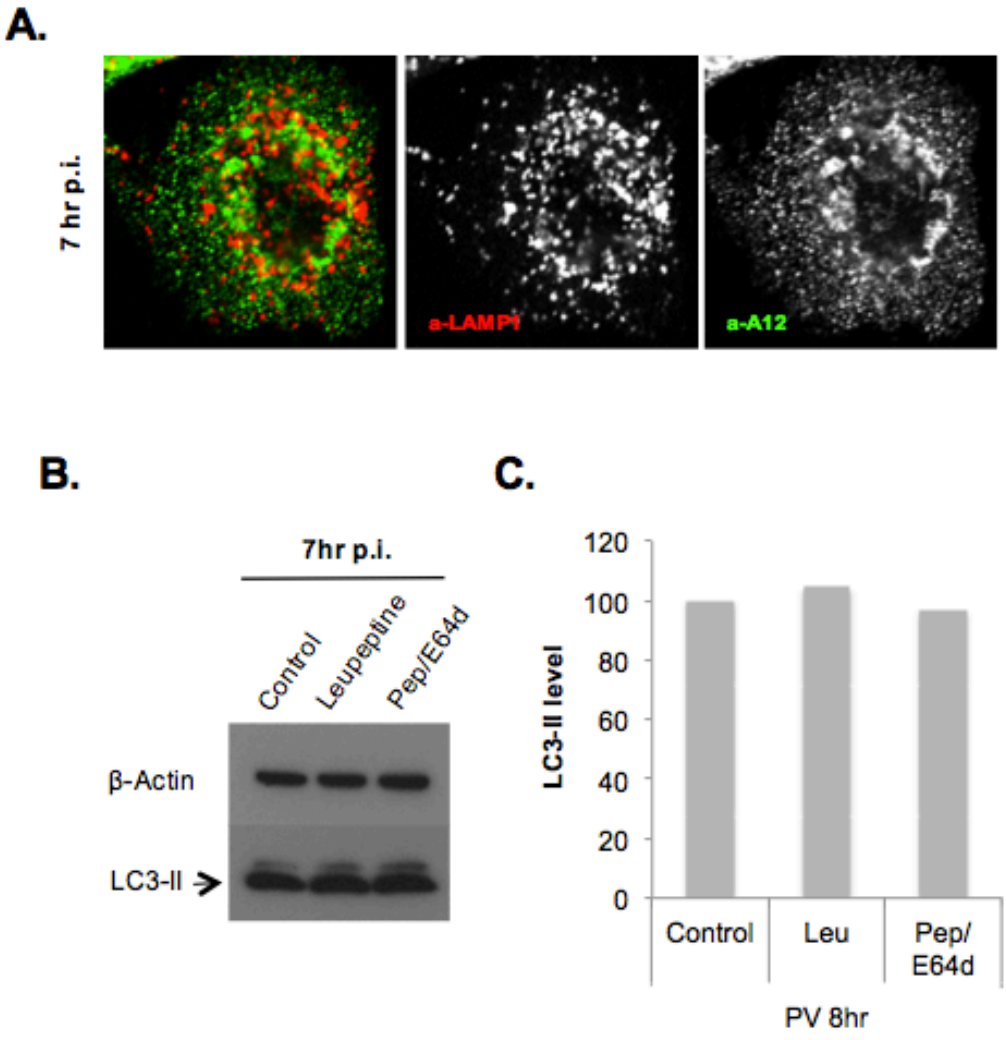

A.
7 hr p.i.
a-LAMP1
a-A12
B.
7hr p.i.
Control
Leupeptine
Pep/E64d
β-Actin
LC3-II
C.
LC3-II level
120
100
80
60
40
20
0
Control
Leu
Pep/
E64d
PV 8hr

Figure 4-6: Syntaxin 17 is not on the virions contained autophagosome-like structures.

A) HeLa cells expressing STX17-GFP were infected with poliovirus for 7 hours. Cells were fixed and immunostained with anti-GFP and A12 antibodies. STX17 was not colocalized with mature virions.

B) HeLa cells expressing STX17-GFP and LC3-RFP were infected with poliovirus for 7 hours. Cells were fixed and immunostained with anti-GFP and RFP antibodies. STX17 was not at the autophagosome structures during infection.

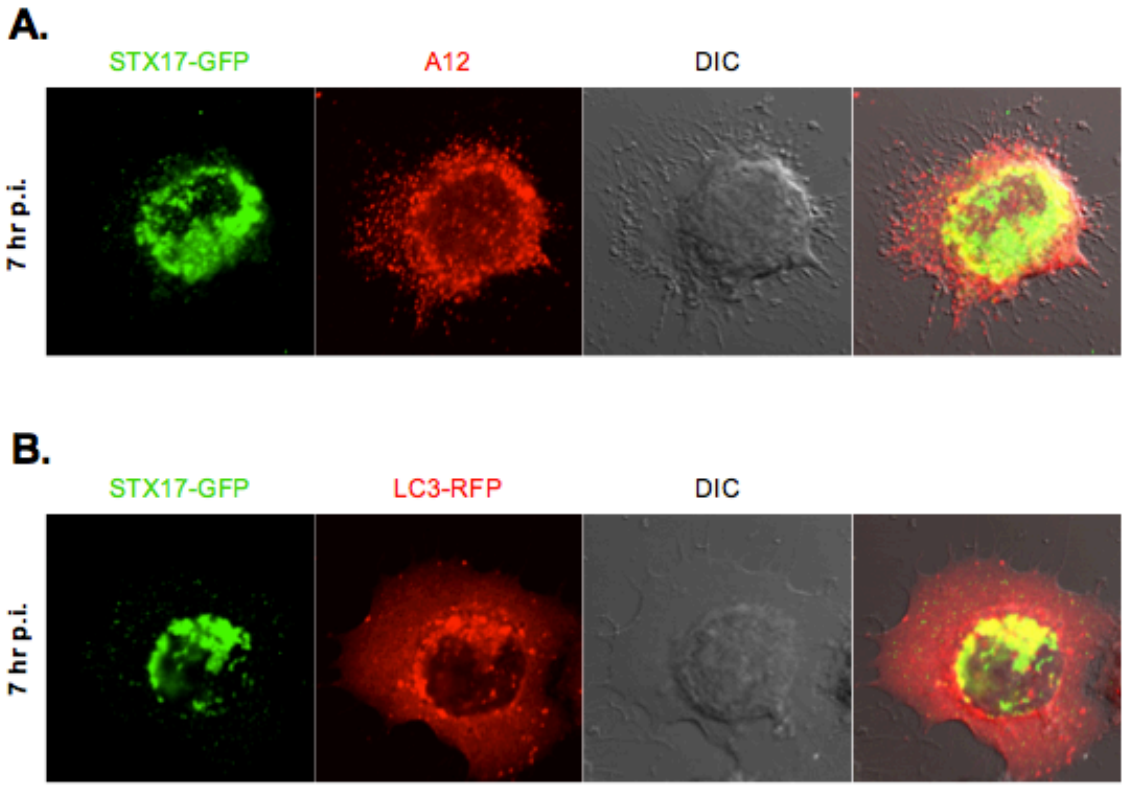

A.
STX17-GFP
A12
DIC
7 hr p.i.
B.
STX17-GFP
LC3-RFP
DIC
7 hr p.i.

Figure 4-7: Syntaxin 17 is recruited to the replication organelles during poliovirus infection.

HeLa cells expressing STX17-GFP and Arf1-RFP were infected with poliovirus. Live imaging was performed with confocal microscopy. STX17 was localized at the replication organelles (Arf-1) from 4 hours post infection.

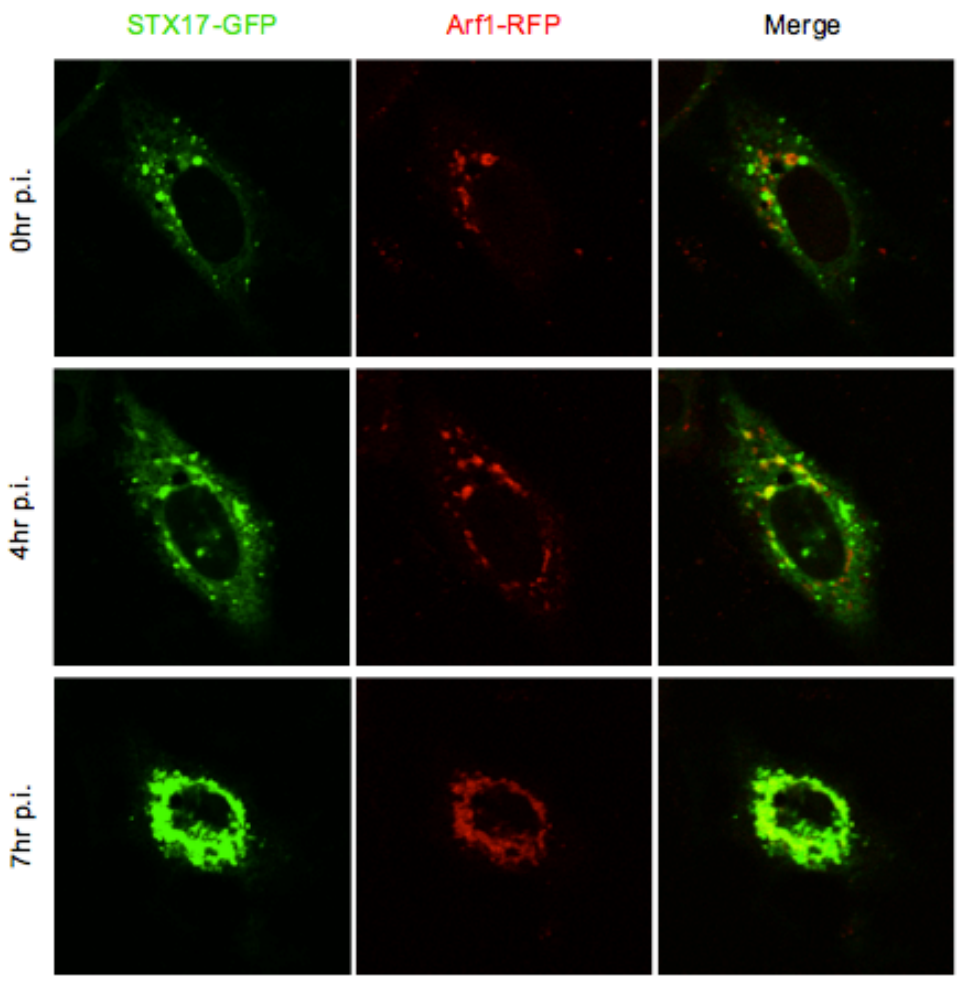

STX17-GFP
Arf1-RFP
Merge
0hr p.i.
4hr p.i.
7hr p.i.

Figure 4-8: Poliovirus release is decreased when autophagy is disrupted.

