ABSTRACT

Title of Dissertation: CHARACTERIZATION OF THE GBF1-ARF1 AXIS IN ENTEROVIRUS RNA REPLICATION

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The *Enterovirus* genus includes many known and emerging pathogens, such as poliovirus, enteroviruses A71 and D68, rhinoviruses, and others. Enterovirus infection induces the massive remodeling of intracellular membranes and the development of specialized domains harboring viral replication complexes, called replication organelles. The cellular protein Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1 (GBF1) is essential for the replication of enteroviruses, but its molecular role in the replication process is unclear. In uninfected cells, GBF1 activates small GTPases of the Arf family and coordinates multiple steps of membrane metabolism, including the functioning of the cellular secretory pathway. The nonstructural protein 3A of poliovirus and other enteroviruses directly interact with and recruits GBF1 to the replication organelles. Moreover, enterovirus infection induces the massive recruitment of all isoforms of the small cellular Arf GTPases to the replication organelles, but the mechanistic role of these proteins in the replication process is not understood either. Here, we sought to characterize the role of the

GBF1-Arf1 axis in enterovirus replication. First, we systematically investigated the conserved elements of GBF1 to understand which determinants are important to support poliovirus replication. We demonstrated that multiple GBF1 mutants inactive in cellular metabolism could still be fully functional in the replication complexes. Our results showed that the Arf-activating property, but not the primary structure of the Sec7 catalytic domain is essential for viral replication. They also suggest a redundant mechanism for recruiting GBF1 to the replication sites. This mechanism depends not only on the direct interaction of the protein with the viral protein 3A but also on elements located in the noncatalytic C-terminal domains of GBF1. Next, we investigated the distribution of viral proteins and Arf1 on the replication organelles and their biochemical environment. Pulse-labeling of viral RNA with 5-ethynyl uridine showed that active RNA replication is associated with Arf1-enriched membranes. We observed that Arf1 forms isolated microdomains in the replication organelles and that viral antigens are localized in both Arf1-depleted and Arf1-enriched microdomains. We investigated the viral protein composition of the Arf1-enriched membranes using peroxidase-based proximity biotinylation. Viral protein biotinylation was detected as early as 3 h.p.i., and the non-cleaved fragments of the viral polyprotein were overrepresented in the Arf1-enriched domains. Furthermore, we show that after 4 h.p.i. viral proteins could be efficiently biotinylated only upon digitonin permeabilization of the replication organelle membranes, while such permeabilization inhibited the Arf1 biotinylation signal at the Golgi in non-infected cells. Together, these data support a model that recruitment of GBF1 to the replication organelles generates foci of activated Arfs on the membranes, which further differentiate into specific microdomains through the recruitment of a specific complex of viral proteins and cellular Arf effectors likely needed to establish the lipid and protein composition required for viral replication.

CHARACTERIZATION OF THE GBF1-ARF1 AXIS IN ENTEROVIRUS RNA REPLICATION

by

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Dedication

This dissertation is dedicated to my family members and friends who were always there for me. I will be forever grateful for their love and unconditional support.

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List of Abbreviations

5-EU: 5-ethynyl-uridine

ACBD3: Acyl CoA-binding domain-containing 3

ACSLs: long-chain Acyl-CoA synthetase enzymes

AFM: Acute flaccid myelitis

APEX2: Ascorbate peroxidase 2

ARF: ADP-ribosylation factor

Arf-GAP: Arf-GTPase activating protein

Arf-GEF: Arf guanine nucleotide exchange factor

ATGL: Adipocyte triglyceride lipase

BFA: Brefeldin A

CNS: Central nervous system

Co-IP: Co-immunoprecipitation

cVDPV: Circulating vaccine-derived poliovirus

CVB3: Coxsackievirus B3

CRE: cis-acting RNA element

DMEM: Dulbecco modified Eagle's medium

dsRNA: Double-stranded RNA

eIF: Eukaryotic initiation factor

ERGIC: Endoplasmic Reticulum-Golgi intermediate compartment

EV-A71: Enterovirus A71

EV-D68: Enterovirus D68

FBS: Fetal bovine serum

GBF1: Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1

GFP: Green fluorescent protein

GPEI: Global Polio Eradication Initiative

HFMD: Hand-food-and-mouth disease

hnRNP: Heterogeneous nuclear ribonucleoprotein

HSL: Hormone-sensitive lipase

HPI: Hours post-infection

IPV: Inactivated poliovirus vaccine

IRES: Internal ribosome entry site

ITAF: IRES trans-activating factor

kDa: Kilodalton

LDs: Lipid droplets

MOI: Multiplicity of infection

NPEV: Non-polio enterovirus.

OPV: Oral polio vaccine

ORF: Open reading frame

OSBP: Oxysterol-binding protein

PFU: Plaque forming unit

PI4KIIIβ: Phosphatidylinositol 4-kinase III β

PI4P: Phosphatidylinositol 4-phosphate

PV: Poliovirus

PVR: Poliovirus receptor

ROs: Replication organelles

RV : Rhinovirus

SDS-PAGE : Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

UTR: Untranslated region

VAPP: Vaccine-associated paralytic poliomyelitis

VDPV: Vaccine-derived poliovirus

VPg: Viral protein genome linked

Chapter 1: Introduction to Enteroviruses

1.1 Enterovirus classification and enterovirus-associated diseases.

The *Enterovirus* genus within the *Picornaviridae* family comprises 15 species: *Enterovirus A* to *L* and *Rhinovirus A* to *C*. Members of a genus in the *Picornaviridae* family are expected to have homologous proteins and their sequences should form monophyletic trees (2). Among enterovirus species, *Enterovirus A* to *D* and rhinoviruses can cause various illnesses in humans, ranging from mild respiratory or gastrointestinal infections to more severe diseases such as asthma, exacerbation of chronic pulmonary disease, encephalitis, meningitis, myocarditis, and poliomyelitis (3-6). Enterovirus species have been defined through phylogenetic analysis reflecting evolutionary relationships. For instance, the *Enterovirus A* species has 25 types, including coxsackievirus A16 and enterovirus A71, both of which can cause hand, foot, and mouth disease. *Enterovirus B* contains 63 types, including coxsackievirus B3, which is associated with illnesses such as type I diabetes, acute myocarditis, and dilated cardiomyopathy. Poliovirus (PV), which is the etiological agent of poliomyelitis, belongs to the *Enterovirus C* species, which contains 23 types in total. *Enterovirus D* has five types and includes the emerging neurotropic enterovirus D68. *Rhinovirus A*, *B*, and *C* have 80, 32, and 57 types, respectively (7-9). *Enterovirus E* to *L* infect animals. *Enterovirus E* and *F* are mostly found in cattle, whereas *Enterovirus G* is found in goats and pigs. They include 5, 7, and 22 types, respectively, associated with diarrhea, respiratory disorders, skin lesions, flaccid paralysis, and infertility (10-13). *Enterovirus J* comprises 6 types that are found in simians (14-16). Rodent enteroviruses belong to the species *K,* with currently 2 types (17) and only one type has been identified in the *Enterovirus* species *H*, *I,* and *L*, which have been found in macaques and dromedary camels (18, 19). It should be noted that

new picornaviruses, including enteroviruses, are discovered regularly, mostly from metagenomics data. Thus, the *Picornaviridae* family is constantly being updated.

1.1.1 Poliovirus.

Poliovirus is the etiological agent of poliomyelitis, a disease that affects the central nervous system (CNS). PV naturally infects humans, whereas non-human primates and transgenic mice engineered to express the human poliovirus receptor can be experimentally infected (20, 21). This host range is determined by the presence of the PV receptor (PVR), also known as CD155, a transmembrane glycoprotein in the immunoglobulin superfamily involved in many different physiological processes ranging from cell adhesion and migration, proliferation, and modulation of immune responses (22-24).

Poliovirus is transmitted via the fecal-oral route. The virus first multiplies in the oropharyngeal and intestinal mucosa, then it reaches the draining lymph nodes and enters the bloodstream, establishing viremia (25-27). In most cases of PV infection, there is a primary minor viremia without clinical manifestation. However, in approximately 4-8% of those individuals, a second major viremia can occur, leading to cold-like symptoms such as sore throat and fever. Only a small number of people with major viremia develop neurological symptoms, which are a rare complication of PV infections. Less than 1% of PV-infected individuals develop poliomyelitis (25, 28). In this case, the virus spreads to the CNS by permeation through the blood-brain barrier (BBB) independently of PVR or by retrograde axonal transport from peripheral nerves (29, 30).

Poliomyelitis is classified according to the specific clinical manifestations in 1) spinal poliomyelitis, which is limited to the extremities and trunk and varies from mild weakness to quadriplegia; 2) bulbar poliomyelitis, where the respiratory muscles are paralyzed; and 3) bulbospinal poliomyelitis, a combination of the two (31).

Over the years, efforts have been made to eradicate poliovirus across the globe. This initiative is led by the Global Polio Eradication Initiative (GPEI), a public-private partnership that is led by national governments and comprises five core partners: the World Health Organization (WHO), Rotary International, the U.S. Centers for Disease Control and Prevention (CDC), the United Nations Children's Fund (UNICEF), and the Bill & Melinda Gates Foundation. Since the launch of the GPEI in 1988, the number of polio cases caused by wild-type polioviruses (WPVs) has reduced by over 99%. Two out of the three wild-type poliovirus (WPV) serotypes have been declared eradicated, WPV2 in 2015 and WPV3 in 2019, with the last known cases of these viruses reported in 1999 in India and 2012 in Nigeria, respectively. Nowadays, there are only two countries with WPV1 transmission, Pakistan and Afghanistan. Wild-type polioviruses are not the only viruses that can cause poliomyelitis. In certain instances, the live-attenuated polioviruses from the oral poliovirus vaccine (OPV) can mutate and spread in populations with low vaccination levels and revert into circulating vaccine-derived polioviruses (cVDPV). These viruses are indistinguishable from wild-type viruses both clinically and epidemiologically (32-35). The three serotypes of the virus can cause cVDPV; however, more than 90% of the cases are associated with type 2 (36, 37).

In 2020, the GPEI revised its plan of action to eradicate poliovirus and implemented the Polio Eradication Strategy 2022–2026, which aims to permanently interrupt all poliovirus transmission in endemic countries and stop cVDPV transmission and prevent outbreaks in non-endemic countries. Many targeted activities have been implemented to effectively achieve these goals, including the development of next-generation vaccines with better genetic stability, increased vaccination coverage for children, and the establishment of intensive disease surveillance in regions with a history of circulating VDPV outbreaks.

