ABSTRACT

Title of Document:	MECHANISM OF UPREGULATION OF PHOSPHATIDYLCHOLINE SYNTHESIS DURING PICORNAVIRUS INFECTION AND ITS ROLE IN THE DEVELOPMENT OF VIRAL REPLICATION ORGANELLES.
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Picornaviruses are a group of human and animal pathogens capable of inflicting serious public health diseases and economic burdens. Treatments options through vaccines for prevention or antivirals to cure infection are not available for the vast majority of these viruses. These shortcomings, in the development of vaccines or antivirals therapeutic, are linked to the genetic diversity and to an incomplete understanding of the biology of these viruses. Despite the diverse host range, this group of positive-strand RNA viruses shares the same replication mechanisms, including the development of membranous structures (replication organelles) in the cytoplasm of infected cells. The development of these

membranous structures, which serve as sites for the replication of the viral RNA genome, has been linked to the hijacking of elements of the cellular membrane metabolism pathways. Here we show that upon picornavirus infection, there is a specific activation of acyl-CoA synthetase enzymes resulting in strong import and accumulation of long chain fatty acids in the cytoplasm of infected cells. We show that the newly imported fatty acids serve as a substrate for the upregulation of phosphatidylcholine synthesis required for the structural development of replication organelles. In this work, we identified that acyl-CoA synthetase long chain 3 (ACSL3) is required for the upregulation of lipids syntheses and the replication of poliovirus. We have shown that the poliovirus protein 2A was required but not sufficient for the activation of import of long chain fatty acids in infected cells. We demonstrated that the fatty acid import is upregulated upon infection by diverse picornaviruses and that such upregulation is not dependent on activation of ER stress response or the autophagy pathways. In this work, we have demonstrated that phosphatidylcholine was required for the structural development of replication organelles. Phosphatidylcholine synthesis was dispensable for the production of infectious particles at high MOI but required at a low MOI for the protection of the replication complexes from the cellular innate immunity mechanisms.

MECHANISM OF UPREGULATION OF PHOSPHATIDYLCHOLINE SYNTHESIS DURING PICORNAVIRUS INFECTION AND ITS ROLE IN THE DEVELOPMENT OF THE VIRAL REPLICATION ORGANELLES

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Dedication

This dissertation is dedicated to my father Andre Nchoutmboube and my mother Elise Ngamo for their unconditional love and sacrifice to make the person I am today

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List of Abbreviations

ACSL: acyl-CoA synthetase long chain

AGPAT: acyl-glycerol phosphate acyl transferase

BCE: Before Common Era

CNS: Central nervous system

cVDPV: Circulating vaccine derived polio-vaccine

CVB: Coxsackie virus B

CCT: CTP: phosphocholine cytidylyltransferase

DNA: Deoxyribonucleic acid

DMEM: Dulbecco Modified Eagle's Medium

DAG: Diacylglycerol

DGAT: Diacylglycerol acyl transferase

DENV: Dengue virus

EMCV: Encephalomyocarditis virus

EV: Enterovirus

EBSS: Earle's Balanced Salt Solution

FA: Fatty acid

FMDV: Foot and mouth disease virus

FATP: Fatty acid transport proteins

FHV: Flock house virus

FBS: Fetal bovine serum

GPAT: Glycerol phosphate acyl transferase

GFP: Green fluorescent proteins

GI: Gastrointestinal tract

HPI: Hours post infection

HA: Hemagglutinin

HCV: Hepatitis C virus

IPV: Inactivated polio-vaccine

IRES: Internal ribosome entry site

LPA: Lysophosphatidic acid

mRNA: Messenger RNA

MOI: Multiplicity of infection

Min: minute

MALDI-TOF-MS: Matrix assisted laser desorption-ionization time-of-flight mass spectrometry

NT: Nucleotide

OPV: Oral polio-vaccine

PV: Poliovirus

PFU: Plaque forming unit

PCR: Polymerase chain reaction

PVR: Poliovirus receptor

PC: Phosphatidylcholine

PE: Phosphatidylethanolamine

PS: Phosphatidylserine

PA: Phosphatidic acid

RI: Replication intermediate

RF: replicative form

RNA: Ribonucleic acid

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

siRNA: short interfering RNA

TLC: Thin layer chromatography

VAPP: Vaccine associate paralytic poliomyelitis

VDPV: Vaccine derived polio-vaccine

WNV: west Nile virus

Chapter 1: Introduction

1.1 Poliovirus history

Poliovirus is the causative agent of poliomyelitis, a human disease characterized by a flaccid paralysis in its acute phase. This is an ancient disease already depicted in Egyptian's art between 1580-1350 BCE, displaying a person with an atrophic leg leaning on a crutch to walk (105). Michael Underwood was the first physician to describe clinical symptoms of this disease in 1789 in children characterized by a debilitation of the lower extremities (36, 120). Karl Landsteiner and Erwing Popper formally identified poliovirus in 1908 as responsible of poliomyelitis in humans. Evidences of infantile paralysis were documented in the early nineteen century when an outbreak erupted in the South Atlantic Island of St Helena and the English town of Workshop around 1835 (7). The disease began to spread in the late nineteen centuries and early twentieth centuries in Europe especially in Norway (>900 cases) Sweden (>1000 cases) in 1905 (7). Poliomyelitis began to manifest itself all over Europe especially in Vienna and Lower Austria (1908-1909), Germany (1909), and England and Wales (1911) (7). The first documented emergence of poliomyelitis in the United States (USA) was recorded in 1841 when 10 cases of poliomyelitis in the West Feliciana in Louisiana had been identified (7).

Fifty years following the discovery of PV as the main agent of poliomyelitis, of the 57000 cases of infection diagnosed, 22000 cases of paralytic forms had been identified in the USA by 1952 (62). A vaccine developed by Jonas Salk, tested and proven effective was licensed in 1955 (36, 127). The Salk vaccine, or the Inactivated Poliovirus Vaccine (IPV), was successful and had reduced the number of paralytic cases of poliomyelitis by more than 95% within five years after its introduction in the USA (102). A second vaccine, the

Oral (or “live”, “attenuated”) Poliovirus Vaccine (OPV), developed by Albert Sabin and licensed in the USA in 1961 also had proven to be effective in stopping the spread of the wild type PV infection (36, 127). The OPV became the preferred vaccine largely used around the globe because of its ability to provide a strong gastrointestinal immunity as well as its ease of use and manufacturing. Because of the introduction of the IPV and the OPV vaccines, the number of poliovirus infections sharply decreased around the globe and the last case of outbreak of wild type poliovirus infection in the USA was recorded in 1979.

1.2 Pathogenesis

Poliovirus infects mostly humans who are the natural host, non-human primates such as chimpanzees and old world monkeys when they are used for experimental disease, and transgenic mice expressing the human poliovirus receptor (127). PV is a highly contagious virus transmitted from person to person through ingestion of food or water contaminated with feces of a person shedding PV particles (105). The incubation period, the time between exposure to the virus and the onset of the disease, may last 1 to 3 days leading to an abortive poliomyelitis or could extent between 2 to 35 days (99). Virus can be recovered from the blood and feces 3 to 5 days after infection (99). Poliovirus is stable in acid milieu, which is necessary for infection and replication in the gastrointestinal (GI) tract where the virus encounters low pH conditions. A determinant to poliovirus infection is the presence of poliovirus receptor (PVR) also named CD155 on the surface of permissive cells of the GI tract (126). CD155, discovered by Mendelsohn *et al*, is a transmembrane glycoprotein which mediates the attachment of PV particles on the cell surface (126). Its discovery had also prompted the generation of transgenic mice expressing the human PVR in order to facilitate the study of PV infection on animal’s

model (129). Following infection, the poliovirus initially multiplies in lymphatic tissues such as Peyer's patches in the small intestine or tonsils in the pharynx (45).

The successful multiplication ultimately allows the PV to enter the bloodstream leading to the development of viremia in the absence of neutralizing antibodies (112). The entry of PV particles in the bloodstream accelerates the propagation of the virus in non-neural tissues such as cervical and mesenteric lymph nodes, bone marrow, liver and spleen (112). 95 % of poliovirus infections are either asymptomatic or may lead to an abortive poliomyelitis where there is a mild occurrence of symptoms due to the development of a minor viremia (126). The minor viremia ends with mild symptoms such as sore throat, fever, gastrointestinal illness and a generalized malaise (126). The development of PV infection into a major viremia spreads the virus to the central nervous system (CNS) where it multiplies in the motor neurons of the spinal cord, the brain stem and the motor complex leading to muscle paralysis or the development of non-paralytic aseptic meningitis (126). There are three serotypes of polioviruses, although all three serotypes are highly infectious, the poliovirus type 1 is responsible of 80% cases of paralysis in its severe form of infection (105).

Three routes of PV entrance to the CNS following infection have been hypothesized: The first one is the crossing of the blood brain barrier (BBB) following the development of a major viremia after PV infections which allow viruses to access the CNS independently of poliovirus receptor (PVR) (36, 127). The second route of PV access to the CNS is the retrograde axonal transport through the sciatic nerve in a PVR dependent manner (113). The third route is the importation of poliovirus into the CNS by infected macrophages (36, 44). The clinical symptoms of non-paralytic meningitis of PV infection

is characterized by the rigidity of the neck, back and lower limbs, and this occurs in 1-2% of PV infections while paralytic cases occur in 0.1-1% of PV infected individuals (36).

The tissue tropism of PV particles and subsequently the occurrence of the acute form of infection leading to meningitis and paralysis are tightly linked to the role of interferon and the production of neutralizing antibodies. PV infection is rapidly suppressed in non-neural tissues because of the strong response of the innate immune system mediated by type1 interferon (T1IFN). Contrary to non-neural tissues, PV infection of neural tissues especially in CNS is accelerated because of the weak response of the T1IFN (108). Buisman *et al* have shown that preexisting immunoglobulin A (IgA) in circulation controlled reinfection and shedding of PV particles and probably its absence could contribute to PV circulation (25).

1.3 Poliovirus vaccines

The development of PV vaccines was made possible by Enders J.F *et al* who in 1949, propagated PV in live cells of non-neural tissues for the first time (46). Jonas Salk began the development of IPV vaccine in 1953 by growing all three PV serotypes in African green monkey (Monkey kidneys cells: Vero) cells (135, 136). These PV infected cells were then treated with lower doses of formaldehyde (also known as formalin) to kill the infectivity of viral particles while preserving the antigenic activity. The PV strains selected to produce the vaccine were Mahoney (type1), MEF-1 (type2) and Saukett (type3). The IPV, administered by intramuscular injection, induces high titers of neutralizing antibodies and effectively prevents the development of viremia or the acute phase of the disease. Although the IPV prevents pharyngeal excretion of virus (51) and it is safe for immunocompromised persons, it cannot stop the infection of the gut which is the major

route of PV transmission, induces lower intestinal immunity compared to natural infection, and is expensive to produce because it requires the wild type virus which can constitute a biohazard (83). The “killed” poliovirus vaccine was the only effective tool to combat the ravaging effect of PV infection until 1961 when the second PV vaccine was licensed.

Many investigators at various institutions were involved in the development of a live attenuated PV vaccine. Among others, we can point out the laboratory of Albert Sabin at the children hospital center in Cincinnati, the Lederle laboratories headed by Herald Cox, and finally the laboratory of Hilary Kaprowski at the Wistart institute in Philadelphia. The live attenuated poliovirus vaccine was generated by serial passages of the virus in cell culture such as monkey testis, kidney and skin at high and low multiplicity of infection (MOI), passage through animal host such as monkey and at suboptimal temperature (36, 102). The live attenuated PV had to satisfy several conditions for a vaccine purpose: (i) ability to replicate effectively in the GI tract; (ii) inability to invade or to replicate in the CNS and (iii) genetic stability to withstand the pressure of replication within the human host without reversion to a neurovirulent phenotype (36). The OPV was developed from all three serotypes of PV: the type 1 also known as (Sabin1) derived from a type 1 Mahoney strain previously modified by Li and Schaefer, the type 2 (or Sabin 2) was isolated from a healthy child and shown to have low virulence and lastly the type 3 (or Sabin 3) was from Leon strain (82, 102). Following its early success, the OPV became the vaccine of choice for mass campaign of vaccination. The OPV, which mimics the natural route of infection, has many advantages over IPV and is more appealing to mass vaccination campaigns: it provides both systemic and intestinal immunity, it is easy to administer through oral

delivery, inexpensive to produce, and it confers herd immunity since the live virus vaccine is excreted in the environment. Although the OPV had become the weapon of choice for poliovirus eradication in many developing countries, it has significant shortcomings: this vaccine is not indicated for immuno-deficient or immunosuppressed individuals and, most importantly, the attenuated virus is genetically unstable.

1.4 Poliovirus Eradication challenges

The IPV and OPV vaccines have been excellent in reducing poliovirus infection around the globe since their introduction in 1955 and 1961 respectively. In 1988, the world health assembly, the legislative branch of the World Health Organization (WHO), decided to launch the global campaign aimed at eradication of poliovirus by the year 2000. This marked the beginning of the Global polio eradication initiative supported by non-governmental agencies, the WHO and national countries around the globe. Although never reaching the ultimate eradication goals, this campaign significantly reduced the burden of poliovirus infections from ~350000 cases in 1988 to 416 cases in 2013 (WHO; http://www.who.int/mediacentre/factsheets/fs114/en/). At the beginning of this initiative, poliovirus was endemic in 125 countries around the globe, but today, Pakistan and Afghanistan are the only remaining countries. The campaign of poliovirus eradication has been largely successful in reducing cases of acute flaccid paralysis (AFP) due to poliovirus infection by 99% (WHO: http://www.who.int/mediacentre/factsheets/fs114/en/). Of the three serotypes, only the wild poliovirus type 2 was successfully eradicated around the globe with the last case registered in 1999 in India (http://www.who.int/mediacentre/factsheets/fs114/en/).

The use of OPV as the primary tool in the eradication campaign has raised significant challenges. The OPV can inflict vaccine-associated paralytic poliomyelitis (VAPP) among vaccine recipients. Because OPV is a live virus vaccine, it can be transmitted from person to person and progress to a circulating vaccine-derived poliovirus (cVDPV). Through mutation and possibly recombination with other enteroviruses, the cVDPV can regain the pathogenicity of the wild type virus and its ability to cause paralytic disease (81). Thus, the type 1 cVDPV has been at the origin of outbreak of poliovirus infection on the island of Hispaniola which had been previously covered by OPV and certified polio-free (81). The continued use of OPV, especially in areas declared free of indigenous polio through a certification of eradication, is the subject of debates giving the risk of reintroducing a live virus with the ability to revert to wild type. The eradication of the wild type polio continues to be a success around the globe and was highlighted by the removal of Nigeria in 2015 from the list of endemic countries. The monitoring is required especially in both certified areas as well as in places marked by poverty, social unrest or civil war, which facilitate migration and prevent health workers to perform vaccination campaign.

Chapter 2: Poliovirus life cycle and genome organization

2.1 The *Picornaviridae* family

Poliovirus is a member of the *Picornaviridae* family of viruses. This family contains non-enveloped, single stranded positive RNA viruses. The name *Picornaviridae*, reflects the small size (Pico=10^{-12}) of both the viral genome and the viral particle characteristics of viruses in this family (128). Picornaviruses are known to inflict human and animal diseases leading to serious economic and public health damages. Among the most notable of these viruses are poliovirus, the Foot and mouth disease (FMDV), Hepatitis A virus, enteroviruses 71 and D68, Coxsackie viruses and human rhinoviruses. Coxsackie virus B3 is believed to be associated with human diabetes and myocarditis, enteroviruses 71 and D68 are known to inflict hand-foot-and-mouth disease and acute flaccid paralysis in children respectively, the Human rhinovirus A, B and C is linked to respiratory diseases (128). The Foot and Mouth Disease virus (FMDV) is known to inflict highly infectious vesicular diseases in cloven-footed animals such as cattle, sheep (85). Viruses in this family have served as objects of many groundbreaking studies in the field of virology. The first animal virus isolated was FMDV (128), poliovirus was the first RNA virus identified with a missing 5' cap structure (128). The first infectious cDNA clone from an animal virus was developed for poliovirus, as well as the plaque assay which is a method used to quantify infectious viral particles (128). The *Picornaviridae* family contains 29 genera, and in addition, viruses in this family are further subdivided to more than 28 species. The poliovirus is the prototype member of the *Picornaviridae* family of the genus *Enterovirus* and is one of the most studied animal viruses.

2.2 Poliovirus structure

Polioviruses particles have an icosahedral shape of around 30nm in diameter and lack a lipid envelop. Viral particles are highly stable and can retain their infectivity at a pH value of 3.0 since they have to gain access to intestine for replication. The poliovirus virion is composed of 60 subunits each of capsids proteins VP1, VP2, VP3 and VP4 (67). These capsid proteins derived from the proteolysis of the P1 domain of viral polyprotein. VP1, VP2, and VP3 constitute the outer structure of the virion whereas VP4 is located in the inner surface of the capsid (128). VP1 proteins are located around the five-fold axes (mesa) where there is also the presence of a deep depression ("canyon") proven to be the binding site of PV receptor, while VP2 and VP3 alternate around two and three-fold axes (53, 128). Beneath the canyon which is located on the viral capsid protein VP1, there is a hydrophobic tunnel or pocket where the sphingosine lipid is found in poliovirus type 1 and 3 virions (128). VP1 to -3 (with an average MW ~ 30 kDa) capsids proteins shared the same topology and each have eight-stranded and antiparallel β-barrels.

Each beta strands of capsid proteins formed a wedge like structure made up of antiparallel β-sheets. The VP1 to -3 capsids proteins are characterized by the presence of loops connecting the β-strand and the N- and C-terminal of β-barrels (128). These loops or amino acid decorate the surface of PV particles and confer distinctive morphology and constitute major sites of antigenicity (128). Antibodies capable of neutralizing PV infectivity are raised against these amino acids (160). The PV particles possess four major neutralizing antigenic sites; however there are only three unique sets of these four sites corresponding to three serotypes of PV (type 1, type 2 and type 3) (128).

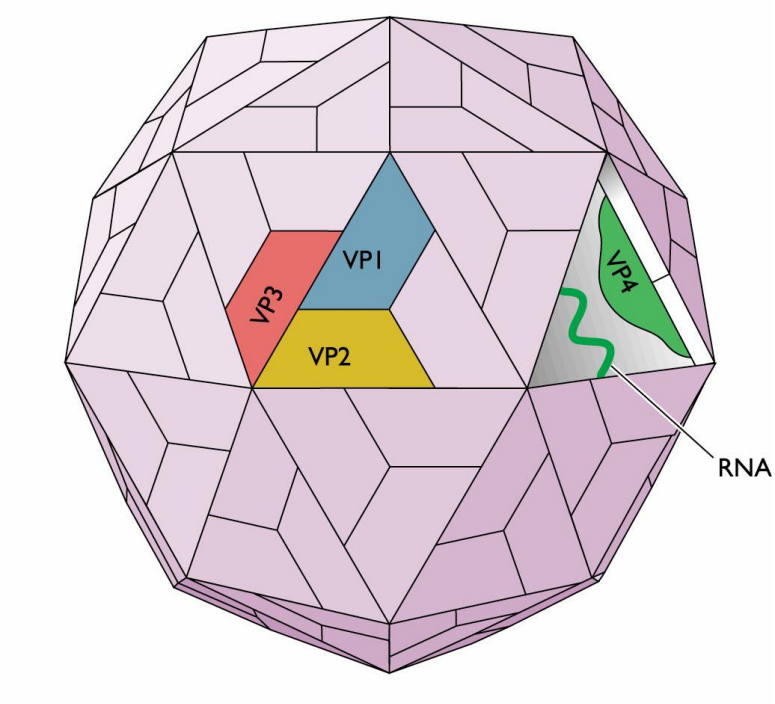


Figure 2.1: Schematic representation of poliovirus structure with capsid proteins and viral genome (49)

2.3 Poliovirus genome organization

The PV RNA genome is composed of 7.5 kilo bases (kb) of nucleotides (nt) and has four distinctive regions: the 5' untranslated region (UTR) has an internal ribosome entry site (IRES) structure covalently linked to a 22 amino acid viral protein VPg (virion protein genome linked); a single open reading frame (ORF) encoding the viral polyprotein; the 3' UTR which is highly structured and possesses a pseudoknot, and finally a poly(A) tract region which has about 60 adenine residues (36, 128).

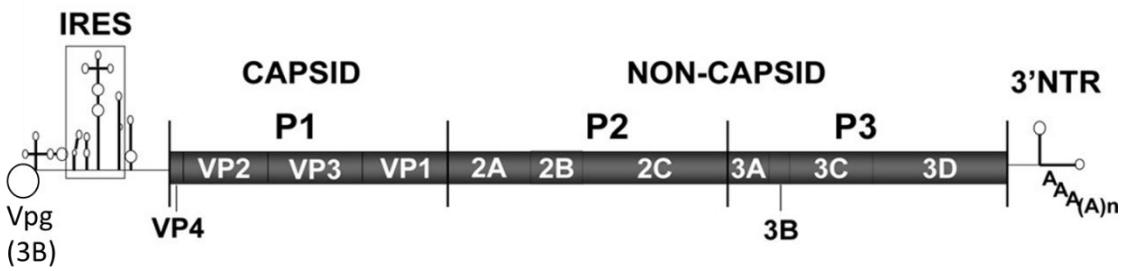


Figure 2.2: Genome organization of poliovirus (Source: courtesy of Dr George Belov)

The 5' UTR of PV is a complex structure characterized by: the presence of an internal ribosome entry site (IRES) which directs the cap-independent translation of the viral mRNA ; a clover-leaf structure indispensable during viral RNA replication, and a polypyrimidine-tract of nucleotides lining upstream of the IRES structure (5). The 3' UTR, an AU rich section of PV RNA, is about 72 nt long and exhibits a secondary structure consisting of two hairpins involved in the replication (23). The 3' UTR serves as a template for the initiation of minus-strand synthesis by the replication complex (114). Another structural component of PV genome is the presence of a cis-acting replication element

(CRE). CRE, which has 61 bases, is located in the 2C coding region of PV and serves as the template for VPg uridylation (58, 59, 119) .

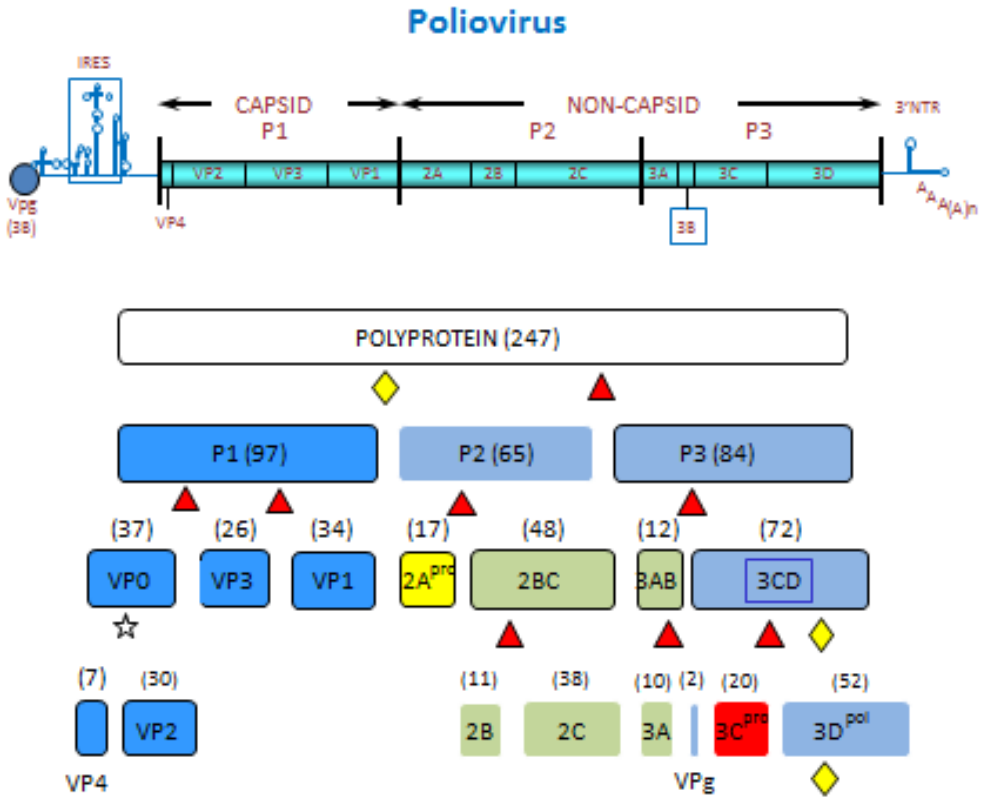


Figure 2.3: Processing of polyproteins and precursor proteins into individual structural and nonstructural proteins. The poliovirus has a genome of a positive-strand RNA of about 7500 nucleotides. The 5' end is covalently linked to a viral protein VPg and contains the internal ribosome entry site (IRES). The open reading frame is divided into three domains: The P1 domain encodes structural proteins or capsid while the P2 and P3 domains encode non-structural proteins required for the replication. The 3' end contains a poly (A) tail of around 60 residues. Upon its released in the cytoplasm of infected cells, the viral genome is translated to yield a large polyprotein, which is then processed by viral proteases 2A, 3C and 3CD into protein precursors and individual proteins. The yellow triangles represent sites cleaved by the viral protease 2A. The red triangles represent sites cleaved by viral proteases 3C and 3CD. The 3CD protease cleaves preferentially structural proteins while 3C cleaves non-structural proteins. The white start presents a site of autocatalytic processing. (Source: courtesy of Dr George Belov)

The ORF codes for a single large polyprotein subdivided into three domains P1, P2 and P3. P1 is the precursor of structural proteins, while P2 and P3 are precursors to non-structural proteins. The P1 domain is cleaved by viral protease $3C^{Pro}$ to yield structural proteins VP0 (precursor to VP2 and VP4), VP1 and VP3. The P2 domain of the polyprotein is cleaved to yield 2A and 2BC proteins and the 2BC is later processed to yield 2B and 2C proteins (137). The P3 domain is cleaved into 3AB and 3CD precursors proteins and are later processed into 3A, 3B (VPg), $3C^{Pro}$ (3C protease) and $3D^{Pol}$ (3D polymerase) the RNA dependent RNA polymerase (RDRP) (137).

2.4 Poliovirus proteins and function

The PV protein $2A^{Pro}$ is a viral proteinase cleaving the newly synthesized polyprotein *in cis* at the junction between the structural precursor polyprotein P1 and non-structural precursor polyprotein P2 during translation (114). The viral protein 2A is also known to inhibit protein synthesis of host cells by cleaving eIF-4G which is required for the initiation of the host protein synthesis (146). The $2A^{Pro}$ protein has a chymotrypsin-like folding pattern with cysteine as the active site nucleophile.