A) Impact of viral replication was checked by viral protein levels. HeLa cells were treated with Beclin1 and LC3 siRNA (100μM) for 48 hours. Beclin1 and LC3 knock down do not affect viral proteins synthesis.

B) The depletion of Beclin1 and LC3 proteins were confirmed by western blot.

C) HeLa cells were treated with Beclin1 and LC3 siRNA for 48 hours. After treatment, cells were infected with poliovirus for 7 hours. Virus titers were determined by plaque assay.

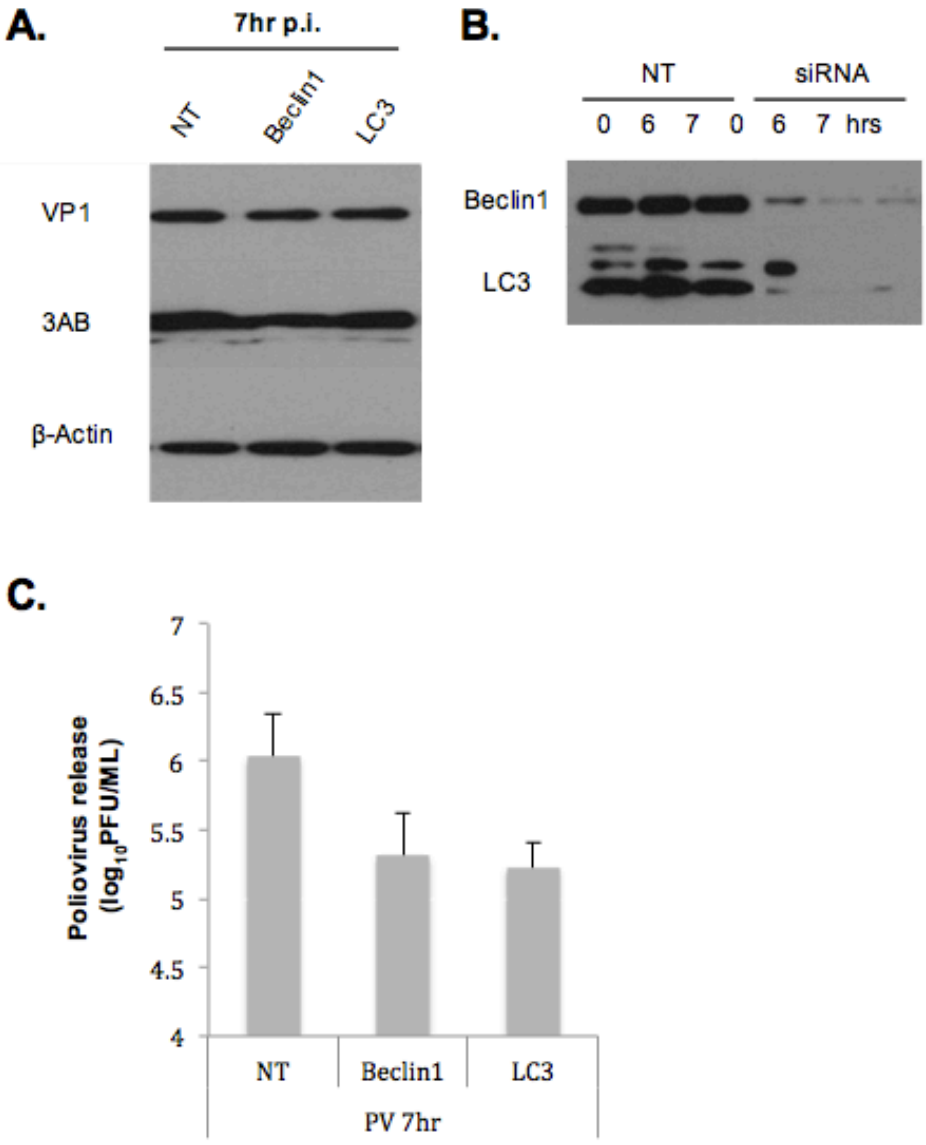
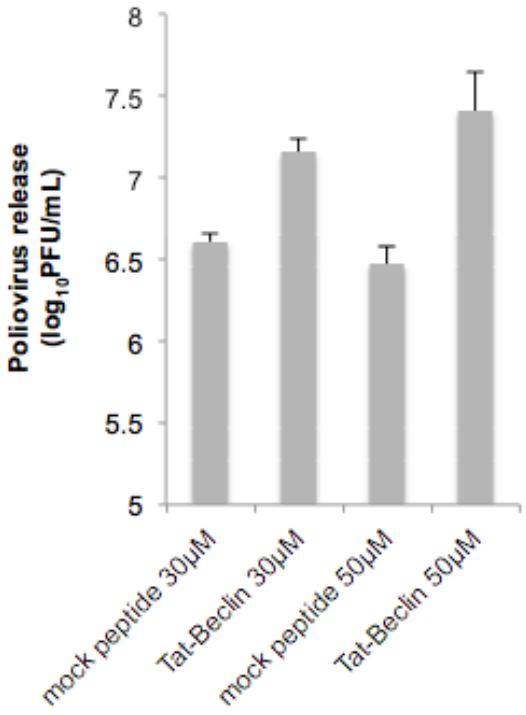

A.
7hr p.i.
NT
Beclin1
LC3
VP1
3AB
β-Actin
B.
NT
siRNA
0 6 7 0 6 7 hrs
Beclin1
LC3
C.
Poliovirus release
(log10PFU/ML)
7
6.5
6
5.5
5
4.5
4
NT
Beclin1
LC3
PV 7hr

Figure 4-9: Extracellular poliovirus yields are stimulated when cells are treated with Tat-Beclin1 peptide.

Tat-Beclin1 peptides (30μM and 50μM) were added into the media at 3.5hr post infection. Poliovirus release from 6 hours post infection was determined by plaque assay. 100 folds increasing of virus release was seen when cells were treated with 50μM Tat-Beclin1 peptide. Tat-Scramble peptides were used as the control treatment.


Poliovirus release
(log_{10}PFU/mL)
8
7.5
7
6.5
6
5.5
5
mock peptide 30μM
Tat-Beclin 30μM
mock peptide 50μM
Tat-Beclin 50μM

CHAPTER 5

The prevalence of viruses within vesicles

Enveloped viruses and non-enveloped viruses are separated by having a lipid based membrane sheath around their protein capsids. The membrane of enveloped viruses is acquired from either intracellular membrane-based organelles or the plasma membrane. For example West Nile virus virions or the human immunodeficiency virus assemble and bud out from the plasma membrane (Ng, Howe et al. 1994) whereas hepatitis C virus (HCV), buds into the endoplasmic reticulum and utilizes the secretory pathway membranes to release from the cells (Tamai, Shiina et al. 2012). In contrast, non-enveloped viruses including Poliovirus are largely released from cells without picking up any membranes. In previous chapter we described how poliovirus hijacks the autophagosomal machinery to assist in its release. However, how the autophagosome-like structures leave the cells is unclear. In this chapter, using Confocal and differential interference contrast microscopy (DIC) we will investigate the dynamics of these autophagosome-like structures during poliovirus infection and we will isolate these structures from the extracellular medium and characterize their proteomic and lipidomic composition.

RESULTS

Poliovirus containing vesicles are released from filopidia of infected cells

In chapter 4, we identified that the autophagosome like structures play a critical role in carrying poliovirus out of the cells. In order to understand how the autophagosome-like structures can leave the cells without lysing them, we utilized Confocal and DIC microscopy. The location of the autophagosome-like structures containing poliovirus was determined by co-immnostaining with anti-LC3 and anti-A12 antibodies. We observed that poliovirus infected cells developed many filopodial plasma membrane extensions (Figure 5-1B). We found that the Poliovirus containing autophagosome like structures were localized to the filopodia (Figure 5-1A). We next examined the dynamics of the autophagosome-like structures during infection using RFP-tagged LC3 expressed in HeLa cells. We observed many LC3-RFP labeled structures moving towards the tips of filopodia (Figure 5-1C), and filopodia became longer suggesting possible fusion of the autophagosome like structures with the filopdial plasma membrane. Filopodial extension contains actin (Mallavarapu and Mitchison 1999). Given this we tested whether depolymerizing actin would inhibit Poliovirus release from cells. We treated cells at 4hrs post infection with Latrunculin A and B, the potent inhibitors of actin polymerization, for 3 hours, then collected and quantified the amount of poliovirus released into the extracellular medium by a plaque assay. We found 100-fold and 70-fold decrease respectively in Poliovirus release (Figure 5-2) suggesting that actin dynamics are required for poliovirus release.

We next utilized correlative light scanning electron microscopy to observe the surface topography of the infected cells at the time window when poliovirions

are being released. HeLa cells were immunostained with anti-A12 antibodies at 7 hours post-infection to localize the poliovirions. The cells with the fluorescent labeling were identified based on their location on a gridded coverslip. The cells were then processed for scanning electron microscopy. By electron microscopy, many vesicular structures, with ~ 400nm size were observed at the tips of filopodia (arrows Figure 5-3) as well as at other regions of the plasma membrane. Given that these structures were labeled with A12 antibody (Figure 5-3) and on the extracellular side of the cell, this data indicated that the Poliovirus is released into the exterior of cells while still in membrane-bound structures.

Poliovirus receptor is necessary for the vesicle virus infection

We next tested the infectivity of Poliovirus in vesicles collected from the extracellular medium. Vesicles from infected cells were collected at 8 hours post infection as described in the methods. Given our previous findings, we tested whether the collected vesicles still contained autophagosomal membrane protein, LC3. LC3 levels in the collected vesicles was assayed by SDS-PAGE and western blotting. We found that the collected vesicles not only contained LC3 (Figure 5-4A), but LC3 was slightly enriched in the vesicles relative to the cell lysate (Figure 5-4B). We also determined whether the collected vesicles contained mature poliovirions. When the Poliovirus capsid is assembled with the genomic RNA inside, the capsid structural protein VP0 becomes cleaved into VP2 and Vp4 proteins (Harber, Bradley et al. 1991). Thus the ratio of VP2 to VP0 is reflective of the degree of maturation of the poliovirus. We found that the

collected vesicles contained a significantly higher VP2/VP0 ratio then the cell lysate suggesting that the vesicles released preferentially are enriched in mature poliovirions (Figure 5-4C and 5-4D).

Next to test the infectivity of the collected vesicles, we collected them, performed several low speed centrifugation steps to get rid of supernatant where free (non-vesicle) poliovirions may be as a result of inadvertent lysing of vesicles or lysing cells. The membranous pellets containing the vesicles were then added on to a new batch of confluent HeLa cells and replication was measured after 4 hours by collecting the cells and performing SDS-PAGE/western blotting with anti-3AB antibodies. A high level of infectivity was observed from the vesicles which implied that the poliovirus within the vesicles could enter and infect cells (Figure 5-5, the first bar).