1.1.2 Non-polio enteroviruses.

In recent years, several non-polio enteroviruses (NPEVs) have emerged as significant public health concerns. These viruses include coxsackieviruses, echoviruses, numbered enteroviruses, and rhinoviruses, which are important pathogens that cause a wide range of diseases with varying symptoms and severity levels. Most commonly, these viruses affect infants, young children, and individuals with compromised immune systems (38).

Hand-foot-and-mouth disease (HFMD) is a viral illness commonly seen in children under 5 years of age. Viruses from *Enterovirus A* species are the most common causes of HFMD epidemics, and the disease is mostly prevalent across the Asia-Pacific region (39). The illness is characterized by painful lesions in the mouth, known as herpangina, and skin rashes on the hands and feet. The main causes of mild cases of HFMD are coxsackievirus A6, A10, and A16, whereas enterovirus A71 has been associated with up to 96% of severe cases (40-43). Clinical studies carried out in Malaysia showed that 10-30% of children with EV-A71-related HFMD hospitalization developed neurological complications such as aseptic meningitis, acute flaccid paralysis, encephalitis, and in more rare instances cerebellar ataxia, and transverse myelitis (40, 44). The main risk factors implicated in the development of neurological complications of HFMD are a history of lethargy, mean peak temperature $\geq 38.5°C$, and total duration of fever ≥ 3 days (45).

Another neurotropic NPEV is enterovirus D68. EV-D68 is transmitted via the respiratory route and can cause mild to severe respiratory symptoms that can result in hospitalization and death (46). EV-D68 infections are associated with several neurological complications, with acute flaccid myelitis (AFM) being the most common. AFM affects the spinal cord's gray matter, which causes muscle weakness and decreased reflexes (47, 48). EV-D68 shows a biennial pattern of

circulation and the vast majority of cases affect children younger than 10 years (49). Echoviruses, also members of the *Enterovirus A* species, are also implicated in neurological disorders. For instance, echovirus 33 can cause AFM (50), and echoviruses 5, 6, and 9 are involved in encephalitis and meningitis (51-53).

Coxsackievirus B3 (CVB3) is known to cause acute viral myocarditis, and it is linked to type I diabetes. CVB3 tissue tropism depends on the coxsackievirus and adenovirus receptor (CAR), a transmembrane protein associated with tight junctions (54). The analysis of CAR mRNA expression in human tissues shows its presence in the heart and pancreas, and lower expression levels in the lungs and liver, which is consistent with the pattern of tissue susceptibility and the clinical presentations of CVB3 infections (55, 56).

Myocarditis is the inflammation of the myocardium, the middle layer of the heart muscle, that can result in the degeneration and death of adjacent myocytes (3). CVB3 is implicated in 3.2% of cases of acute heart disease. Enterovirus-induced cardiomyopathy primarily affects young patients, potentially leading to sudden death or lifelong heart failure (57). CVB3 is also associated with chronic myocarditis and dilated cardiomyopathy (58). Evidence shows that the virus continues to replicate in later stages of the disease, as demonstrated by the identification of negative-strand and positive-strand enteroviral RNA, as well as enteroviral proteins in the heart muscle of chronic patients with cardiomyopathy (59, 60).

Type 1 diabetes is a long-term autoimmune disease that involves the destruction of insulin-producing beta cells in the pancreas. A complex interaction between genetic predisposition and environmental factors can contribute to the development of the disease. Several studies have found enteroviruses in the blood and pancreas of patients with type 1 diabetes where no other viruses were found (61-63). CVB3, CVB4, and CVB5 can replicate in human pancreatic islets *in vitro*,

and some CVB4 isolates can cause diabetes in certain mouse strains and non-human primates (64, 65). Several mechanisms have been proposed to contribute to type 1 diabetes pathogenesis, including direct cytolysis, molecular mimicry, and persistent infection (66). For instance, activation of autoreactive T-lymphocytes can be caused by the presentation of viral antigens, such as the protein 2C of CVB4, due to its high homology with the human islet autoantigen GAD-65 (67). Viral persistence can also lead to the dysfunction of β-cells in type 1 diabetes. Persistent enterovirus infections of pancreatic islets can result in a continuous presentation of viral peptides and the production of proinflammatory cytokines that progressively promote the development of islet autoimmunity (66).

Rhinoviruses (RV) are the main causative agent of the common cold, with symptoms including a runny nose, congestion, sore throat, and cough. RVs circulate worldwide and the infections are most common in infants and young children. RVs are highly contagious and mainly cause upper respiratory infections, although in certain cases, they can invade the lower parts of the respiratory tract leading to hospitalization (4). Children who wheeze when infected with rhinoviruses face a higher risk of developing asthma. Once asthma develops, rhinovirus infections can act as strong triggers for acute airway obstruction in children and adults. RV-A and RV-C viruses are associated with a higher incidence of illness and wheezing than RV-B viruses. The greater virulence of the A and C species may be attributed to their faster rates of viral replication, which elicit more significant inflammatory responses than the RV-B species (68).

Enteroviruses also cause acute hemorrhagic conjunctivitis, a highly contagious eye infection that affects the conjunctiva and is characterized by redness, swelling, and pain. The disease is caused by enterovirus D70, coxsackievirus A24, and echovirus type 7. The conjunctivitis

caused by enteroviruses is often indistinguishable from the one caused by adenoviruses (31, 69, 70).

1.2 Enterovirus vaccines.

Nowadays, licensed vaccines are available only against enterovirus A71 and poliovirus.

There is no FDA-approved vaccine for EV-A71 in the United States. However, the China National Medical Products Administration has approved three different commercially available vaccines for EV-A71 in China, which were developed by Sinovac Biotech, Beijing Vigoo, and the Chinese Academy of Medical Science. These vaccines are formalin-inactivated, whole-virus vaccines and their efficacy and safety have been confirmed through phase III clinical trials. The trials showed that the vaccines are 94.8% effective against HFMD associated with EV-A71, and 100% effective against HFMD with neurologic complications (71, 72). Live-attenuated vaccines against EV-A71 have not been licensed due to an incomplete understanding of the mechanisms of viral pathogenesis, although determinants of pathogenicity such as the amino acid position 145 of the capsid protein VP1 have been identified (73). Other types of vaccines to combat enterovirus A71 are under development, such as recombinant VP1 vaccine, synthetic peptide vaccines, and multivalent vaccines against enterovirus A71 and other HFMD-related enteroviruses like Coxsackievirus A6, A10, and A16 (74).

The two vaccines against poliovirus are the inactivated Salk vaccine (IPV) and the live-attenuated Sabin vaccine (OPV). The inactivated poliovirus vaccine was developed in 1955 by Dr. Jonas Salk. It is administered by intramuscular or intradermal injection and triggers humoral immunity in the vaccine recipient. The live-attenuated Sabin vaccine, on the other hand, is administered orally and induces both humoral and mucosal immunity (75). Compared to IPV, OPV confers strong systemic and intestinal immunity, is easier to administer through oral delivery, and

is less costly. Moreover, since the live-attenuated virus mimics the natural route of infection and is excreted into the environment, it provides herd immunity to unvaccinated individuals in close contact with the vaccine recipients (31). Nevertheless, the main disadvantage of OPV is that it can revert to the wild-type neurovirulent phenotype, cause vaccine-associated paralytic poliomyelitis (VAPP), and lead to the circulation of vaccine-derived poliovirus causing outbreaks of poliomyelitis.

There are three categories of vaccine-derived polioviruses: 1) circulating vaccine-derived poliovirus (cVDPV), 2) immunodeficiency-associated vaccine-derived poliovirus (iVDPV), and 3) ambiguous vaccine-derived poliovirus (aVDPV). The source of infection for aVDPV is not known or it can be associated with a case of acute flaccid paralysis without clear evidence of VDPV circulation or immunodeficiency (76). To prevent vaccine-associated poliomyelitis, novel oral poliovirus vaccines (nOPVs) with improved genetic stability are being developed. In November 2020, the type 2 novel OPV (nOPV2) was recommended by the WHO Emergency Use Listing for outbreak response to cVDPV2 (77), and experiments conducted on mice with type 1 and 3 nOPV showed that the vaccine candidates are highly immunogenic and remain attenuated due to an enhanced genetic stability (78).

1.3 Anti-enteroviral drugs

Despite the wide range of enterovirus species and their associated diseases, no FDA-approved anti-enteroviral drugs are available. Antiviral drugs usually target viral proteins. Such compounds targeting viral proteins are generally less cytotoxic as they do not interfere with cellular metabolism. Examples are compounds that target capsid proteins (pleconaril, pirodavir, BTA798, BPR0Z-194, vapendavir, V-073), proteases (rupintrivir, telaprevir, AG7404, telaprevir), and the polymerase (favipiravir, azvudine, ribavirin, DTriP-22, amiloride) (79-81). However, the

emergence of resistant viruses represents a major drawback of using anti-enteroviral drugs targeting viral proteins.

An alternative strategy to fight enterovirus infection involves the development of antiviral compounds that target cellular proteins that are crucial for viral replication. Targeting host factors to inhibit viral infection offers several advantages. This approach can increase the threshold for the development of viral resistance and provide broad-spectrum antiviral action against different viruses that rely on the same host protein for their replication. However, the main challenge of this approach is the potential cytotoxicity of these compounds (82).

Many compounds targeting host proteins to inhibit enterovirus infection have been identified, but none of them have been approved for clinical use. For example, geldanamycin is a capsid assembly inhibitor that targets the cellular chaperone HSP90. Enterovirus capsid proteins require HSP90 to fold correctly for capsid assembly and virion maturation, and geldanamycin has been found to impair enterovirus replication in infected mice (83). Another HSP90 inhibitor called VER-50589 has antiviral activity against CVB3, CVB4, and echovirus 11 in cell culture and promotes survival by reducing viral load in EV-A71-infected mice (84).

Other examples include enviroxime-like compounds. These compounds are classified as major enviroxime-like compounds (PIK93, GW5074, MDL-860, and T-00127-HEV1) and minor enviroxime-like compounds (OSW-1, itraconazole, AN-12-H5, and T-00127-HEV2) depending on whether they target the cellular proteins phosphatidylinositol 4-kinase III β (PI4KIIIβ) or oxysterol-binding protein (OSBP), respectively (85-87). PI4KIIIβ inhibitors like MDL-680 have antiviral activity against a broad spectrum of viruses. However, they have displayed potential toxicity and side effects *in vivo* (88-90). Another compound, OSW-1, has shown prophylactic antiviral activity against coxsackievirus A9 and echovirus 2 in cell culture by reducing the cellular

abundance of OSBP (91, 92). Nevertheless, the use of enviroxime-like compounds for therapeutic purposes is hindered by the emergence of resistant viruses, as well as the toxicity of some of the candidate drugs (79).