The viral protein 2B and its precursor 2BC have been associated with the membrane remodeling and production of membranes vesicular structures on which the synthesis of viral RNA takes place in infected cells (32). The presence of hydrophobic domains characterized by amphipathic α-helix allows both 2B and 2BC proteins to interact with or integrate into membranes organelles such as Golgi, ER complex and virus-induced membranes in PV infected cells (32). It was shown that the expression of PV 2B/2BC inhibits the secretion and trafficking of proteins between Golgi and the ER complex and lowers the Ca^{2+} level (6, 41, 152), induces membrane permeabilization (1) and the

disassembly of the Golgi complex (139). The viral protein 2C has NTPase activity (132) and just like 2B and its precursor 2BC, is also associated with the induction, remodeling and proliferation of membranous vesicles in PV infected cells (32). Structural prediction studies and conserved sequences analysis show that $2C^{ATPase}$ has amphipathic helix at its NH_2-terminal domain, a nucleotide binding domain and a COOH-terminal zinc-binding domain (150). The $2C^{ATPase}$ protein possesses RNA binding activity and is the target of guanidine-HCl, an inhibitor known to block the initiation of the negative-strand RNA synthesis (8).

The viral proteins 3A and its precursor 3AB are implicated in the induction and the proliferation of membranous structures in the cytoplasm of PV infected cells. The expression of PV protein 3A disrupts the cellular protein trafficking from the ER complex to Golgi (40, 41). The presence of hydrophobic residues in the 3A section of the precursor protein 3AB mediates its association with cytoplasmic as well as PV-induced membranes during infection (151). The viral protein 3A specifically recruits ADP-ribosylation factor (arf) to membranes via the recruitment of Golgi-specific brefeldin A resistance factor 1 (GBF1). 3AB also possesses RNA-chaperone activity, which may facilitate poliovirus RNA replication and support recombination between different genomes and may be important for pathogenesis of picornavirus infections (37, 38, 55). It was proposed that the precursor protein 3AB may serve as a substrate for 3D polymerase for VPg uridylylation (131), however other experiments suggested that other precursors proteins such as 3BC and 3BCD may be used preferentially (117). Following its uridylylation by the PV polymerase $3D^{Pol}$ (118), the VPg protein is relocated at both the 3' end and the 5' end to serve as a primer for the synthesis of both the negative- and the positive-strand RNA

synthesis (124). 3B (VPg) is covalently attached to the 5' end of PV genome via a 5' tyrosyluridine bond.

The viral protein $3C^{Pro}$ and its precursor 3CD have protease activities. The precursor protein 3CD is preferentially involved in the processing of the structural polyprotein fragment P1 whereas 3C is responsible for most of the other polyprotein cleavages. Processing of cellular proteins by $3C^{Pro}$ and 3CD modifies intracellular processes such as nuclear transcription or host mRNA translation and enhances PV replication in infected cells. The $3C^{Pro}$ protein possesses an RNA binding motif and has a Cys-reactive proteinase active nucleophile site with chymotrypsin-like fold (114). The 3CD viral protein interacts with 3AB and poly(C)-binding protein 2 (PCBP2) at the 5' cloverleaf structure to initiate the synthesis of minus-strand PV RNA (64, 157, 163). The 3CD protein binds to the CRE RNA site, which serves as a template for the synthesis of VPg-pUpU (116). The viral protein $3D^{Pol}$ is the RNA-dependent RNA polymerase (RDRP) and catalyzes the synthesis of both the minus-strand and plus-strand of PV RNA genome. The $3D^{Pol}$ protein has 461 amino acids and the three-dimensional "palm" subdomain structure is similar to other polymerases while its "thumb" and "fingers" subdomains are different from those of other polymerases (63). The PV protein $3D^{Pol}$ has RNA-binding properties, it relaxes the double-stranded RNA for elongation and possesses a terminal adenyltransferase activity (30, 48). In the course of PV replication, the viral protein $3D^{Pol}$, along with other non-structural proteins such as 3AB, 2B and 2C, associates with the replication complexes localized on the cytoplasmic surface of virus-induced membranous vesicles.

2.5 Poliovirus life cycle

2.5.1 Poliovirus entry

Endocytosis is the principal route of PV entry into the host cell. The uptake of infectious PV particles is dependent on ATP, tyrosine phosphorylation of the cytoplasmic tail of the poliovirus receptor or actin but independent of clathrin, caveolis, micropinocytosis and is insensitive to microtubule polymerization inhibitors (22, 88). It starts with the attachment of poliovirus to the poliovirus receptor (also called PVR or CD155) on the surface of susceptible cells. The PVR is a transmembrane immunoglobulin-like (Ig) protein with three extracellular Ig-like domains (100, 128). The attachment on viral particles occurs within the hydrophobic pocket beneath the canyon located on the PV surface (128). The attachment of PV particles with the receptor triggers conformational changes, leading to the exposition of internal sequence of the myristoyl-VP4 and the N-terminal sequence of the capsid protein VP1 (52). The externalization of VP4 and the hydrophobic N-terminal of VP1 sequences alter the structure of the virion and produce a particle called the "A particle". The N-terminal sequence of VP1 is inserted into the cell membrane where it creates a pore, thereby facilitating the attachment of PV particles and uncoating of viral RNA in the cytoplasm (52, 68, 128).

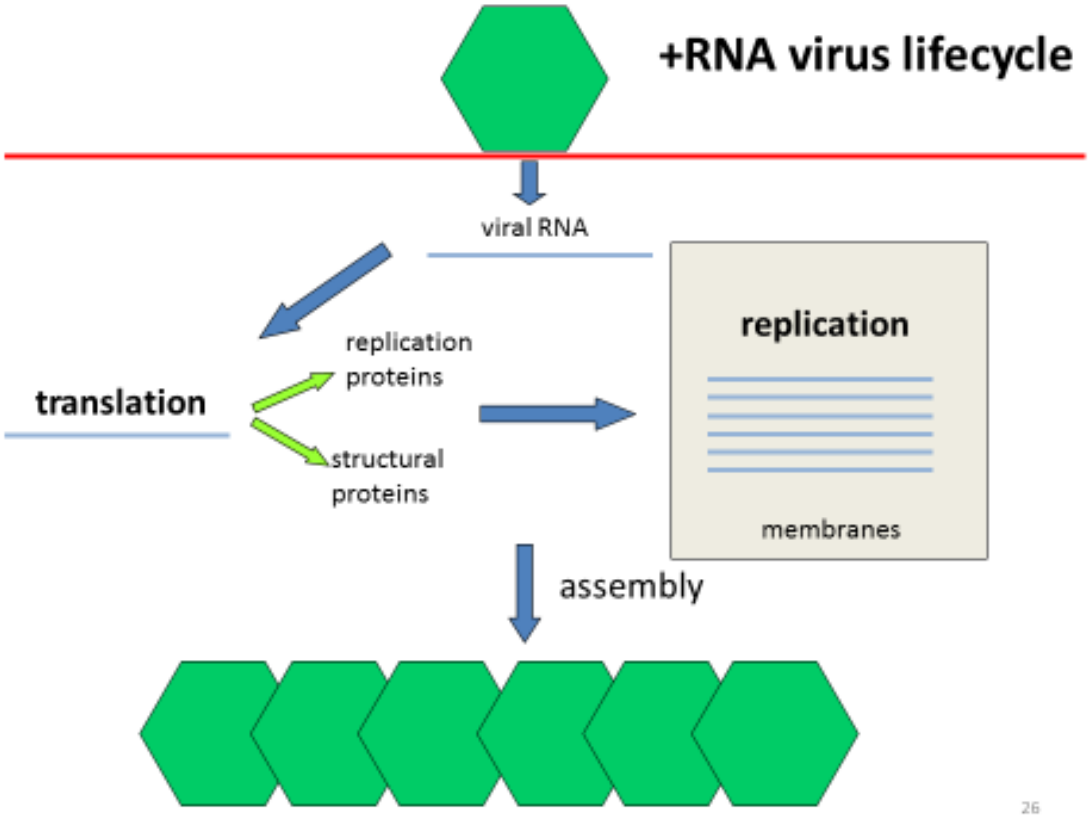


Figure 2.4: Poliovirus life cycle. (Source: courtesy of Dr George Belov)

2.5.2 Poliovirus translation and replication

Following delivery of the viral mRNA in the cytoplasm, the VPg-linked protein is detached from the genome by a host cell enzyme (4, 93, 156). The first step in the replication cycle of PV is the translation of the viral mRNA. The translation of PV mRNA is regulated by the IRES structure, the binding site of the 40S ribosomal subunits. The 40S ribosomal subunits are recruited at the IRES site via interaction with the complex of eIF3 and C-terminal region of eIF4G (128). Since the PV mRNA codes for a single long ORF, the translation produces a long polyprotein which is processed co- and post-translationally by viral proteases $2A^{Pro}$ (which cleaves the P1 and P2 junction) and $3C^{Pro}$ and $3CD^{Pro}$ into intermediates and mature structural and non-structural proteins. During viral proteins syntheses, there are also profound modifications of intracellular processes such as transcription of host genes, translation initiation of host proteins as well as nucleo-cytoplasmic trafficking (43). The cellular proteins synthesizing machinery is incapacitated by the cleavage of the translation initiation factor eIF4G by PV protease $2A^{Pro}$ (43).

The PV genome serves as a template for both the translation and minus-strand RNA synthesis in infected cells. The translation of PV genome and the minus-strand RNA synthesis are two processes that cannot be carried out simultaneously (9, 54). Following the synthesis of sufficient amounts of viral proteins, translation is reduced and the PV genome serves as a template for the synthesis of negative-strand RNA. It was shown that a switch from translation to minus-strand RNA synthesis occurred when an RNA structure, locates at the 5' end non-coding region of PV genome and next to the IRES, binds the viral protein $3CD^{Pro}$ and displaces the host poly r(C)-binding proteins (PCBP) (20). The translation and replication mechanisms in poliovirus infected cells are also regulated by

the cleavage of PCBP by the viral protease $3C^{Pro}$ (128). The host PCBP binds stem-loop IV during the IRES-driven translation and stem-loop I during viral RNA synthesis (128).

The replication complex of PV is located on the surface of membranous structures proliferating in the cytosol. In the course of PV RNA replication, there are various forms of viral RNAs synthesized in infected cells: the single stranded RNAs, the replication intermediate (RI) and the replicative form (RF) (128). A few minus-strand RNAs serve as templates for the synthesis of the positive single-stranded RNAs (128). The RF is a double-stranded RNA (dsRNA) formed by full length copies of positive and the negative strands while the RI is an RNA on which multiple nascent positive single-stranded RNAs are synthesized (128).

2.5.2 Poliovirus assembly and release

The P1 domain, the viral polyprotein fragment that contains all structural proteins, is preferentially cleaved by viral protease $3CD^{Pro}$ to form three polypeptides VP1, VP3 and the immature VP0 (160). The three polypeptides VP1, VP3 and VP0 form protomers. In the course of viral assembly, five protomers aggregate to generate pentamers of which twelve spontaneously assemble to form a procapsid (36, 160). Procapsid particles are made up of 60 copies each of VP1, VP2 and VP0. It remains unknown whether the viral genome is inserted into the procapsid or enclosed during assembly of pentamers. The packaging of PV genome leads to the formation of a provirion, which contains the RNA. The immature polypeptide VP0 likely undergoes autoproteolytical cleavage to yield VP2 and VP4 capsids proteins, thus converting the provirion into an infectious particle. Poliovirus is the most

studied virus in the *picornaviridae* family, it can serve as a model to understand the biology of other existing related virus with high public health and economic impacts.

2.6 Origin and functioning of poliovirus replication organelles

2.6.1 Overview

The replication complexes of poliovirus are intimately associated with membranous structures (also known as replication organelles (RO)) in the cytosol (101). These membranous structures were first detected by Kallman *et al.* in poliovirus-infected rhesus monkey kidney cells. The authors observed the appearance of dense materials they called cytoplasmic bodies, or U bodies, proliferating in great numbers in the cytosol of polio-infected cells (78). Membranous structures induced in poliovirus-infected cells appeared as vesicles or rosette-like structures and varied in size between 70-400 nm (101). Belov *et al.* have shown that these structures had complex spatial morphology and featured single membrane at the early stage of replication and double membranes in the late stage of replication (Figure 2.5) (14).

All positive strand RNA viruses of eukaryotes share this reorganization of intracellular membranes upon viral infection. Viruses from the *Flaviviridae* (Dengue virus, Hepatitis C virus, and West Nile virus), *Togaviridae* (Semliki forest virus, Sindbis virus, and Rubella virus), *Bromoviridae* (Brome mosaic virus), and *Coronaviridae* (Severe acute respiratory syndrome coronavirus, Mouse hepatitis virus) families induce profound remodeling of intracellular organelles and proliferation of membranous structures that support the replication complexes (57). These groups of positive-stranded RNA viruses largely induce two types of membranous structures upon infection: invaginated vesicles (InV) or spherule type and the double membranes vesicles (DMV) type (57). In the case

of picornaviruses, single membrane branching tubular structures filled with cytoplasmic materials are detected early in the infection and then collapse into DMV (14).

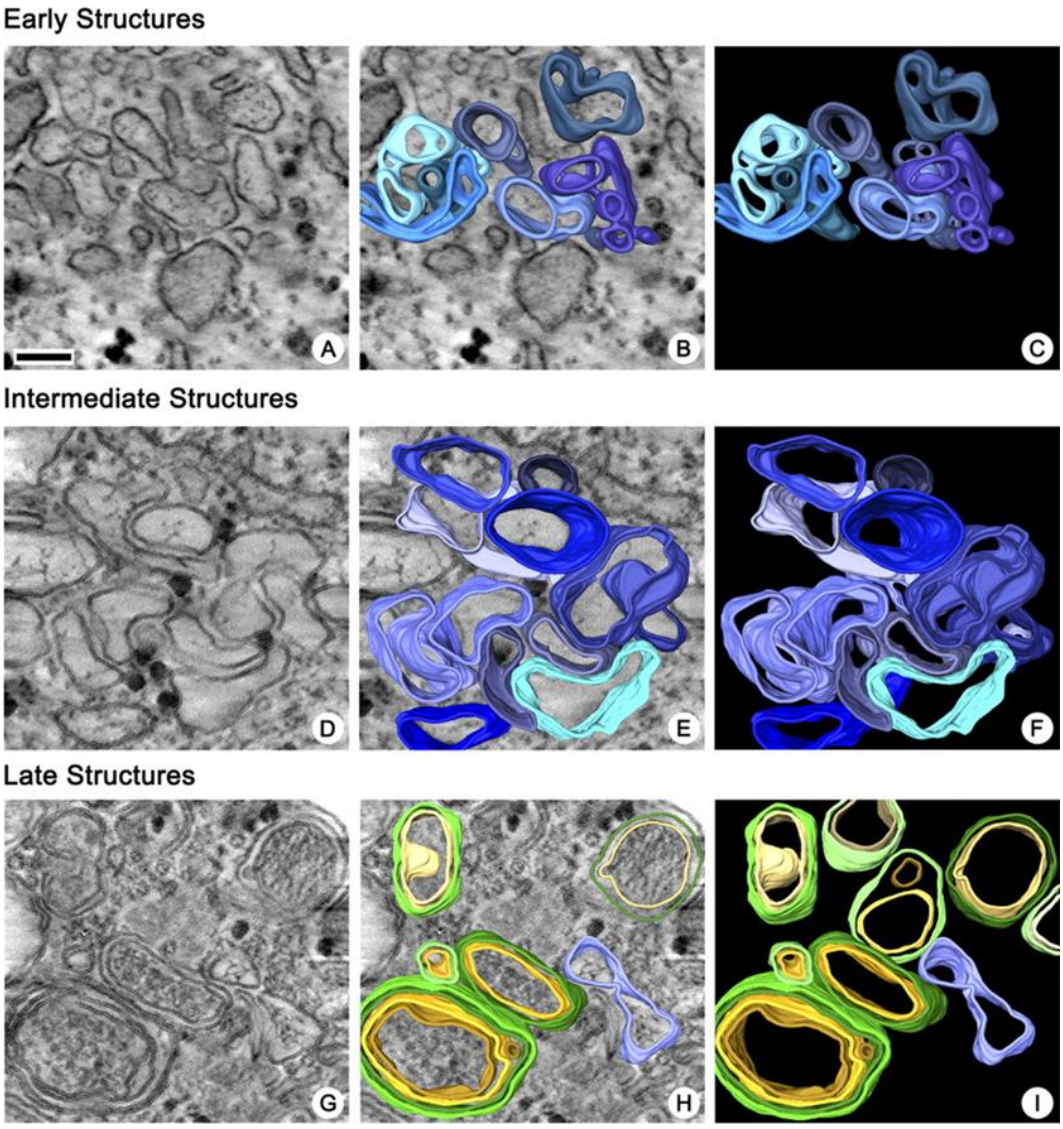


Figure 2.5: Reconstructions showing the 3-D architecture of poliovirus membranous replication complexes at the early (3 h.p.i.), intermediate (4 h.p.i.), and late (7 h.p.i.) stages of development. (A, D, and G) Central slices in tomographic volumes. (B, E, and H) Central slices with segmented overlays. (C, F, and I) Segmented volumes, with blue indicating single-membrane structures and yellow and green indicating inner and outer membranes of double-membrane structures, respectively. Bar, 100 nm. Figure adapted from George A. Belov et al (14)

Although the functional role of these membranous structures in the viral life cycles is not well understood, it is hypothesized that they may serve multiple purposes in the development of infection. Thus, they may increase local concentration of viral and host proteins associated with the replication, serve as a structural support for the proper assembly of the replication complexes and hide the replication complexes from cytosolic sensors of viral infection such as PKR, RGI-l and MDA5 (101).

2.6.2 Role of poliovirus proteins in the development of replication organelles

The expression of non-structural proteins of picornaviruses can induce the remodeling of intracellular membranes similar to those observed during infection (148). The poliovirus proteins 2B, 2C and 3A have membrane-targeting domains. Thus, to understand their role in the rearrangement of intracellular membranes, several group expressed various poliovirus polyprotein fragments or individual proteins with the help of a recombinant vaccinia virus. The expression of poliovirus non-structural proteins 2C or 2BC stimulated the modification and the proliferation of membranous structures similar to those found in infected cells (32). Interestingly, the introduction of mutations in the membrane-binding motif of either 2C or 2BC did not prevent the rearrangement and proliferation of membranous vesicles (32).

The expression of poliovirus proteins 3A alone or in combination with 2BC generated membranes vesicles structurally similar to those observed in infected cells (145). In other families of positive strand RNA viruses, the remodeling of pre-existing membranes could also be driven by the expression of non-structural proteins with hydrophobic signaling. In the family *Flaviviridae*, the rearrangement of ER is induced by the expression of the viral protein NS4A in the case of DENV and WNV. In the case of HCV, it was

shown that the expression of the combination of structural proteins core-E1-E2-p7 or the non-structural proteins NS3-4A and NS4B were capable to induce the formation of DMV or " membranous-web" (161). While the expression of the viral membrane-targeted proteins is capable of inducing membrane alterations, it is not clear if such membranous structures fairly represent membrane remodeling observed during infection or just share superficial morphological resemblance with the replication organelles.

2.6.3 Origin of poliovirus membranous replication organelles

The origin of the replication membranes vesicles is not well understood, although several proteins markers from various intracellular organelles (endoplasmic reticulum, lysosomes, cis- and trans-Golgi) have been found associated to these replication organelles in the cytosol of PV infected cells (141). This suggests that either these replication organelles may come from multiple sources, or that extensive mixing of the intracellular membrane materials is induced during PV infection. The mechanism of how cellular membranes are transformed into the replication organelles is not known. To date, the following intracellular pathways have been hypothesized to contribute to the development of these membranous structures: the secretory pathway, the autophagy pathway and the lipid synthesis and trafficking pathways.

2.6.3.1 The secretory pathway

The secretory pathway allows the transport of proteins and lipids between the ER where they are synthesized through Golgi complex to plasma membrane and other intracellular organelles. The COPII coated vesicles mediate the anteretrograde transport of proteins and lipids from the ER to the ER-Golgi intermediate compartment (ERGIC) (107).

COPI-coated vesicles mediate the retrograde transport between the ERGIC compartment and the ER and also within the Golgi complex (107).

Maynell *et al*. have shown that the replication of poliovirus was severely inhibited when cells were treated with brefeldin A (BFA) (97). BFA, a fungal metabolite, blocks the transport of proteins between the ER and Golgi complex via the prevention of the COPI-coated vesicle formation. The primary target of BFA is a family of guanine nucleotide exchange factors (GEFs) GBF1, BIG1 and BIG2 involved in the activation of Arf GTPases from GDP-bound inactive to the GTP-bound active form. Belov G *et al*. have shown that the depletion of GBF1 with siRNA significantly inhibited the replication of poliovirus whereas overexpression of GBF1 rescued the viral replication in BFA-treated cells (72). In another study, Belov *et al*. demonstrated that viral protein 3CD specifically promoted binding of BIG1/2 to the membranes, thus confirming the involvement of elements of the intracellular secretory pathway to poliovirus replication (10). Rust *et al*. have shown that Sec13, a component of COPII coat, co-localized with viral protein 2B in poliovirus-infected cells (134). Gazina *et al*. have demonstrated differential sensitivity of three genera of picornaviruses to brefeldin A (BFA).

The replication of echovirus 11 (EV11) was strongly inhibited while parechovirus 1 was partially sensitive to the presence of BFA (56). The replication of encephalomyocarditis virus (EMCV), a cardiovirus, was not altered in the presence of BFA (56). Similarly, O'Donnell *et al*. demonstrated that the replication of the Foot and Mouth Disease virus (FMDV), an aphtovirus, was insensitive to the presence of BFA (111). Surprisingly, although BFA was able to block the replication of poliovirus and some other picornaviruses, Belov *et al*. showed that the expression of poliovirus proteins from a non-

replicating RNA was able to generate membranous vesicles in the presence of BFA (72). Thus, while elements of the secretory pathway are important for poliovirus replication, they are not likely to be directly involved in the structural development of the replication organelles.

2.6.3.2 The autophagy pathway

The cellular autophagy pathway has also been proposed to be an important source of membranes supporting the replication of poliovirus (145). Autophagy is an intracellular catabolic pathway targeting damaged organelles and proteins for recycling (9). This pathway is activated by cellular starvation, pharmacological agents such as rapamycin, tamoxifen or viral infections (87). Autophagy is also a key branch of the innate immune response, involved in targeting cytosolic pathogen such as viruses and bacteria for degradation (9). During autophagy, damaged organelles and protein aggregates are engulfed in a crescent shape membranes termed phagophores in the cytosol (9). The extremity of these phagophores will eventually fuse to form enclosed double membranes vesicles termed autophagosomes (9). These autophagosomes will fuse with endosomes to form amphisomes or with lysosomes to generate autolysosomes. Following the formation of autolysomes, their contents will be degraded by lysosome enzymes (9).

The appearance of DMV that resemble cellular autophagosomes in the cytoplasm of PV-infected cells prompted Schlegel *et al.* to hypothesize that some elements of the autophagy pathway were involved in the formation of the replication organelles (141). Jackson *et al.* have shown that LC3, a key regulator of autophagosome formation was recruited to the replication complex of poliovirus and co-localized with viral protein 3A (73). In the same study, they demonstrated that the stimulation of autophagy in polio-

infected cells with pharmacological inducers such as tamoxifen or rapamycin, promoted viral replication, whereas knockdown of LC3 or atg12 proteins, inhibited the extracellular yield of poliovirus (73). With these results, Kirkegaard and Jackson hypothesized that the DMV or autophagosomes-like structures appearing in the cytoplasm of enteroviruses-infected cells may participate in a non-lytic release of cytoplasmic viruses (83). In this regard, recent work by Bird *et al.* showed via quantitative time-lapse microscopy the release of cytoplasmic materials of PV-infected cells in the absence of cells lysis (19). The result further supports the idea that the autophagy pathway could be involved in a non-lytic release of PV infectious particles.

Other members of the picornaviruses family have also been shown to use elements of this pathway to support their replications. The Coxsackie virus group B3 (CVB3), another enterovirus closely related to poliovirus, uses elements of the autophagy pathway to promote its replication. Wong *et al.* have observed the proliferation of DMV in the cytoplasm of CVB3 infected cells and increased processing of LC3 proteins (162). The induction of the autophagy with rapamycin increased the replication while its inhibition with 3-methyladenine or siRNAs targeting key proteins required for autophagosomes formation severely reduced the replication of CVB3 (162). In that same study, while they observed a strong activation of autophagy during CVB3 infection, they also showed that the inhibition of autophagosome-lysosome fusion significantly improved the replication (162). The increased lipidation of LC3-I proteins to LC3-II, the association of elements of this pathway with viral proteins, the proliferation of DMV were observed in various picornaviruses-infected cells such as FMDV, EMCV and EV71 (70, 110).

Although, the appearance of double membrane vesicles had suggested a possible role of components of autophagy in the development of infection, other studies produced conflicting data on the role of this pathway in the replication of picornaviruses. Whereas Klein and Jackson demonstrated that the activation of the autophagy with rapamycin efficiently improved the replication of the human rhinovirus type 2, Zaruba *et al* showed that the induction of autophagy does not affect the production of the same virus (84, 148). More recently, Alirezaei *et al.* demonstrated that coxsackievirus exploited the LC3 protein in an autophagy dependent or independent manners to provoke a massive rearrangement of membranes for replication (109). These observations demonstrated that at least some elements of the autophagy pathway are involved in the picornavirus life cycle but whether they directly support the development and/or functioning of the replication organelles or primarily facilitate virion maturation and/or non-lytic virus release at the end of infection is still actively debated.

2.6.3.3 The lipid metabolism pathways.

Poliovirus infection profoundly alters cellular lipid metabolism pathways. Cornatzer *et al.* were the first to show the modification of phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and phosphatidylinositol metabolism during the development of poliovirus infection (92). In that study, they demonstrated that the synthesis of various phospholipids species increased dramatically after 240 minutes following infection with poliovirus type 1 (92). Other studies by Penman in 1965 and by Dales and Amako in 1967 showed that infection with poliovirus or mengovirus enhanced the uptake of lipid precursors such as choline (35, 91). The imported choline was readily incorporated in the plasma membrane, endoplasmic reticulum, nuclear envelope and

mitochondria membranes and was also found in membranous fraction associated with the viral replicase activity (35, 91).

Mosser *et al*. demonstrated that the incorporation of lipid precursors such as choline and glycerol in poliovirus-infected cells correlated with smooth-surfaced membrane proliferation associated with formation of the viral replication complexes (49). Vance *et al*. later demonstrated that poliovirus infection stimulated the rate of phosphatidylcholine biosynthesis in Hela cells (153). The study was followed by Choy *et al*, who showed that the surge of phosphatidylcholine synthesis in poliovirus-infected cells correlated with an increased concentration of CTP nucleotides, which in turn stimulated the activity of the phosphatidylcholine rate limiting enzymes

CTP: phosphocholine cytidylyltransferase (CCT) (34). In the same direction, Guinea and Carrasco have shown that the continuous synthesis of phospholipids was required for an efficient replication of poliovirus (61). The alteration of the lipid metabolic pathway and the corresponding proliferation of membranous structures in the cytoplasm seem to be shared by many picornaviruses. Thus, Schimmel and Traub observed an increased synthesis of phosphatidylcholine in mengovirus-infected cells. These observations led them to speculate that the observed upregulation of lipids could be associated to the proliferation of smooth cytoplasmic membranes needed for the replication (70). Recent studies have shown that membranes enriched with specific glycerophospholipid facilitate the non-lytic release of infectious poliovirus particles. In this regard, Chen *et al* have shown that following infection, mature poliovirus particles were packaged in phosphatidylserine-enriched vesicles then non-lytically released from infected cells (24).