Poliovirus has been shown to bind the CD155 cell surface protein, so called the Poliovirus Receptor (PVR) in order to infect cells (Brandenburg, Lee et al. 2007). We next tested whether the infection carried out by Poliovirus within vesicles could bypass the receptor. PVR on the HeLa cells was blocked or neutralized with the anti-PVR antibody (Kaplan, Freistadt et al. 1990) prior to the addition of the collected vesicles. We found that the infection was almost entirely blocked by the PVR antibodies indicating that the infection was still dependent on the PVR. (Figure 5-5, the third bar). (Brandenburg, Lee et al. 2007).

Poliovirions within vesicles cause a “patch infection” phenotype and have an increased efficiency of infection relative to free virus.

We next examined more carefully the phenotype of the cells after infection with the vesicles. Remarkably, we observed that cells were infected collectively in a patch pattern (Figure 5-6A). In contrast, when the vesicles were freeze-thawed, to release the poliovirus, and added to the cells, the distribution of the infection was sporadic, with single cells infected in a scattered pattern (Figure 5-7). When we diluted the vesicles, only the number of patches decreased while the patch pattern remained (Figures 5-6A and 5-6B). On the other hand when we varied the multiplicity of infection of the free virions by a 100 fold (MOI 0.1-10), we never observed a patch infection (Figure 5-7). The simplest explanation for the patch infection phenotype is a lysis of the vesicle to release a bolus of virus to collectively infect a cluster of cells. From our correlative light scanning electron microscopy results of vesicle size and the known size of a single poliovirion (~30nm in diameter), we predict that a single vesicle released could harbor hundreds to thousands of virions.

We next compared the infection efficiency of virions within vesicles to free virions. We collected the vesicles and left them either intact or freeze thawed them to release virus before adding them to HeLa cells for 4 hours. We normalized the protein level by plaque numbers to ensure that the amount of viruses from vesicle viruses and free viruses was equal. The infection efficiency was then determined by the collecting the cells, running an SDS-PAGE and western blotting with anti-3AB antibodies. We found that for a given number of poliovirions, when they were concentrated within vesicles the efficiency of

infection was ~40% greater than for free virus (Figure 5-8A).

We next investigated how cell density would impact the efficacy of infection for poliovirions when they were vesicles versus free. The vesicles and free virions (normalized for number) were added onto different densities of HeLa cells for 4 hours and infection efficiency was determined as described above. Note that the same numbers of virions were added to the cells at each density and the numbers of virions added were at a high multiplicity of infection so that even at the highest cell density all cells would be predicted to become infected. We found a direct relationship between the efficacy of infection of poliovirus within vesicles and cell density (Figure 5-8B, right panel for vesicle infection). In contrast cell density did not affect the efficacy of infection of free poliovirus (Figure 5-8B, left panel for free virus infection). One potential explanation for these findings is that the vesicles have a limited sampling capacity such that when they encounter cell free regions they cannot deliver their viral contents whereas the free virions are able to diffuse and sample large swaths of the dish. The evidence suggested that the infection efficiency of the vesicle viruses greatly depended on the density of the cells.

Figure 5-1: Autophagosome-like structures are localized at the elongated filopodia.

A) HeLa cells were infected with poliovirus for 7 hours and immunostained with anti-A12 and anti-LC3 antibodies. The images were taken by confocal and DIC imaging.

B) Live imaging was performed with DIC microscopy. The arrows indicate the elongated filopodium at 7 hours post infection.

C) HeLa cells expressing LC3-RFP were infected with poliovirus. Live imaging was performed with confocal and DIC microscopy from 5 hours post infection. Dynamics of autophagosome-like structures were seen at the tip of filopodia.

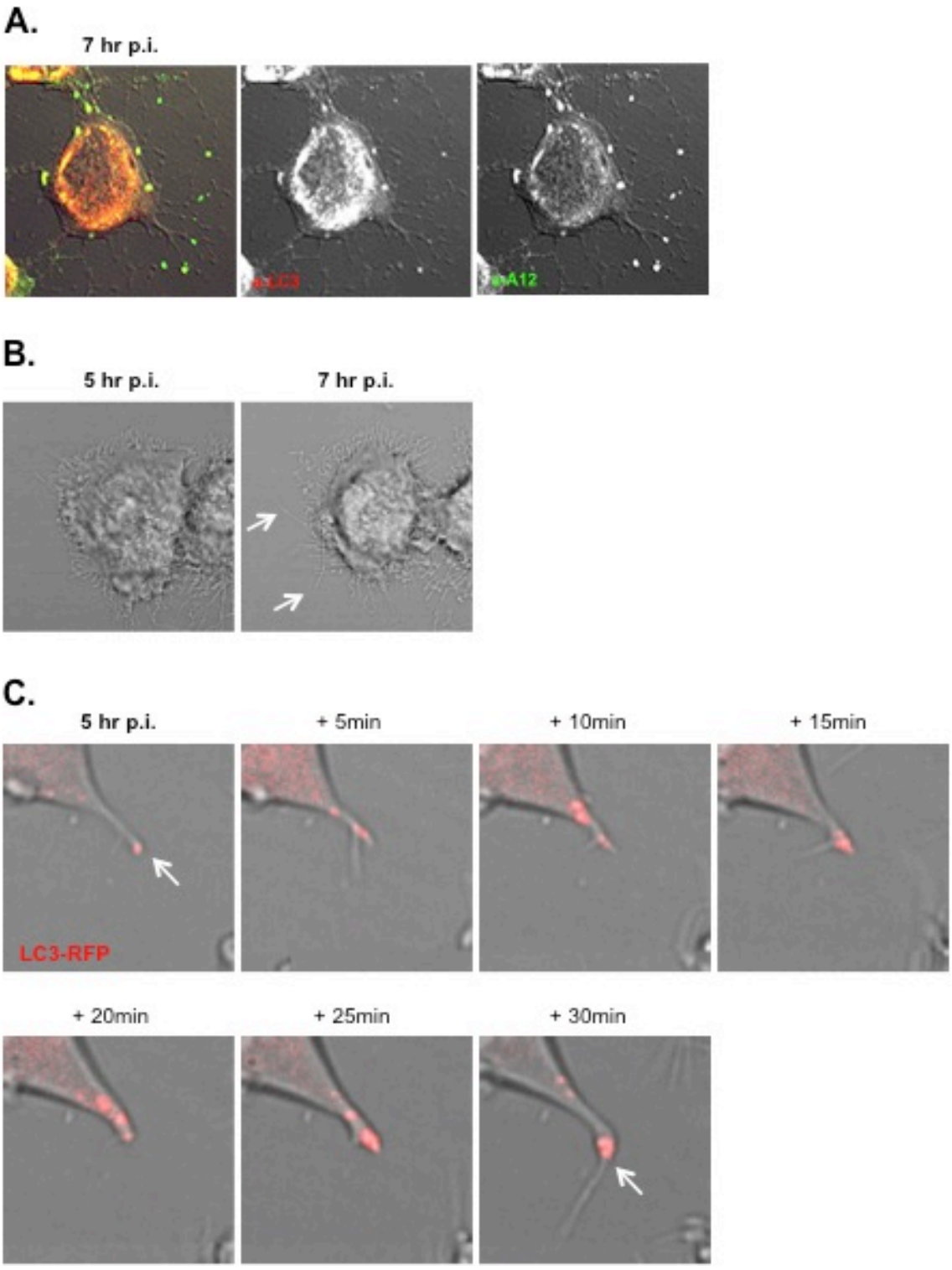

A.
7 hr p.i.
αLC3
αA12
B.
5 hr p.i.
7 hr p.i.
C.
5 hr p.i.
+ 5min
+ 10min
+ 15min
LC3-RFP
+ 20min
+ 25min
+ 30min

Figure 5-2: Vesicle release is actin-dependent.

Latrunculin A and B were added into the media at 4hr post infection. Poliovirus release from 7 hours post infection was determined by plaque assay. 100-fold and 70-fold decrease of virus release were seen when the actin was depolymerized.

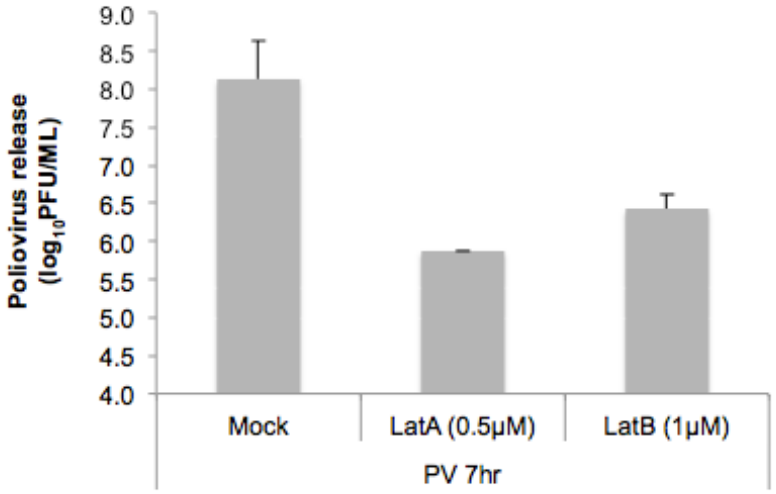

Poliovirus release
(log10PFU/ML)
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
Mock
LatA (0.5μM)
LatB (1μM)
PV 7hr

Figure 5-3: Correlative Light Scanning Electron Microscopy imaging demonstrates released vesicle viruses at the tip of filipodia.

HeLa cells were infected with poliovirus for 7 hours and immunostained with anti-A12 antibody. The images were performed with correlative light scanning electron microscopy. The arrows indicated the released vesicles.