One crucial strategy for developing effective antiviral therapies is to understand the virus replication cycle and the cellular proteins that support infection to target these host-virus interactions.

1.4 Enterovirus genome organization, virion structure, and replication cycle.

1.4.1 Genome organization.

All enteroviruses have a similar genome organization consisting of a positive, single-stranded RNA that ranges from 7200 to 8500 nucleotides. Poliovirus is the prototype member of the genus. The enterovirus genome has five distinctive regions: a 5' untranslated region (UTR), two open reading frames (ORFs), a 3' untranslated region, and a poly(A) tail of around 60 adenine residues (Figure 1.1) (34, 93).

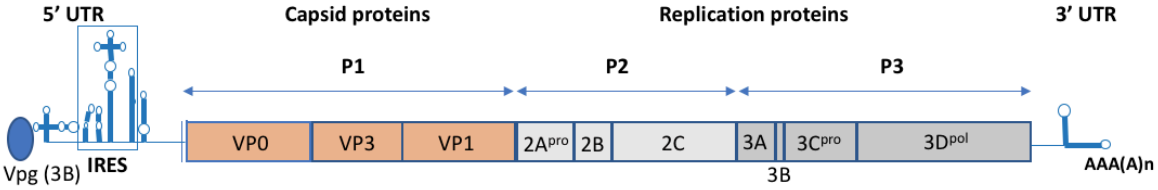


Figure 1.1: Poliovirus genome organization.

The poliovirus 5' UTR is composed of two highly conserved secondary structures. It is 742 nucleotides long and is covalently linked to the viral protein VPg (viral protein genome linked), also called 3B (93, 94). The first 108 nucleotides form a cloverleaf-shaped structure with three stem-loops (B, C, and D) branching from a single stem. The cloverleaf is involved in viral RNA replication through the formation of viral ribonucleoprotein complexes and protects the genome

from degradation by cellular nucleases (95, 96). The other secondary structure of the 5'UTR is the internal ribosome entry site (IRES), which consists of five domains (II, III, IV, V, and VI) composed of several stem-loops that direct the initiation of mRNA translation in a cap-independent manner (97).

Furthermore, the 5' UTR contains two spacer regions, one between the cloverleaf and the IRES, called spacer I; and the other located between the 3' end of IRES and the initiation codon of the polyprotein, called spacer II. Short oligo(C) segments in Spacer I are crucial for ribonucleoprotein complex formation in the cloverleaf, which is necessary for RNA replication. Mutations in this region lead to attenuation of poliovirus neurovirulence (98-100). The spacer II is associated with the neurovirulent phenotype of poliovirus in mice and non-human primates (101, 102). Spacer II is absent in rhinovirus genomes.

The main ORF encodes a single polyprotein that is cleaved co- and post-translationally by viral proteases and is divided into three regions - P1, P2, and P3. P1 is the precursor of the structural proteins that form the capsid, whereas P2 and P3 are precursors of non-structural proteins that participate in viral replication. A small upstream open reading frame (uORF) has been discovered within the 5' UTR of poliovirus type 1, enterovirus A71, and echovirus 7. This uORF encodes a polypeptide that is predicted to have a transmembrane domain and is believed to play a role during the release of viruses from intestinal cells (103).

Another important functional element of the enterovirus genome is a cis-acting replication element (CRE), which serves as a template for the synthesis of the Vpg-pU-pU primer for RNA replication. The CRE function is independent of the location in the genome, and it varies among viruses. For instance, the CRE of the members of *Enterovirus A, B*, *C,* and *D* is in the coding region

of the protein 2C, whereas in members of *Rhinovirus A*, *B,* and *C* the CRE is located in 2A, VP1, and VP2, respectively (104-106).

The 3' UTR is about 72 nucleotides and consists of secondary structures forming a pseudoknot that plays a role in viral RNA synthesis. The 3' UTR is followed by a poly(A) tail of about 60 adenine residues that are involved in viral RNA translation and replication (107, 108).

1.4.2 Virion Structure.

The enterovirus virion is an icosahedral, non-enveloped particle of about 30 nm in size (Figure 1.2). The viral particle contains 60 copies of a heteromeric structural unit, called the protomer, made of one copy of the proteins VP1, VP2, VP3, and VP4 (109). Five protomers form a pentamer, and 12 pentamers assemble into a capsid with an icosahedral symmetry. Each pentamer contains two-fold, three-fold, and five-fold axes of symmetry. VP2 and VP3 alternate between two- and three-fold axes, while VP1 is located around the five-fold axes. The C-termini of VP1, VP2, and VP3 are located on the outer surface of the virion, and the N-termini are in the interior. VP4 lies exclusively internally. Although VP1, VP2, and VP3 have no amino acid sequence homology, they share the same topology, the β-barrel jelly roll. The β-barrel jelly roll is a wedge-shaped structure of two four-stranded antiparallel β-sheets. The β-barrel domains of these proteins have a similar shape, facilitating their interaction to assemble the capsid, while the interactions between N-terminal domains inside the capsid reinforce the β-barrel packing. VP4 differs from the other proteins in that it has an extended conformation and contains myristic acid covalently linked to glycine at the N-terminus (93).

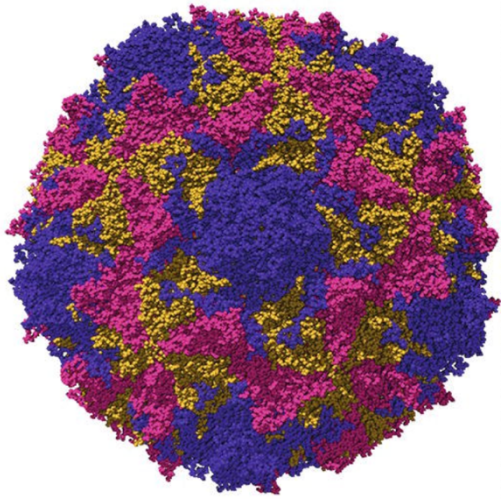


Figure 1.2: Crystal structure of poliovirus type I Mahoney at 2.2A of resolution (1)

An important structural feature of the enterovirus particle is the presence of a canyon, which serves as the receptor binding site. The canyon is created by the arrangement of VP1 around the five-fold axes of symmetry, which results in a pronounced crest that wraps around the axis creating a depression. The structure of the canyon may vary among enteroviruses. For example, the canyon of enterovirus D68 is narrower, and the capsid of rhinovirus C contains protrusions on its surface creating shallow and non-continuous canyons (110, 111). The floor of the canyon contains a small hydrophobic pocket that harbors a pocket factor, which consists of a lipid molecule. For instance, the pocket factors of poliovirus type I, coxsackievirus B3, and rhinovirus A16 are sphingosine, C16 lipids, and C12 lipids, respectively (111-113). On the other hand, some enteroviruses like rhinovirus B3, B14, and C15 do not have a pocket factor (93, 110).

1.4.3 Replication cycle.

1.4.3.1 Attachment and entry.

Enteroviruses start infecting cells by binding to a receptor on the plasma membrane. The interaction between the virion and the receptor triggers intracellular signals that activate the entry

pathways and the necessary conformational changes in the viral particle for genome release. Enteroviruses use various types of receptors, including immunoglobulin (Ig)-like receptors (PV and CVBs), mucin-like receptors (EV-A71 and CV-A16), integrins (CV-A9, and echovirus 1 and 8), sialic acid (EV-D68 and EV-D70), proteoglycans (echovirus 5), complement control proteins (CV-A21, echovirus 3, 6 and 7), and low-density/very low-density lipoprotein receptor (LDLR and VLDLR, used by some rhinoviruses) (93, 114).

Enteroviruses are subsequently internalized through receptor-mediated endocytosis, mainly clathrin-mediated endocytosis and caveolin-mediated endocytosis. Clathrin-mediated endocytosis starts with cargo binding to receptors on the cell surface. This binding triggers the recruitment of clathrin and adaptor proteins such as AP-2, which promotes membrane bending and the assembly of clathrin-coated pits. Then, the protein dynamin facilitates the excision of the coated pit from the plasma membrane. Once the clathrin-coated vesicle is formed, it sheds its clathrin shell and fuses with endosomes (115, 116). Caveolin-mediated endocytosis creates small invaginations called caveolae in the cell membrane, which are formed by the assembly of a membrane protein that binds directly to cholesterol, known as caveolin. Once the caveolar vesicles are excised from the plasma membrane, they can merge with early endosomes or transport their contents directly to other specific destinations within the cell (117).

The canyon in the viral particle usually serves as the receptor's binding site during enterovirus entry. Binding to the receptor at physiological temperature triggers conformational changes in the capsid, producing an altered particle called the A particle. Compared to the native virion, this particle has a different sedimentation coefficient and antigenic properties. It is characterized by the externalization of VP4 and the N-terminal domain of VP1, forming an

amphipathic helix inserted into the endosomal vesicles' membranes through which the RNA can be released into the cytoplasm (118-121).

The poliovirus receptor (PVR), CD155, is a type I transmembrane glycoprotein belonging to the Ig superfamily of proteins. It has three extracellular Ig-like domains, and its N-terminal Ig-like domain binds to the canyon of all three serotypes of PV (122-124). Orthologs of CD155 are found in multiple mammalian species, including those that are not susceptible to poliovirus. The amino acid sequence of the N-terminal domain of the receptor that binds to poliovirus differs in non-susceptible hosts, which explains the restriction in the host range (125). The mechanism of PV entry depends on the cell type. For instance, poliovirus entry into HeLa cells occurs via an endocytic pathway that depends on actin, ATP, and the phosphorylation of the cytoplasmic tail of CD155 by a tyrosine kinase but is independent of clathrin, caveolin, microtubules, flotillin, and pinocytosis (126). However, viral entry into human brain microvascular endothelial cells occurs via dynamin-dependent caveolin-mediated endocytosis (127).

There are two groups of rhinoviruses, minor and major, based on the type of receptor they use for entry. The minor group of rhinoviruses uses LDLR, which binds to VP1 in a conserved lysine (Lys224) located near the five-fold axis outside the canyon. In contrast, members of the major rhinovirus group lack this conserved lysine and use the intracellular adhesion molecule 1 (ICAM-1) as a receptor that binds to the canyon (128-132). LDLR mediates rhinovirus entry via clathrin-mediated endocytosis, and the release of viral RNA is triggered by endosome acidification (133, 134). However, the exact entry mechanism used by ICAM-1-binding rhinoviruses remains unclear. The receptor for *Rhinovirus C* has been identified as cadherin-related family member 3 (CDHR3) (135).