Together with upregulation of synthesis of structural phospholipids such as phosphatidylcholine and phosphatidylserine, picornavirus infection modifies the distribution and synthesis of a signaling lipid phosphatidylinositol-4-phosphate (PI4P) and cholesterol. Hsu *et al* have shown that during poliovirus and CVB3 infection, host factor phosphatidylinositol-4-kinase IIIβ (PI4KIIIβ) is selectively recruited to the replication organelles resulting in high enrichment of these structures in PI4P (66). Co-immunoprecipitation demonstrated that PI4KIIIβ associates with 3A, 3AB, and 3D in the replication complexes in CVB3-infected cells. Depletion of PI4KIIIβ with siRNA treatments or the inhibition of its activity with pharmacological agent PIK93 profoundly reduced replication (66). IInytska *et al.* found that PV and CVB3 hijacked the clathrin-mediated endocytosis pathway to direct cholesterol from the plasma membrane and the extracellular medium to the replication organelles, where it regulates polyproteins processing and promotes viral RNA synthesis (71). The same group demonstrated that the depletion of intracellular or the plasma membrane free cholesterol inhibited the replication of PV, CVB3 as well as the development of the replication organelles (71).

The alterations of the lipid metabolism coupled to the remodeling of intracellular membranes have been reported for a wide range of families of positive-strand RNA viruses. It was shown that following HCV infection, a member of the *Flaviviridae* family, the phosphatidylinositol-4-kinase IIIα (PI4KIIIα) enzyme was recruited to the replication complexes to synthesize the PI4P (15). Berger *et al.* also showed that the inhibition of PI4KIIIα with pharmacological agents or silencing with siRNA treatments severely decreased HCV replication and membranous web formation (15). HCV infection

upregulates the synthesis of phosphatidylcholine and phosphatidylethanolamine to stimulate membranes syntheses in infected cells (39). It was shown that the fatty acid synthetase (FASN) and the Acetyl-CoA carboxylase 1 (ACACA) enzymes, two key regulators of fatty acid synthesis, were required for the replication DENV and WNV, the other members of the *Flaviviridae* family (65, 94). The activity and the protein level of FASN increased dramatically in HCV infected cells. The flock-house virus (FHV), a positive-stranded RNA virus of the *Nodaviridae* family is known to induce formation of invaginated vesicles or spherules serving as replication organelles (80). It was shown the FHV modulated the glycerophospholipid metabolic pathway and the inhibition of phospholipid synthesis severely decreases its replication as well as that of the Tomato bushy stunt virus (TBSV), a plant positive-strand RNA virus (80, 143).

Taken together, the accumulated data demonstrate that activation of phospholipid synthesis and retargeting of lipid trafficking pathways support structural development of the replication organelles of diverse positive RNA viruses.

2.7 The phospholipid synthesis pathway

2.7.1 Overview

Cellular membranes are made of a combination of different lipids. Glycerophospholipids, sterols, and sphingolipids are the principal structural lipids of eukaryotic membranes (47). Among the glycerophospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) constitute 50% and 25%, respectively, of the phospholipid mass in eukaryotic cells (66). The synthesis of glycerophospholipids, which happens in the ER and Golgi, requires the supply of long chain fatty acids and head groups

such as choline, ethanolamine or serine. Fatty acids are the main constituent of the hydrophobic domains of membranes.

There are three major sources of fatty acids available for lipid synthesis: mobilization from lipid droplet storage depot, the *de novo* synthesis of fatty acids by FASN and import from the extracellular medium. For fatty acids to enter the metabolic or the catabolic pathways, the synthesis of fatty acyl-CoA is a prerequisite step, and this process is facilitated by acyl-CoA synthetases. This activation confers hydrophilic properties to the previously hydrophobic molecule. Composition of long chains fatty acids determines the physical properties of cellular membranes such as fluidity, permeability and flexibility. The head group of phospholipids such as choline must be provided as a vitamin, while serine and ethanolamine can be synthesized *de novo*, at least by some cell types, but are also actively imported from the extracellular medium. Upon their importation, they must be phosphorylated to enter the lipid metabolic pathway.

2.7.2 Glycerophospholipid biosynthesis

2.7.2.1 Acyl CoA synthetase enzymes

The long chain acyl-CoA synthetases (ACSL), the very long chain fatty acid transport protein (FATP), and the acyl-CoA synthetases bubblegum (ACSBG) are enzymes responsible for the transport and activation of long chain fatty acids. The human genome possesses thirteen genes encoding these enzymes (123). The ACSL family has five isoforms, the FATP family has six isoforms and the ACSBG two isoforms each encoded by a separate gene (123). The acyl-CoA synthetase enzymes differ by their substrate specificities for FAs, sub-cellular locations, and tissue expression (65). ACSLs and FATPs are located on almost all intracellular organelles: the ER, mitochondria, peroxisomes, lipid

droplets and the plasma membrane (65). ACSBG is mainly localized in the cytoplasm, the mitochondria or microsomes of brain cells (123). The long chain acyl-CoA synthetases control the transport of FAs across cellular membranes. The import of fatty acids across the plasma membranes proceeds via passive diffusion and can be facilitated by fatty acids transporter proteins located in the plasma membranes (21). Contrary to ACSL, FATP and ACSBG enzymes, FAs transporter proteins such as acyl-CoA binding proteins (ACBP), fatty acid binding proteins (FABP) and fatty acid translocase (FAT/CD36) do not possess acyl-CoA synthetase activities. However, they regulate the concentration of free fatty acids and free fatty acyl-CoAs in the cytoplasm.

The import of fatty acids from extracellular milieu is a reversible process unless they are activated by ACSL, FATP and ACSBG enzymes or bound by cytosolic fatty acids transporter proteins (21). The activation of FAs by acyl-CoA synthetases is a two-step reaction requiring energy form ATP hydrolysis (65).

FA + ATP→ acyl- AMP + PPi

Acyl - AMP + CoA → Acyl - CoA + AMP

Fatty acyl-CoAs are transported towards various metabolic sites such as ER for the synthesis of phospholipids, or mitochondria for β- oxidation. Overexpression studies and knock-down experiments of acyl-CoA synthetases have shown that some isoforms channel fatty acids to specific metabolic pathways (123).

2.7.2.3 Phosphatidic acid biosynthesis

The endoplasmic reticulum is the major site of glycerophospholipid biosynthesis. This process starts with the conversion of glycerol-3-phosphate (G3P) into phosphatidic acid (PA) by acyl transferase enzymes (79). Similarly to acyl-CoA synthetases, these enzymes differ by their substrate specificities, sub-cellular locations, and tissue expression (147). The glycerol-3-phosphate acyltransferase (GPAT) and the acylglycerolphosphate acyltransferase (AGPAT) enzymes are membranes-associated proteins with multiple isoforms, mainly located in the ER and the mitochondria. The GPAT protein has four isoforms (GAPT1-4) and AGPAT protein has close to ten isoforms (AGPAT1-10) (147). The glycerolphosphate acyltransferase (GPAT) catalyzes the synthesis of lysophosphatidic acid (LPA) by attaching a fatty-acyl to the sn-1 position of the G3P (147). Following the synthesis of LPA, the acylglycerolphosphate acyltransferase enzymes (AGPAT) will synthesize the phosphatidic acid (PA) by catalyzing the attachment of the second fatty acyl chain at the sn-2 position of the LPA (147).

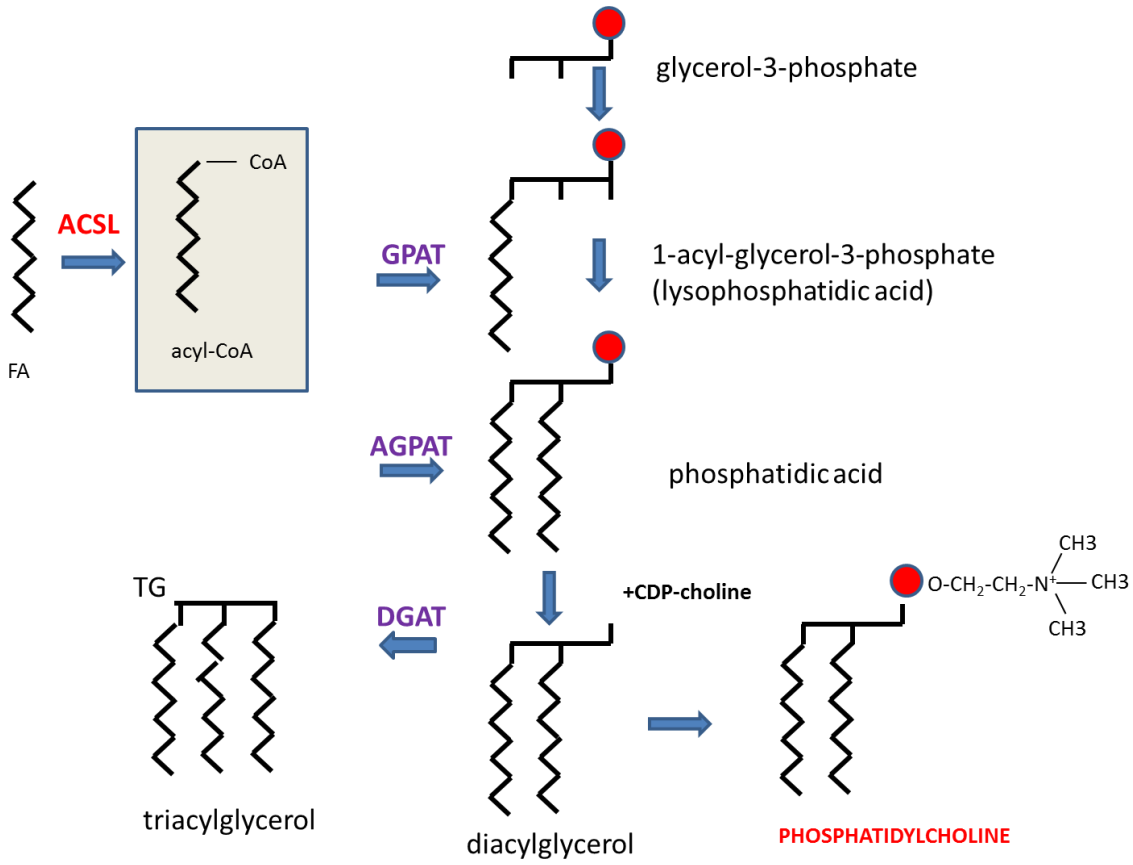


Figure 2.6: Scheme of the glycerophospholipid biosynthesis (Source: courtesy of Dr George Belov)

2.7.2.4 Phosphatidylcholine biosynthesis

Following the synthesis of phosphatidic acid, the phosphatidic acid phosphatase (PAP) enzyme catalyzes the dephosphorylation of PA to yield the diacylglycerol (DAG). The DAG serves as a substrate for the transfer of phosphocholine, phosphoethanolamine or the phosphoserine group, for the synthesis of phosphatidylcholine, phosphatidylethanolamine or phosphatidylserine respectively (147). The DAG molecule can also serve as a substrate for the synthesis of triacylglycerol (TAG) which is then stored in lipid droplets and this process is catalyzed by the diacylglycerolphosphate (DGAT) (147). Two pathways lead to

the biosynthesis of PC in cells, the CDP-choline pathway also termed the Kennedy pathway, and the phosphatidylethanolamine-methylation pathway, which is significant only in hepatocytes (80).

Three enzymes are involved in the CDP-choline pathway: the choline kinase (CK), the phosphocholine cytidylyltransferase (CCT) which is the rate limiting factor in this pathway and finally the CDP-choline phosphotransferase enzymes (80). The CK initiates the first step in the phosphorylation of choline, and then the CCT catalyzes the transfer of a phosphate molecule to choline-phosphate to yield the CDP-choline (154). Finally, the CDP-choline phosphotransferase enzyme catalyzes the transfer of phosphocholine from the CDP-choline donor molecule to a diacylglycerol molecule to form the phosphatidylcholine (154). This enzyme is mainly located on the ER membranes and on the Golgi, MAM as well as nuclear membrane.

CCT enzymes are expressed from two distinct genes: PCYT1a, which encodes CCT alpha (CCTα), and PCYT1b which encodes up to three isoforms CCT beta 1, 2 and 3 (CCTβ1, CCTβ2, CCTβ3) (72). CCTα, the predominant form regulating PC syntheses is mostly found in the nucleus in its soluble state while CCTβ is a cytosolic resident protein (91, 92). CCTα, upon activation, relocates from the nucleus to intracellular organelles such as the ER where it drives the synthesis of PC. The CCTα gene has approximately 26 kb spanning 9 exons encoding specific domains. Exon 2 encodes the nuclear localization signal as well as the translation start site, exons 4-7 encode the catalytic domain; the membrane-biding domain which has an alpha helical structure is encoded by exon 8 and finally, the C-terminal phosphorylation domain is encoded by exon 9 (154). The C-terminal phosphorylated domain and the membrane-binding domain regulate CCTα activity. The

inactive form of CCTα is extensively phosphorylated, however, it is dephosphorylated following activation and association with intracellular membranes (154).

2.8 Goals of the study

The upregulation of phospholipids syntheses and the proliferation of replication membranes is a shared hallmark of picornaviruses-infected cells. The goals of this project were:

i. To understand the mechanism of the upregulation of phospholipid synthesis upon picornavirus infection.
ii. To investigate the involvement of the ER stress response and the autophagy pathways in the modulation of lipid metabolism in infected cells.
iii. To investigate the role of the activation of phosphatidylcholine synthesis in the development of the replication organelles and poliovirus propagation.

Chapter 3: Materials and Methods

3.1 Cells and viruses

Hela cells and Human embryonic kidney (HEK) 293 cells were grown in Dulbecco Modified Eagle's Medium (DMEM) containing 1X of penicillin/streptomycin, sodium pyruvate (1mM, Sigma), L-glutamine (2mM, Sigma) and supplemented with 10% heat-inactivated fetal bovine serum (FBS). Poliovirus types 1 Mahoney (PV1), Coxsackievirus B3 (CBV3) and Encephalomyocarditis virus (EMCV) were propagated on Hela cells and the titer was determined by the standard plaque assay. Cells in monolayer were washed once with a serum-free DMEM, and then the virus was added with the desired multiplicity of infection (MOI) for 30mins in the room temperature. The infectious medium consisted of a serum-free DMEM supplemented with 50mM of Hepes at a PH 7.4. Following the adsorption, infected cells were incubated in a standard growth medium or serum-free medium according to the experimental design.

To monitor poliovirus infection in the absence of PC synthesis, Hela cells were maintained in DMEM supplemented with 10% of heat-inactivated fetal bovine serum (FBS). The next day, the cultured medium was replaced by choline-free Earle's balanced salt solution (EBSS) supplemented with non-essential amino acid mix (Sigma) and L-glutamine (Sigma). After 48h of incubation in a choline-depleted medium, cells were washed once with EBBS solution then infected with a purified poliovirus kept in a 1X PBS and at the required MOI. The purified poliovirus was isolated via the cesium chloride centrifugation assay. The infectious medium was made of EBSS solution with 50mM of Hepes pH 7.4. After 30mins of adsorption, infected cells were incubated according to the experimental design in the absence or in the presence of 200μM of choline in a standard

medium, in a serum-free EBSS or in EBSS supplemented with10 % of FBS. At the required time post infection, infected cells were processed for microscopic imaging

3.2 Viral titration

Hela cells infected grown in choline-free EBSS for 48h on a 12 wells plate were infected at 10PFU/cell then incubated in the presence or absence of choline. At 6hpi, the infectious supernatant was collected to assess extracellular infectious particles and infected cells were processed (freeze and thaw) to release intracellular infectious particles. For the plaque assay, Hela cells grown in a 6cm plate were infected with various dilutions of supernatant or intracellular infectious particles between 10^{-5} and 10^{-9}**.** The viral titer was evaluated by counting the number of plaques and the average from two wells were assessed. This formula was used to assess the titer:

pfu/mL = Average of number of plaques/ Dilution factor * Volume of diluted virus added to the plate

3.3 Plasmids.

Plasmids pTM-2A-3D, pTM-2B-3D, pTM2C-3D coding for the corresponding fragments of poliovirus cDNA under transcriptional control of T7 promotor and translational control of EMCV IRES were a gift from Dr. Natalya Teterina (NIH). PXpA-RenR plasmid coding for polio replicon with *Renilla* luciferase gene substituting capsid region of polio genome was described elsewhere (10). Plasmid pTM-2Amut-3D containing mutation C109A in the 2A sequence was produced by point mutagenesis of the 2A sequence in pXpA-P2P3 (12) and recloning the PstI-SpeI fragment into pTM-2A-3D. For plasmid pXpA-SH-PV2A-HA coding for the full length polio cDNA containing 2A with HA tag the SpeI-SnaBI fragment containing 2A-HA was generated by two sequential

overlapping PCRs and recloned into pXpA-SH (12). Plasmid pTM-2A-HA for vaccinia-based expression of 2A protein with HA tag PmlI-SalI fragment containing part of EMCV IRES and the whole coding sequence for 2A-HA was synthesized by Life technologies and was cloned into pTM-1 vector (gift from Dr. Natalya Teterina, NIH). All polio constructs were verified by sequencing. Plasmid pGFP-Acsl3-HA was generously provided by Dr. Joachim Füllekrug, University of Heidelberg, Germany.

3.4 Fatty acids import assay

To monitor the import of fatty acids, infected and mock-infected cells were incubated in the presence or absence of serum according to the experimental design. At a specific time, post infection, the incubation media was replaced by a pre-warm serum free media containing 0.4 μM of bodipy 500/520 C4-C9 (Molecular Probes) for 30mins at 37°C. Cells were fixed with 4% paraformaldehyde for 20 mins, washed with 1XPBS pH 7.4 and processed for microscopic imaging or fluorescence reading with TECAN infinite M1000 plate reader.

3.5 Fatty acid import competition assay.

HeLa cells were grown on a 96 well tissue culture plate with transparent bottom overnight and the next day infected with poliovirus at 50 PFU/cell. After infection the cells were incubated either in standard or serum-free media as indicated. At 4 h p. i. the media was replaced with the same type of pre-warmed media supplemented with 0.4 μM of bodipy-FA label, 50 μM of the competitor fatty acid and 1μg/ml of cell-permeable DNA stain Hoechst 33342. After 30 min incubation the cells were washed with PBS and fixed with 4% formaldehyde in PBS and the Hoechst and bodipy-FA fluorescence were read in TECAN Infinite M1000 plate reader at excitation/emission 340/455 and 490/520

respectively. The bodipy-FA signal was normalized to Hoechst to account for variability in cell density and the data for each fatty acid are averaged form 12 wells. The data are expressed as percentage of the signal from control mock-infected cells incubated without any competitor fatty acid.

3.6 Acyl-CoA synthetase activity measurement.

HeLa cells grown on T75 flasks were harvested, washed 3 times with cold PBS and re-suspended in STE buffer (8.5% sucrose, 10 mM Tris-HCl pH8. 0.5 mM EDTA) supplemented with protease inhibitors cocktail (Sigma-Aldrich). Cells were lysed by twice freeze-thawing and the protein concentration of the lysates was determined by Bradford method. The assay mix containing 500 nM Bodipy 500/510 C4-C9 substrate solubilized with α-cyclodextrin (10 mg/ml in 10mMTris-HCI pH 8.0), 40mM Tris-HCI pH 7.5, 10mM ATP, 10mM $MgCl_2$, 0.2 mM CoA, and 0.2 mM dithiothreitol was assembled on ice, and the reaction was started by addition of an aliquot of cell suspension containing 60 µg of total protein. Duplicate reactions were incubated at 37C for 20 min and terminated by the addition of Dole's solution (isopropanol: heptane: 2N H_2SO_4 40:10:1). Newly synthesized fluorescent acyl-CoA was recovered in aqueous phase after 4 extractions with heptane and the fluorescence was measured by TECAN Infinite M1000 plate reader.

3.7 Lipid extraction.

Lipid extraction was performed according to Folch method (50). Cells grown on T75 flasks were harvested, resuspended in STE buffer (8.5% sucrose, 10 mM Tris-HCl pH8. 0.5 mM EDTA) and the protein concentration of cell suspension was determined by Bradford method. An aliquot of cell suspension containing 1500 µg protein was adjusted

to 250 µl by STE in a glass tube and 3.75 ml of chloroform: methanol (2:1) with 5 mM HCl mix was added. The tube was vortexed for 30", then 0.75 ml H2O was added, and the tube was vortexed again for 30" and centrifuged at 1500 rpm in a tabletop centrifuge for 5 min. The top aqueous phase was discarded and lower organic phase was extracted 3 more times with Folch's theoretical upper phase (chloroform: methanol: H2O 3:48:47). After extractions lipid-containing organic phase was dried under a stream of nitrogen gas. Lipids were re-suspended in 50 µl of chloroform before loading on TLC plates.

3.8 Butanol extraction of Bodipy-FA

To extract the fluorescent bodipy-FA from poliovirus- and mock-infected cells or cells treated with tunicamycin, thapsigargin and mock-treated, labelled cells were washed once then incubated with 1 mL of versen at 37 degree C. Following the incubation, 100uL of cells from each sample was collected to assess the concentration of proteins and the remaining 900ul was spun down at max speed for 5mins to collect cells pellet and discard the supernatant. Then, 500ul of butanol was added in each sample then incubated at room temperature. Following 10 min incubation, these samples were spun down and the supernatant was collected to measure the fluorescence. Then, 50ul of supernatant was distributed in a 96 wells plate which was then placed in a plate reader Infinite Tecan M1000 machine to assess the fluorescence of bodipy-FA at excitation/emission of 490/520.

3.9 Thin-layer chromatography (TLC).

Thin layer chromatography glass silica plates (Analtech) were prewashed with chloroform: methanol (1:1) and air-dried. Polar lipids were separated in chloroform: ethanol: water: triethylamine (30:35:7:35) and neutral lipids were separated in hexane:

ether: acetic acid (80:20:1). Phospholipids were stained with Phospray (Sigma Aldrich) and neutral lipids were detected with bromothymol blue spray (Sigma Aldrich).

3.10 Propargylcholine labelling

To monitor the synthesis of phosphatidylcholine, monolayers of Hela cells were infected with poliovirus at a required multiplicity of infection. At 5hpi, polio-infected cells were supplied with a medium containing 200uM of propargylcholine plus Hoechst in a mixed of L-glutamine (Sigma), amino acid (sigma) and the Earl's balanced salt solution (EBSS). After 1 hour of incubation, the labelled cells were fixed with 4% of paraformaldehyde (PFA) for 25mins then washed three times with 1X PBS. The next day, labelled cells were stained with 5uM of Alexa Fluor 488 (lifetechnologies) via the click it "chemistry" protocol (lifetechnologies).

3.11 Western Blot analysis

Confluent monolayer cells were infected or mock infected with virus at a specific MOI or treated or mock treated with different drugs and incubated for a specific amount of time. According to the experimentation design, cells were washed with a cold PBS once then incubated with a mild cells lysis buffer (0.1M Tris-HCL pH 7.8; 0.5% Triton-X100) containing a protease inhibitor (Sigma-Aldrich) at a room temperature on a shaking incubator for 20mins. Cells lysates were collected and centrifuged at 4 C at a maximum speed for 5mins to collect the supernatant. The lysate was kept at -80 C for further processing for a SDS-PAGE western blot analysis. The western blot analysis was done on a 12-15% SDS-PAGE gels composed of H2O, ultra-pure 40% Protogel or Acrylamide: Bis-Acrylamide solution (Rational diagnostic), TEMED or N,N,N',N'-tetramethyl-

ethylenediamine (Rational diagnostic), 10% SDS (Cellgro), 1.5M Tris-HCL, pH8.8 and 10% ammonium persulfate or APS (Rational diagnostic).

Proteins were transferred on an immune-blot polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Following the transfer, the membrane was sequentially incubated with the blocking buffer for then with the primary antibodies. Multiple membrane stripping was performed to analyze the expression of both viral and host proteins. The attachment of the primary antibodies was identified with either an anti-mouse or an anti-rabbit antibody attached to horseradish-peroxidase. The detection of proteins on the Immune-Blot membrane was done with ECL Select western blotting detection reagent (GE Healthcare).

3.12 Digitonin permeabilization assay

To examine membranes-associated proteins, Hela cells grown on a 12 wells plate were infected and incubated for 4hours in growth media. Following the incubation, infected and mock-infected cells were washed once with KHM buffer (110 mM K-acetate, 2 mM MgCl2, 20 mM HEPES-KOH, pH 7.4) then incubated for 5 mins in 50ug/mL of fresh digitonin solution in KHM buffer. After that cells were washed twice with KHM buffer then lysed with mild lysis buffer (0.1M Tris-HCl pH 7.8; 0.5% Triton-6100) supplemented with protease inhibitors cocktail (Sigma-Aldrich). The lysate cleared by low-speed centrifugation was used for western blot analysis.

3.13 Vaccinia-T7 expression system

Purified recombinant vaccinia virus expressing T7 RNA polymerase (VT7-3 [40]) was a gift from Dr. Natalya Teterina, NIH. The day before experiment HeLa cells were transfected with pTM- or pXpA-based plasmids coding for fragments of polio cDNA under transcriptional control of T7 RNA polymerase promoter and translational control of EMCV

or polio IRES respectively, with Mirus 2020 DNA transfection reagent according to manufacturer's protocol. The next day cells grown on glass cover-slips were infected with vaccinia VT7-3 virus at a multiplicity of 10 PFU/cell in serum-free DMEM for 1 hour at 37 C and then incubated for 4 more hours in standard growth media. After that the media was changed to pre-warmed serum free media supplemented with 5 mM of fluorescent fatty acid analog Bodipy 500/510 C4–C9 and the cells were incubated for 30 more minutes, then washed once with PBS and fixed with 4% formaldehyde in PBS and processed for microscopy analysis.

3.14 Polio replicon replication assay.

Polio replicon assay was performed as described in (11) with minor modifications. Briefly, HeLa cells grown on 96-well plates were transfected with polio replicon RNA with mRNA trans-it kit (Mirus) and incubated in TECAN Infinite M1000 plate reader at 37C for 16 hours in standard growth media supplemented with 30□M live cell Renilla substrate EnduRen (Promega). Measurement of Renilla luciferase activity was taken every hour post transfection, and the signal for each sample is averaged from at least 8 wells of the 96 well plates.

3.15 Antibodies.

Anti-Acsl3 mouse polyclonal antibodies were from Abnova; anti-Acsl5 mouse monoclonal antibodies were from Abcam; rabbit polyclonal anti-FATP3, anti-FATP4, anti-Acs bubblegum 2 were described in (75, 121, 122). Mouse monoclonal anti-polio 2B, 2C and 3A antibodies were a gift from Prof. K. Bienz, University of Basel, Switzerland. Rabbit polyclonal anti-polio 3D antibodies were made by Chemicon using recombinant polio 3D protein as immunogen. Secondary anti-mouse and anti-rabbit highly cross-

adsorbed goat antibodies conjugates with Alexa 350, Alexa 498 and Alexa 595 were from Molecular Probes.

3.16 Reagents.

Fluorescent fatty acid Bodipy 500/510 C4-C9 (bodipy-FA) was from Molecular probes. Unlabeled long chain fatty acids, Coenzyme A (CoA), ATP and neutral and phospholipid standards were from Sigma-Aldrich. Phosphatidylinositol 4 phosphate from porcine brain was from Avanti Polar Lipids. Fatty acid stock solutions were prepared in DMSO. Neutral and phospholipids were dissolved in chloroform.