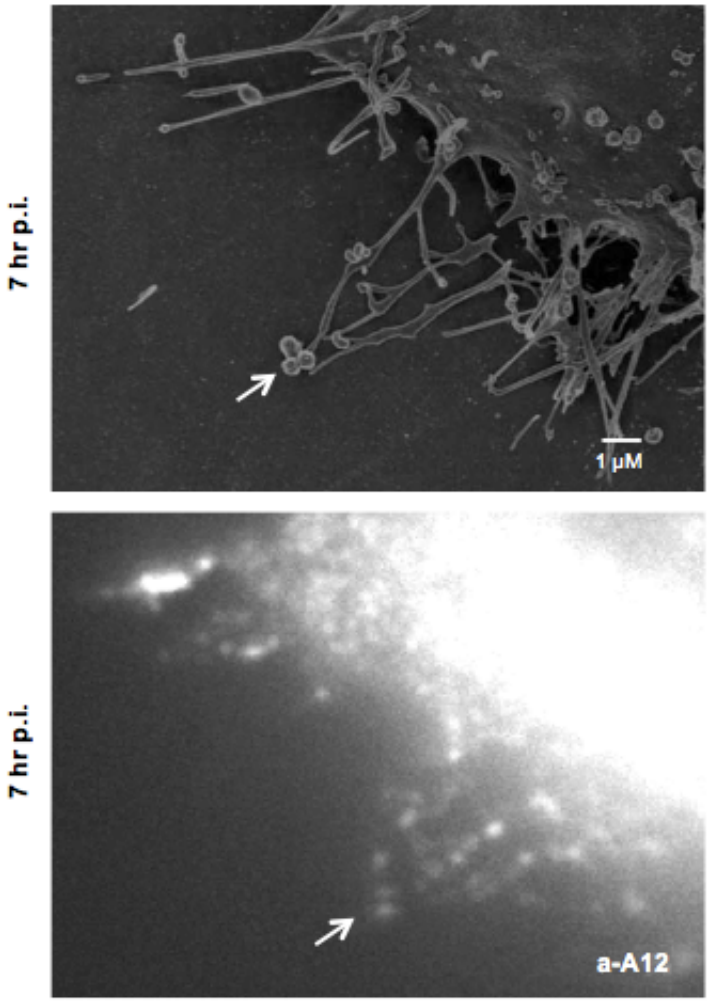

7 hr p.i.
1 µM
7 hr p.i.
a-A12

Figure 5-4: Vesicles are enriched with autophagosome membrane proteins and mature virions.

A) Cell lysate and vesicles were collected from the cells and supernatant of 8 hours post infection respectively. The proteins were blotted with LC3 antibody.

B) Quantification of LC3-II levels.

C) Intracellular virion proteins and vesicle virion proteins were from the cells and vesicles respectively. The proteins were blotted with VP2 antibody to determine the mature virion levels.

D) Quantification of VP2/VP0 levels.

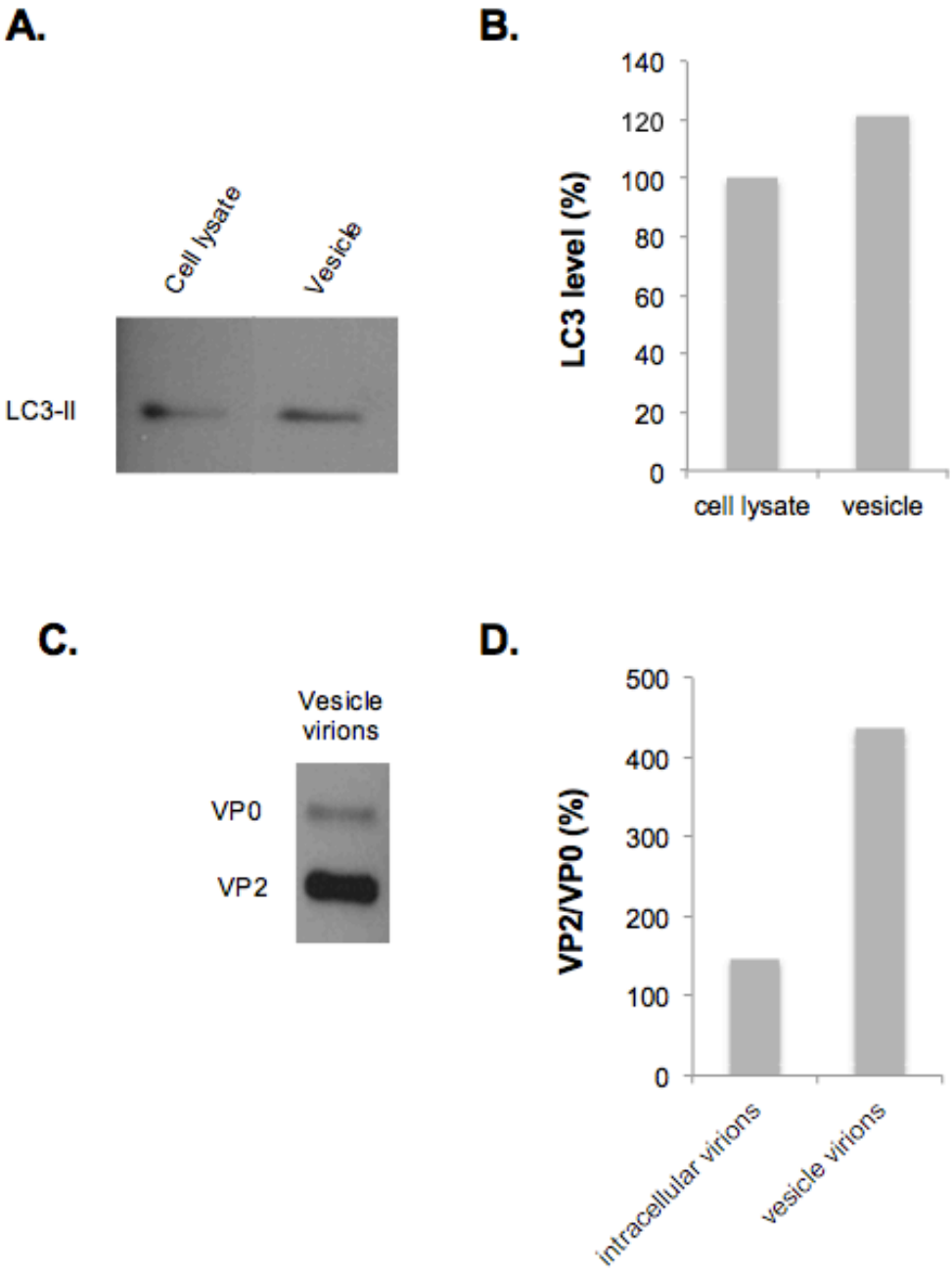

A.
Cell lysate
Vesicle
LC3-II
B.
LC3 level (%)
140
120
100
80
60
40
20
0
cell lysate
vesicle
C.
Vesicle virions
VP0
VP2
D.
VP2/VP0 (%)
500
400
300
200
100
0
intracellular virions
vesicle virions

Figure 5-5: Poliovirus receptor is necessary for vesicle virus infection.

Confluent HeLa cells were infected with collected vesicles from poliovirus infection. A12 or poliovirus receptor (PVR) antibodies were added together with vesicles when infection. Infectivity was measured by western blotting of 3AB protein levels.

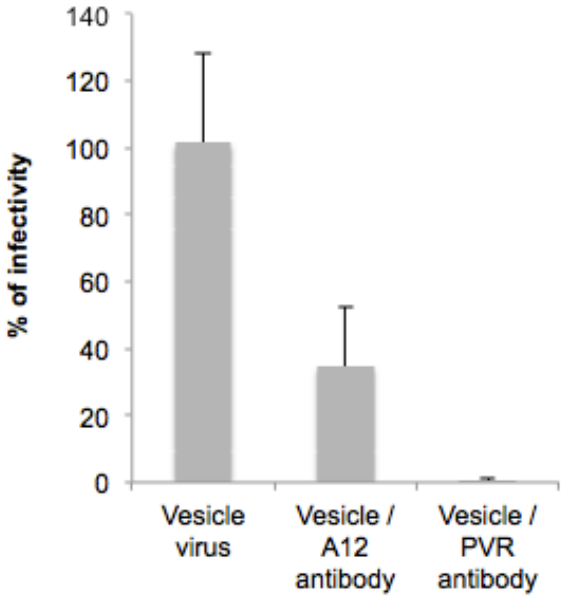
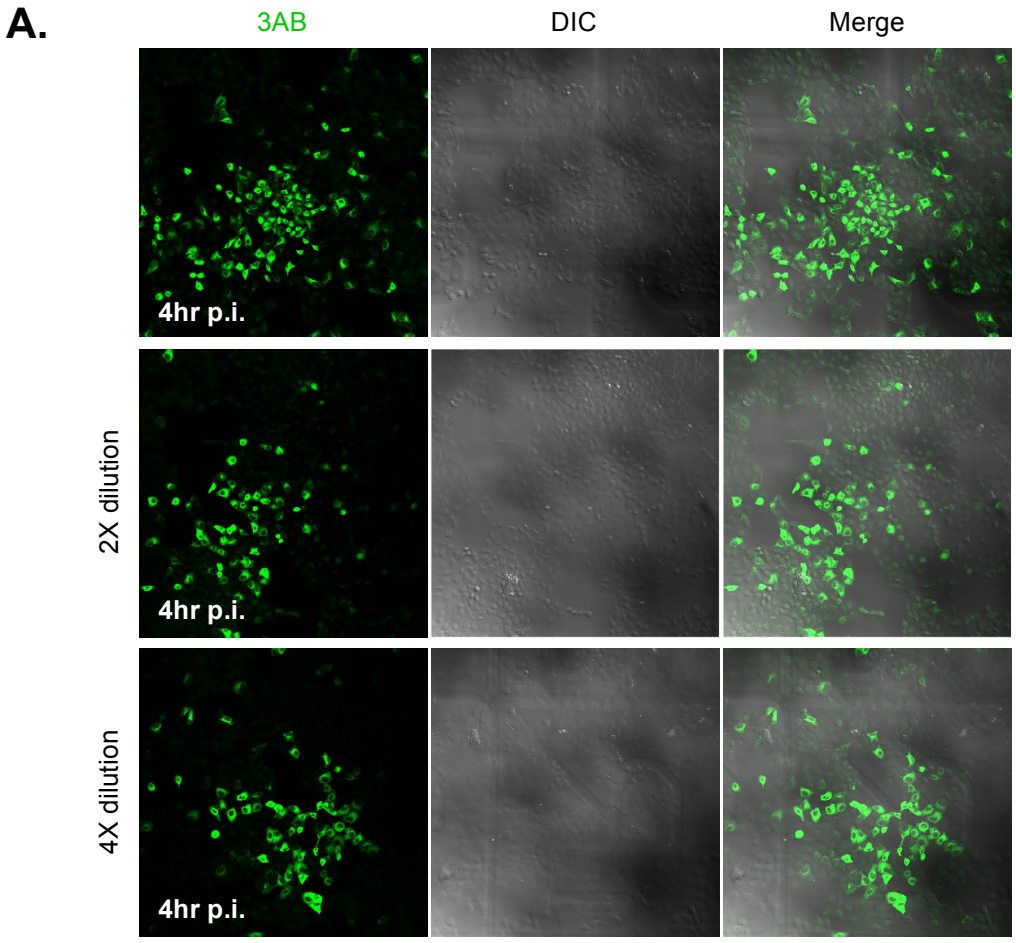

140
120
100
80
60
40
20
0
% of infectivity
Vesicle virus
Vesicle / A12 antibody
Vesicle / PVR antibody

Figure 5-6: Viruses in vesicles infect patch of cells.

A) Confluent Hela cells were infected with poliovirus of different dilution for four hours and immunostained with anti-3AB antibody. 3AB staining indicated infected cells. The images were performed with confocal and DIC microscopy.