Two receptors have been identified for enterovirus A71: P-selectin glycoprotein ligand 1 (PSGL1) and scavenger receptor class B member 2 (SCARB2). PSGL-1 is a mucin-like protein found in leukocytes, but it has also been reported to be expressed in human neuronal tissue. SCARB2 is a type III integral membrane protein with two transmembrane domains, two short intracellular domains, and an N-glycosylated central extracellular domain. Neurons express SCARB2, and transgenic mice that express human SCARB2 develop similar neurological symptoms as humans, suggesting that SCARB2 is the primary determinant for the development of neurological disease (136, 137). SCARB2 triggers clathrin-mediated endocytosis (138), and PSGL-1 can bind to EV-A71 and internalize it via caveolin-mediated endocytosis (139).

Other enteroviruses require more than one molecule for entry. Such is the case of members of the *Enterovirus B* species. For example, CVB3 binds to the decay accelerating factor (DAF), a member of complement proteins composed of four extracellular short consensus repeats attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor located on the apical surface of polarized epithelial cells. CVB3 binding to DAF does not cause conformational changes and virus internalization but instead triggers actin rearrangements to move the virus-DAF complex to the tight junctions where the virus can bind to the coxsackievirus-adenovirus receptor (CAR) to enter the cells via caveolin-mediated endocytosis (140, 141). The human neonatal Fc receptor (FcRn) serves as an uncoating receptor for the members of the *Enterovirus B* species (142).

1.4.3.2 Genome translation and polyprotein processing.

Once the positive, single-stranded RNA genome is released into the cell, it is translated by the cellular translation machinery to produce the non-structural proteins needed for viral replication and the structural proteins that form the virion (93).

1.4.3.2.1 Initiation of translation.

Proteins are synthesized by ribosomes from an mRNA template read in the 5'-3' direction. Eukaryotic mRNAs have a 5' cap structure consisting of a 7-methylguanosine linked to the mRNA by a 5'-5' triphosphate bond, followed by a 5′ UTR, a protein coding sequence and a 3′ UTR that ends in a poly(A) tail. In eukaryotic cells, the canonical translation pathway starts with the formation of a 43S pre-initiation complex composed of the eukaryotic initiation factor 1 (eIF1), eIF1A, eIF3, eIF5, the 40S ribosomal subunit, and a ternary complex made of eIF2, GTP and methionyl-tRNA. In parallel, the eIF4F complex is assembled at the 5' end of the capped mRNA. This complex comprises the scaffolding protein eIF4G, the RNA helicase eIF4A, and the cap-binding protein eIF4E. Additionally, the poly(A) binding protein (PABP) binds to the poly(A) tail at the 3' end of the mRNA. It interacts with eIF4G in the eIF4F complex, resulting in the circularization of mRNA, which enhances translation efficiency. The eIF4F complex recruits the 43S pre-initiation complex, forming the 48S complex. The 48S complex then scans the 5′ UTR until it finds a start codon, which triggers the release of most eIFs. Finally, the binding of eIF5B to the 40S ribosomal subunit recruits the 60S ribosomal subunit at the start codon, forming the 80S initiation complex to begin protein synthesis (143, 144).

The enterovirus genome is uncapped, and viral protein synthesis is mediated by the internal ribosome entry site (IRES). Following the delivery of the viral RNA into the cytoplasm, the viral protein VPg is cleaved from the 5' end of the genome by a cellular DNA repair enzyme called 5′-tyrosyl–DNA phosphodiesterase-2 (TDP2) (145-147), although this cleavage is not essential for the functioning of the viral RNA as a template in translation (148). Then, translation initiation occurs when the IRES recruits the 40S ribosomal subunit through its interaction with eIF3 and the C-terminal domain of eIF4G. eIF1, eIF4A, and the eIF2-GTP-met-tRNA ternary complex are also

required, but not the cap-binding protein eIF4E. Additionally, the poly(rC)-binding protein 2 (PCBP2) acts as an IRES trans-activating factor (ITAF). PCBP2 is a cellular RNA-binding protein that contains three heterogeneous nuclear ribonucleoprotein (hnRNP) K-Homology domains (KH1, 2, and 3) which are crucial in forming the initiation complex at domain IV of the IRES (149). Once it is formed, the initiation complex scans the RNA until the start codon is located.

Enterovirus infection inhibits 5' cap-dependent translation of cellular mRNAs. This is achieved by $2A^{pro}$-mediated cleavage of eIF4G into the N-terminal eIF4E-binding domain and the C-terminal domain that binds eIF3 and the IRES, and by cleavage of PABP by $3C^{pro}$ (150-154).

1.4.3.2.2 Polyprotein processing cascade.

The enteroviral proteins are encoded in an ORF that is translated into a single polyprotein of approximately 250 kDa. The viral proteases $2A^{pro}$, $3C^{pro}$, $3ABC^{pro}$, and $3CD^{pro}$ proteolytically process it in *cis* and *trans*, yielding several intermediates and mature proteins with different functions during infection (Figure 1.3) (155). The cleavage site for $2A^{pro}$ is between tyrosine and glycine in poliovirus, rhinovirus, and other enteroviruses. In certain coxsackieviruses and echoviruses, the cleavage occurs between threonine–glycine and phenylalanine–glycine (93). $3C^{pro}$ and $3CD^{pro}$ cleave between glutamine-glycine sites and show a preference for an additional hydrophobic amino acid at the fourth residue N-terminal to the cleavage site (93, 156, 157). The $3ABC^{pro}$ intermediate has a preference for the 2C-3A and 3B-3C junctions *in vitro* (158).

The full-length polyprotein is not observed in infected cells during viral infection because it is processed both co- and post-translationally. The enterovirus structural protein precursor, P1, is situated at the N-terminal part of the polyprotein. The viral protein $2A^{pro}$ cleaves P1 from the polyprotein in *cis*. After that, P1 processing is carried out in *trans* by the viral protein $3CD^{pro}$, yielding the proteins VP0, VP3, and VP1. VP0 is autocatalytically cleaved into VP4 and VP2

during virion maturation. The P2-P3 domain of the polyprotein can undergo processing via two different pathways. In the first pathway, which is detected in the soluble fraction of infected cells and occurs slowly, the P2-P3 junction is cleaved by the viral protein $3C^{pro}$, generating the P2 and P3 precursors that are further processed into multiple intermediates and mature peptides. P2 initially generates 2A and 2BC, and then 2BC is cleaved to generate 2B and 2C. The cleavage of the P3 precursor can occur through the major pathway, which generates the viral proteins 3AB and $3CD^{pro}$, or the minor pathway, which produces 3A and the precursor 3BCD (159). Fully processed P3 yields the mature peptides 3A, 3B, $3C^{pro}$, and the viral RNA-dependent RNA polymerase $3D^{pol}$. The second pathway of P2-P3 processing is associated with membranes and involves the rapid cleavages of P2 proteins by $3C^{pro}$ to generate 2A, 2BC, and the P3 precursor without the generation of P2. The existence of different pathways of polyprotein processing may facilitate locating replication proteins on the membranes to assemble the replication complexes and alternatively generate diffusible proteins that perform functions related to the production of virus particles and the modulation of the host gene expression and metabolism (160-162).

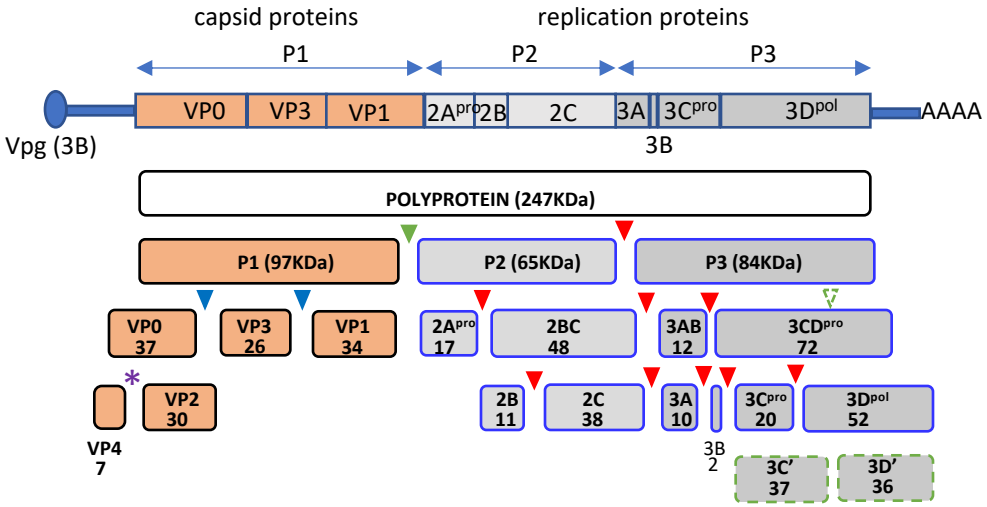


Figure 1.3: Poliovirus polyprotein processing. The molecular weights of the precursor and mature viral proteins are presented next to their names in kDa. The red triangles (▼) are the cleavage sites for the viral protease $3C^{pro}$; the green triangle (▼) shows the site cleaved by protease $2A^{pro}$, and the blue triangles (▼) are the active sites for the protease $3CD^{pro}$. The purple star * indicates the autocatalytic site between the structural proteins VP2 and VP4.

Enterovirus polyprotein also contains alternative cleavage sites. For instance, cleavage within 3C is mediated by its catalytic activity (163), and cleavage within 3CD is mediated by $2A^{pro}$ at a tyrosine-glycine pair located in 3D, which generates the peptides 3C' and 3D' (Figure 1.3, green dashed triangle). Mutational analysis conducted in poliovirus demonstrated that a threonine positioned adjacent to the tyrosine at the cleavage site within 3D plays a crucial role in $2A^{pro}$ recognition, as a threonine to alanine substitution (T147A) eliminates $2A^{pro}$-mediated cleavage. Poliovirus carrying the T147A substitution has no defect in growth, which indicates that the fragments 3C' and 3D' are dispensable for virus replication (164, 165).

Cellular proteins may also assist enterovirus polyprotein processing. For example, *in vitro* studies have shown that a group of papain-like cysteine proteases called calpains can cleave the P1 precursor of *Enterovirus B* species at the VP3-VP1 junction. However, whether they assist in polyprotein processing during natural infection remains to be determined (166). Moreover, Turkii *et al.* have demonstrated that efficient processing of viral nonstructural proteins during *Enterovirus B* infection in cell culture depends on the intermediate filament vimentin since inhibition of vimentin reorganization decreased the production of 2A, 3C, and 3D but not VP1 (167).