3.17 siRNAs.

SiGenome siRNA pools and corresponding individual siRNA oligos targeting human long chain acyl-CoA synthetases were from Dharmacon. HeLa cells were plated at 10000 cells/well in a 96 well plate and transfected with siRNA with Dharmafect 1 transfection reagent (Dharmacon) according to manufacturer's recommendations. After 72 hours of incubation with siRNA the cells were used for polio replicon replication assay. Toxicity of siRNA treatment was assessed with Cell-Titer Glo luminescent assay (Promega).

3.18 Microscopy.

For immunofluorescent microscopy cells were fixed with 4% formaldehyde in PBS for 20 min. The cells labeled with Bodipy 500/510 C4-C9 were incubated with primary and secondary antibodies in 0.02% saponin in PBS with 5% FBS for 1 hour and were washed 3 times with PBS after each incubation. Secondary antibodies conjugated to Alexa 350 were used for Bodipy 500/510 C4-C9-labeled cells since Bodipy 500/510 emits strong

fluorescence in both green and red spectra. Images were taken with Zeiss Axiovert 200M fluorescent microscope equipped with Axiocam Mrm monochrome digital camera.

To observe the association of newly synthesized PC with membranes, polio and mock-infected cells labelled with propargylcholine were fixed with 4% PFA. Propargylcholine-labelled cells were incubated for 1 hour each with primary then secondary antibodies in 0.02% saponin in 1X PBS with 5% FBS. Cells were washed 3 times each with 1X PBS between incubation. Because propargylcholine staining with Alexa fluor 488 had a green signal, the detection of viral antigen was done with Alexa fluor 595 (red signal) and the detection nucleus done with Hoechst 33342 (Blue signal).

3.19 Electron microscopy

Hela cells were seeded in at a concentration of 125000cells/well in a 24 wells plate. The next day, the growth medium was changed from a choline-enriched DMEM plus FBS to choline-deprived Earle's balanced salt solution (EBSS) plus L-glutamine and amino-acid. Following a 48 hours of starvation in a choline-depleted medium, cells were polio and mock-infected at a MOI of 50PFU/cell with a purified poliovirus for 30 minutes then incubated in the presence or absence of choline. At 4 h.p.i, the incubation medium was replaced by a fixing solution of CaCl2 in PIPES.

Chapter 4: The development of replication organelles is linked to a strong import of fatty acids and the increased activity of acyl-CoA synthetase enzymes in poliovirus-infected cells

Parts of this chapter were published in Nchoutmboube JA, Viktorova EG, Scott AJ, Ford LA, Pei Z, et al. (2013) Increased Long Chain acyl-CoA Synthetase Activity and Fatty Acid Import Is Linked to Membrane Synthesis for Development of Picornavirus Replication Organelles. PLoS Pathog 9(6)

4.1 Introduction

Picornaviruses like all eukaryotic (+) RNA viruses, require association of the replication machinery with cytosolic membranes also called replication organelles (RO). It has been hypothesized these RO served multiple purposes in the course of the viral life cycle to facilitate the replication and propagation. They may serve as a structural scaffold for the formation of the replication complex, protect the replication complex from the cytosolic sensors of innate immune system and may help to increase the local concentration of viral and host proteins associated with the replication complexes. In case of picornaviruses, the development of these membranous structures has been attributed to the modification of the autophagy pathway, the cellular secretory pathway and the lipid metabolic pathway.

The modification of these intracellular processes in poliovirus-infected cells has been described in the literature review (section 2.6.3). Not all picornaviruses rely on the secretory pathway for their replication, and similarly conflicting data support the importance of the autophagy pathway in the development of picornaviruses infections. Contrary to the secretory and the autophagy pathways, modification of the phospholipid synthesis has been consistently associated with the development of picornavirus infections. Synthesis of phosphatidylcholine, the most abundant phospholipid of cellular membranes,

is upregulated in many picornavirus-infected cells (27, 35, 70, 153). However, the mechanism of phosphatidylcholine and other phospholipid synthesis and its role during infection are not well understood.

In this report, we demonstrated that poliovirus-infected cells manifest a dramatic fast increase in import of long chain fatty acids. In infected cells, imported fatty acids are rapidly incorporated into newly synthesized membranes, while in mock-infected cells they are destined for storage in lipid droplets. Mock-infected cells preferentially import long saturated fatty acids while in infected cells the spectrum shifts towards shorter and unsaturated fatty acids. Transport of fatty acids is intrinsically linked to their activation by acyl-CoA synthetases. We demonstrate a strong increase of acyl-CoA synthetase activity in infected cells and provide evidence that long chain acyl-CoA synthetase 3 (ACSL3) is the host protein involved in activation of fatty acids in infected cells.

We identify poliovirus protein 2A as responsible for activation of fatty acid transport independent of its protease activity. Increased acyl-CoA synthetase activity in infected cells shifts the cellular lipid-synthesizing machinery towards production of new membranes and allows mobilization of fatty acids from all available sources – imported from external media, synthesized within the cell or released from lipid droplets. Moreover, we show that import of long chain fatty acids is strongly activated upon infection of diverse picornaviruses in different cell types. Our data explain earlier observations of increased phospholipid synthesis in infected cells, provide a simple model for development of the membranous scaffold of replication complexes, and show how poliovirus can change overall cellular membrane homeostasis by targeting one critical process.

4.2 Results

Poliovirus infection increases the import of exogenous fatty acids and prevents their targeting to lipid droplets. The synthesis of phospholipids, the major constituents of cellular membranes, requires supply of precursor molecules including long chain fatty acids (154). To understand the metabolism of fatty acids upon infection, we used a fluorescent fatty acid analog bodipy 500/510 C4, 9 (bodipy-FA). Bodipy-FA is a synthetic fluorescent fatty acids which mimics a FA of 18 carbons backbone and is readily metabolized by living cells to synthesize various lipid species (98). Hela cells were poliovirus-infected at a multiplicity of infection (m.o.i) of 50 PFU/cell to ensure simultaneous development of infection, then labelled at 4 hours post infection (h.p.i) with bodipy-FA for 30mins in serum-free media.

The result showed a strong import of bodipy-FA in poliovirus-infected cells (Figure 4.1 A and B) compared to mock-infected cells (Figure 4.1 A and B). The imported bodipy-FA accumulated in a characteristic perinuclear ring in poliovirus-infected cells (Figure 4.1 C). In mock-infected cells, the label was distributed in multiples dots-like structures around the nucleus in the cytoplasm (Figure 4.1 E mock and D). These dots-like structures were later identified as lipid droplets as evidenced by co-localization of bodipy-FA with Adipocyte Differentiation-Related Protein (ADRP) a known marker of lipid droplets (159) (Figure 4.1 D).

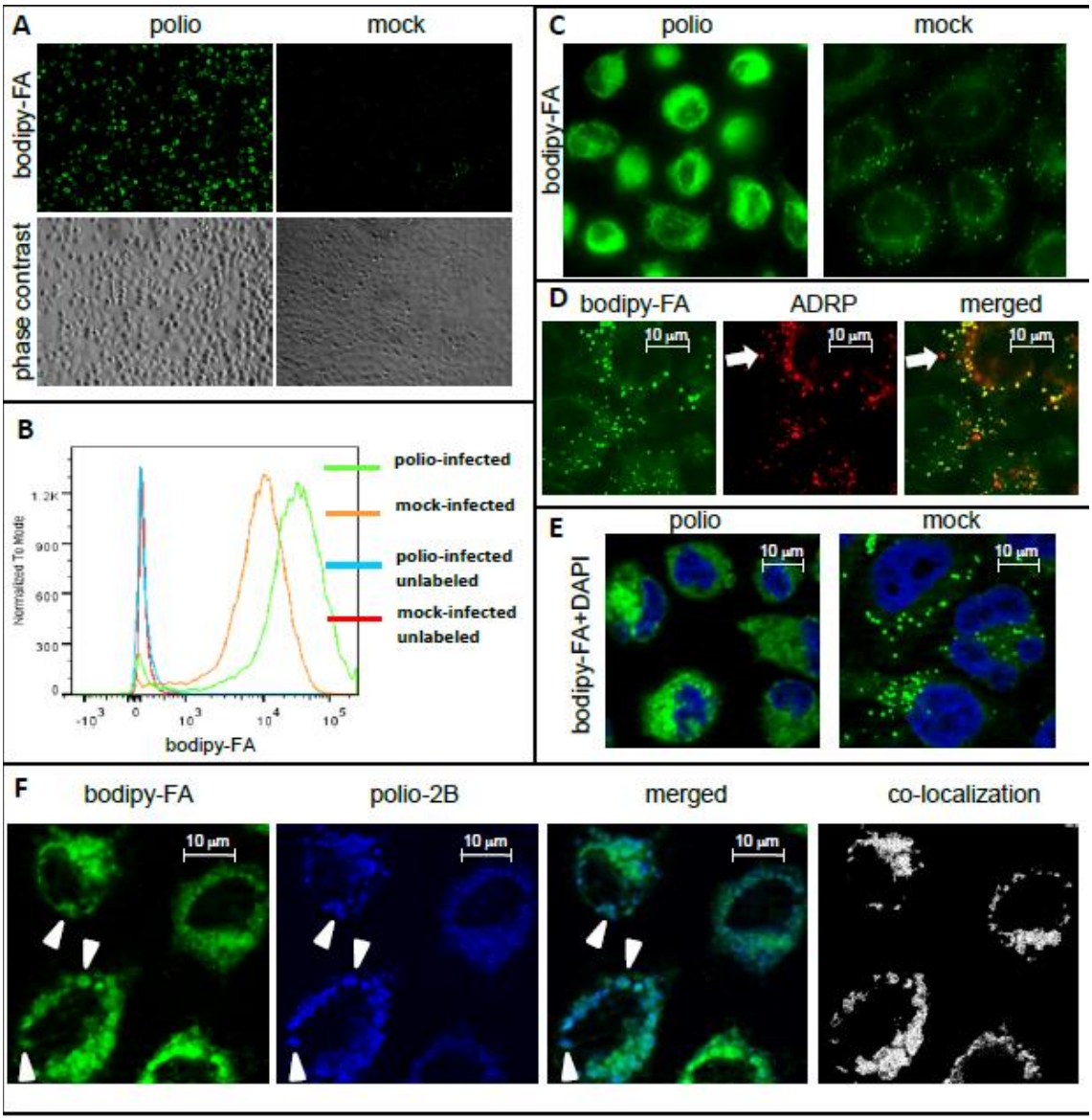

A
polio
mock
bodipy-FA
phase contrast
B
polio-infected
mock-infected
polio-infected unlabeled
mock-infected unlabeled
Normalized To Mode
bodipy-FA
C
polio
mock
bodipy-FA
D
bodipy-FA
ADRP
merged
10 µm
E
polio
mock
bodipy-FA+DAPI
10 µm
F
bodipy-FA
polio-2B
merged
co-localization
10 µm

Figure 4.1: **Poliovirus induces a strong import of fatty acids. A)** low magnification view of mock and polio-infected cells; **B)** FACS analysis of mock and polio-infected cells; **C)** Higher magnification of mock and polio-infected cells showing bodipy-FA probe distributed in lipid droplets of mock and into membranes of infected cells; **D)** Confocal images of Hela cells transfected with pmCherry-ADRP and labeled with bodipy-FA; **E)** Confocal images of mock and polio-infected Hela cells labeled with bodipy-FA and Hoechst 33-342; **F)** confocal images of mock and polio-infected Hela cells labeled with bodipy-FA then processed and stained for polio protein 2B

To determine whether the imported fatty acids were used for the synthesis of membranes of the replication organelles, we stained cells for poliovirus membrane protein 2B, a component of poliovirus replication complex (32). Staining of cells for 2B revealed an extensive co-localization of the fluorescent bodipy-FA and the replication organelles (Figure 4.1 F). This result demonstrated that import of long chain fatty acids is upregulated in polio-infected cells and that they were channeled towards the membranes of the replication organelles, whereas in mock-infected cells, the imported FA accumulated in lipid droplets.

Imported fatty acids in infected cells are channeled towards the synthesis of phosphatidylcholine. To identify the metabolic destination of the imported fatty acids, HeLa cells were infected with poliovirus (mock-infected) at 50 PFU/cell and pulse-labeled for 30mins with the bodipy-FA at 4hpi in the presence or in the absence of serum. Following the incubation, labeled cells were processed for lipid extractions. The extracted lipids were resolved by thin layer chromatography (TLC) using two solvent systems optimized for the separation of either neutral or polar lipids. The chromatogram was photographed in a fluorescence imager to identify newly synthesized lipids and then stained for total lipid content. We did not observe significant differences in the fluorescent label targeting cells incubated in the presence of serum, other than a weaker signal as it could be expected because of competition of serum lipids with bodipy-FA import.

The signal from cells labeled in the absence of serum in the TLC optimize for the resolution of neutral lipids revealed targeting of Bodipy-FA to triglyceride synthesis in mock-infected cells (Figure 4.2 A, compare lanes 3 and 4), consistent with microscopic observations. Virtually no fluorescent compounds were resolved in neutral lipid system

from the material isolated from poliovirus-infected cells; the lipids extracted from infected cells remained as bright spots located at the loading position (Figure 4.2: A lanes 1 and 3). This result indicated that the synthesis of neutral lipids was inhibited in poliovirus-infected cells.

Conversely, in the TLC system optimized for polar lipid resolution, poliovirus-infected cells revealed a strong signal of phosphatidylcholine synthesis (Figure 4.2: B lanes 1 & 3). Similar to neutral lipids, the total of polar lipids content extracted from polio and mock-infected cells incubated was not significantly different. Thus, PV infection does not only increase the level of FA import but modifies their metabolic channeling by down-regulating the synthesis of neutral lipids and redirecting the newly imported FAs for the highly up-regulated production of PC.

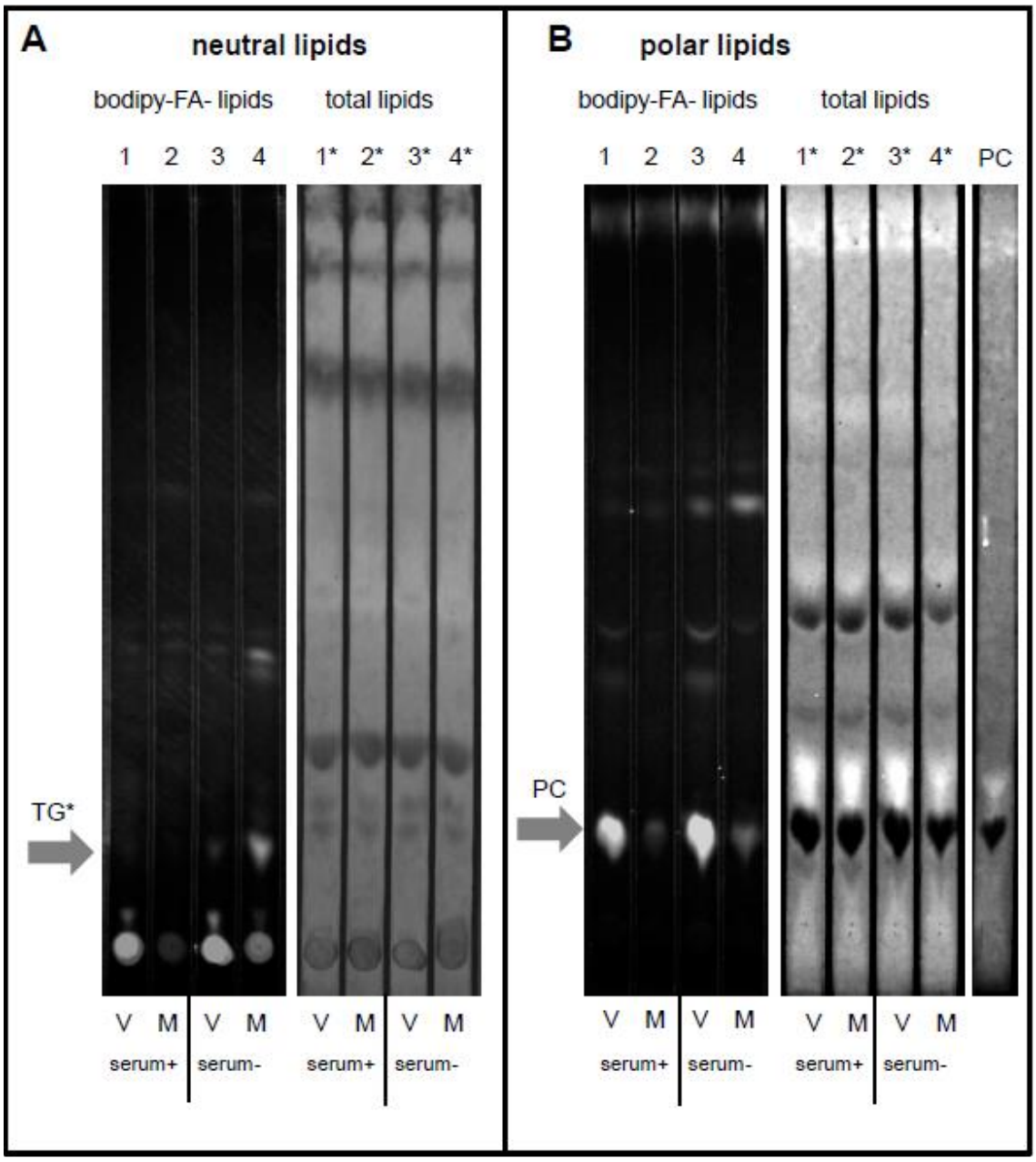

A
neutral lipids
bodipy-FA- lipids
total lipids
1 2 3 4 1* 2* 3* 4*
TG*
V M V M V M V M
serum+ serum- serum+ serum-
B
polar lipids
bodipy-FA- lipids
total lipids
1 2 3 4 1* 2* 3* 4* PC
PC
V M V M V M V M
serum+ serum- serum+ serum-

Figure 4.2: **In infected cells, synthesis of neutral lipids is shut down and imported fatty acids are directed to strongly stimulated synthesis of phosphatidylcholine**. HeLa cells were infected (V) or mock-infected (M) with poliovirus at 50 PFU/cell and incubated in medium with or without serum as indicated. Bodipy-FA was added for 30 min at 4 h p. i.; after that total lipids were extracted and resolved by thin layer chromatography. **A**. Neutral lipids resolved in hexane: ether: acetic acid (80:20:1) system. Bodipy-FA-lipids represent fluorescent fatty acid-containing lipids synthesized during 30 min labeling period. Total lipids show all neutral lipids stained with bromothymol. **B**. Polar lipids resolved in chloroform: ethanol: water: triethylamine (30:35:7:35) system. Bodipy-FA-lipids represent fluorescent fatty acid-containing lipids synthesized during 30 min labeling period. Total lipids show all phospholipids stained with Phostain. PC: 2-Oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (phosphatidylcholine marker)

Long chain acyl-CoA synthetase activity increases in poliovirus-infected cells and exhibits substrates preference for shorter fatty acids. The import and sequestration of long chain fatty acids in the cytosol is intimately linked to the activity of long chain acyl-CoA synthetase enzymes (ACSL) (123). The ACSL enzymes catalyze the activation of hydrophobic fatty acids to yield hydrophilic acyl-CoA molecules, which are then channeled towards various cellular metabolic pathways including synthesis of membrane lipids (123). To monitor the activity of ACSL enzymes, lysates of Hela cells infected at a multiplicity of 50 PFU/cell and incubated at different times post infection in the absence of serum were collected. Bodipy-FA was added to the lysates, incubated for 20 mins, and the hydrophilic acyl-CoAs were separated from the hydrophobic input bodipy-FA. Lysates of infected Hela cells displayed an increase in ACSL activity as early as 2 h.p.i and continued to steadily increase through 6 h.p.i (Figure 4.3: A).

The cellular ACSL enzymes preferentially activate certain types of fatty acids however the substrate specificity is not strict (123). To identify the substrate preference of ACSL activated upon infection, we performed a fatty acids competition assay. Infected (mock-infected) Hela cells were incubated at 4 h.p.i for 30 mins with bodipy-FA in the presence of a 125x molar excess of a competitor FAs. In this assay, if the FA competitor was the preferred substrate, this would result in decrease of bodipy-FA import and thus in reduction of the fluorescence signal. The control poliovirus-infected cells displayed the strongest incorporation of bodipy-FA of more than 250% relative to mock-infected cells regardless of the presence or absence of serum. The unsaturated fatty acids added in mock-infected cells incubated with serum did not affect the import of bodipy-FA (Figure 4.3: C). Conversely in the absence of serum, we observed a noticeable increase of bodipy-FA

import in mock-infected cells incubated with linolenic (C18:3) or linoleic (C18:2) fatty acids compared to control (Figure 4.3: E) and which likely reflects stimulation of triglyceride synthesis by non-saturated fatty acids, a well-known effect. In poliovirus-infected cells incubated with oleic (18:1), linoleic (C18:2) and linolenic (C18:3) fatty acids there was no stimulation of bodipy-FA incorporation but rather a small reduction of bodipy-FA import in the absence or presence of serum (Figure 4.3: C and E).

In mock-infected cells incubated with serum, saturated fatty acids did not influence the incorporation of the bodipy-FA (Figure 4.3: B). In mock-infected cells incubated in the absence of serum, the strongest competitor was the palmitic acid (C16:0) (37% of mock control) followed by myristic acid (C14:0) (22% of mock control) (Figure 4.3: D). In contrast, myristic acid (C14:0) was the best competitor in polio-infected cells reducing the import of FAs in poliovirus-infected cells from 270% to 150% in the presence of serum and from 250% to 130% in the absence of serum (Figure 4.3: B) followed by palmitic acid which reduced the import of bodipy-FA from 250% to 190% in the presence of serum and from 270% to 220% in the absence of serum (Figure 4.3: D). This result showed that total ACSL activity is increased in poliovirus-infected cells and that its substrate specificity is modified showing that different enzymes are responsible for the bulk of ACSL activity in infected and non-infected cells.

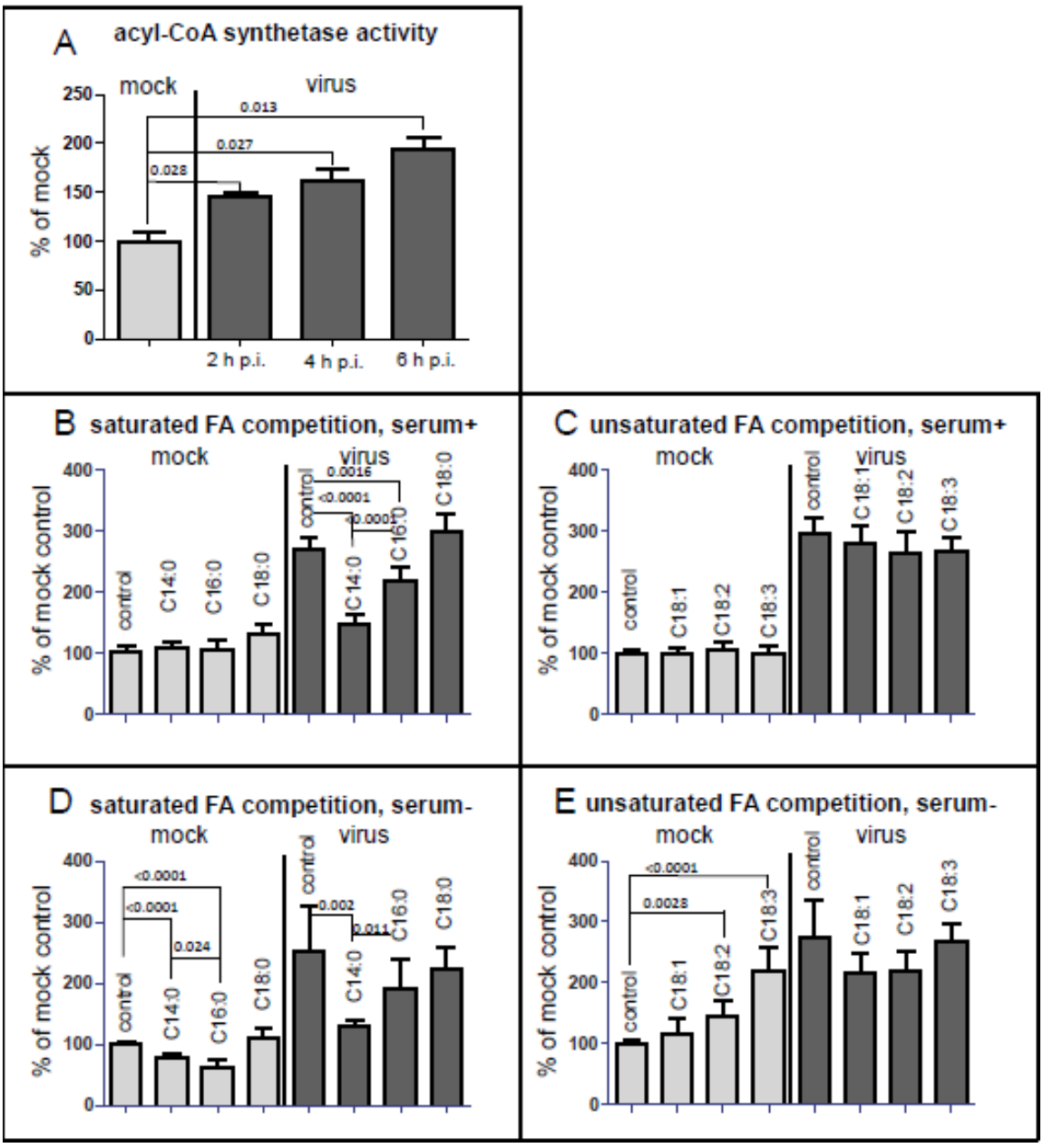
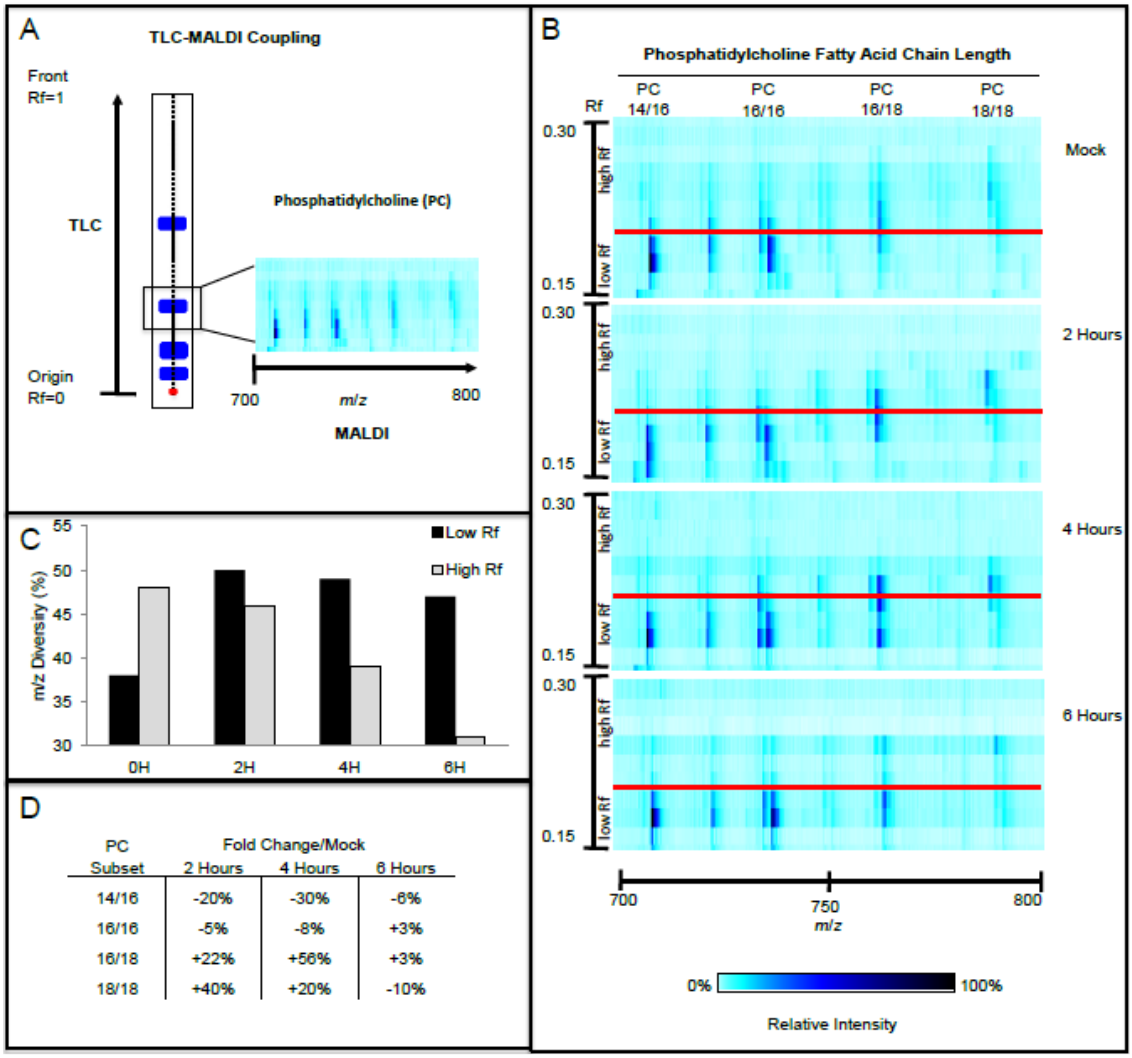