B) Patch numbers were counted from origin and 4X dilution samples. Patch infection was still seen at low amount of viruses.


A.
3AB
DIC
Merge
4hr p.i.
2X dilution
4hr p.i.
4X dilution
4hr p.i.

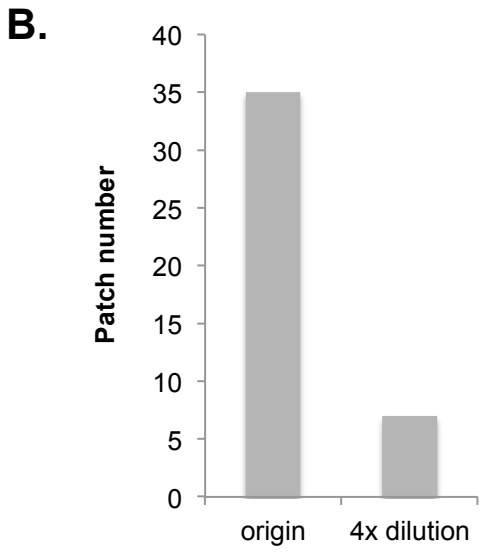

B.
Patch number
40
35
30
25
20
15
10
5
0
origin
4x dilution

Figure 5-7: Free viruses do not cause patch infection.

Confluent Hela cells were infected with poliovirus of different MOI for four hours and immunostained with anti-3AB antibody. 3AB staining indicated infected cells. The images were performed with confocal and DIC microscopy.

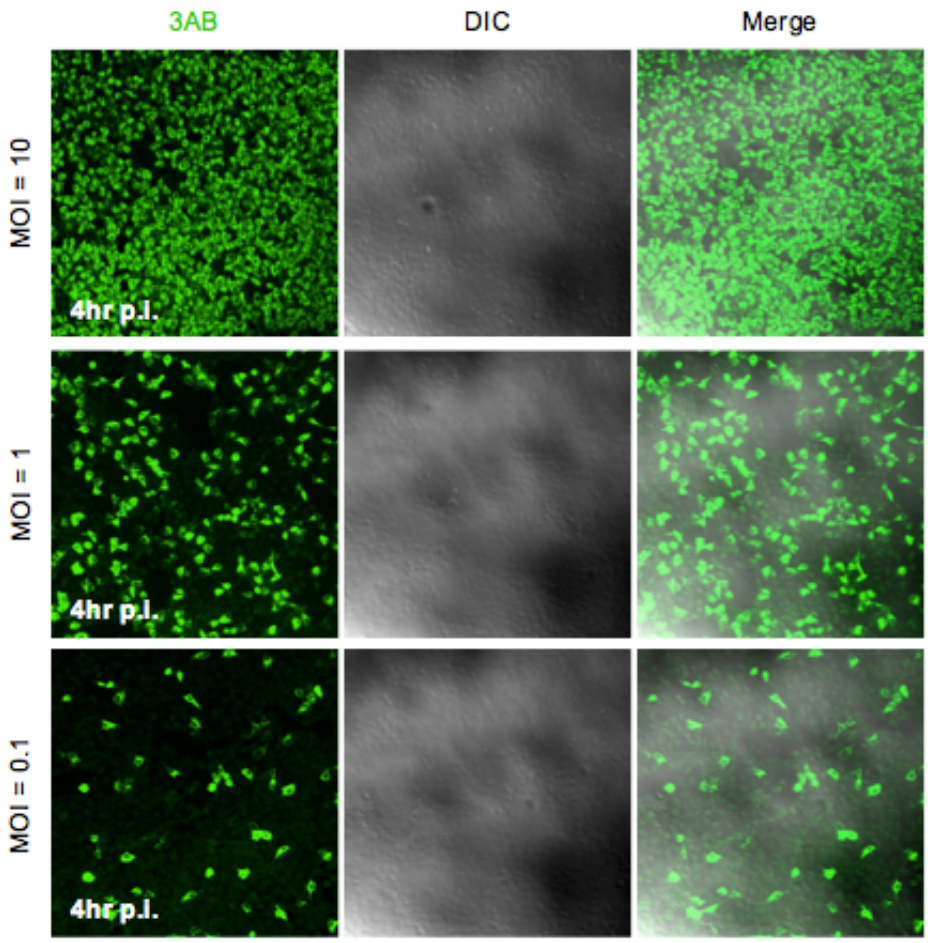
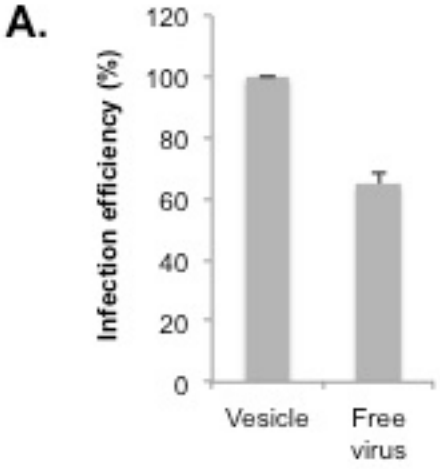
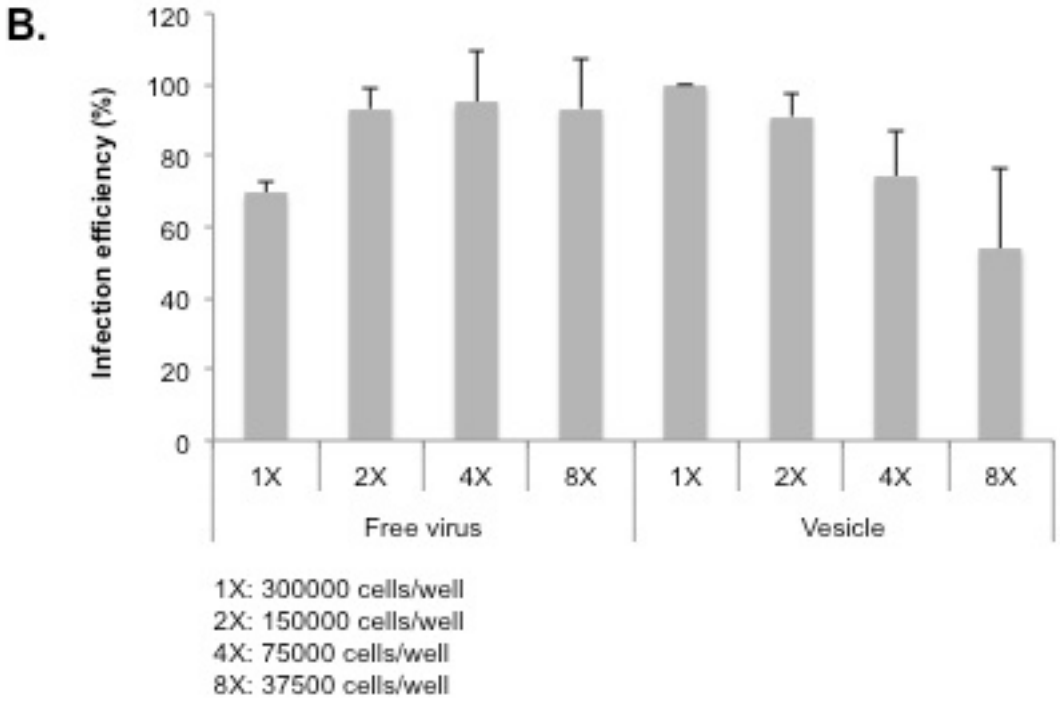

3AB
DIC
Merge
MOI = 10
MOI = 1
MOI = 0.1
4hr p.i.
4hr p.i.
4hr p.i.

Figure 5-8: Clustered viruses within vesicles increase infection efficiency by ~40% and the infection efficiency is in a cell density manner.

A) Confluent HeLa cells were infected for four hours with collected vesicles or free virus from poliovirus infection. Infectivity was measured by western blotting of 3AB protein levels.

B) Different amounts of HeLa cells were infected either with collected vesicles or free virus for four hours. Two-fold dilution of cells was used for infection. Infectivity was measured by western blotting of 3AB protein levels.





1X: 300000 cells/well
2X: 150000 cells/well
4X: 75000 cells/well
8X: 37500 cells/well

CHAPTER 6

The role of phosphatidylserine enriched membrane vesicles during viral infection

Both prokaryotic and eukaryotic cells are known to secrete membrane-bound vesicles, into their extracellular environments. These vesicles can come in various sizes depending on the cell type from which they are secreted and can be of unique composition. They are often formed from the plasma membrane but also can originate from intracellular compartments such as multivesicular bodies. For instance, exosomes which are ~50-100nm in size are from endosomal origins, with membranes enriched in cholesterol, sphingomyelin, and phosphatidylserine (PS) (Thery, Amigorena et al. 2006). Microvesicles on the other hand have a broader size range (100 to 1000 nm), bud from the plasma membrane and their major lipid composition is phosphatidylserine (Heijnen, Schiel et al. 1999). Both microvesicles and exosomes have been shown to be important for intercellular communication by transferring membranes, lipids, cytosolic proteins and RNA between cells. However the mechanisms of how these vesicles can travel long distances and deliver their protein/lipid cargo to distant cells remains unknown.

Many types of viral infection have been reported to induce membrane vesicle production (Meckes and Raab-Traub 2011). Exosomes are released from HIV infected cells and contain HIV co-receptors such as CCR5, for enhancing HIV entry the cells (Mack, Kleinschmidt et al. 2000). Moreover, herpes simplex

virus also induces the cells to secret noninfectious microvesicles that contain parts of viral proteins (Loret, Guay et al. 2008).

In general, phosphatidylserine lipids are always found on the inner leaflet of the plasma membrane and the cytosolic surface of endocytic vesicles (Leventis and Grinstein 2010). When cells undergo apoptosis, phosphatidylserine is flipped outward to the extracellular leaflet on the cell surface where it becomes an important signal for cell distress, attracting and then triggering macrophages to engulf the dying cells (Verhoven, Schlegel et al. 1995). Given the frequent presence of phosphatidylserine in vesicles secreted from cells and our findings that membrane vesicles were released from poliovirus-infected cells, here in this chapter, we investigated whether PS lipids were present on the Poliovirus containing vesicles and whether the PS had a functional significance for poliovirus infections.

RESULTS

A wide spread increase in the release of phosphatidylserine positive membrane vesicles from cells infected with non-enveloped viruses

The human vascular anticoagulant, annexin V, is a phospholipid-binding protein that has a high affinity for phosphatidylserine (Andree, Reutelingsperger et al. 1990). We used annexin V labeled with a fluorophore as a live-cell fluorescent reporter to monitor the dynamics of phosphatidylserine lipids during poliovirus infection. Annexin V was added onto the cells at 4 hours post infection and the dynamics of phosphatidylserine was imaged by Confocal/DIC

microscopy. A significant increase in annexin V labeled vesicles being released from cells was seen at the later stages of infection (~8 hours post infection) (Figure 6-1A). Notably, the plasma membrane of cells was not labeled with Annexin V indicating the lack of apoptotic flipping of PS, however we observed localized “hot spots” of annexin V labeling at the filopodia of infected cells. The annexin V labeling at the filopidia were of brief duration indicating the localized exposure of PS potentially due to the release of the autophagosome like vesicles containing the poliovirions (Figure 6-1B).