1.4.3.3 Genome replication.

Viral RNA replication is associated with remodeled cellular membranes in the cytoplasm, called replication organelles (ROs), which provide the structural and biochemical environment for genome replication (168, 169). For further description of the ROs see Chapter 2.

The positive single-stranded RNA genome is used as a template for negative-strand RNA synthesis. To initiate the process, a ribonucleoprotein complex is formed at the 5' end of the genome through the interaction of the viral protein $3CD^{pro}$ with the cloverleaf's stem-loop d and the cellular protein poly(rC)-binding protein 2 (PCBP2) with the stem-loop b, along with the adjacent C-rich region in spacer I. Additionally, Poly(A)-binding protein (PABP) binds to the poly(A) tail at the end of the 3' UTR and interacts with PCBP2 and $3CD^{pro}$. This results in the circularization of the genome, which promotes the interactions between the 5' and 3' ends, thereby facilitating the negative-strand RNA synthesis (170). Additionally, the P3 precursors 3BC and/or 3BCD interact with CRE and $3D^{pol}$ to produce uridylylated VPg (VPg-pU-pU) using the sequence AAA located in CRE as a template (159). The complex is then transferred to the 3' end of the genome, and $3D^{pol}$ utilizes VPg-pU-pU as a primer for negative-strand RNA synthesis (171, 172). During the process, a long double-stranded RNA called replication form (RF) is created, made of one positive and one negative strand (173).

The next step is the synthesis of the positive-strand RNA genome from a negative-strand RNA template. This process requires the formation of ribonucleoprotein complexes in the replicative form. Apart from the interactions between PCBP2, $3CD^{pro}$, and the cloverleaf, a host protein called heterogeneous nuclear ribonucleoprotein C (hnRNP-C) binds to both ends of the replicative form. This helps preserve a single-stranded structure at the ends of the replication form. As a result, the ribonucleoprotein complex is stabilized, which paves the way for the initiation of positive-strand synthesis by the 3D polymerase with uridylylated VPg as a primer (174-176).

The enterovirus genome serves as a template for both the translation and minus-strand RNA synthesis in infected cells. Protein synthesis and RNA replication are two processes that cannot be carried out simultaneously because the viral polymerase moves along the RNA template

in the 3'-5' direction and the ribosomes do it in the opposite direction (93). *In vitro* experiments have shown that inhibitors such as cycloheximide, which inhibit polypeptide chain elongation and "freezes" the ribosome in the viral RNA, also inhibit viral RNA replication. In contrast, inhibitors that cause polypeptide chain termination and induce the dissociation of ribosomes from the RNA stimulate viral RNA replication (177). The mechanism for regulating viral RNA translation and replication involves the binding of $3CD^{pro}$ to the cloverleaf and the $3C^{pro}$-mediated cleavage of PCBP2, which participates in both processes. Cleaved PCBP2 lacks the KH3 domain and cannot bind domain IV of the IRES but can participate in the ribonucleoprotein formation needed for RNA synthesis (178). This mechanism is strengthened by the binding of $3CD^{pro}$ to CRE, which increases the affinity of PCBP2 to the CRE and decreases it for the IRES (179).

1.4.3.4 Virion morphogenesis.

The P1 domain of the enterovirus polyprotein contains the structural proteins that form the capsid. The viral protease $3CD^{pro}$ cleaves P1, which is myristoylated at the N-terminus, to produce the polypeptides VP0, VP3, and VP1. This process requires the heat shock protein 90 (HSP90). HSP90 interacts with P1 and is necessary for its correct folding, which allows $3CD^{pro}$ to recognize it and cleave it (83). Then, VP0, VP3, and VP1 form the structural unit of the capsid, called the protomer. The protomer self-oligomerizes to form pentamers stabilized by myristate groups and glutathione, which interacts with capsid proteins to provide stability to precursors and mature virions. Enteroviruses such as EV-A71 have a surface-exposed methionine in VP1 located at the interface between protomers and do not require glutathione for virion assembly (180, 181). Twelve pentamers interact and assemble to form a procapsid. Packaging signals have not been identified in the genome of enteroviruses. Instead, it is currently believed that the viral RNA is encapsidated through protein-protein interactions between 2C, which is an essential component of the

replication complexes, and the capsid protein VP3 (182-186). Finally, viral RNA packaging results in virion maturation, during which VP0 is autocatalytically cleaved into VP4 and VP2. During the cleavage process of VP0, a histidine residue that is located near the cleavage site plays an important role. Specifically, Histidine 195 of VP2 activates local water molecules, which leads to a nucleophilic attack at the VP0 scissile bond with the coordination of carbonyl oxygen atoms in the RNA (187, 188). Thus, the mature virion contains the viral RNA genome packaged into a non-enveloped icosahedral capsid made of 60 copies of VP1, VP2, VP3, and VP4.

1.4.3.5 Virion release.

Enteroviruses may be released from infected cells via lytic and non-lytic pathways. Lytic virion release happens upon cell death, which can occur by multiple mechanisms including necrosis and apoptosis. Necrosis is a form of uncontrolled cell death triggered by external stimuli that destroy the plasma membrane integrity, releasing cellular content to the extracellular environment, and resulting in a strong inflammatory response (189). For instance, CVB3 release from polarized intestinal epithelial cells happens through necrotic cell death and requires the Ca^{+2}-activated protease calpain-2 (190). Apoptosis is a form of programmed cell death that occurs through the formation of apoptotic bodies and is characterized by the activation of proteases known as caspases. Poliovirus replication and the injury of the central nervous system in mice infected are linked to apoptosis, which can be induced by viral proteins $2A^{pro}$ and $3C^{pro}$ (191-193).

Enteroviruses can exit the infected cell through non-lytic pathways. In this scenario, enteroviruses are enclosed in vesicles and released without destroying the cell. One example of this is poliovirus, which has been observed to exit intact cells through a process called autophagosome-mediated exit without lysis. During this process, the vesicles containing virions fuse with the plasma membrane and egress from the cell. Other enteroviruses such as CVB3 and

EV-A71 induce autophagy and use this non-lytic pathway for virion release (194). Another mechanism of non-lytic egress is via exosomes, which are generated by the fusion of plasma membrane with specialized endosomes called multivesicular bodies. The exosomal pathway is used by EV-A71 to exit cells, and infection with exosome-enclosed viruses is more efficient in human neuroblastoma (195). The contribution of different pathways of enterovirus virion release is likely cell-type dependent.

Chapter 2: Origin and Functioning of Enterovirus Replication Organelles

2.1 Overview.

Enteroviruses replicate their genome associated with remodeled intracellular membranes called replication organelles (ROs). These ROs are believed to 1) help increase the local concentration of viral and cellular proteins required for replication; 2) provide a structural scaffold for the assembly of the replication complexes, which may confer proteins the appropriate topology to carry out functions needed for replication; 3) confine RNA replication to a specific location within the infected cell; and 4) shield the double-stranded RNA intermediates of viral replication from pattern recognition receptors (such as RIG-I, PKR, and MDA-5) that can activate host defense mechanisms (196). The composition and structure of ROs change dynamically during the course of infection (197). These changes are crucial to provide the necessary lipid and protein composition required to support different stages of the replication cycle.

2.2 Structural features of membranous replication organelles.

All single-stranded positive-sense RNA viruses induce membrane remodeling upon infection. Viruses investigated in detail from several families including *Flaviviridae* (Dengue virus, Zika virus, West Nile virus, hepatitis C virus), *Togaviridae* (Sindbis virus, Semliki forest virus), *Bromoviridae* (brome mosaic virus, cucumber mosaic virus), *Coronaviridae* (severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)), and *Nodaviridae* (flock house virus) induce significant structural changes of intracellular organelles and the proliferation of membranous structures that support their replication (198-200). The structure and composition of ROs depend on the virus and the membrane source. For instance, the Hepatitis C

Virus (HCV) forms a membrane web in infected cells, which is enriched in phosphatidylcholine (PC), sterols, and phosphatidylinositol 4-phosphate (PI4P) and contains single-membrane and double-membrane vesicles derived from the endoplasmic reticulum (ER) (201-204). Another example is a plant virus, Tomato bushy stunt virus (TBSV), a member of the *Tombusviridae* family that replicates in peroxisome-derived multi-vesicular bodies enriched in phosphatidylethanolamine (PE) (205, 206).

Ultrastructural analysis of enterovirus-infected cells shows that the process of membrane remodeling starts with the formation of convoluted tubular structures with a single membrane. As the infection progresses, these structures develop into round or irregularly shaped double-membrane vesicles (197, 207). In poliovirus infection, the single-membrane structures are associated with the *cis*-Golgi marker GM130, indicating that they may originate from this organelle (197). Live-cell imaging and scanning electron microscopy of whole cells infected with coxsackievirus B3 revealed that RO biogenesis occurs at various points within the ER, followed by RO formation at *trans*-Golgi membranes (208).

2.3 Rewiring of host cell membrane metabolism during enterovirus infection.

2.3.1 Overview.

The remodeling of cell membranes during enterovirus infection implies that host proteins involved in membrane metabolism should participate in establishing and supporting infection. Current knowledge shows that the structural development of enterovirus ROs and the functioning of replication complexes require two different but related components: 1) the activation of phospholipid synthesis required for membrane remodeling and replication organelle formation (203); and 2) the recruitment of components of the secretory pathway to the viral replication complexes to create the biochemical environment conducive for RNA replication (209).

2.3.2 The role of the cellular secretory machinery.

2.3.2.1 Overview.

The cellular secretory pathway is a complex trafficking network that modifies, controls both the quality and movement of proteins from the ER to other organelles, including the plasma membrane. This pathway comprises the ER, ER-Golgi intermediate compartments (ERGIC), the Golgi complex, endosomes, and the plasma membrane (Figure 2.1) (210).