A
acyl-CoA synthetase activity
mock
virus
% of mock
0.013
0.027
0.028
2 h p.i.
4 h p.i.
6 h p.i.
B saturated FA competition, serum+
% of mock control
control
C14:0
C16:0
C18:0
C16:0
0.0016
C C unsaturated FA competition, serum+
C18:1
C18:2
C18:3
D saturated FA competition, serum-
0.024
0.002
0.011
E unsaturated FA competition, serum-
0.0028

Figure 4.3: Modulation of long chain acyl-CoA synthetase activity in poliovirus-infected cells. **A**. Acyl-CoA synthetase activity is stimulated as early as 2 h p. i. and continues to increase during the time-course of infection. Acyl-CoA synthetase activity in vitro assay was performed with lysates of HeLa cells collected at indicated times post infection, the data are normalized to the activity of the lysate from mock-infected collected at 2 h p. i., p-values are shown. **B. and C**. Fatty import competition assay performed with the cells incubated in serum- supplemented medium during the whole experiment. HeLa cells were infected (or mock-infected) with poliovirus at 50 PFU/cell; at 4 h.p.i. bodipy-FA was added for 30 min in the presence of 1256 molar excess of the indicated long chain fatty acids. No competitor fatty acid was added to control samples. The data are normalized to the signal from the mock-infected control sample; p-values are shown. **D. and E**. Fatty import competition assay performed with the cells incubated in serum-free medium during the whole experiment. HeLa cells were infected (or mock-infected) with poliovirus at 50 PFU/cell; at 4 h.p.i. bodipy-FA was added for 30 min in the presence of 1256molar excess of the indicated long chain fatty acids. No competitor fatty acid was added to control samples. The data are normalized to the signal from the mock-infected control sample; p-values indicating significant differences are shown

Spectrum of PC molecules synthesized in poliovirus-infected cells is different form that in non-infected cells. We observed a strong import of bodipy-FA, an increased ACSL activity, a change in its substrate specificity and an upregulation of PC synthesis in poliovirus-infected cells. We further investigated whether the change in the ACSL substrate specificity could result in the synthesis of different species of PC molecules. To this end, we combined two methods: first, the TLC (used above) to identify and characterize phospholipids through their pattern of migration and second, the matrix assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) which allows mass identification of molecules. The PC molecules were identified and characterized through their pattern of migration on TLC then the reflection positive mode MALDI-TOF-MS was used to scan the TLC lane (Figure 4.4: A).

The mass to charge ratio (*m/z*) was used to secondarily identify the major PC molecules and acyl chain variants (Figure 4.4: B). Early in the infection, we observed an increased diversity of PC molecules with short acyl chain variants at low Rf chromatography zone of the TLC lane whereas at high Rf, there was a drop in the diversity of PC molecules containing C18 chains (Figure 4.4: C). The analysis of the individual PC classes demonstrated a fast shift in the composition of PC species following poliovirus infection. Thus at 2 h.p.i, we observed a significant increase of PCs species with C18/C18 and C16/C18 acyl chains variant and conversely a sharp drop with C16/C16 and C14/C16 compared to mock-infected cells. This trend continued later in the infection at 4 h.p.i where PC molecules with C16/C18 acyl chains significantly increased compared to mock-infected cells (Figure 4.4: D). Changes in lipid abundance at 6 h.p.i do not follow the

general trends observed at 2 and 4 h.p.i likely due to the significant degree of cell lysis observed at this late stage of infection at high MOI.



PC Subset	Fold Change/Mock 2 Hours	4 Hours	6 Hours
14/16	-20%	-30%	-6%
16/16	-5%	-8%	+3%
16/18	+22%	+56%	+3%
18/18	+40%	+20%	-10%

Figure 4.4: Shift in phosphatidylcholine spectrum following infection demonstrated by TLC-MALDI. **A**. Schematic representation of TLCMALDI, blue spots represent phospholipid migration on TLC plate, hash marks represent stepwise MALDI data capture. **B**. HeLa cells were infected with poliovirus at 50 PFU/cell and processed for the total lipid extraction at the indicated time points post infection. TLC-MALDI data shown at phosphatidylcholine (PC) migration (Rf) range, fatty acid chain lengths noted, intensity of signal at respective mass to charge ratios (m/z) (blue scale). **C**. PC diversity (unique m/z signatures) shifts in abundance from higher Rf to lower Rf during time course of infection. **D**. Percent change of PC subset/PC total ratio compared to mock. Results from a representative experiment are shown

Once activated, the increase of FA import in infected cells does not depend on active viral RNA replication or translation and is independent of new cellular genes expression. Poliovirus infection rapidly shuts down intracellular metabolisms such as the host gene transcription, nucleo-cytoplasmic trafficking and translation initiation (43). Here, we investigated whether the continuing expression of viral proteins and the viral RNA synthesis is required for increased import of bodipy-FA by infected cells. To this end, we monitored the import of bodipy-FA in the presence of guanidine-HCL (GUA), a known inhibitor of poliovirus replication, or cycloheximide (CHI), an inhibitor of eukaryotic translation (2, 104). Cells were infected at the MOI of 50 PFU/cell and incubated for a period of 3.5h, then we replaced the medium with a fresh medium containing 2mM of GUA or 10µg of CHI. After 30 mins of incubation to allow inhibitors to enter cells, the medium was again replaced with the labelling medium also containing the respective inhibitors and bodipy-FA. After 30mins of incubation, cells were fixed and processed for microscopic imaging.

Thus, the cells were exposed to GUA or CHI for a total of 1h (control cells were incubated without the inhibitors but underwent the same media changing procedures). The import of bodipy-FA in cells treated with an inhibitors of poliovirus replication GUA or translation inhibitor CHI was similar to that in control (Figure 4.5: D, E and F). We also monitored the import of bodipy-FA in poliovirus infected cells treated with actinomycin D (AMD), a known inhibitor of nuclear transcription, which was shown not to affect poliovirus replication (Figure 4.5: A) (69, 140). Prior to poliovirus infection, cells were pre-incubated for 30mins in the presence or absence (control) of AMD and the inhibitor was present for the whole time thereafter during infection and bodipy-FA labeling. The

activation of bodipy-FA import was not affected by the presence of AMD (Figure 4.5: B and C).

These results show increase of FA import in infected cells does not depend on new expression of cellular genes and likely relies on activation of pre-existing cellular factors by viral proteins

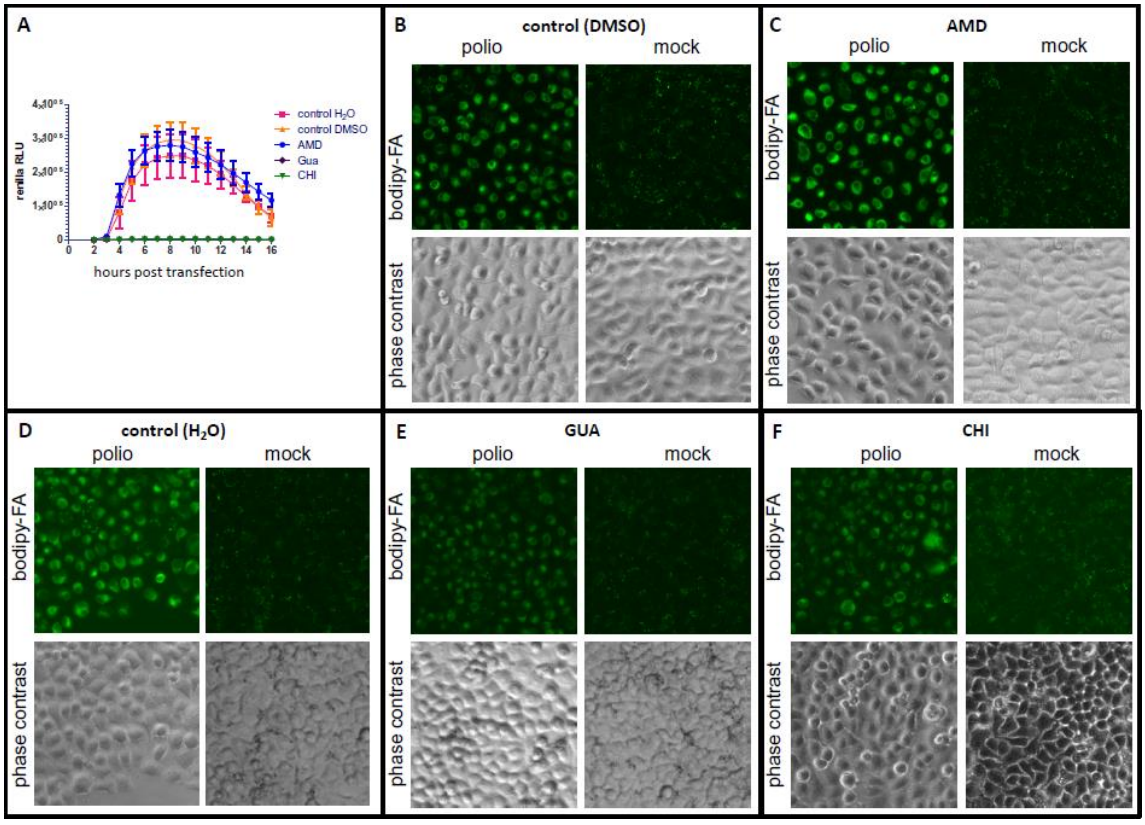


Figure 4.5: The fatty acid import does not depend on the active viral replication, translation and is independent of genes expression. A. Effect of the inhibitors on polio replicon replication. HeLa cells grown on a 96 well plate were transfected with a polio replicon RNA with the Renilla luciferase gene substituting capsid region. Luminescence was monitored in live cells incubated with Endu-Ren substrate added in the media. Actinomycin D (AMD, an inhibitor of nuclear transcription) was added to the cells for 30 min before the replicon transfection and was present in the incubation media thereafter at 5 µg/ml. Cycloheximide (CHI, an inhibitor of mRNA translation) and Guanidine-HCl (Gua a specific inhibitor of polio replication at this concentration) were added at the time of transfection at 10 µg/ml and at 2 mM respectively. Equivalent amount of DMSO (solvent for AMD) and water (solvent for CHI and Gua) were added to the control cells**. B. and C**. HeLa cells were pre-incubated for 30 min with 5 µg/ml AMD) (equivalent amount of the DMSO

solvent was added to the control cells) and infected with poliovirus at 50 PFU/cell. After 4 hours incubation in the standard media in the presence of AMD (DMSO in control) the cells were labeled for 30 min with 0.4 μM bodipy-FA in pre-warmed serum-free media also in the presence of the inhibitor (DMSO in control). After the incubation with the label, the cells were washed with PBS and fixed with 4% formaldehyde in PBS. Fluorescence (bodipy-FA) and phase contrast images are shown. **D–F**. HeLa cells were infected with poliovirus at 50 PFU/cell and incubated in standard growth media for 3.5 h; after that, the media was replaced with pre-warmed media containing: **D**. (control) equivalent amount of water (solvent for CHI and Gua) **E.** 10 μg/ml CHI. F. 2 mM GUA. The cells were incubated for 30 more min, and then the media was replaced with pre-warmed serum-free media containing the same inhibitors (water in control) and supplemented with 0.4 μM of bodipy-FA label. After 30 min incubation with the label, the cells were washed with PBS and fixed with 4% formaldehyde in PBS. Fluorescence (bodipy-FA) and phase contrast images are shown.

ACSL3 is required to support effective poliovirus replication. To understand the role of acyl-CoA synthetase enzymes in the replication of poliovirus, we performed poliovirus replicon replication assay in Hela cells treated with pools of siRNAs targeting all thirteen human long chain acyl-CoA synthetase genes. Of all treatments, only siRNA targeting FATP5 gene was severely toxic to cells thereby eliminated for further consideration (Figure 4.6). We observed a strong decrease of poliovirus replication of about 80% following knockdown of ACSL3, FATP3 and the ACSBG2 genes expression without compromising the viability of the cells (Figure 4.6). However, treatment of cells with individual siRNAs from FATP3 targeting pool did not result in a significant reduction of FATP3 expression.

Similarly, the effect of anti-ACSBG2 siRNAs was non-specific since the enzyme is not expressed in HeLa cells. Anti-ACSL3 siRNAs on the other hand demonstrated specific inhibition of ACSL3 expression (not shown), thus the siRNA knock-down experiments suggested an important role of ACSL3 enzyme in poliovirus replication. To further characterize the inhibitory effect of ACLS3, additional treatments of Hela cells with various pools of siRNAs targeting ACSL3 significantly reduced the replication of poliovirus replicon (Figure 4.7: B).

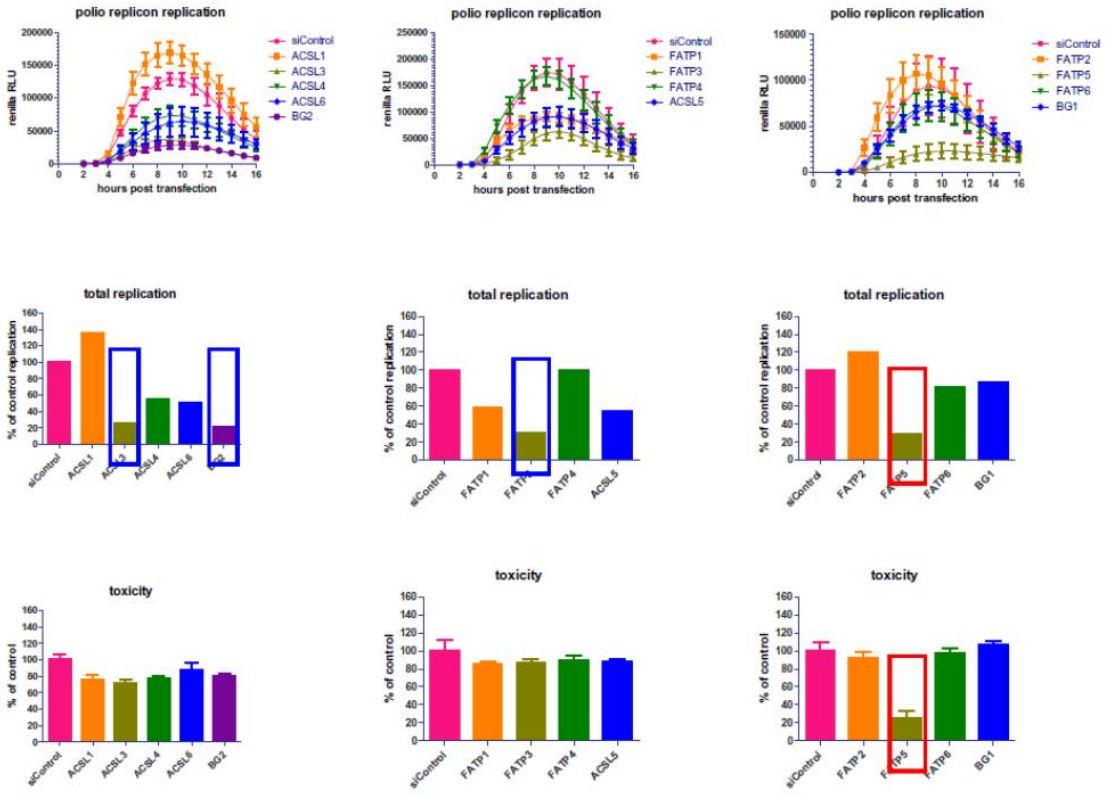


Figure 4.6: HeLa cells grown on 96 well plates were transfected with siGenome siRNA pools targeting all known human long and very long chain acyl-CoA synthetases, 16 wells for each siRNA pool. siControl scrambled siRNA (Dharmacon) served as a control. After 72 h incubation with siRNA polio replicon replication assay was performed. Total replication is calculated as area under curve using Prizm software and the data are displayed as percentage of control. Toxicity was measured after replicon replication assay. siRNAs exhibited the strongest effect on replication (Acsl3, Acs BG2 and FATP3) are outlined in blue boxes. The toxic FATP5 siRNA is outlined by the red box.

Our previous results demonstrated that the infection-induced increase of long chain fatty acid import does not require new cellular gene expression, suggesting that it relies on post-translational activation of pre-existing ACSL enzymes. Here we wanted to monitor possible post-translational modifications and/or changes in membrane association of ACSLs upon poliovirus infection. The amount of fatty acid transport protein 4 (FATP4) and ACSL5 protein did not vary in the course of infection (Figure: 4.7: A), however at the same time we observed proteolytic cleavage of FATP3 and to a lesser extend ACSL3 protein (Figure 4.7: A). In spite of some cleavage, the total amount of FATP3 and ACSL3 did not vary significantly between control and poliovirus-infected cells. Activity of ACSL enzymes depend in many cases on their association with specific membranes (95). Poliovirus infection induces the reorganization of intra-cellular membranes and the redistribution of host proteins.

To monitor a possible redeployment of these enzymes following poliovirus infection, we performed a digitonin permeabilization assay. Digitonin extracts cholesterol from membranes thus permeabilizing cholesterol-reach plasma membrane and leaving intracellular cholesterol-poor membranes relatively intact. Cytosolic proteins are eluted from digitonin-treated cells; thus this method allows assessment of changes in membrane association of proteins. The viral membrane-associated proteins 2C and 2BC were recovered and the soluble protein $3D^{Pol}$ was partially depleted as expected in digitonin-treated cells, confirming the efficiency of permeabilization (Figure 4.7: A + digitonin). The association of ACSL3, FATP4 and ACLS5 with intracellular membranes remained unchanged in the course of infection since these proteins were recovered following the digitonin treatment (Figure 4.7: A + digitonin). On the other hand, we observed a depletion

of FATP3 at 4 and 6 h.p.i, suggesting that its association with intracellular membranes was unstable in the course of poliovirus infection (Figure 4.7: A + digitonin, arrowhead).

To further characterize the specific role of ACSL3 on the replication of poliovirus, a rescue experiment was performed to thwart the inhibitory effect of anti-ACSL3 siRNA by expressing a resistant ACSL3. Hela cells were co-transfected with the anti-ACSL3 siRNA and pCI-ACSL3^{r} plasmid coding for an ACSL3 sequence with a mutated siRNA targeting site. The polio replicon assay performed following a DNA transfection revealed that the expression of the resistant ACSL3 genes rescued the polio replication (Figure 4.8).

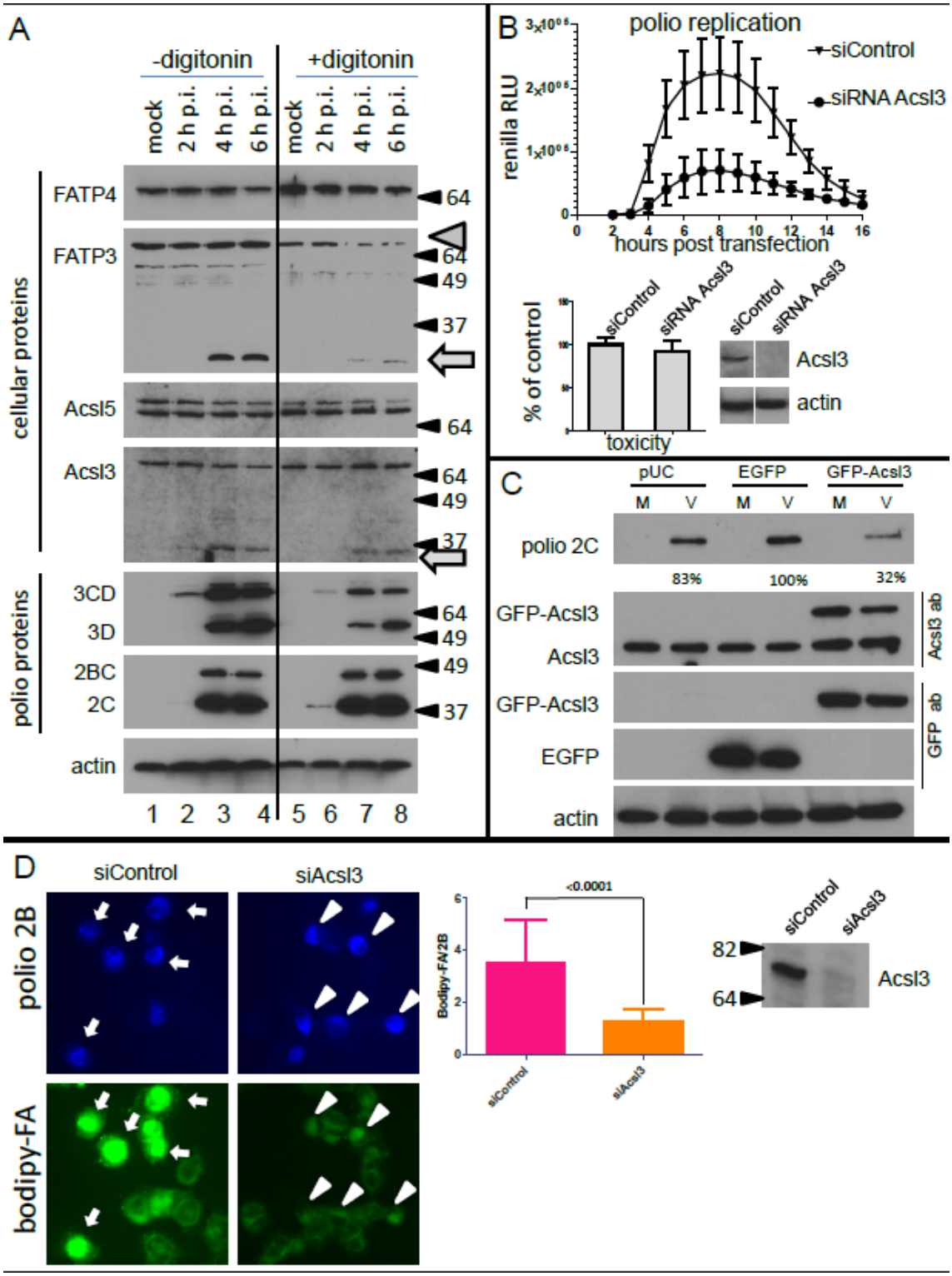

A
-digitonin
+digitonin
mock
2 h p.i.
4 h p.i.
6 h p.i.
cellular proteins
FATP4
FATP3
Acsl5
Acsl3
polio proteins
3CD
3D
2BC
2C
actin
64
49
37
1 2 3 4 5 6 7 8
B
polio replication
siControl
siRNA Acsl3
renilla RLU
hours post transfection
% of control
toxicity
Acsl3
actin
C
pUC
EGFP
GFP-Acsl3
M
V
polio 2C
83%
100%
32%
GFP-Acsl3
Acsl3
Acsl3 ab
EGFP
GFP ab
actin
D
siControl
siAcsl3
polio 2B
bodipy-FA
Bodipy-FA/2B
<0.0001
82
64

Figure 4.7: Cleavage and redistribution of long chain acyl-CoA synthetases in infected cells and requirement of functional Acsl3 for polio replication and FA import. **A.** HeLa cells infected at 50 PFU/cells were incubated for 2, 4, and 6 hours post infection and collected for Western blot after permeabilization with digitonin for 5 min at room temperature (lanes 5–8); control cells (lanes 1–4) underwent the same treatment but without the detergent. Proteins were detected by multiple western blots of the same membrane after stripping of previous antibodies. Actin is shown as loading control. Results from a representative experiment are shown. Arrows indicate cleavage products detected with anti-FATP3 and Acsl3 antibodies. Arrowhead points to the loss of FATP3 after digitonin treatment from infected cells. **B**. Acsl3 knock-down severely impairs polio replicon replication (top panel) while showing minimal cytotoxicity (lower panel). siRNA knock-down efficiency of Acsl3 protein is shown. **C**. Expression of a fusion protein GFP-Acsl3-HA reduces poliovirus replication. HeLa cells were transfected overnight with either empty pUC plasmid, pEGFP-N1 plasmid or pGFP-Acsl3-HA plasmid. Cells were infected (V) with poliovirus at 50 PFU/cell or mock-infected (M) and collected for analysis at 4 h.p.i. Polio 2C band intensity is normalized to the EGFP expressing sample. Expression of GFP-Acsl3 protein is detected with either anti-Acsl3 antibodies (second panel) or anti-GFP antibodies (third panel) which also show expression of EGFP (forth panel). Actin is shown as loading control. **D**. Knock-down of ACSL3 expression reduces activation of FA import upon expression of poliovirus proteins. HeLa cells were transfected with control or ACSL-3-targeting siRNA and 48 h later they were transfected with the plasmid pTM-2A-3D coding for the entire poliovirus non-structural polyprotein fragment P2P3. The next day expression of polio proteins was induced by infection of cells with vaccinia-T7 virus. Bodipy-FA label was added for 30 min at 4 h post vaccinia-T7 infection. Statistical analysis of, 150 cells from each sample shows bodipy-FA signal normalized to poliovirus antigen 2B fluorescence, p value is shown. Western blot shows ACSL3 knock-down, actin is shown as a loading control

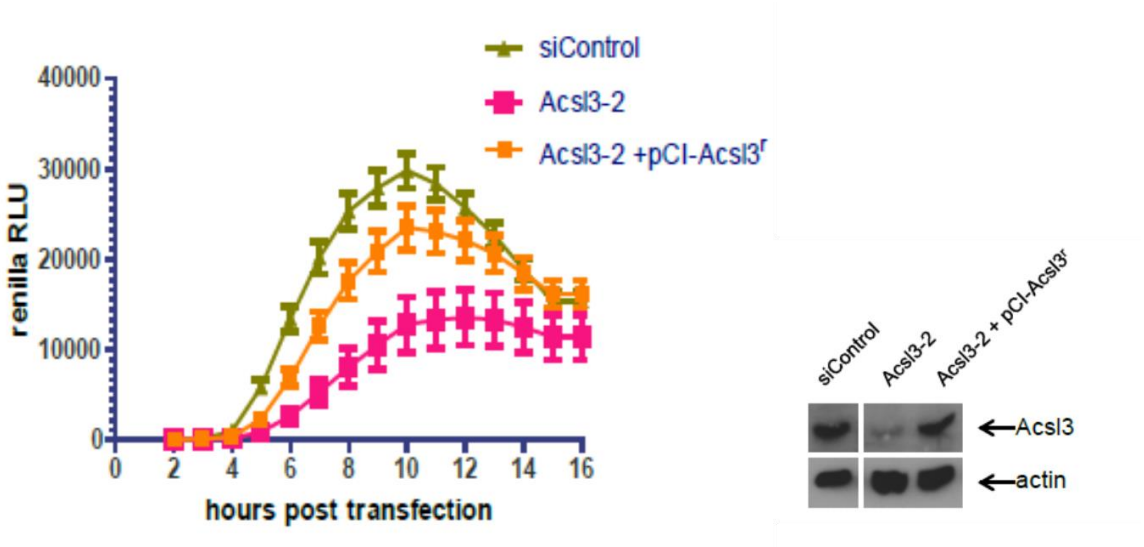


Figure 4.8: rescue of the inhibitory effect of anti-Acsl3 siRNA#2 by expression of a resistant Acsl3. For the siRNA rescue experiment cells were transfected with the most potent anti-Acsl3 siRNA #2 (or control siRNA) and in ~48 hours they were transfected with pCI-Acsl3r plasmid coding for the Acsl3 sequence with mutated siRNA targeting site (control samples were transfected with an empty vector). The next day after DNA transfection polio replicon assay was performed.