We next investigated whether these phosphatidylserine-enriched vesicles were exclusively released from poliovirus-infected cells, or whether it was a more general phenomena associated with non-enveloped virus production. We tested Coxsackie virus B3 (CVB3) and rhinovirus, both of which are members of the single positive strand RNA virus family as well as Rotavirus, a member of the double stranded RNA virus family. HeLa, HeLa-Ohio and MA104 cells were infected with CVB3, rhinovirus and rotavirus respectively. At different intervals post infection, membrane vesicles were collected from the supernatant of the infected cells. Vesicles were then incubated with the fluorescent annexin V, collected, washed and fluorescence intensity measured with a spectrofluorometer. Surprisingly, relative to uninfected cells, we found significant increases in the amount of phosphotidylserine enriched vesicles released from poliovirus (9-fold increase), CVB3 (3-fold increase), rhinovirus (3-fold increase) and rotavirus (64-fold increase) infected cells (Figure 6-2). Thus non-enveloped virus infections seemed all stimulate PS-enriched membrane vesicle release

from cells.

PS dynamics in cells

We next investigated the origin of PS on the autophagosomal like structures containing poliovirions. We expressed in cells ectopically LactC2-GFP, a protein that binds PS present on the cytosolic leaflet of intracellular membranes (Kay, Koivusalo et al. 2012) as well as Arf1-RFP, the latter as an indirect indicator of the replication site organelles (Hsu, Ilnytska et al. 2010). Uninfected cells expressing LactC2-GFP exhibited a PS distribution similar to what has been reported with other methods, including plasma membrane staining and endosomal compartments. We then infected the cells with PV and took time lapse confocal imaging series. During the course of infection we saw an increase in intracellular compartments labeled with PS and notably an increase in PS at membranes surrounding the replication sites (Figure 6-3A). We then fixed the cells and co-stained with antibodies against A12 and GFP, the latter to localize the PS. We found that the A12 labeled mature poliovirions were nestled among PS rich regions of the replication sites between 4-6 hrs post and later at 7hrs post infection were in the cytoplasm surrounded by PS rich membranes (Figure 6-3B and 6-3C). This indicated that the replication organelle membranes which are endoplasmic reticulum derived become enriched in PS over the course of infection with the PS exposed also to the cytosolic leaflet of the ER membrane. Furthermore the virions once dispersed from the replication organelles continue to carry with them membranes containing PS.

The origin of the increase in PS at the replication organelle membranes is currently unclear. It can be due to at least two sources: an increase in ER based PS synthesis; flipping of luminal ER PS to the cytosolic leaflet of the ER; and/or recruitment of PS from intracellular endosomes. Indeed we had previously shown that recycling endosomes are fusing with the replication organelle membranes to bring cholesterol to the sites (Ilnytska, Santiana et al. 2013).

Given the presence of PS on the autophagosome like structures surrounding the poliovirions we next asked whether autophagosomes in general contain PS. To test this we treated Hela cells, expressing LactC2GFP and LC3-RFP, with Rapamycin for 16 hours. After treatment we found a significant increase in LC3 positive structures, indicating a stimulation of autophagy. Remarkably these structures were also co-labeled with LactC2-GFP, indicating the presence of PS (Figure 6-3D). Thus it appears that autophagosomes are enriched in PS lipids both in non-infected and viral infected cells.

Phosphatidylserine enriched vesicles contain poliovirus and phosphatidylserine is required for vesicle infection

In order to characterize the phosphatidylserine-enriched vesicles released from the poliovirus-infected cells, we investigated whether the vesicles contained the poliovirus. To determine this we incubated the vesicles with annexin V coated magnetic microbeads and sorted the vesicles away from the unbound beads by a magnetic field separator. We then collected the annexin V bound phosphatidylserine-enriched vesicles and determined the content of the vesicles

by western blotting. Capsid protein, VP2, was detected from the phosphatidylserine-enriched vesicles (Figure 6-4A). It suggested these phosphatidylserine-enriched vesicles did consist of the polioviruses.

Next, we tested whether the phophatidylserine on the vesicles was required for infection of cells. We coated the surface of the vesicles with annexin V in order to “neutralize” the PS domains. We then added these annexin V coated vesicles on to a fresh layer of confluent HeLa cells. Replication was measured after 4 hours by collecting the cells and performing SDS-PAGE/western blotting with anti-3AB antibodies. No infection was observed from the vesicles which implied that neutralization of the phosphatidylserine on the vesicles interfered with the infection. Importantly, we could rescue infection when we froze and thawed the vesicles to release the poliovirions (Figure 6-4B). Collectively this data indicate that phosphatidylserine on the vesicles plays an important role in facilitating viral infection.

Figure 6-1: Release of phosphatidylserine positive membrane vesicles from poliovirus infected cells.

HeLa cells were infected with poliovirus and annexin V was added onto the cells at 4 hours post infection. The dynamics of phosphatidylserine was imaged by Confocal/DIC microscopy.

A) The arrow indicated the released vesicle labeled by annexin V.

B) The arrow indicated the filopodia labeled by annexin V.

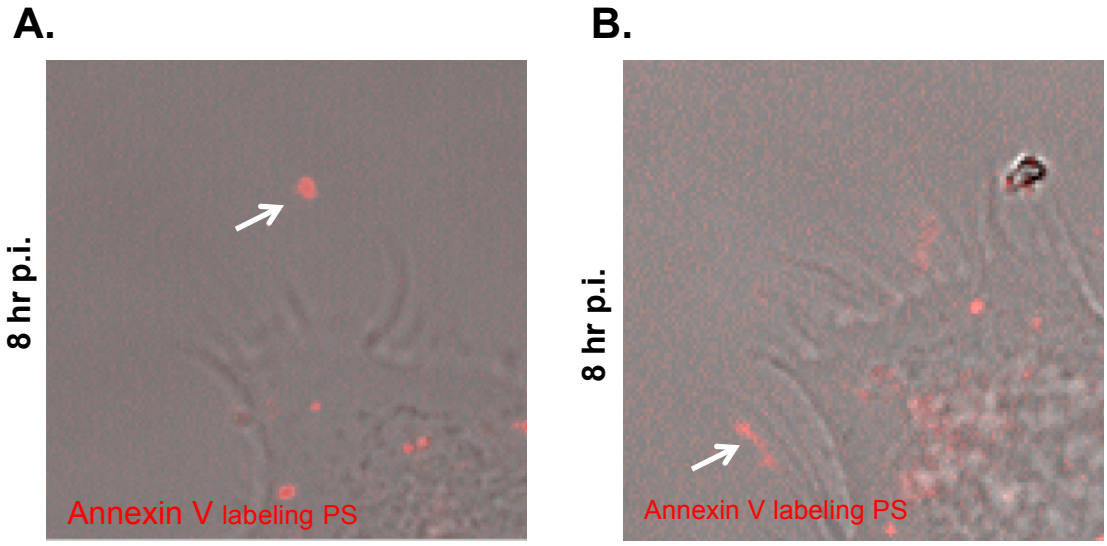

A.
8 hr p.i.
Annexin V labeling PS
B.
8 hr p.i.
Annexin V labeling PS

Figure 6-2: Annexin V labeled vesicles are increased when PV, CVB3, Rhino and Rota viruses infection

Cells were infected until late stages of infection. Vesicles were collected and stained with Annexin V proteins conjugated with Alexa 568 fluorophore. Intensity of fluorescence from non-infected cells and infected cells were measure by spectrofluorometer. The increasing fold was determined by the ratio of the intensity from infected cells versus non-infected cells.

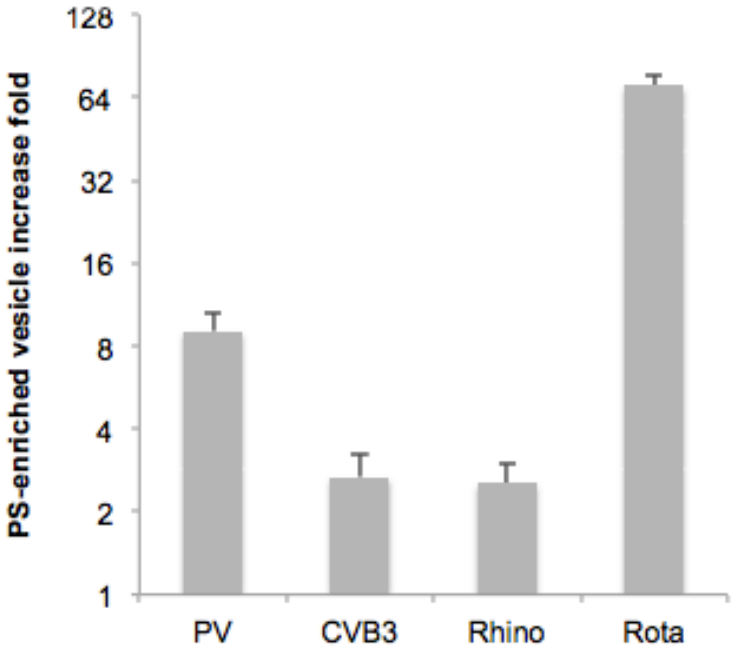

PS-enriched vesicle increase fold
128
64
32
16
8
4
2
1
PV
CVB3
Rhino
Rota

Figure 6-3: Phosphatidylserine lipids are enriched in replication organelles and autophagosme-like structures during polioviral infection.

A) HeLa cells expressing LactC2-GFP and Arf1-RFP were infected with poliovirus for 7 hours. All fluorescence images were confocal images of optical slice thickness~ 1 μm.

B) HeLa cells expressing LactC2-GFP were infected with poliovirus for 5 hours. Cells were fixed and immunostained with anti-GFP and A12 antibodies.

C) HeLa cells expressing LactC2-GFP were infected with poliovirus for 7 hours. Cells were fixed and immunostained with anti-GFP and A12 antibodies.

D) HeLa cells expressing LactC2-GFP and LC3-RFP were treated with rapamycin for 16 hours.