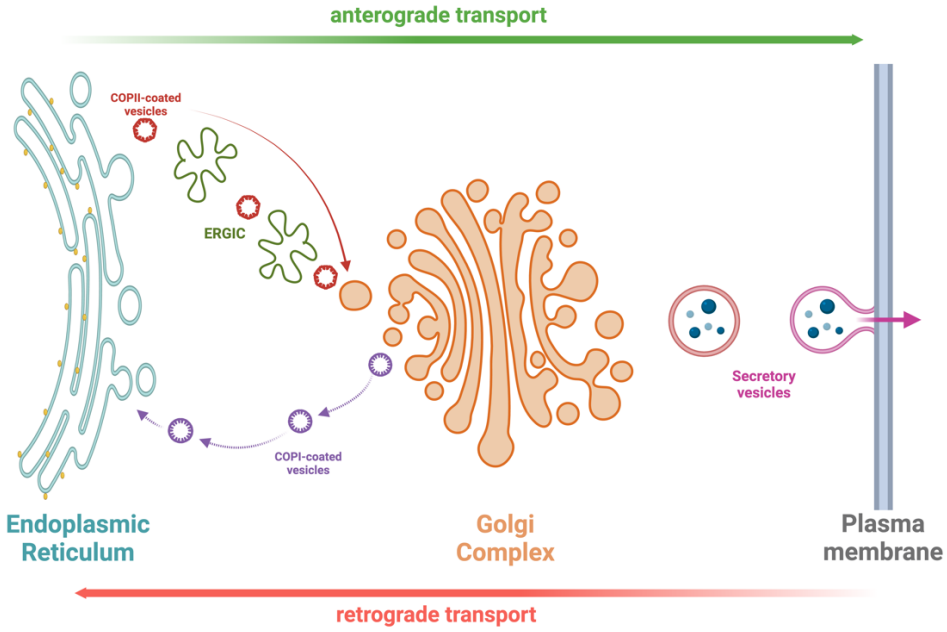


Figure 2.1: Scheme of the cellular secretory pathway. The pathway comprises multiple compartments including ER, Golgi cisternae, endosomes, and plasma membrane. Nascent proteins synthesized in the ER are transported via COPII-coated vesicles (small red circles) to the ERGIC and cis-Golgi during anterograde trafficking. They are either secreted from the cell to the extracellular environment or function in intracellular metabolic pathways. During retrograde trafficking, the misfolded proteins and ER-resident proteins are sent back to the ER via COPI-coated vesicles (small purple circles).

All secreted proteins are synthesized by ER-associated ribosomes and are translocated across the membrane into the ER lumen. While in the ER, the newly synthesized proteins interact with ER-resident proteins that facilitate the proper folding and perform quality control. Then, during anterograde transport, proteins exit the ER at the ER exit sites (ERES) where they are

packaged into COPII-coated vesicles via signal sequences that mediate their interaction with cargo receptors and adaptors, thus differentiating secretory proteins from ER-resident proteins. COPII vesicles deliver their cargo to the ER-Golgi Intermediate Compartment (ERGIC), and from there, the proteins are transported to the Golgi. The dynamic steady-state equilibrium between the ER and the Golgi is maintained by the retrograde transport of cargo that recycles membrane and ER-resident proteins from the Golgi back to the ER in COPI-coated vesicles. The delivery of secretory proteins to the plasma membrane can occur through the fusion of endosomal compartments derived from the *trans*-Golgi network (TGN) and/or through the release of TGN-derived secretory granules (211). During the transition through the secretory pathways, secretory proteins may undergo posttranslational modifications such as complex glycosylation.

Early during infection, poliovirus replication organelles are associated with the *cis*-Golgi marker GM130, and components of the COPII and COPI coats are found on enterovirus replication structures. Whole-cell tomography of coxsackievirus-infected cells showed that the ROs originate at various points within the ER and are formed in *trans*-Golgi membranes (168, 197, 208, 212, 213). Moreover, enterovirus infection is inhibited by brefeldin A (BFA), a fungal metabolite that blocks the secretory pathway by targeting a group of large guanine nucleotide exchange factors (GBF1, BIG1, and BIG2) responsible for activating Arf GTPases (214). Taken together, these observations imply that enterovirus replication depends on components of the secretory pathway.

2.3.2.2 Arf GTPases in the secretory pathway.

The ADP-ribosylation factor (Arf) family of proteins is part of the Ras superfamily of small GTPases. Arf GTPases regulate the vesicular traffic needed for protein secretion and endocytosis by recruiting the components of the vesicle coat. There are six mammalian Arf proteins (Arf1 to Arf6), with Arf2 missing in primates. They are classified into three classes based on their primary

structure: class I includes Arf1 and Arf3; Arf4 and Arf5 are part of Class II, and Arf6 is the only member of Class III. Arf GTPases act as molecular switches that cycle between their active, membrane-bound GTP form and inactive, cytosolic GDP-bound form. The spatiotemporal regulation of Arf GTPase activity is mediated by guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), which facilitates the GDP for GTP exchange and the hydrolysis of bound GTP, respectively (Figure 2.2) (215-217).

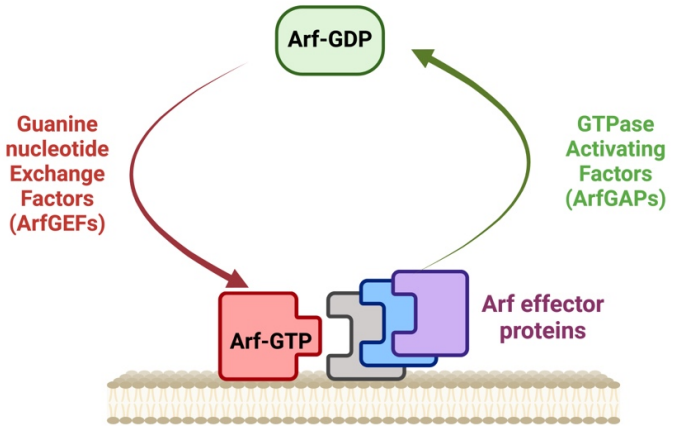


Figure 2.2: Scheme of Arf GTPases activation cycle.

Arf GTPases feature an N-terminal myristoylated amphipathic helix, which is autoinhibitory in the GDP-bound state but facilitates membrane association in the GTP-bound state. They also have a G domain that includes switch 1 (SW1) and switch 2 (SW2) loops that directly participate in GTP binding. A defining feature of members of the Arf family is the presence of an inter-switch that connects switches 1 and 2 and acts as an allosteric communicator between the N-terminal helix and the nucleotide-binding sites (Figure 2.3). The inter-switch mediates the conformational changes between the GDP- and GTP-bound forms. GEFs, GAPs, and Arf effectors generally bind to switch 1, switch 2, and/or the inter-switch (218-220).

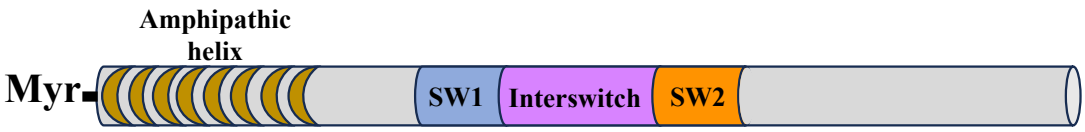


Figure 2.3: Domain organization of Arf GTPases.

Activated Arf GTPases function by interacting with and recruiting effector proteins that create a unique biochemical environment in Arf-enriched membranes. Arf-GTP recruits vesicle coat proteins, adaptors, lipid-modifying enzymes, and other molecules that remodel membranes and facilitate vesicle trafficking. For example, the formation of COPI-coated vesicles for retrograde transport relies on the activity of Arf1-GTP, which interacts with and recruits coatomer subunits like β-COP and γ-COP. Additional effectors include lipid-modifying enzymes such as phosphatidylinositol phosphate kinase and phospholipase D, which generate phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidic acid (PA) to regulate cytoskeleton activity and vesicular traffic (221, 222).

2.3.2.3 GBF1 in the secretory pathway.

Golgi-specific brefeldin A-resistance factor 1 (GBF1) is a large protein of about 206 kDa that acts as a guanine nucleotide exchange factor for small GTPases of the Arf family (ArfGEF). Human ArfGEFs are categorized into six families based on sequence similarity, evolutionary relationship, and domain structure: GBF1, BFA-inhibited GEFs (BIGs), Arf nucleotide binding site opener (ARNO)/Cytohesins, exchange factor for Arf6 (EFA6/Psd), BFA-resistant Arf GEF (BRAG/IQSec), and F-box only protein 8 (FBX8) (217, 223). Although all ArfGEFs share a common mechanism of action, they differ in their regulation, cellular localization, and interacting partners.

GBF1 domain organization consists of an N-terminal dimerization and cyclophilin-binding (DCB) domain, a homology upstream of Sec7 (HUS) domain, the catalytic Sec7 domain, and the

C-terminal homology downstream of Sec7 (HDS1-3) domains (224) (Figure 2.4). Structural analysis of the Sec7 domain shows that it comprises ten α-helices (A to J) forming a rod-shape structure with a hydrophobic groove, which serves as the Arf-binding site and contains a conserved glutamate in the loop between helix F and G that is critical for catalytic activity (225-227).

Figure 2.4: Domain organization of GBF1.

When GBF1 binds to Arf, the inter-switch in the GTPase facilitates the displacement of the autoinhibitory N-terminal myristoylated amphipathic helix of Arf. This allows the helix to associate with membranes, enabling the interaction between the glutamate in the Sec7 domain and the GDP in Arf. The electrostatic repulsions between the glutamate and the phosphate expel the GDP from the nucleotide-binding site, resulting in a nucleotide-free complex that can bind GTP (217, 224). The fungal metabolite BFA binds at the GBF1-Arf-GDP interface and inhibits the conformational changes in Arf that are required to release the GDP molecule, stabilizing the transient complex on the membranes and inhibiting secretion (228, 229).

GBF1 is primarily located in the ERGIC and the cis-Golgi, where it activates Arf to facilitate COPI vesicle formation (230). It also functions at the TGN to assist in recruiting BIG1 and BIG2 by activating Arf4 and Arf5 (231). In addition to vesicular trafficking, GBF1 is involved in various metabolic processes, including lipid homeostasis, Golgi biogenesis, mitochondrial positioning, and cell motility (232-235). It achieves this by interacting with proteins involved in these pathways, such as the membrane transport factor p115 (236), the clathrin adaptor GGA (237),

the GTPase Rab1b (238), the γ-COP subunit of the COPI vesicle (239), the lipid droplet-associated lipase ATGL (240), the mitochondrial protein Miro (235), and AMP-activated protein kinase (241).