Since siRNA knockdown demonstrated an important role of ACSL3 in polio replication, we investigated if its overexpression may stimulate the infection. Hela cells were transfected with plasmids coding for GFP-ACSL3-HA, this enzyme was fully functional in acyl-CoA synthetase activity assay (125), GFP or the empty vector pUC and then infected with poliovirus the next day at 50PFU/cells. A western blotting analysis of viral proteins showed that overexpression of the recombinant GFP-ACSL3-HA retarded the expression of poliovirus proteins, thus working as a dominant negative against polio replication (Figure 4.7: C). The result shows that either lack or excess of ACSL3 is detrimental to the replication of poliovirus.

To understand if ACSL3 is the enzyme responsible for activation of long chain fatty acid import upon infection, we expressed the viral P2P3 polyprotein fragment with the help of vaccinia virus expressing a T7 RNA polymerase, in cells transfected with control and the anti-ACSL3 siRNAs. At 4hpi, vaccinia-infected cells were incubated in the presence of bodipy-FA in serum free media for 30mins to monitor the import of FAs. The result revealed a strong activation of FA import in cells treated with siRNA-control and positive for the viral antigen while accumulation of bodipy-FA was significantly lower in cells with ACSL3 knockdown (Figure 4.7: D).

These data showed specific limited proteolysis of ACSL3 and FATP3 in infected cells, accompanied by the loss of membrane association of FATP3, which likely contributes to modulation of bulk ACSL specificity in infected cells, and demonstrate that functional ACSL3 is required for polio replication and is directly involved in activation of FA import upon expression of polio proteins.

Poliovirus protein 2A is required for the activation of fatty acid import independently of its protease activity. To identify viral proteins associated with the increased import of bodipy-FA in poliovirus- infected cells, we expressed various polyprotein fragments of poliovirus with the help of a recombinant vaccinia virus which provided the T7 RNA polymerase (15). This system, which had been used previously to study the role of poliovirus proteins in the development of membranous structures (32), ensured a fast expression of viral proteins independently of RNA replication. Cells were transfected with plasmids coding poliovirus polyprotein fragments under the transcriptional control of a T7 RNA polymerase promoter. The next day, they were infected with the recombinant vaccinia virus which expressed the T7 RNA polymerase. Cells were incubated with the fluorescent bodipy-FA for 30mins at 4 hours post vaccinia virus infection and processed for microscopic observation or FACS analyses.

The P1 domain coding capsid proteins is dispensable for poliovirus replication thereby, only fragments of the P2P3 portion of the polyprotein were expressed (Figure 4.8: A). Cells expressing the polyprotein fragment 2A-3D displayed a strong import of fatty acids compared to cells expressing the polyprotein fragments 2B-3D or 2C-3D (Figure 4.8: B) which demonstrated the level of bodipy-FA import comparable to that in control cells transfected with an empty vector pUC (Figure 4.8: B). These results show that the presence of poliovirus protein 2A is required for activation of the fatty acid import.

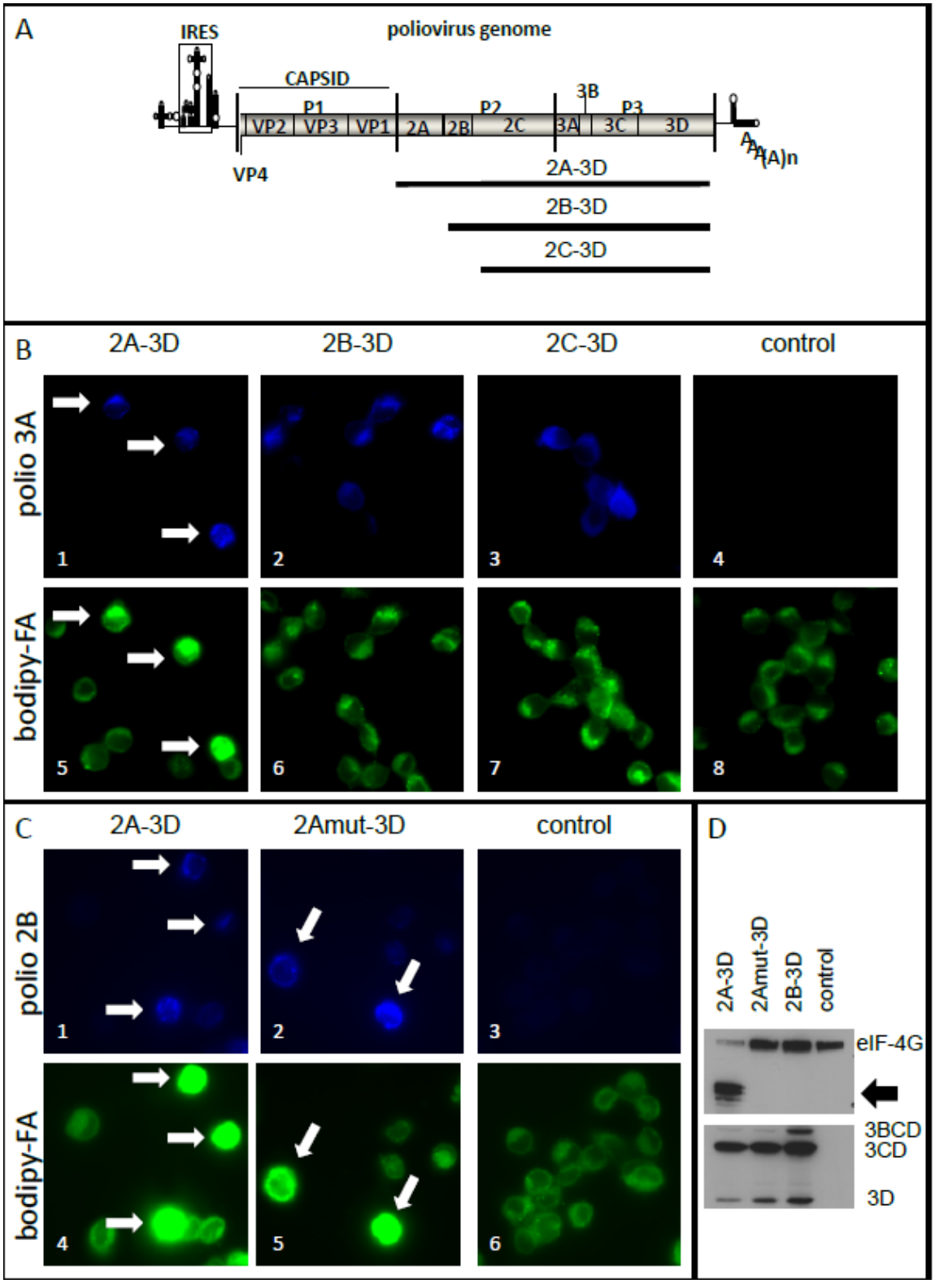

A
IRES
poliovirus genome
CAPSID
P1
P2
3B
P3
VP2
VP3
VP1
2A
2B
2C
3A
3C
3D
VP4
A
A
(A)n
2A-3D
2B-3D
2C-3D
B
2A-3D
2B-3D
2C-3D
control
polio 3A
bodipy-FA
1
2
3
4
5
6
7
8
C
2A-3D
2Amut-3D
control
polio 2B
bodipy-FA
1
2
3
4
5
6
D
2A-3D
2Amut-3D
2B-3D
control
eIF-4G
3BCD
3CD
3D

Figure 4.9: Activation of fatty acid import requires polio protein 2A. **A**. Schematic representation of poliovirus genome and truncated constructs used for expression of polio proteins. **B**. Only expression of the full P2P3 region activates import of bodipy-FA label (Arrows). HeLa cells were transfected with plasmids coding for the indicated polyprotein fragments under control of T7 promoter (empty vector for the control sample) The next day the cells were infected with vaccinia-T7 virus and labeled with bodipy-FA for 30 min at 4 h.p.i. Poliovirus antigen 3A is detected as a marker of expression of viral polyprotein fragments 2A-3D (complete P2–P3), 2B-3D and 2C-3D. **C**. Protease activity of 2A is dispensable for activation of fatty acid import (arrows). HeLa cells were transfected with plasmids coding for the indicated polyprotein fragments under control of T7 promoter (empty vector for the control sample) The next day the cells were infected with vaccinia-T7 virus and labeled with bodipy-FA for 30 min at 4 h p. i. Poliovirus antigen 2B is detected as a marker of expression of the wt and 2A-mut containing P2–P3 polyprotein. **D**. Parallel samples to those shown in C were collected and analyzed for 2A protease activity and expression of viral proteins. Processing of eIF-4G (black arrow) is detected only in the sample expressing functional 2A protease (top panel). Accumulation of viral proteins detected by viral antigen 3D is comparable in all samples showing that the lack of 2A protease activity is because of the mutation, not because of the insufficient expression (lower panel).

2A is a protease responsible for the separation of the capsid protein precursor P1 from the P2P3 polyprotein and is also involved in cleavage of a number of cellular substrates rendering host cell permissive for polio replication (28). To determine whether the protease activity of 2A was required for the stimulation of fatty acid import, we introduced a point mutation in 2A sequence substituting the catalytic amino acid C109 to A to inactivate the proteolytic function (39). The expression of the P2P3 fragment with the 2A mutant strongly stimulated fatty acids import to the level similar to wt. P2P3 fragment (Figure 4.8: C). The western blotting analysis showed that eIF4G, a known target of wild type polio protein 2A in infected cells (146), was not cleaved in cells transfected with the mutant P2P3 2Amut-3D (Figure 4.8: D). To investigate if the expression of poliovirus proteins 2A alone could stimulate the import of fatty acids, a plasmid expressing the 2A protein with an HA tag and the substituted catalytic domain C109A was constructed. The level of fatty acid import in cells expressing 2A-HA C109A mutant was similar to that in control cells (not shown).

These data show that expression of poliovirus protein 2A is required but not sufficient for activation of fatty acid import and that its protease activity is dispensable.

Activation of fatty acids import is a universal attribute of picornavirus infection. To determine whether the activation of fatty acid import in poliovirus-infected cells was a phenomenon specific to HeLa cells, we used different cell lines: Vero (green African monkey kidney) cells, 293 HEK (human embryonic kidney) cells and SH-S5Y5 (human neuroblastoma) cells. These cells were infected at a multiplicity of 50 PFU/cell and pulse-labeled with bodipy-FA for 30min at 4hpi in a serum free medium. The result showed an increase of bodipy-FA import upon infection in all cell lines tested and the

formation of lipid droplets in mock-infected cells (Figure 4.9: A-C). An example of infected Vero cells shows that expression of the viral proteins correlates with a strong activation of fatty acid import (figure 4.9: D). To determine whether the increase of fatty acid import can also be induced by other members of the *Picornaviridae* family, Hela cells were infected with Coxsackie virus B3 (CVB3) and the Encephalomyocarditis virus (EMCV). CVB3 is a close relative of poliovirus belonging to the same *Enterovirus C* species, while EMCV is a distantly related picornavirus from the *Cardiovirus* genus. Following the infection at 50PFU/cell and pulse-labeling with bodipy-FA for 30mins, we observed a strong increase of fatty acid import in infected cells (Figure 4.9: E-F).

These results show that the activation of fatty acids import could be a universal mechanism to alter lipid metabolism in picornaviruses-infected cells.

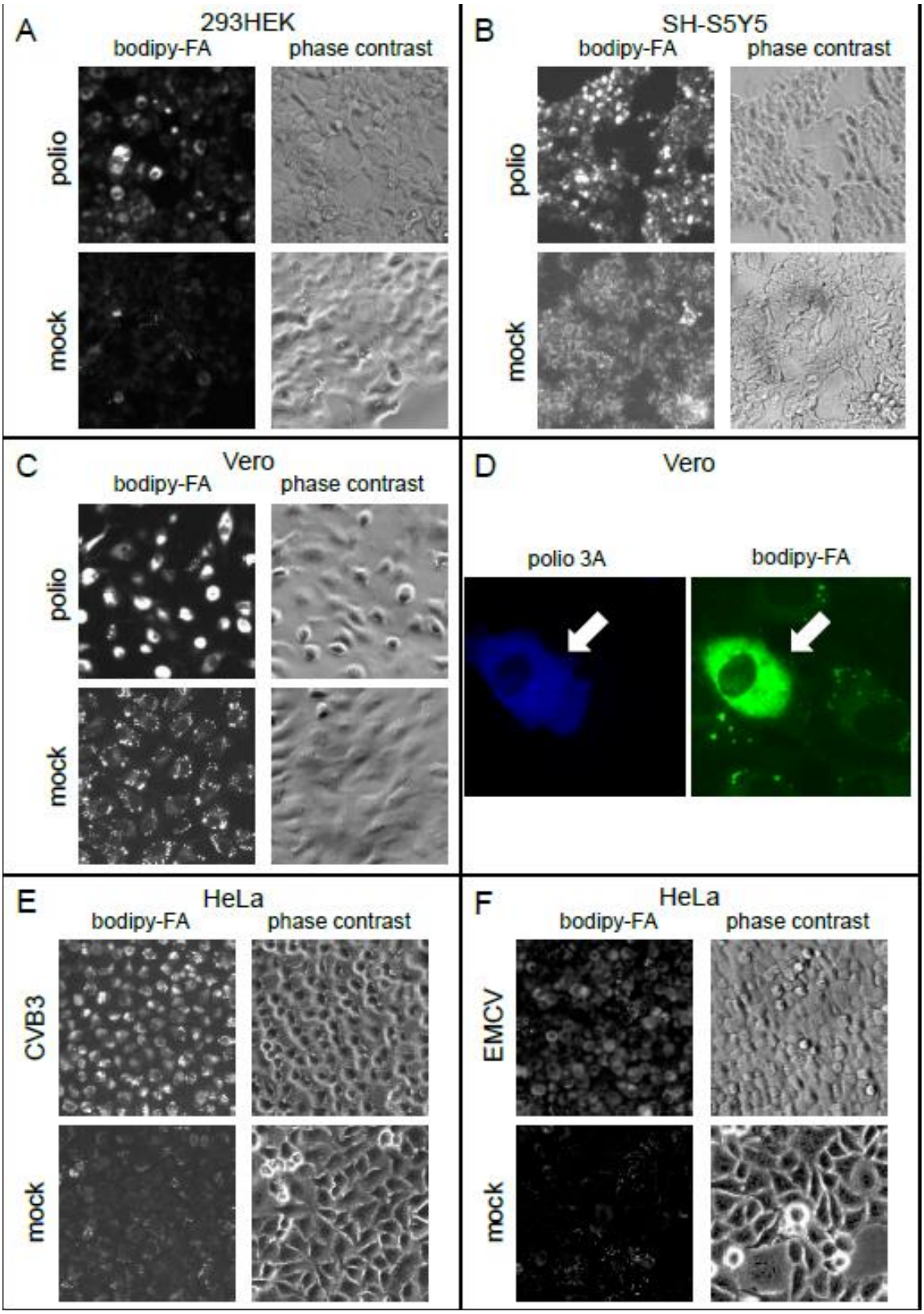

A
293HEK
bodipy-FA
phase contrast
polio
mock
B
SH-S5Y5
bodipy-FA
phase contrast
polio
mock
C
Vero
bodipy-FA
phase contrast
polio
mock
D
Vero
polio 3A
bodipy-FA
E
HeLa
bodipy-FA
phase contrast
CVB3
mock
F
HeLa
bodipy-FA
phase contrast
EMCV
mock

Figure 4.10: Activation of long chain fatty acid import is a general phenomenon of picornavirus infection. **A–C**. 293HEK (human embryonic kidney), SH-S5Y5 (human neuroblastoma) or Vero (green African kidney) cells were infected with poliovirus at 50 PFU/cell and incubated for 4 hours before 30 min label with bodipy-FA. Fluorescent and phase contrast images of infected and mock-infected cells are shown. **D**. Higher magnification of Vero cells infected with poliovirus and labeled with bodipy-FA like in C, showing that bodipy-FA accumulation is activated only in cells actively expressing viral proteins (arrow). **E, F**. HeLa cells were infected with either Coxsackie B3 virus or encephalomyocarditis virus at 50 PFU/ cell and incubated for 4 hours before 30 min label with bodipy-FA. Fluorescent and phase contrast images of infected and mock-infected cells are shown.

4.3 Discussions

The remodeling of intracellular membranes is an indispensable step in the development of picornavirus infection, however the underlying mechanism(s) remain unknown. In this report we demonstrated that the up regulation of lipid synthesis in picornaviruses-infected cells is accompanied by a rapid increase of long chain fatty acid import catalyzed by the activation of acyl-CoA synthetase activity. In mock-infected cells the fluorescent long chain fatty acid analog bodipy-FA accumulated in lipid droplets whereas in polio-infected cells it was immediately channeled for the synthesis of PC and distributed to the replication organelles.

Thus, in infected cells the newly imported FAs were utilized for the synthesis of membranous structures that support the viral replication. In the course of infection, the increased activity of the acyl-CoA synthetases resulted in the synthesis of species of PCs with distribution of long chain acyl chains different from that in non-infected cells. Changes in PC species could determine the distinctive biophysical properties of the replication organelles. Although the fatty acid competition assay showed a strong preference for the import of the myristic acid C14, the synthesis of PCs species preferentially accumulated the palmitic acid C16 and the stearic acid C18, likely reflecting the relative abundance of different fatty acid species available for lipid synthesis. This result demonstrated that the development of replication organelles with unique lipid composition in PV infected cells to the large part depends on synthesis of new membranes and not only on the remodeling of pre-existing organelles.

ACSL enzymes have distinct tissue expression patterns and subcellular locations. A western blot analysis monitoring the expression patterns and membranes association following polio infection revealed some differences among ACSL enzymes. Thus, the expression of FATP4 and ACSL5 did not change significantly overtime and the treatment with digitonin showed they were forming stable associations with intra-cellular structures. Both ACSL5 and FATP4 proteins are located on the ER and the mitochondrial membranes (94, 123) and it is possible these associations and their acyl-CoA synthetase activities are not inhibited in the course of polio infection. Contrary to ACSL5 and FATP4, FATP3 and ACLS3 were cleaved in the course of infection and FATP3 significantly lost its association with membranous structures in polio infected cells.

The accumulation of cleaved products of FATP3 and ACSL3 did not translate into a significant loss of the full length proteins. This suggested that possibly, a small fraction of these proteins were affected, probably in a specific subcellular location. At this stage, we do not know if viral proteinases 2A and 3C or intra-cellular proteases are involved in the proteolysis of ACSL3 and FATP3. The cap-dependent translation of host proteins and the nuclear transcription of host genes are rapidly shut down upon polio infection (43) thus, it is likely that the activation of pre-existing acyl-CoA synthetases is responsible for activation of fatty acid import and PC synthesis in picornaviruses infected cells. This also explains insensitivity of this process to an inhibitor of nuclear transcription Actinomycin D.

Thirteen acyl-CoA synthase enzymes are involved in the activation and the transport of long and very long chain fatty acids with overlapping substrate specificity (65). The siRNA screening of all 13 human acyl-CoA synthetases showed that knockdown of

ACSL3 was the most potent in reducing polio replicon replication without inducing cellular toxicity. Curiously, overexpression of the recombinant GFP-ACSL3-HA also reduced the replication of poliovirus suggesting that the level of ACSL3 activity should be strictly regulated during infection or, alternatively that the recombinant protein worked as a dominant negative mutant due to the presence of GFP and/or HA elements. Although ACSL3 has the most significant effect on the reduction of polio replication following siRNA treatments, other acyl-CoA synthetases also modulated the infection albeit to a lesser effect.

It was reported that siRNA knock-down of ACSL3 decreased the expression of several lipogenic transcription factors such as the peroxisome proliferator activation receptor-γ (PPAR-γ), carbohydrate-responsive element-binding protein (ChREBP), sterol regulatory element-binding protein-1c (SREBP1-c) and liver X receptor-α (LXR-α) (21). Here in our experiment, knock-down of ACSL3 significantly decreased fatty acid import and could also presumably perturb the activity of key enzymes involved in the synthesis of PC and other lipids associated to polio replication. The down regulation of ACSL3 via siRNA treatments was shown to inhibit the incorporation of fatty acids to PC and block the secretion of Hepatitis C virus (HCV) in human hepatoma Huh7-derived cells (143). Thus in our experiment, it is possible that siRNA knock-down of ACSL3 expression redirect FA away from PC synthesis and thus interfering with the development of poliovirus membranous replication organelles.

We also observed that activation of bodipy-FA import required poliovirus protein 2A. Through unknown mechanism, 2A could stimulate acyl-CoA synthetase activity only in concert with other viral factor(s) from the P2P3 polyprotein fragment. Interestingly,

while 2A is mostly known for its proteolytic functions in the viral life cycle, the proteinase activity of 2A was dispensable for the activation of fatty acid import. Thus, activation of acyl-CoA synthetase activity represents a novel function for this protein. Currently, the identity of host and viral factors that are involved in 2A-driven activation of ACSL activity is the subject of investigation.

The fatty acid import seems to be universally activated in picornaviruses infected cells. Our data showed the import of FA was upregulated in CVB3 and EMCV infected cells as well as in various cell lines such as Vero cells, 293 HEK cells infected with poliovirus. It is possible that diverse picornaviruses rely on different acyl-CoA synthetase enzymes for activation of fatty acid import similarly to other enzymes involved in lipid synthesis. For example, the synthesis of PI4P for different genera of picornaviruses can be driven by different phosphatidylinositol-4-kinases. It was shown that poliovirus relied on the Golgi-localized phosphatidylinositol-4-kinase IIIβ (PI4KIIIβ) whereas EMCV relied on ER-localized PI4KIIIα to promote the synthesis of PI4P on replication organelles (47, 66).

Our data suggest a new model of the structural development of picornavirus replication organelles through the upregulation of synthesis of structural phospholipids, especially PC. Here, upon picornavirus infection, the increased ACSL activity provides an excess of long chain acyl-CoAs that drives the upregulation of phosphatidylcholine synthesis. The distinct substrate specificity of ACSL activity in infected cells results in synthesis of a different pool of phosphatidylcholine molecules which likely underlies the unique three dimensional shape of the replication organelles which have no analogues in non-infected cells (13). The modulation of fatty acid metabolism in infected cells

represents an attractive target for development of future broad spectrum antiviral therapeutics.

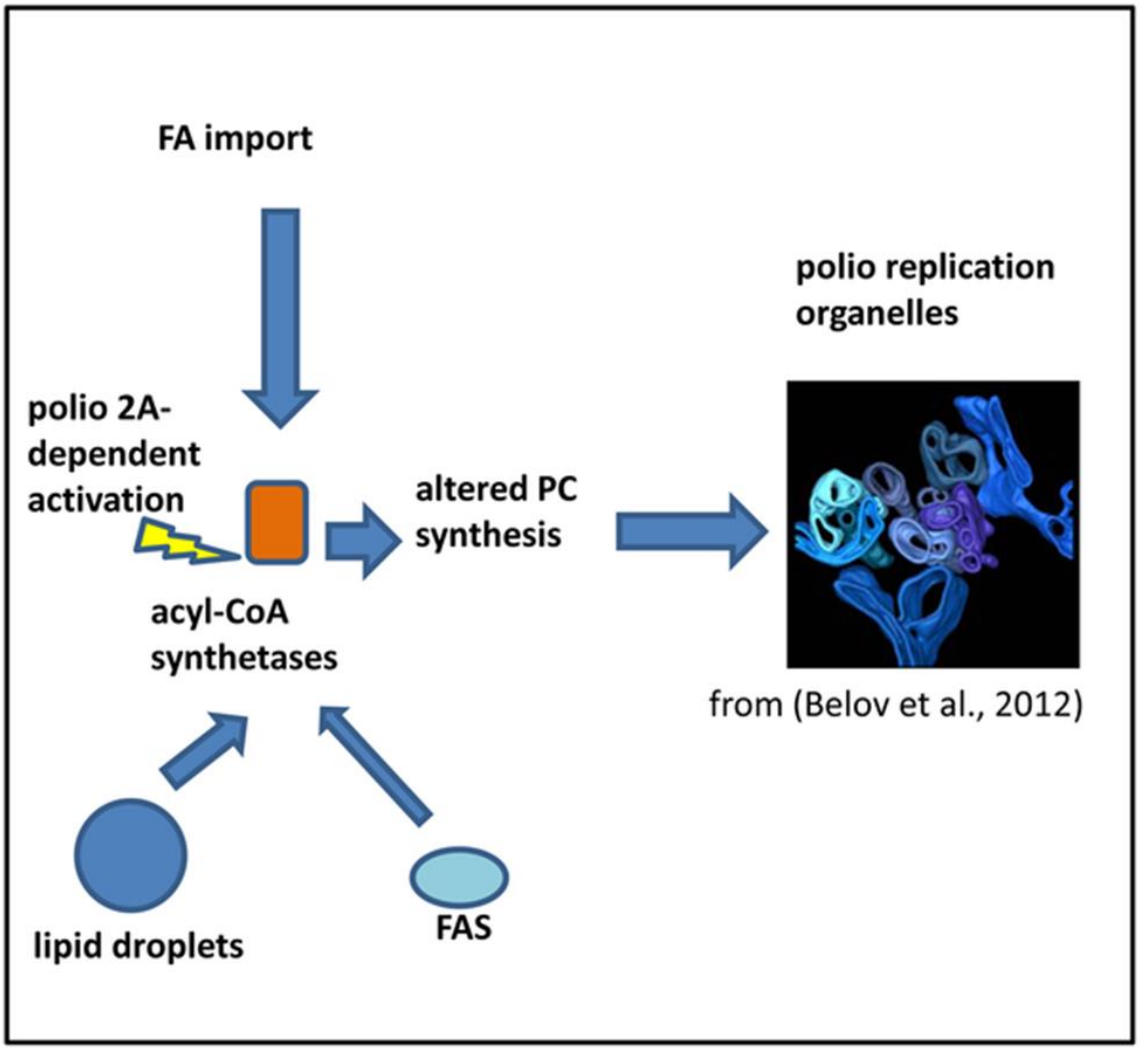


Figure 4.11: model of the structural development of picornavirus replication complexes based on activation of cellular acyl-CoA synthetase activity by viral proteins (2A is required, but is not sufficient in case of polio).