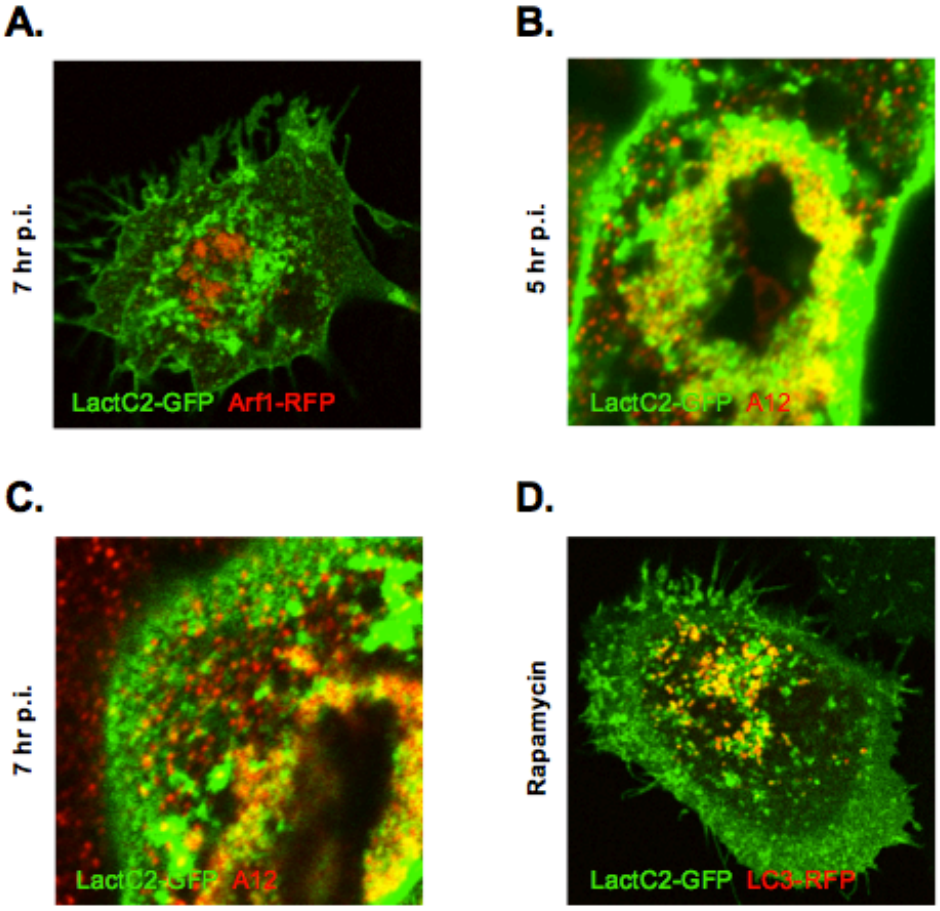
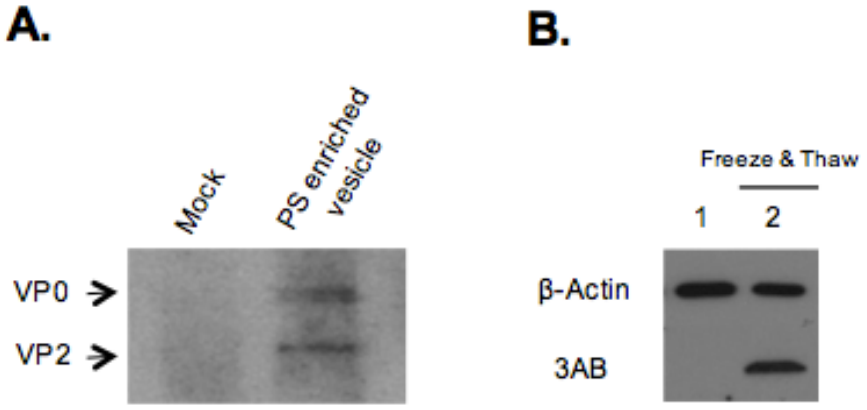

A.
7 hr p.i.
LactC2-GFP Arf1-RFP
B.
5 hr p.i.
LactC2-GFP A12
C.
7 hr p.i.
LactC2-GFP A12
D.
Rapamycin
LactC2-GFP LC3-RFP

Figure 6-4: Phosphatidylserine enriched vesicles contain poliovirus and phosphatidylserine is required for vesicle infection.

A) Vesicles were sorted by annexin V microbeads with the magnetic field separator. Phosphatidylserine enriched vesicles then were collected and the content of the vesicles was determined by western blot with anti-VP2 antibodies. **B)** Confluent HeLa cells were infected for four hours with annexin V coated vesicles (1) or membrane disrupted annexin V coated vesicles (freeze and thaw three times). Infection was determined by western with anti-3AB antibodies.


A.
Mock
PS enriched vesicle
VP0
VP2
B.
Freeze & Thaw
1
2
β-Actin
3AB

Chapter 7

Discussion

Summary and Model

The work presented in this thesis is primarily focused on how a non-enveloped virus, namely poliovirus, is able to hijack the autophagy pathway to traffic itself non-lytically out of the host cell. I demonstrated that the plasma membrane remains intact when poliovirus exits the cells. A dramatic change of plasma membrane integrity would be observed if viruses release from the cells via a lytic pathway. Based on our findings in Chapters 3-6 I propose the following model for the non-lytic release of poliovirus via the autophagy pathway (Figure 7-1). In my model poliovirions are formed at the PI4P/cholesterol enriched replication organelles and remain at those sites until viral RNA synthesis is slowed or stopped (~ 5-6 hrs post infection). By 7 hours post infection poliovirions are dispersed into the cytoplasm and are closely associated with membranes containing LC3. Overall there is an increase in membranes containing LC3-II suggesting an acute stimulation of autophagy. These membranes I found to be also enriched in phosphatidylserine lipids. These autophagosome-like structures containing mature poliovirions did not fuse with lysosomes likely due to the absence of a key component, syntaxin 17 (STX17), which is required for fusion. Instead I found that STX17 was sequestered at replication organelles. Then we showed that these autophagosome-like structures containing the virions egressed from the cell into the extracellular environment, primarily via the

filopidial extensions. The vesicles containing the mature poliovirions could be collected and subsequently used to infect fresh new host cells. Remarkably the infectivity of the virus when inside a vesicle was higher then when free likely due to the vesicles containing many virions which collectively infected multiple cells, forming so called “patch infections”. Finally I showed that the exported viral vesicles were highly enriched in phosphatidylserine lipids and that the infection on subsequent host cells was dependent on not only the poliovirus receptor but also the phosphotidylserine lipids on the vesicles.

Formation of autophagosome-like structures

Cytosolic surfaces of single-membraned structures are the sites where viral RNAs replicate at 3-4 hrs post infection. An increase of LC3-II level has been shown from 5 hrs post infection. Meanwhile, the autophagosome-like structures with mature poliovirions are formed in the cytoplasm. The origin of autophagosome membrane is still controversial. Recently, it was proposed that the isolation membranes are from the mitochondria-associated endoplasmic reticulum (ER) membrane (MAM) (Hamasaki, Furuta et al. 2013). However, in the case of poliovirus infection, the autophagosome-like structures may be derived either from the ER membranes or the single-membraned replication organelles. 3AB, a single protein encoded by poliovirus, has been shown to induce double-membraned liposomes sufficiently through the invagination of single membraned liposomes (Wang, Ptacek et al. 2013). Therefore, the viral protein 3AB alone could coordinate other autophagy-related proteins and drive the curvature of

preautophagosome isolation membranes. In addition, how the mature poliovirions could be engulfed into the autophagosome-like structure when it is forming is still unclear. Instead of being captured in the cytoplasm randomly, the polioviruses more likely require selective sequestration of the specific cargo into autophagosomal membrane. Autophagy receptors and ubiquitylated cargos are the key components in selective autophagy (Kirkin, McEwan et al. 2009). However, up to now, there is no evidence showing that the capsids of poliovirus are able to be modified by ubiquitin. It remains to be determined what mechanisms bring the virions into autophagosome-like structures.

Lack of fusion of autophagosome like structures with lysosomes

Finding STX17, a member of the SNARE family, to be sequestered at the replication organelles was notable given that normally it is on autophagosomes mediating fusion with lysosomes. Replication organelles are highly enriched in cholesterol due to viral hijacking of the endosomal pathway to deplete cholesterol from the plasma membrane and track it to the replication sites (Ilnytska et al., 2013). SNARE proteins have been shown to cluster within membranes via a cholesterol-dependent mechanism (Lang, Bruns et al. 2001). Thus the viral enrichment of cholesterol at the replication sites to facilitate RNA synthesis may also aid the virus by hijacking STX17 away from the autophagosome like structures containing poliovirions.

Filopodial extensions during viral infection

Accompanying poliovirus infection over time, I discovered that Hela cells not only increased their number of filopodial projections but these filopodia become longer and more dynamic. I frequently observed autophagosome-like structures containing poliovirions moving to the tips of the filopodia which correlated with the extension of the filopodial projections. Filopodia are full of actin and I found that actin is also important for the egress of the viral vesicles since acute treatment with the potent actin depolymerizing agent, Latrunculin A and B, significantly decreased the amount of virus release to the extracellular environment.

I observed many vesicles containing mature poliovirus, on the extracellular side of the filopodia but still attached. These data suggest possible fusion of the limiting membrane of the autophagosome-like structure with the plasma membrane at the filopodia resulting in release of the interior unilamellar vesicle packet containing the virions. Alternatively the autophagosome-like structures may be budding from the tips of filopodia thus picking up additional lipid layers en route to the extracellular side. To resolve this and quantify how many membranes there are surrounding the vesicles we are currently doing transmission correlative electron microscopy on the collected extracellular vesicles.

Notably filopodia have been shown to play a role in the release and spread of several other types of viruses. For instance in human immunodeficiency virus (HIV) infected dendritic cells, more than 90% of the dendritic filopodia bear HIV on their tips. It is widely believed that positioning the

virus on the long filopodial tips facilitates its spread from dendritic cell to CD4+ helper T cells (Aggarwal, Iemma et al. 2012). Another example is the deadly negative strand RNA virus, Marburg virus (MARV), which buds out exclusively from the filopodia of cells. Similar to what I found with poliovirus, inhibiting actin-polymerization in MARV-infected cells also significantly decreases viral particle release (Kolesnikova, Bohil et al. 2007). It remains to be determined however if the elongated filopodia during poliovirus infection also plays a role in spreading the virus by directly targeting it to the cells.

Efficiency of infection when virus is in vesicles

I found that when poliovirus was presented inside vesicles to new host cells, the cells were infected in patches. On the other hand when virus was presented outside the vesicle, as free virus, the infection was sporadic. The simplest explanation for the patch phenotype and the increase in efficiency is that the vesicles contain many poliovirions that end up lysing outside the cells and simulatenously infecting a small population. This is a novel type of viral infection which may not allow for cells to alert neighbors to viral danger (via interferons or other antiviral defenses) and thus result in a more efficient infection. As predicted we found the efficiency of the virus when in vesicles to be directly proportional to the density of cells. The presence of phosphatidylserine lipids on the vesicles may allow the vesicles to bind to phosphatidylserine receptors on the cell surface and either destabilize and lyse or be endocytosed, lysed and eventually recycled back out to the extracellular medium to infect the neighboring cells. Indeed

neutralizing the phosphatidylserine with Annexin V completely blocked the infection but freeze thawing the vesicles to release the virus while in the presence of annexin V does not block infection. The mechanism is currently under investigation and remains to be determined.