2.3.2.4 Arf GTPases in enterovirus infection.

Enterovirus infection induces the recruitment of all isoforms of Arf GTPases (Arf1 to 6) to the RO membranes. Moghimi *et al.* have demonstrated that poliovirus induces a dynamic redistribution of Arf isoforms during infection, starting with the recruitment of Arf1 to the replication organelles during the early/middle stages of infection (3 h.p.i.), followed by all other isoforms later during infection (4 to 6 h.p.i.). Interestingly, viral antigens show different degrees of association with Arf in the replication membranes, with 2B-containing antigens showing the highest co-localization with Arf-enriched membranes and the signal from the dsRNA of the replicative form being excluded from Arf-enriched regions. The sequential recruitment of Arf isoforms, plus the distinct pattern of association of viral antigens with Arfs, create unique biochemical microdomains in the replication organelle membranes that are likely required to support the different stages of the replication cycle (242).

Arf1 is the most important isoform for enteroviruses. It is the first isoform to be recruited to the replication organelles, and its knockdown greatly increases enterovirus sensitivity to BFA (242). Synthetic lethality studies also support this observation, where Arf1 is the only synthetic lethal partner of GBF1 (243). Moreover, Arf1 plays a role in the development of enterovirus resistance to BFA. Mutations in the viral protein 2C lead to resistance to high concentrations of BFA and the replication of BFA-resistant poliovirus is significantly compromised in Arf1 knockout HeLa cells. Co-immunoprecipitation experiments showed that the BFA-resistant mutations in 2C enable the protein to strongly interact with activated Arf1. Interestingly, the 2C

of rhinovirus 1A, which is less sensitive to BFA, interacts with activated Arf1 stronger than the 2Cs of BFA-sensitive enteroviruses such as poliovirus and CVB3. This suggests a connection between the strength of 2C-Arf1 interaction and the virus's sensitivity to BFA (244).

The mechanistic role of Arfs during enterovirus replication is not well understood. The association of Arf-GTPases with the replication organelle membranes likely results in the recruitment of effector proteins that may contribute to the functioning of the replication complexes. The proteomic characterization of enterovirus replication organelles revealed multiple Arf1 effectors recruited to the replication complexes, including the BFA-insensitive ArfGEF Cytohesin2/ARNO and the glycolytic enzymes L-lactate dehydrogenase chain A (LDHA), but the specific role that these Arf1 effectors play in enterovirus infection requires further investigation (245).

2.3.2.5 GBF1 in enterovirus infection.

Enterovirus infection is inhibited by brefeldin A (BFA), a fungal metabolite that blocks the secretory pathway by targeting a group of large guanine nucleotide exchange factors (GBF1, BIG1, and BIG2) responsible for activating Arf GTPases (214). GBF1 is the only ArfGEF responsible for enterovirus sensitivity to the drug. Previous studies have shown that only GBF1 overexpression can rescue poliovirus replication from inhibition by BFA, whereas siRNA-mediated knockdown of GBF1 severely impairs viral replication (246, 247).

The initial model of GBF1 activity during enterovirus infection proposed the activation and subsequent recruitment of Arfs to the replication membranes, which in turn recruit effector proteins required to support viral replication, such as phosphatidylinositol-4-kinase III β (PI4KIIIβ) (168). However, evidence supporting this straightforward model is lacking. It has been shown that recruitment of PI4KIIIβ to the replication organelles of coxsackievirus B3 and human

rhinovirus is independent of GBF1 and Arf1 (248, 249), and that known GBF1-interacting partners like p115 and Rab1b are dispensable for viral replication (250), suggesting that during infection GBF1 engages in a specific set of interactions that differs from the ones in uninfected cells. Experiments testing if GBF1-mediated Arf activation is needed for enterovirus replication also generated controversial results. Previous reports showed that poliovirus replication may not require the catalytic Sec7 domain of GBF1 (250), and BFA inhibition could not be rescued by overexpression of any wild-type or constitutively activated Arf mutants (247). Thus, the complete characterization of domains/functions of GBF1 required to support enterovirus replication needs further investigation.

GBF1 is recruited to the replication organelles by enterovirus non-structural protein 3A. The nonstructural protein 3A from poliovirus and coxsackievirus B3 strongly interact with GBF1, leading to its recruitment to the viral replication organelles and the inhibition of protein secretion. Yeast two-hybrid and co-immunoprecipitation experiments show that the first 37 amino acids of the N-terminal domain of GBF1 directly interact with a conserved region in the N-terminus of the viral protein 3A. However, the functional significance of this interaction needs clarification, as enterovirus mutants that are severely defective in this interaction show only minor growth defects in cell culture (247, 251, 252).

2.3.3 Lipid metabolism.

2.3.3.1 Overview.

Lipids are a diverse group of nonpolar or amphipathic molecules with crucial functions in signal transduction pathways, the regulation of energy metabolism, and the structural integrity of cellular membranes (253, 254).

The modulation of lipid metabolism coupled with remodeling intracellular membranes is a hallmark of several families of positive-strand RNA viruses. For example, HCV infection promotes the accumulation of PI4P, which is believed to generate and maintain the integrity of the membranous webs that characterize HCV replication organelles. It does so by recruiting the enzyme phosphatidylinositol-4-kinase III α (PI4KIIIα) via interaction with the viral non-structural protein 5A (NS5A) (255-257). Plant viruses also hijack lipid metabolism for replication. The p33 protein of Tomato bushy stunt virus (TBSV) interacts and recruits the endosomal Rab5 GTPase to enrich the replication organelle with phosphatidylethanolamine (206, 258).

Enterovirus infection significantly alters the synthesis of structural phospholipids such as phosphatidylcholine and it modifies both the distribution and synthesis of signaling lipids like phosphatidylinositol-4-phosphate (PI4P) and cholesterol, to support the structural development of replication organelles (168, 259-263).

2.3.3.2 Upregulation of phospholipid synthesis in enterovirus infection.

Poliovirus reprograms the metabolic targeting of fatty acids (FAs), preventing their accumulation in triglycerides and cholesterol esters stored in lipid droplets and increasing phosphatidylcholine biosynthesis during infection. Phosphatidylcholine is the most abundant phospholipid of eukaryotic membranes, accounting for more than 50% of the membrane phospholipids (264). The mechanism of upregulation of phosphatidylcholine synthesis during infection involves an increase in long-chain acyl-CoA synthetase (ACSL) activity and the recruitment of CTP-phosphocholine-cytidyl transferase alpha (CCTα) to poliovirus ROs.

Acyl-CoA synthetases (ACSs) catalyze the activation of FAs to CoA esters, maintaining the concentration gradient of FAs and promoting their uptake by the cell (265). There are 13 isoforms of ACSs in humans, divided into three subfamilies: five members of the long chain acyl-

CoA synthetase (ACSL) family; six proteins of the very long chain acyl-CoA synthetase family also known as fatty acid transport proteins (ACSVL or FATP); and two bubblegum acyl-CoA synthetases (ACSBG) (266-268). The ACSL family comprises 5 isoforms (ACSL1 and 3 to 6), which differ by tissue expression, intracellular localization, and substrate specificity (269). Knockdown experiments demonstrated that ACSL3 is required to support poliovirus replication. The upregulation of long-chain acyl-CoA synthetase activity during enterovirus infection requires the viral protein 2A independent of its protease activity (270).

CCTα is the enzyme responsible for the rate-limiting step in phosphatidylcholine synthesis (271). Its activity is regulated by phosphorylation/dephosphorylation, with the dephosphorylated form showing greater membrane affinity and increased activity (272). During poliovirus infection, CCTα is released from the nuclear depot and associates with the replication organelles, where it controls the activation of PC synthesis. The translocation of CCTα from the nucleus requires the activity of the viral protease 2A. The accumulation of the dephosphorylated form during the course of infection correlates with an increase in enzymatic activity in the replicating membranes, leading to an increased PC synthesis in the replication organelles (273).

2.3.3.3 PI4P-Cholesterol pathway in enterovirus infection.

Enteroviral replication organelles are enriched in cholesterol. Cholesterol is involved in many cellular processes, from maintaining membrane fluidity and permeability to modulating signaling pathways and synthesis of steroid hormones and vitamin D (274). Under normal cellular metabolism, cholesterol is taken up via low-density lipoprotein (LDL) through clathrin-mediated endocytosis, facilitated by the LDL receptor (LDLR). Cholesterol derived from LDL is released in lysosomes and then transported to the plasma membrane, where it plays a structural role. It is

also transported to the ER membrane, where it regulates its synthesis and uptake and is esterified with long-chain fatty acids for storage as cholesteryl esters in lipid droplets (275-277).

The enrichment of replication organelles with cholesterol promotes viral RNA replication. The underlying mechanism involves the regulation of $3CD^{pro}$ proteolytic processing. Depletion of cholesterol in the replication organelles greatly stimulates the proteolytic cleavage of $3CD^{pro}$ into $3C^{pro}$ and $3D^{pol}$, and the subsequent decrease in levels of $3CD^{pro}$ impairs the initiation of viral RNA replication and the proteolytic processing of structural proteins to form the capsid. Enteroviruses harness several metabolic pathways to modulate the cholesterol landscape in infected cells. Coxsackievirus B3 takes over clathrin-mediated endocytosis to increase the intracellular levels of cholesterol and to prevent its recycling to the plasma membrane by recruiting endosomes to the ROs via interaction between Rab11, a component of recycling endosomes, and the viral protein 3A (261). In contrast, rhinovirus A16 obtains cholesterol from the cholesteryl esters stored in lipid droplets (278).

Cholesterol transfer to the enteroviral replication organelle membranes occurs in a PI4P-dependent manner, and it involves the cellular proteins phosphatidylinositol 4 kinase III beta (PI4KIIIβ) and oxysterol binding protein (OSBP). PI4P is synthesized by PI4 kinases (PI4Ks). In mammalian cells, there are four PI4Ks divided into two groups, type II (PI3KIIα and PI4KIIβ) and type III (PI4KIIIα and PI4KIIIβ), which differ in their cellular localization and mechanism of membrane recruitment (279). Enteroviruses utilize PI4KIIIβ, which is composed of two ordered domains comprising a helical domain and a bi-lobal kinase domain; and three disordered linkers located at the N-terminus, between the helical and kinase domain, and within the kinase domain (280, 281). The disordered linkers are sites of post-translational modifications and are involved in binding with regulatory partners. Two different pathways of PI4KIIIβ recruitment to the

replication organelles have been proposed; one relies on the viral protein $3CD^{pro}$ and depends on the cellular proteins GBF1 and the GTPase Arf1 (282), and the other involves the cellular protein ACBD3 and is mediated by the viral protein 3A (283, 284).