Chapter 5: Activation of fatty acid import in poliovirus-infected cells does not depend on autophagy or ER stress response

5.1 Introduction

Picornavirus infection results in massive remodeling of intracellular membrane architecture. Cells infected with poliovirus and other related picornaviruses rapidly develop clusters of novel membranous structures (replication organelles) harboring the viral RNA replication machinery. The replication organelles ultimately occupy almost all the cytoplasmic volume by the end of the infection cycle (13, 17, 18, 90). It was shown that in picornavirus-infected cells, the synthesis of structural phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) is highly activated and that the newly synthesized lipids are found in membranes associated with the RNA replication activity (3, 103). We recently identified that increase of PC synthesis in infected cells is associated with strong activation of import of long chain fatty acids (FAs) which constitute the hydrophobic part of the phospholipid molecules.

This activation of FA import was attributed to post-translational up-regulation of activity of the cellular long chain acyl-CoA synthetases which thioesterify FA moieties to coenzyme A (CoA) generating fatty acyl-CoAs (106). Acyl-CoAs serve as substrates in downstream metabolic reactions, including phospholipid synthesis. Thus picornavirus infection induces profound changes in the basic cellular lipid metabolism, however what regulatory mechanisms are engaged upon infection to activate the lipid synthesis and ultimately the development of the membranous replication organelles is not clear.

Poliovirus genome RNA codes for one large polyprotein which is processed by viral proteases into about a dozens of mature peptides. Expression of membrane-targeted poliovirus proteins such as 2B, 2C, 3A and intermediate cleavage products containing these sequences was shown to significantly alter morphology of the intracellular membranes (31, 149) and to inhibit normal trafficking of membranes and proteins through the cellular secretory pathway (16, 33, 40, 42), likely resulting in the activation of protective cellular mechanisms including ER stress response.

Moreover, poliovirus infection stimulates autophagy which was shown to promote maturation of infectious viral particles (130). Both ER stress response and autophagy are intimately linked to membrane metabolism (142, 144) and thus it is possible that changes in the lipid synthesis observed in infected cells, including activation of long chain FA import, are the results of activation of these pathways upon infection.

5.2 Results and discussions

To understand the role of the autophagy pathway in the import of exogenous FAs, HeLa or 293HEK cells were seeded on 12 well plates and the next day incubated with 5μM of autophagy inducer STF-62247 (Calbiochem) (group 1); corresponding amount of the DMSO solvent (group 2); or left untreated (groups 3 and 4) overnight. The next day the cells left untreated were infected with 10 PFU/cell of poliovirus type 1 Mahoney (group 3) or mock-infected (group 4). At 6 hours post infection medium from 4 wells of each group was replaced with pre-warmed DMEM containing 0.4 μM of Bodipy 500/510 C4, C9 (bodipy-FA) (Molecular Probes) and 1 μg/ml Hoechst 33342 (Sigma), a cell-permeable DNA stain. Bodipy-FA is an analog of a long chain FA with 18 carbon atom backbone, and this and similar fluorescent probes are extensively used in membrane metabolism

research (77, 133). After 30 mins of incubation, cells were fixed and processed for microscopy observations and for fluorescence analysis in a Tecan Infinite M1000 plate reader as described in (155).

Bodipy-FA fluorescence which reflects the import of long chain FAs from the medium was normalized to the Hoechst fluorescence which is proportional to the number of cells in the well (Fig. 1A). Parallel samples of cells from all groups were processed for western blot analysis. To follow the autophagy process, we monitored the processing of LC3B protein, which is required for the initiation of formation of autophagosomes, and the degradation of the polyubiquitin-binding protein P62/SQSTM1 (76). The P62 protein directly binds to LC3 on autophagosomal membranes and is degraded upon subsequent fusion of autophagosomes and lysosomes, thus the degradation of P62/SQSTM1 reflects the autophagosome maturation or "autophagy flux". (87, 115). P62/SQSTM1 degradation in poliovirus-infected cells was reported previously (130).

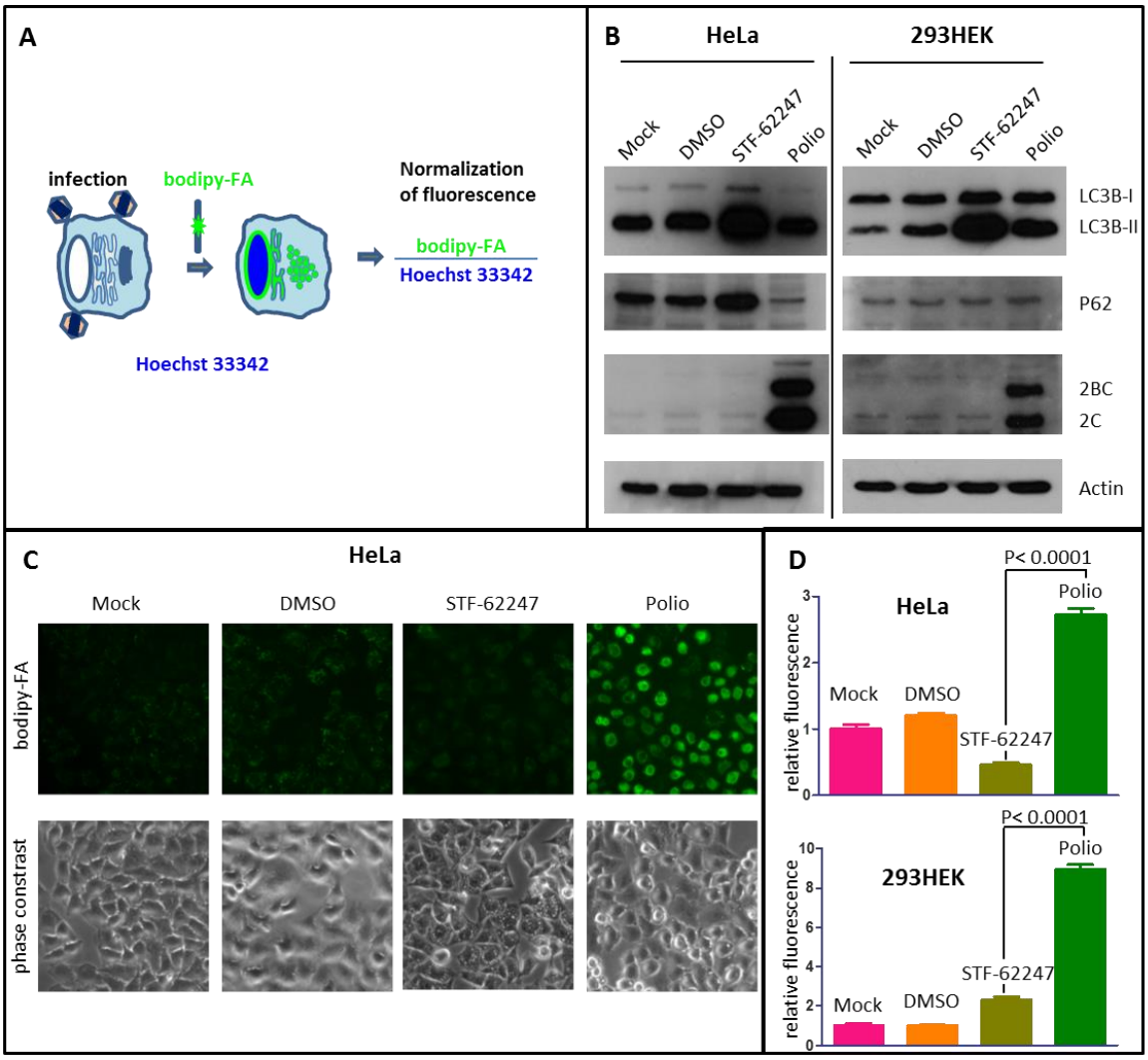

A
infection
bodipy-FA
Hoechst 33342
Normalization of fluorescence
bodipy-FA
Hoechst 33342
B
HeLa
293HEK
Mock
DMSO
STF-62247
Polio
LC3B-I
LC3B-II
P62
2BC
2C
Actin
C
HeLa
Mock
DMSO
STF-62247
Polio
bodipy-FA
phase constrast
D
HeLa
P< 0.0001
Polio
Mock
DMSO
STF-62247
relative fluorescence
293HEK
P< 0.0001
Polio
STF-62247
Mock
DMSO
relative fluorescence

Figure 5.1: Activation of autophagy is not responsible for stimulation of the long chain FA import in poliovirus-infected cells. **A.** Scheme of the long chain FA import assay. **B.** Western blotting analysis of the protein expression in HeLa and 293HEK cells upon poliovirus infection or autophagy induction (STF-62247). Control cells were either mock-infected or treated with DMSO (a solvent for STF-62247). Expression of the viral proteins 2B and 2C is shown as the evidence of infection. Actin is shown as a loading control. **C.** Microscopy images of HeLa cells showing import of bodipy-FA (as outlined in **A**, at ~20 hours of treatment with autophagy inducer STF-62247 or 6 hours post infection). **D.** Quantitative analysis of long chain FA import at ~20 hours of treatment with autophagy inducer STF-62247 or 6 hours post infection. Bodipy-FA fluorescence is normalized to that of Hoechst 33342, and the result is expressed relative to the mock-infected cells. Statistically significant differences are indicated. Statistical analysis was performed with Graph Pad Prizm package unpaired t-test module.

In both cell types, we observed a strong accumulation of the processed LC3B-II form upon treatment with STF-62247, confirming the efficiency of autophagy induction with this compound (Fig. 1B). HeLa cells treated with STF-62247 also showed an increased level of p62 compared to control sample, likely reflecting up-regulation of its expression during prolonged treatment with the autophagy inducer. The level of the unprocessed LC3B-I form was significantly lower in control HeLa than in 293HEK cells, indicating higher steady state level of autophagy in HeLa (Fig 1B). Accordingly, induction of LC3B processing upon poliovirus infection was much more noticeable in 293HEK than in HeLa cells. At the same time, degradation of p62 was observed only in HeLa and not in 293HEK cells infected with poliovirus (Fig1B).

Thus, it seems that poliovirus can efficiently induce autophagy in 293HEK cells (processing of LC3B) but not a significant maturation of autophagosomes (degradation of p62). In HeLa cells, on the other hand, the increase in autophagy induction is moderate, compared to the already high level of LC3B processing in non-infected cells, while these cells are much more conductive to completion of the autophagy cycle upon infection as revealed by strong degradation of p62. Both cell types support high level of poliovirus replication, as evidenced by robust expression of poliovirus proteins 2BC and 2C (Fig. 1B). Maturation of autophagosomes in infected cells was proposed to stimulate production of mature virions (130), it would be interesting to see if poliovirus replication in HeLa and HEK 293 cells would yield significantly different ratio of mature vs. immature viral particles. The level of FA import by either HeLa or 293HEK cells treated with STF-62247, and control DMSO-treated and mock-infected cells was not statistically different, while poliovirus-infected cells showed strong stimulation of FA import (Fig. 1C and D), in

accordance with the previously reported data (106). Thus, poliovirus-induced activation and completion of the autophagy program is likely cell type specific, and does not account for the activation of long chain FA import upon infection.

To characterize activation of ER stress response upon poliovirus infection and possible contribution of this pathway in stimulation of long chain FA import HeLa cells were either infected (mock-infected) with 10 PFU/cell of poliovirus type I Mahoney or treated with 1 µg/ml tunicamycin or 0.5 µM thapsigargin, two well-established ER-stress inducers (138). The cells were grown in 6cm dishes to provide sufficient material for RNA isolation to monitor splicing of the XBP1 mRNA as an indicator of ER stress induction (see below). At 6 hours post infection or addition of the ER-stress inducing compounds parallel samples were processed for: 1) long chain FA import assay as described in the previous section and (155) with some modification. The cell incubated with 0.4 µM of bodipy-FA in DMEM for 30 mins were collected in 1000 µl of PBS and 100 µL of this suspension was used to measure the protein concentration for normalization, while the cells from the remaining 900 µl were collected by centrifugation and resuspended in 500uL of butanol to extract the lipids with incorporated bodipy-FA.

The butanol extract was clarified by centrifugation and divided into 5 replicates for fluorescence measurement in a Tecan Infinite M1000 plate reader. 2) RNA extraction with RNAeasy columns and subsequent mRNA enrichment with oligotex mRNA mini kit (Qiagene) following by RT-PCR to detect splicing of XBP-1 mRNA, a hallmark of ER stress response activation (138). 3) Preparation of the cytoplasmic lysate to analyze expression of ER-stress related cellular proteins and viral proteins in western blot. Splicing of XBP-1 mRNA is initiated by the ER-resident endonuclease IRE-1 which is activated in

response to overload of the ER protein folding and processing capacity (26). XBP-1 protein expressed from the spliced mRNA is a potent transcription activator controlling expression of multiple genes including ER-targeted chaperons. Expression of these genes helps to resolve the ER stress conditions and restore the secretory capacity of the cells (86).

We observed a very modest increase of the spliced XBP-1 mRNA in poliovirus-infected compared to mock-infected cells (Fig. 2A) suggesting that either the ER homeostasis is not significantly perturbed upon infection in spite of the extensive membrane remodeling and high expression of membrane-targeted viral proteins leading to the inhibition of cellular secretion, or that poliovirus can actively counteract the cellular signaling machinery.

In contrast, the cells treated with either tunicamycin or thapsigargin demonstrated a very strong processing of XBP-1 mRNA, as expected (Fig. 2A). Accordingly, we observed high expression of the transcription factor C/EBP homologous protein (CHOP) in cells treated with thapsigargin and tunicamycin, but not in infected cells (Fig2B). Expression of CHOP is activated upon induction of the ER stress response (138). Strong activation of the ER stress by tunicamycin or thapsigargin did not result in noticeable increase of the long chain FA import, which was only observed in poliovirus-infected cells (Fig. 2C and D).

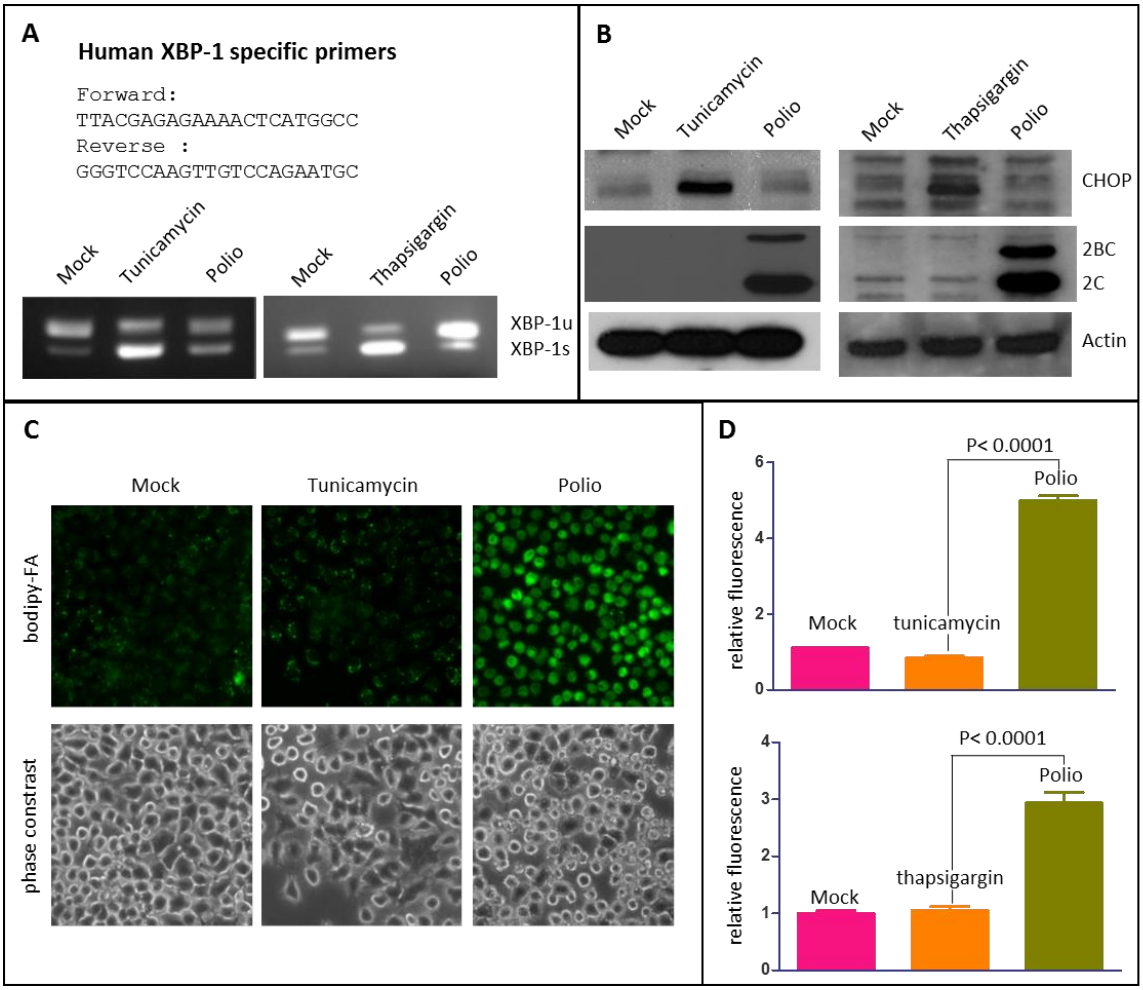

A
Human XBP-1 specific primers
Forward:
TTACGAGAGAAAACTCATGGCC
Reverse :
GGGTCCAAGTTGTCCAGAATGC
Mock
Tunicamycin
Polio
Mock
Thapsigargin
Polio
XBP-1u
XBP-1s
B
Mock
Tunicamycin
Polio
Mock
Thapsigargin
Polio
CHOP
2BC
2C
Actin
C
Mock
Tunicamycin
Polio
bodipy-FA
phase constrast
D
P< 0.0001
Polio
relative fluorescence
Mock
tunicamycin
P< 0.0001
Polio
relative fluorescence
thapsigargin
Mock

Figure 5.2: ER stress response is not activated upon poliovirus infection and does not result in increase of FA import. **A.** RT-PCR analysis of the splicing of XBP-1 mRNA in HeLa cells. XBP1u indicates the PCR fragment amplified from the un-spliced mRNA, XBP-1S is the fragment amplified from the spliced mRNA. Primers used in the PCR reaction are shown. **B.** Western blot analysis of the protein expression in HeLa cells upon induction of the ER stress (tunicamycin and thapsigargin) or poliovirus infection. Expression of the viral proteins 2B and 2C is shown as the evidence of infection. Actin is shown as a loading control. **C.** Microscopy images of HeLa cells showing import of bodipy-FA (as outlined in Fig. 1A, at 6 hours post infection or induction of the ER stress). **D.** Quantitative analysis of long chain FA import at 6 hours post infection or induction of the ER stress. Bodipy-FA fluorescence is normalized to that of Hoechst 33342, and the results are expressed relative to the mock-infected cells. Statistically significant differences are indicated.

It was previously reported that infection of RD cells with enterovirus 71 (EV71), a picornavirus related to poliovirus, induces implementation of some elements of the ER stress response, however such responses were detected at relatively late time points (9 hours post infection and later) and were much less pronounced than those observed in control cells treated with pharmacological ER stress inducers (45). Interestingly, poliovirus replication was shown to be insensitive to treatment of COS-1 cells with 5 μg/ml of tunicamycin, sufficient to inhibit ER-specific N-glycosylation of proteins, and likely to induce strong ER stress response, although activation of the ER stress was not tested directly in this study (40).

These data and our observations show that poliovirus and related picornaviruses neither significantly induce, nor are sensitive to the induction of the cellular ER stress, suggesting that their replication does not depend on the normal ER metabolism. Overall, our results demonstrate that activation of the long chain FA import and, consequently, activation of the structural phospholipid synthesis in picornavirus-infected cells is an infection-specific process that does not depend on cellular stress pathways such as autophagy and the ER stress response, and thus may represent an attractive target for anti-viral interventions.

Chapter 6: Role of phosphatidylcholine synthesis in the development of replication organelles and poliovirus life cycle

Parts of this chapter were published in Jiantao Zhang, Zhenlu Zhang, Vineela Chukkapalli, Jules A. Nchoutmboube, Jianhui Li, Glenn Randall, George A. Belov, and Xiaofeng Wang (2016). Positive-strand RNA viruses stimulate host phosphatidylcholine synthesis at viral replication sites PNAS 2016 113: E1064-E1073.

6.1 Introduction

The origin of membranous structures supporting positive strand RNA viruses replication has been attributed to both the synthesis of new membranes and the complex rearrangement of preexisting intra-cellular organelles (57, 60). In our previous work, we have demonstrated that activation of PC synthesis upon infection relies on activation of acyl-CoA synthetase activity and that infected cells synthesize different spectrum of PC molecules than non-infected one. The upregulated phosphatidylcholine synthesis in infected cells likely supports the rapid structural expansion of the replication organelles, however, it is possible that certain enzymatic reactions of the replication cycle require specific phospholipid environment.

For example, phospholipids have been shown to influence the replicase protein activities of positive RNA viruses from as diverse families as alphaviruses and nodaviruses (96, 158). Most recently, it was shown that an increased level of phospholipids such as phosphatidylethanolamine (PE) or phosphatidylserine (PS) enhanced the replication of tomato bushy stunt virus (TBSV) in PE-enriched vesicles or promoted the non-lytic release of infectious poliovirus particles packaged in PS-enriched vesicles (24, 89).

Here, we investigated the role of PC synthesis in the development of replication organelles and the effect of inhibition of PC synthesis on propagation of poliovirus.

6.2 Results

Phosphatidylcholine synthesis is required for the structural development of the replication organelles. Phosphatidylcholine is the most abundant lipid in cellular membranes and its synthesis is highly upregulated in poliovirus infected cells (106, 154). To understand the requirement of PC synthesis for the development of replication organelles, we first monitored the distribution of newly synthesized PC in polio-infected cells. Hela cells were infected with poliovirus (mock infected) at a MOI of 10 PFU/cell. The cells were incubated at 4 h.p.i with a 200uM of propargylcholine for 1h of incubation. Propargylcholine is a choline analogue that is readily utilized by cells in PC synthesis and propargylcholine-containing molecules can be detected by click-chemistry (74).

The next day, the cells were processed for click chemistry detection of phosphatidylpropargylcholine with Alexa fluor 488 azide and immunostaining for a poliovirus antigen 2B as a marker of the replication complexes. The result showed an increased upregulation of PC synthesis in the cytoplasm of polio-infected cells compared to mock-infected and that the newly synthesized PC co-localized with the replication complexes as evidenced by 2B staining (Figure: 6.1). This result confirms previous observations made by us and the others that PC synthesized during infection is immediately incorporated into the replication membranes.