Viral tropism

Even though poliomyelitis has been used as a viral disease model to understand viral pathogenesis for the past 50 years, there remain many important unanswered questions. Poliovirus lifecycle in the human body starts at the gastrointestinal track. After it is ingested and travels to the gut, it infects the gut epithelial cells. These cells are highly specialized for cholesterol absorption and given our earlier finding of the cholesterol dependence of this virus; it is highly advantageous site for the initial replication. After replicating in the gut cells, the virus enters the bloodstream via lymphatic vessels (Bodian 1955). In some cases, the virus then spreads to the central nervous system (CNS) where it may end up paralyzing its host. Although the poliovirus receptor is ubiquitously expressed, the virus selectively replicates in only a few cell types such as the gut cells, macrophages, and motor neurons. Why the virus selectively infects and replicates; what routes the virus uses to enter the CNS; and how it can cross the blood-brain barrier are all yet unanswered important questions that have implications for not just poliovirus but for viral tropism in general.

Although only low level of poliovirus can be detected from the skeletal muscle of poliovirus receptor transgenic mice if the virus is inoculated by oral

administration, Ren and Racaniello have shown that poliovirus invades the CNS by an axonal pathway when the virus is inoculated into the leg muscle of poliovirus receptor transgenic mice (Ren and Racaniello 1992). A receptor-dependent and -independent axonal retrograde transport of poliovirus in motor neurons in vivo has been observed when the virus is injected intramuscularly into the mice (Ohka, Sakai et al. 2009). The poliovirus is incorporated into the sciatic nerve and causes an initial of paralysis. However, the neural dissemination pathway from the skeletal muscle is not the primary route of the circulating virus to the central nervous system. The circulating PV crosses the blood-brain barrier at a high rate after intravenous inoculation (Yang, Terasaki et al. 1997).

Phosphotidylserine is present on the inner leaflet of the plasma membrane; on endosomal membranes and on the luminal leaflet of the endoplasmic reticulum (Leventis and Grinstein 2010). Exposure of phosphatidylserine on the surface of apoptotic cells is a specific signal for macrophages to hone in on the dying cell and engulf it (Fadok, Voelker et al. 1992). Given this, the phosphatidlserine vesicles containing the poliovirus may be an attractor for macrophages to engulf the virus and lead to it to replicate within this migratory cell that can access multiple tissues in the body including the CNS. Indeed poliovirus has been shown to infect dendritic cells and macrophages productively, i.e. replicate within the cells (Wahid, Cannon et al. 2005). Notably activated macrophages are known to be capable of crossing the blood-brain barrier (Fitch and Silver 1997); therefore the poliovirus-infected macrophages may deliver the poliovirus to the neurons. In this way, poliovirus not

only has a carrier in the form of the macrophage to bring itself to the CNS, but also avoids other immune responses when traveling around the bloodstream. Similarly, HIV infected monocytes paly the same role in carrying the viruses through blood brain barrier and establish local infection (Yadav and Collman 2009).

The phosphotidylserine receptor family is large with many family members. While all the receptors recognize the lipid, what differentiates the receptors is not clear and may be due to additional lipid and protein cofactors in conjunction with phosphotidylserine. Most cells in the body contain these receptors and the cells of the motor neurons or macrophages may contain specific types of the receptor that recognize the phosphatidylserine associated with the vesicles containing poliovirus.

In addition, PV crossing the blood-brain barrier is poliovirus receptor-independent and fast as cationized rat serum albumin which permeates the blood-brain barrier at a high rate (Yang, Terasaki et al. 1997). Similarly, blood-brain barrier permeation of PV has been demonstrated by in vitro BBB model with mouse brain capillary endothelial cells (MBEC4) (Ohka, Nihei et al. 2012). These evidences suggest that a new PV receptor for blood-brain barrier permeation is necessary. The phosphotidylserine receptors may also play a role in recognize the phosphatidylserine associated with the vesicles containing poliovirus directly on the blood-brain barrier. Then the virus can be transcytosed through capillary endothelial cells into the brain. Moreover, the majority of the poliovirus after penetrating the blood-brain barrier are still intact infectious

particles in the brain (Yang, Terasaki et al. 1997). It indicates that PV does not lose its infectivity during the blood-brain barrier permeation; thus the vesicles containing poliovirus could be a way to maintain viral particles intact while transcytosis through capillary endothelial cell layers.

Remarkably we found a significant increase in the release of phosphatidylserine enriched microvesicles from not only Poliovirus infected cells but also CVB3, Rhinovirus and Rotavirus. It is yet to be determined whether the viruses are in the microvesicles enriched in this lipid or whether the viruses are in vesicles released in parallel. Nevertheless these phosphatidylserine enriched vesicles could be an active immune response trigger to attract macrophages to the infection site which may allow the virus to piggy back on to a new host cell and spread around the body; it may also be a defense mechanism by the host to alert the immune system to the presence of invadors. Thus the phosphatidyserine-enriched membrane vesicles from infected cells may have benefits for both the host and the virus.

Novel Therapeutic targets

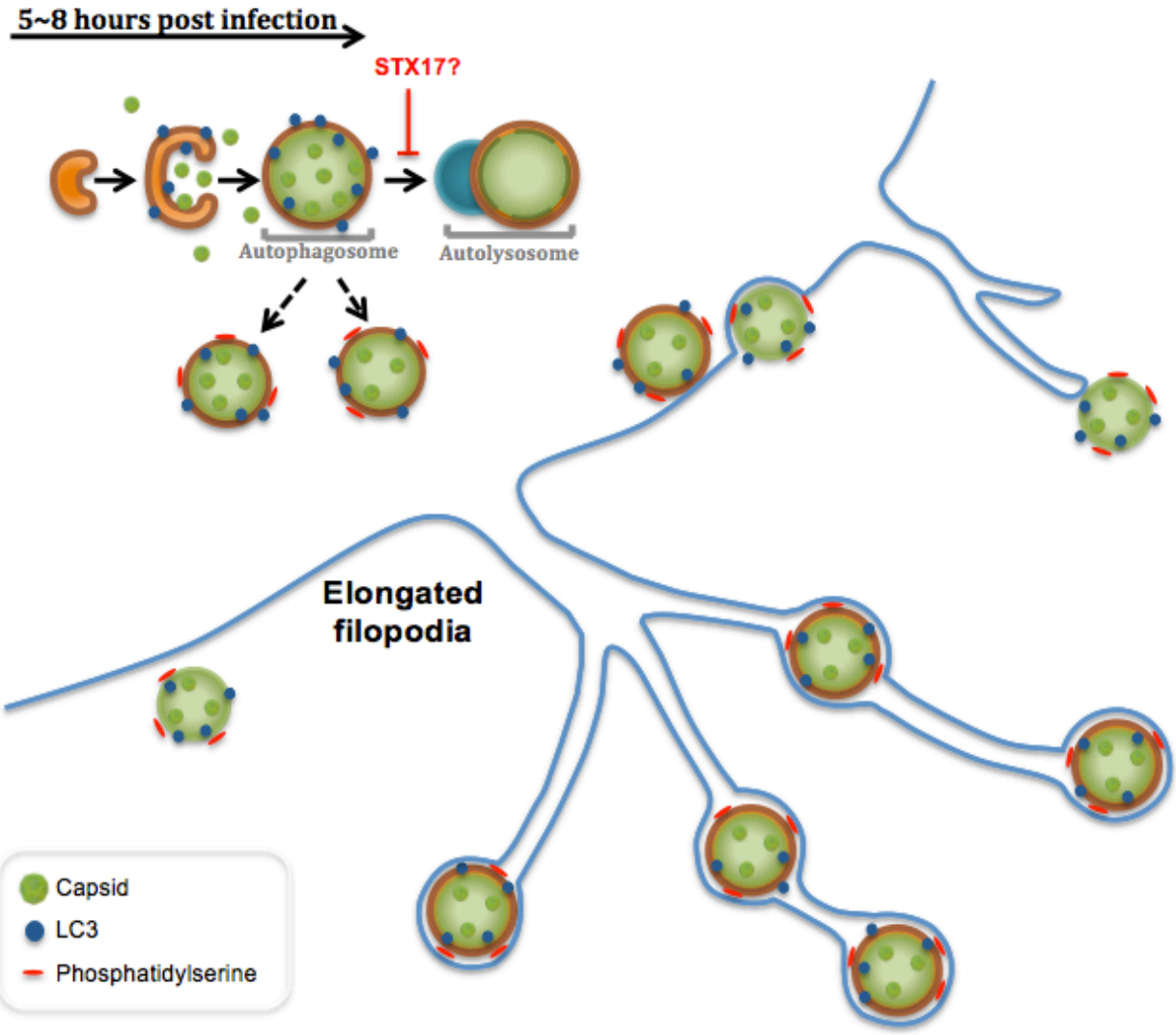
These studies have potential in the development of novel therapeutic approaches to treating viral infections. Inhibiting the autophagy pathway or more specifically preventing the sequestration of STX17 may provide avenues for therapy development. Neutralizing the phosphotidylserine on poliovirus containing vesicles or identifying the specific receptor that it binds to and hence block that receptor may be a promising therapeutic approach which may also

have implications for rhinovirus, CVB3 and even rotavirus infections.

In summary, much like enveloped viruses, my findings suggest that non-enveloped viruses also carry a piece of the host with them on their pathogenic journey. The lipid and protein components within these membranes potentially modulate the pathogenesis of non-enveloped viruses within the host and provide a new paradigm for viral spread and tropism.

Figure 7-1: The proposed model for the non-lytic release of poliovirus via the autophagy pathway

Autophasomes are stimulated by poliovirus infection. By 5 to 8 hours post infection, mature virions are formed and turn out to release from the replication organelles and are dispersed in the cytoplasm. Isolation membrane of autophagosomes engulfs the virions from the cytoplasm and encloses to form the double membrane vesicles. Mature virions then are in the autophagosome-like structures. The structures not only contain autophagosome marker LC3 and poliovirus, but also are enriched in phosphatidylserine. Because lacking of STX17, autophagosomes do not fuse with lysosomes. The degradative process of autophagy is perturbed by the poliovirus infection. Accompany with poliovirus infection over time, the filopodia of the cells become longer and more dynamic. Autophagosome-like structures containing poliovirions are observed at or released from the filopodia.


5~8 hours post infection
STX17?
Autophagosome
Autolysosome
Elongated
filopodia
Capsid
LC3
Phosphatidylserine

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