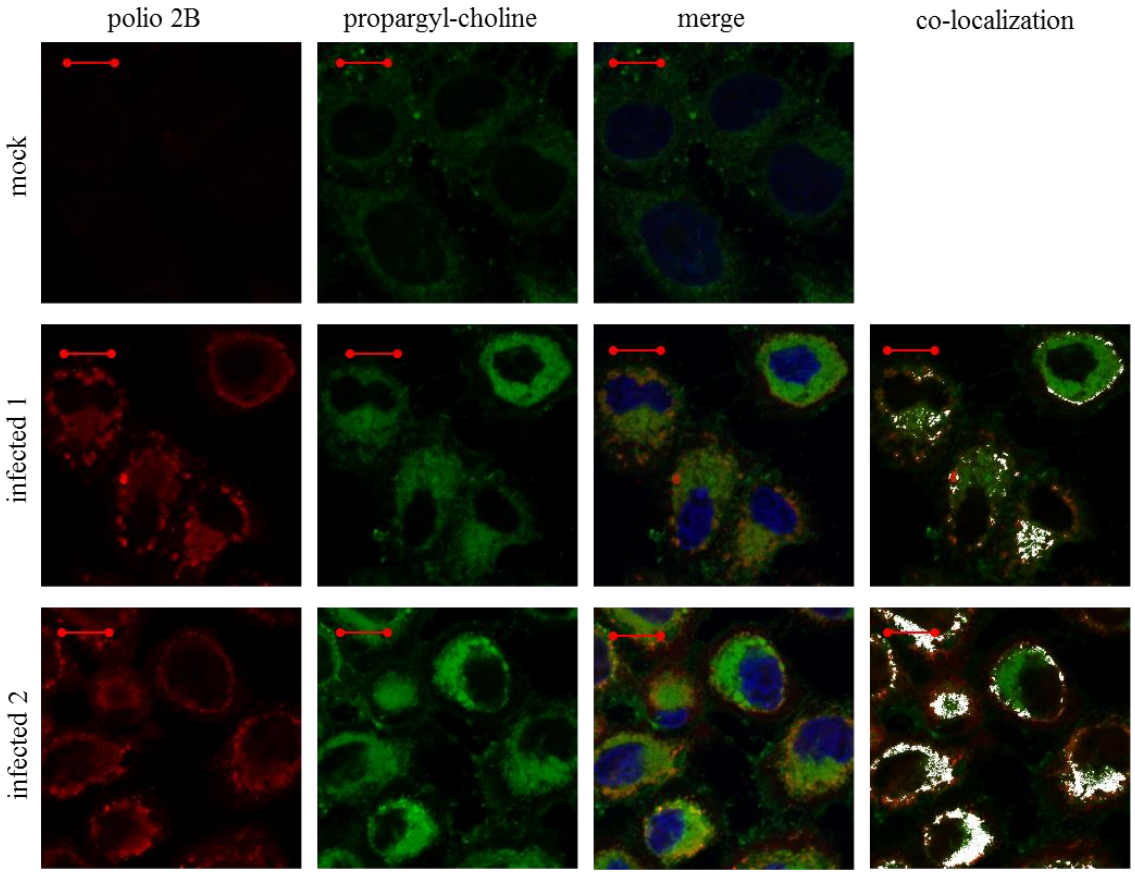

polio 2B
propargyl-choline
merge
co-localization
mock
infected 1
infected 2

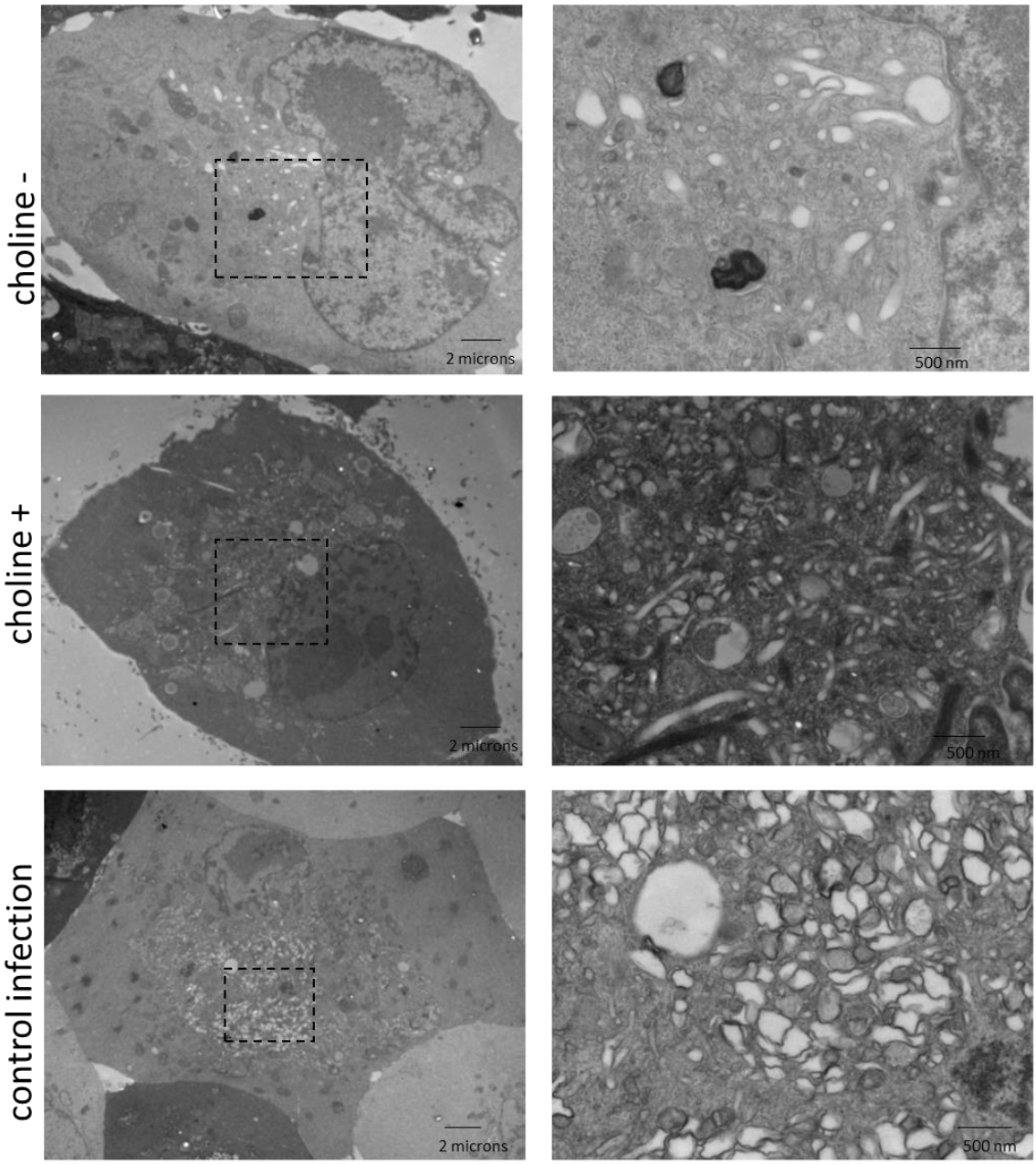
Figure 6.1: The newly synthesized PC co-localized with PV-2B in the replication complexes. Monolayers of Hela cells were infected at MOI of 10pfu/cells with poliovirus. Then at 4 hours post-infection, the incubation medium was replaced by a labeling medium containing 200uM of propargyl-choline for 1 hour of incubation. PC was detected via click it chemistry protocol with Alexa 488 and the viral protein 2B was stained with Alexa fluor 594 and the nucleus stained with Hoechst-33342. The co-localization panel shows the colocalized red and green pixels of 2B and PC identified with ImageJ software. (Scale bar, 10 μm.)

To investigate whether the structural development of replication organelles required PC synthesis, we monitored their formation and distribution upon incubation of infected cells in choline-deficient conditions. Human cells cannot synthesize choline and it needs to be supplied, as a vitamin in the medium, in its absence, no *de novo* synthesis of phosphatidylcholine is possible. Hela cells were incubated in a choline-free medium for 48h prior to infection to let them exhaust their internal choline depot. After that, they were infected with poliovirus (mock-infected) at a MOI of 50 PFU/cells and then either further incubated in choline-deficient medium or in the medium supplemented with 25uM of choline.

Control infection was performed in cells incubated all the time in standard growth medium supplemented with 10% fetal bovine serum. At 4h post-infection, cells were fixed and processed for transmission electron microscopy (EM) imaging. As expected, we observed strong proliferation of membranous replication organelles in control cells incubated in the presence of FBS; however, we observed almost no such proliferation was evident in cells incubated in choline-deprived medium. Virus-induced membrane alterations in such cells were represented by sparsely distributed enlarged cisternae- or vesicular-shaped structures likely representing dilated ER tubules. This phenotype could be largely rescued in choline-containing medium, although the replication structures in this case were still less tightly packed and had more elongated shape than those formed in control cells that did not undergo incubation in choline-deprived medium at any stage of the experiment (Figure 6.2A).

Interestingly, although the inhibition of PC synthesis via choline starvation blocked the development of membranous structures, the western blot analysis showed that the expression pattern of viral proteins 2C in choline-depleted or choline-supplemented infected-cells was similar (Figure 6.2B). This result demonstrates that PC synthesis was required for the structural development of replication organelles in polio-infected cells but at the same time, PC synthesis is dispensable for the virus replication, at least in cell culture conditions at high MOI of infection.

A


choline -
2 microns
500 nm
choline +
2 microns
500 nm
control infection
2 microns
500 nm

B

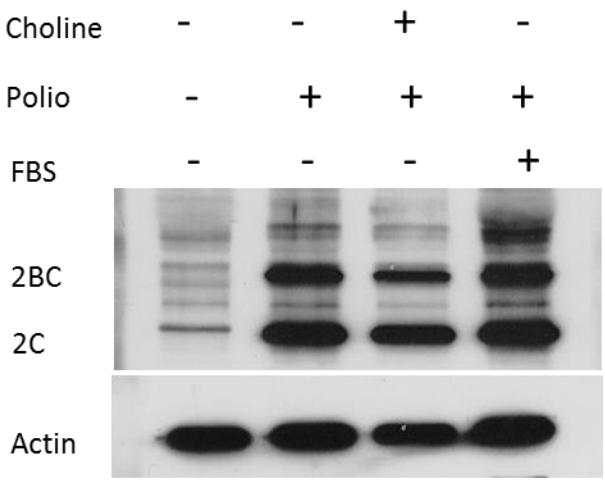
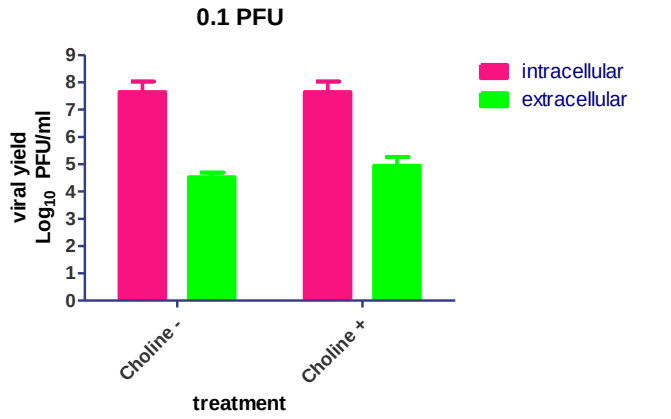


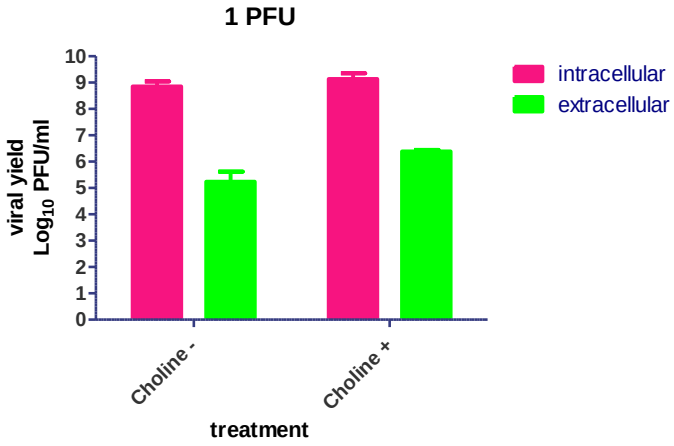
Figure 6.2: PC synthesis is required for the structural development of membranes structures supporting the replication complexes. Hela cells grown for 48h in a choline-depleted medium were polio-infected at a MOI of 50pfu/cell then followed by incubation in choline-enriched or –depleted medium, or in the presence of 10% of FBS. At 4 h post infection, **A)** infected cells were either processed with a fixing solution for an electron microscopic imaging. Original image on the left with inset (Scale bar 2 microns) then inset magnified (Scale bar 500 nm) or **B**) cell lysates were collected to run a western blot using monoclonal antibodies against 2C and actin.

Inhibition of phospholipid synthesis is dispensable for viral replication but is essential for protection against innate anti-viral mechanisms. The apparent uncoupling of the efficient poliovirus replication from the massive development of membranous replication organelles observed in choline-deprived cells prompted us to further investigate the role of membrane remodeling in poliovirus life cycle. First, we monitored if inhibition of PC synthesis would affect the non-lytic release of virus particles, a phenomenon that may depend on specific phospholipid composition of membranes (29). HeLa cells were pre-incubated for 48 h in choline-free medium to exhaust the cellular choline depot and infected with poliovirus at MOIs of 10, 1 and 0.1 PFU/cell. After infection, the cells were either incubated in choline-free medium or in medium supplemented with 25uM of choline. At 6 h.p.i, the supernatant was collected to assess the amount of released extracellular infectious particles and simultaneously infected cells were collected to measure the intracellular virus accumulation.

The amount of extracellular particles recovered at 6 h.p.i. (i.e. representing the single round of infection) in all cases was ~3 logs lower than the amount of virus still associated with the cells. Moreover, the amount of intracellular and extracellular infectious particles did not vary significantly upon incubation of cells in choline-free or choline-supplemented medium at any MOI (Figure 6.3 A, B and C).

Thus, the non-lytic release of poliovirus is an inefficient process compared to the total virus yield and it does not depend on active phospholipid synthesis.


0.1 PFU
intracellular
extracellular
viral yield
Log10 PFU/ml
0
1
2
3
4
5
6
7
8
9
Choline -
Choline +
treatment


1 PFU
intracellular
extracellular
viral yield
Log10 PFU/ml
0
1
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3
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5
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7
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9
10
Choline -
Choline +
treatment

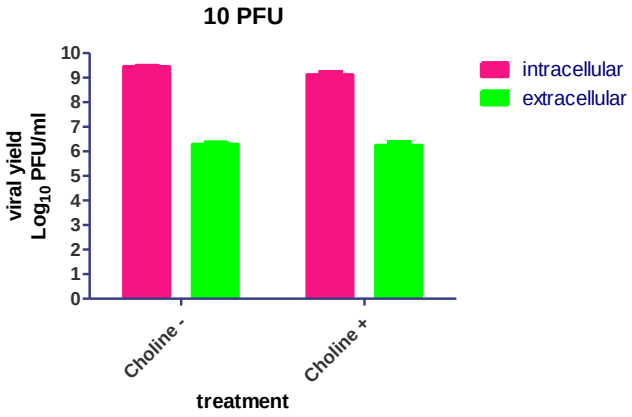
10 PFU
intracellular
extracellular
viral yield
Log10 PFU/ml
0
1
2
3
4
5
6
7
8
9
10
Choline -
Choline +
treatment

Figure 6.3: The upregulation of PC synthesis is dispensable for the development of poliovirus infection at high MOI. Hela cells grown for 48h in a choline-depleted medium were polio-infected at MOI of **A)** 0.1 PFU/cell, **B)** 1PFU/cell and **C)** 10 PFU/cell then followed by incubation in the presence or absence of 25uM of choline. At 6 hours post infection, infected cells were processed via a plaque assay to quantitate the concentration of intracellular (freezing/thawing of infected cells three times) and extracellular (infectious medium collected) infectious particles**.**

Next, we assessed the accessibility of the viral replication complexes in conditions of active and inhibited PC synthesis by immunostaining of a viral protein 2B, a marker of the replication complexes, in mild permeabilization conditions. Hela cells were pre-incubated and cultured in the absence of choline for 48h then infected with poliovirus (mock infected) at an MOI of 10 PFU/cell and incubated for 4h in either choline-free or choline-supplemented media. At 4hpi the cells were fixed and processed for an immunofluorescence assay preceded by a mild treatment with 0.02% of saponin. Saponin selectively interacts with and removes cholesterol from membranes thus creating pores; saponin treatment largely preserves overall membrane architecture as opposed to strong detergents used for cell permeabilization like Triton-X100 that dissolve membranes.

In infected cells incubated in the presence of choline, the replication complexes formed a typical continuous perinuclear ring-like structure, this structure was efficiently stained only on the periphery, showing that the inner area is inaccessible to antibodies (Figure: 6.4). Contrary to the choline-supplemented medium, replication complexes formed in choline-free conditions appeared as evenly stained punctate scattered around the cytoplasm, demonstrating that they are readily accessible to antibodies (Figure: 6.4).

This result demonstrated that the inhibition of PC synthesis prevents formation of tightly packed membranous structures of replication organelles and leaving the viral replication complexes more accessible by cytoplasmic proteins, including cellular sensors of infection.

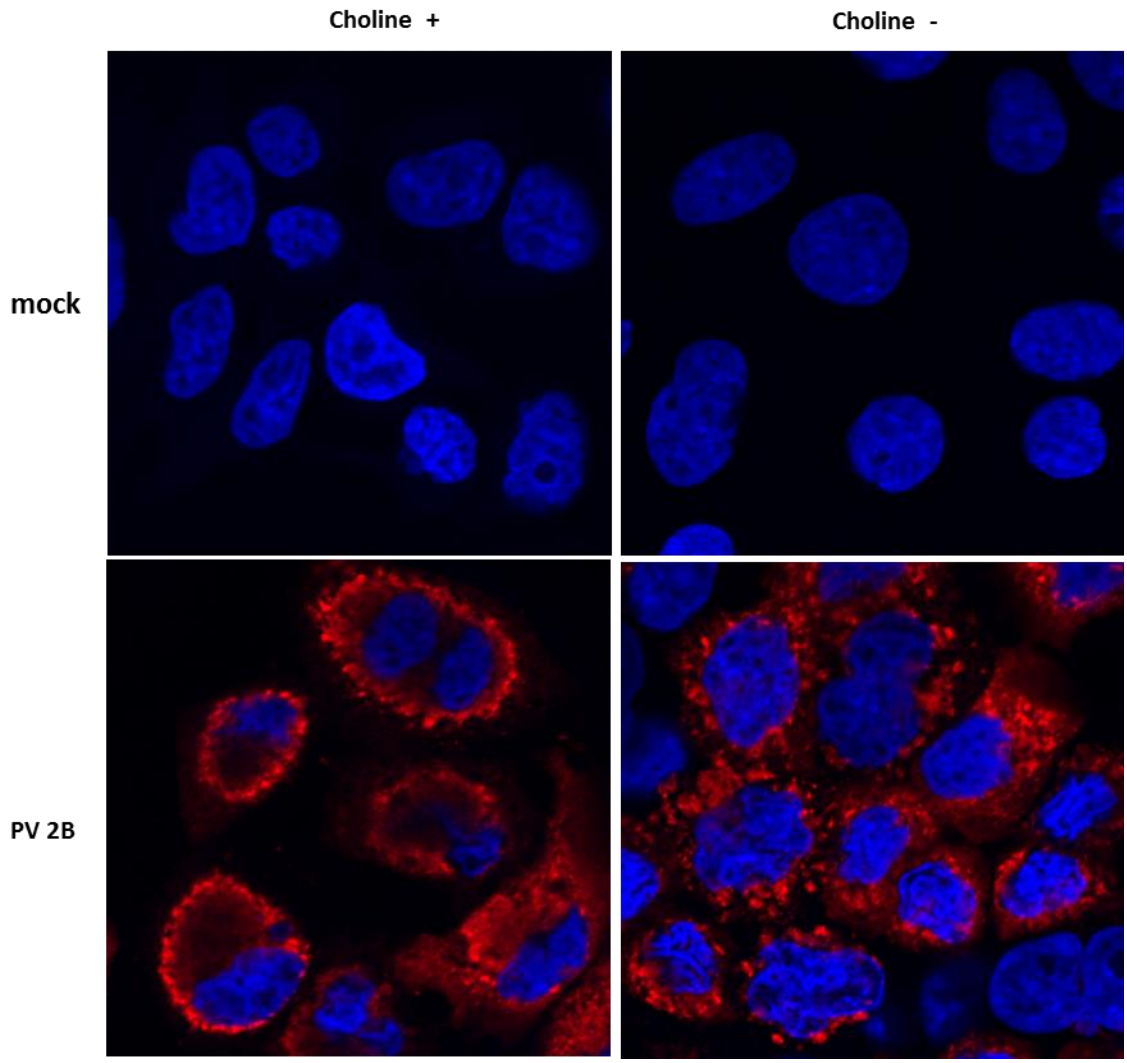

Figure 6.4: The upregulation of PC synthesis is required for the protection of the replication complexes from cytosolic factors. Hela cells grown for 48h in a choline-depleted medium were polio-infected at a MOI of 50PFU/cell then incubated in the presence of absence of 25uM of choline. At 4h post infection, infected cells were treated with 0.02% of saponin for 5mins then processed for an immunoassay staining with monoclonal antibodies targeting the membrane viral protein 2B.

Infection in a natural host inevitably involves multiple rounds of infection ultimately resulting in activation of immune response. Our results suggest that activation of PC synthesis in infected cells while dispensable for a single round of replication is important for forming proper spatial structure of the replication membranes, limiting access to the sites of the RNA replication. Thus, its inhibition should likely allow the cells to detect the infection earlier and initiate the anti-viral signaling leading to activation of immune response. To investigate if PC synthesis and by extension the development of membranous replication organelles are important for suppressing cellular response to infection we infected cells previously incubated for 48 h in choline-free medium at low MOI of 0.1, 0.01and 0.001 PFU/cell followed by an overnight incubation in either choline-free or choline-supplemented media. Since poliovirus replication time in HeLa cells is ~6 h, multiple rounds of infection develop under these conditions. After that total virus yield was assessed.

It should be noted that no toxicity was observed in mock-infected cells incubated in choline-free medium for the whole duration of the experiment. The concentration of infectious particles recovered after infection at the MOI of 0.1 PFU/cell was similar for either choline-free or choline-supplemented medium. At the MOI of 0.01 PFU/cell virus yield from cells incubated in choline-depleted medium was slightly lower, but not significantly, compared to that from the choline-enriched medium. At the lowest MOI of 0.001PFU/cell the virus yield in choline-free medium was more than 2 logs lower than that in choline-supplemented medium (Figure 6.5).

Thus, inhibition of PC synthesis significantly reduced virus yield in conditions of multiple rounds of infection, likely because the replication complexes were not protected from the cytosolic sensors of infection allowing the cells to mount anti-viral response.

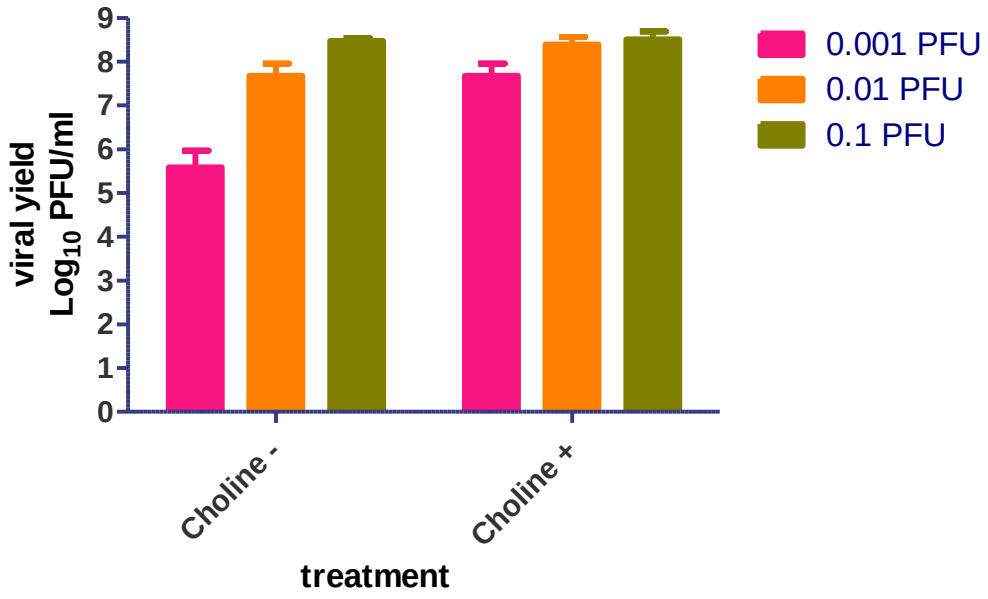

Figure 6.5: The development of poliovirus infection is sensitive to the inhibition of PC synthesis at low MOI. Hela cells grown for 48h in a choline-depleted medium were polio-infected at MOI of 0.001, 0.001 and 0.1 PFU/cell then followed by an overnight incubation in the presence or absence of 25uM of choline. The next day, the concentration of the total infectious particles in each condition was assessed via a plaque assay.

6.3 Discussions

In this project, we investigated the role of PC synthesis in the membrane remodeling in infected cells and the replication of poliovirus. The inhibition of PC synthesis via the choline starvation blocked the formation of membranous structures in the cytoplasm of poliovirus-infected cells. At the same time, the expression of viral proteins was similar in cells incubated in choline-depleted and choline–supplemented media. In line with this observation, active PC synthesis was dispensable for production of infectious viral particles in one round of infection at the broad range of MOIs.

However, since PC synthesis was required for the development of membranous structures, its inhibition resulted in increased accessibility of the viral replication complexes to cytosolic proteins. Accordingly, under conditions of multiple rounds of infections cells incubated in the absence of choline produced significantly less infectious virus than those in choline-supplemented medium, likely due to earlier detection of the infection and successful mounting of the anti-viral response.

Chapter 7: General Conclusion

Positive RNA viruses constitute an important group of animal, and plant pathogens capable of inflicting serious economic damages and human and animal diseases. Although treatments options are available to prevent or cure infections from a few of these pathogens, the vast majority lacks vaccines or antivirals therapeutic. The limitation to the development of vaccines or inhibitors targeting specific viral proteins is linked to the incomplete understanding of the host-pathogen interactions and rapid development of drug resistance. Although positive RNA viruses are different in their size, genome organization or respective hosts, they share one common feature that is the remodeling and induction of membranous structures in infected cells.

These membranous structures, which are specialized sites of the viral RNA replication, derive from various intracellular organelles and have different shapes and structures depending on the specific virus (57, 141). The mechanism of development of these membranous structures has been attributed to the modification of intracellular processes associated with membranes metabolism such as secretory and the autophagy pathways. However, these pathways are differentially required for the replication of these viruses and do not fully explain the development of these replication membranes.

The aim of this dissertation was to investigate the mechanism and role of activation of phospholipid synthesis in the development of membranous replication organelles of picornaviruses. In the chapter 4, we have shown that picornaviruses, upon infection, stimulate a strong import of exogenous fatty acids in infected cells. The newly imported fatty acids targeted lipid droplets in mock-infected cells while in infected cells they were

used for the upregulation of PC synthesis and served as a substrate for the synthesis of replication organelles supporting the replication complexes.

The most important aspect of this result was the identification of ACSL3 enzyme as a novel host factor required for the replication of poliovirus and the import of exogenous fatty acids in polio-infected cells. The ACSL3 enzyme belongs to the group of long and very long chain acyl-CoA synthetases (123). It was interesting to see that overexpression of the recombinant GFP-ACSL3-HA delayed the expression of viral proteins acting like an antagonist to replication. This result suggested that a functional ACSL3 with the normal structural configuration was required for the replication of poliovirus. Since ACSL3 was required for the upregulation of lipid synthesis and the replication of poliovirus, it may represent a potential target for anti-viral therapeutics.

Another remarkable result of this section was the discovery of a novel function of the poliovirus proteinase 2A, which was required but not sufficient for the activation of fatty acid import. The mechanism of action of 2A in the import of fatty acids is not yet understood, but it was independent of its protease activity. The activation of fatty acid import is shared by diverse picornaviruses. It will be interesting as a future goal, to investigate the specific fatty acids composition of membranes structures supporting the replication complexes of different picornaviruses, how they affect the physical properties of the replication organelles such as shape, size, permeability and fluidity as well as the functioning of the replication complexes.

In chapter 5 of this project, we showed via pharmacological treatments, that the ER stress response and the autophagy pathway, two intracellular processes associated with membranes metabolism, were not responsible for the activation of fatty acid import of in polio-infected cells. The processing of X-box protein 1 (XBP1) gene, a transcription factor associated with the ER stress response and the expression of its downstream target CHOP protein were blocked in polio-infected cells. It is well documented that poliovirus, upon infection, shut down the host proteins translation machinery via the cleavage of eIF4GI and eIF4GII by the viral proteinase $2A^{PRO}$ (43).

Poliovirus also blocks host transcription and mRNA splicing processing machinery through the degradation of host factors by viral proteinases $2A^{PRO}$ and $3C^{PRO}$ in infected cells (43). Thus, the inhibition of these intracellular processes upon infection may explain the absence of ER stress response activation in polio-infected cells. Similarly, the activation of autophagy with a pharmacological agent also failed to stimulate the import of fatty acids comparable to poliovirus-infected cells. The activation of autophagy has been linked to the development of double membrane structures or the non-lytic release of infectious particles in picornaviruses-infected cells (73, 110). Therefore, it is safe to say that the upregulation of phospholipids observed in polio-infected cells is an infection-specific process.

In chapter 6, we examined the development of membranous structures and the replication of poliovirus in the absence or presence of PC synthesis. Newly synthesized PC was associated with the replication complexes and was required for the proper development of membranous replication structures in polio-infected cells. At high MOI, PC was dispensable for the production of infectious particles while at a low MOI, the infection was

sensitive to the inhibition of PC synthesis. We speculated that the development of poliovirus infection at low MOI was compromised due to a lack of membranes protecting the replication complexes from cytosolic sensors and therefore activating the production of IFN. Accordingly, we showed that monoclonal antibodies could easily access the viral replication complexes formed in choline-deprived cells but not those in cells incubated in choline-supplemented medium.

In the future it is important to understand the mechanistic details of phospholipid synthesis activation upon picornavirus infection. How do the viral proteins induce rapid posttranslational upregulation of the cellular long chain acyl-CoA synthetase activity? Do different picornaviruses rely on the same or different mechanisms of lipid metabolism modulation? What is the major source of long chain fatty acids to sustain the phospholipid synthesis in infected cells – import from the extracellular medium, mobilization of neutral lipids from lipid droplets, or *de novo* synthesis by fatty acid synthase? What are the critical elements in the phospholipid synthesis in infected cells that can be targeted as a novel broad spectrum anti-viral strategy to make the infection more vulnerable to the natural host defenses?

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