Banerjee *et al.* have shown that the expression of $3CD^{pro}$ induces the synthesis of PI4P in HeLa cells, which involves the GBF1-Arf1-GTP-mediated recruitment of PI4KIIIβ. The genetic analysis of $3CD^{pro}$ shows that the protein plays a role both before and after the activation of Arf1. The 3D region is involved in events before activation, while the 3C region is involved in events after activation. In this pathway, $3CD^{pro}$ may be involved in 1) recruiting GBF1 to membranes, leading to Arf1 activation, and 2) recruiting Arf1 effectors that are responsible for PI4KIIIβ activation (282). The identification and functional characterization of 3CD-interacting partners responsible for inducing PI4P synthesis may clarify the mechanism and regulation of this pathway.

The other pathway of PI4KIIIβ recruitment involves the cellular protein ACBD3 and the viral protein 3A. Acyl-CoA-binding domain-containing 3 (ACBD3) is a scaffolding protein essential for maintaining Golgi structure. It participates in multiple cellular processes, including intracellular trafficking between the ER and the Golgi, embryogenesis, neurogenesis, iron uptake, and metabolic homeostasis. ACBD3 is a multidomain protein containing an Acyl-CoA-binding domain (ACBD), a charged amino acid region (CAR), a glutamine-rich domain (Q), and a GOLD domain that mediates targeting to the Golgi via interaction with giantin, a protein that regulates interconnections between Golgi stacks (285, 286). Lyoo *et al.* have demonstrated that ACBD3 facilitates the interaction between the viral protein 3A and PI4KIIIβ during enterovirus infection. Knocking out ACBD3 impairs the localization of 3A to the Golgi, the recruitment of PI4KIIIβ, and inhibits the replication of EV-A71, CVB3, PV, EV-D68, and rhinovirus A and B; these phenotypes can be reversed by reconstituting ACBD3 expression (283). Structural characterization

revealed that the interaction between ACBD3 and 3A involves the GOLD domain of ACBD3 and the cytoplasmic domain of 3A, while the interaction between ACBD3 and PIK4IIIβ is mediated by the Q domain of ACBD3 and the N-terminal domain of PI4KIIIβ (287, 288).

The other protein involved in the PI4P-cholesterol exchange cycle during enterovirus replication is oxysterol binding protein (OSBP). OSBP consists of an N-terminal disordered region, a pleckstrin homology domain (PHD), a coil-coil region (CCR) that includes the dimerization domain and two phenylalanines in an acidic tract (FFAT) motif, and ligand-binding domain (ORD) (289). Under normal cellular metabolism, OSBP localizes to the ER-Golgi membrane contact sites because it interacts with PI4P from the Golgi membranes and the vesicle-associated membrane protein-associated protein (VAP) from the ER membranes. These interactions require the PH domain and the FFAT motif, respectively. During this process, OSBP transfers PI4P to the ER and cholesterol to the Golgi, which is facilitated by a gradient in the concentration of PI4P created by the phosphatase Sac1 at the ER. The phosphatidylinositol (PI) is then transferred back to the Golgi by the PI transfer protein PITP-b, where PI4K regenerates PI4P to continue the exchange cycle (203, 290, 291). Enteroviruses take over lipid transfer at membrane contact sites to support the structural development of replication organelles (292). Rhinovirus infection requires OSBP, VAP-B, Sac1, and PITP-b (278), and poliovirus replication requires the pleckstrin homology domain and the ligand-binding domain of OSBP, but not the N-terminal disordered region, the CCR, or the FFAT motif (289). Inhibition of the PI4P-cholesterol exchange cycle with drugs that target PI4KIIIβ and OSBP blocks enterovirus replication (293-295).

2.3.3.4 The role of lipid droplets during enterovirus infection.

Lipid droplets (LDs) are highly dynamic organelles that contain a core of neutral lipids made of triglycerides and cholesteryl esters. These lipids are surrounded by a phospholipid

monolayer associated with multiple peripheral and integral proteins. The lipids stored in lipid droplets can be released through the autophagic degradation of lipid droplets, a process known as lipophagy, or through lipolysis, which is mediated by lipases associated with the lipid droplets. Both processes release long-chain fatty acids that can be used to fuel metabolic pathways and membrane biosynthesis (296).

Enterovirus replication organelles form membrane contact sites and develop in close association with lipid droplets (208, 278). During rhinovirus infection, LDs are clustered near the replication sites, and the LD-associated hormone-sensitive lipase (HSL) catalyzes the hydrolysis of cholesteryl-esters to provide the cholesterol needed for the replication organelles (278). The phospholipid synthesis activated upon poliovirus infection is fueled by fatty acids released from LDs through LD-associated lipases, including HSL and adipose triglyceride lipase (ATGL) (273).

Furthermore, Laufman *et al*. demonstrated that the ectopically expressed enteroviral proteins 2B, 2C, and 2BC localize to LD, and their localization is mediated by the N-terminal amphipathic helices of 2B and 2C. Additionally, the clustering of lipid droplets during poliovirus infection requires oligomerization of 2C. Moreover, co-immunoprecipitation experiments showed that the viral protein 3A interacts with ATGL and HSL. However, it is still unclear whether this interaction occurs directly. Notably, 3A interacts with GBF1 and previous studies have reported interactions between GBF1 and ATGL (240). Therefore, it is possible that 3A indirectly pulled down ATGL through its interaction with GBF1 (297). Accordingly, inhibitors of ATGL or HSL activity prevented the development of ROs and blocked PV, EV-A71, and CVB3 replication, although the specificity of these inhibitors is often contested and their off-target activity cannot be excluded (297, 298).

2.4 Goals of the study.

The lack of understanding of the detailed biochemical environment of the replication organelles necessary to support infection, including the contribution of GBF1 and Arf1, prevents the development of antiviral therapies that could target such infection-specific environments/interactions. To clarify the role of the GBF1-Arf1 axis in enterovirus replication, we:

A- Defined the role of GBF1-mediated Arf activation in enterovirus replication by manipulating GBF1 enzymatic activity through structure-directed mutagenesis.

B- Identified the domains of GBF1 important to support enterovirus replication by systematically analyzing conserved functional elements in each domain.

C- Characterized the functional significance of the GBF1-3A interaction.

D- Characterized the viral protein composition of Arf1-enriched domains of the replication organelle membranes.

Chapter 3: Materials and Methods

3.1 Cells.

HeLa cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, and 10% fetal bovine serum (FBS). Retrovirus packaging cell line GP2-293 (TaKaRa) was maintained in DMEM supplemented with 10% FBS and nonessential amino acids. Stable HeLa cell lines expressing individual human Arfs fused to Venus fluorescent protein were described in (242).

3.2 Plasmids.

All GBF1 expression plasmids were made based on the pYFP-GBF1 plasmid described previously (229). Numbers in the names of truncated GBF1 constructs indicate the last amino acid. An ARNO Sec7 domain fragment and ARNO Sec7-PH fragment were amplified from a plasmid coding for a FLAG-tagged human ARNO protein kindly provided by Julie Donaldson (NIH). Fragments with Ala scanning mutations and N-terminal GBF1-BIG2 chimeras were synthesized by the GeneArt service (Invitrogen) and cloned into the pVenus-GBF1 plasmid. The pXpA-RenR plasmid coding for a poliovirus replicon with *Renilla* luciferase substituting for the capsid proteins was described previously (299). pCMV-GLuc, coding for *Gaussia* luciferase, was from New England BioLabs. The plasmid pCI-3A-FLAG, coding for poliovirus 3A with FLAG-Y tag under the control of cytomegalovirus (CMV) promoter, is described in reference (300), and plasmids pCI-3A and pCI-3A-2 coding for the 3A wt and 3A-2 mutant, respectively, were constructed similarly. For the construction of the retroviral vector expressing FLAG-tagged APEX2 C-terminally fused to Arf1, the FLAG-APEX2 coding sequence was amplified by PCR using as a template the plasmid pLNCX2-FLAG-APEX2-GARG-1060 described in (245), and cloned into the AgeI and NotI restrictions sites of the plasmid pLNCX2-Arf1-EGFP described in (242). The

plasmid coding the vesicular stomatitis virus (VSV) envelope glycoprotein was purchased from TaKaRa.

3.3 Reagents and antibodies.

Brefeldin A (BFA) was from Sigma-Aldrich, and a 4 mg/mL stock solution was prepared in dimethyl sulfoxide (DMSO) and stored at −80°C. EnduRen cell-permeable substrate for *Renilla* luciferase was from Promega. DNA and RNA transfection reagents Trans-it 2020 and Trans-it mRNA/mRNA Boost, respectively, were from Mirus. Opti-MEM Reduced-Serum Medium was from Gibco. Polybrene was from Sigma-Aldrich. Biotin phenol was from Chemodex, and hydrogen peroxide was from Sigma Millipore. 5-ethynyl-uridine was purchased from Cayman Chemical. Hoechst 33342 was purchased from Thermo Fisher. The proteinase inhibitor cocktail was from Sigma-Aldrich. The antibodies used in this study are listed in Table 3.1.

Table 3.1: List of antibodies used in this study, with references.

Antibody	Source
Mouse monoclonal anti-poliovirus 3A	(301)
Mouse monoclonal anti-poliovirus 2B	(301)
Mouse monoclonal anti-poliovirus 2C	(301)
Rabbit polyclonal anti-poliovirus 3D	(250, 270)
Mouse monoclonal anti-dsRNA J2	English & Scientific Consulting Kft
Mouse monoclonal anti-Arf	Affinity BioReagents
Rabbit polyclonal anti-GFP	Abcam
Rabbit polyclonal anti-FLAG	Thermo Fisher
Agarose beads conjugated with anti-FLAG mouse monoclonal M13	Sigma-Aldrich
Mouse monoclonal anti-β-actin conjugated with horse radish peroxidase	Sigma-Aldrich
Streptavidin Alexa Fluor 555 conjugate	Molecular Probes (Thermo Fisher)
Secondary antibody conjugated with Alexa fluorescent dyes (488, 568 and 633)	Molecular Probes (Thermo Fisher)
Secondary antibody conjugated with horse radish peroxidase	Amersham

3.4 Virus strain and infection protocol.

Poliovirus type I Mahoney was propagated in HeLa cells, and its titer was determined by plaque assay. For infection, HeLa cells were seeded in 12-well plates (with or without a coverslip depending on the future analysis). For adsorption, the cells were incubated with the required amount of the virus resuspended with DMEM to a total volume of 200 µL in each well for 30 minutes at room temperature and then at 37°C in DMEM with 10% FBS for the indicated times post-infection.

3.5 Plaque assay.

HeLa cells were seeded into a 6-well plate and incubated at 37°C. The next day, a ten-fold serial dilution was prepared from the viral stock to be tittered in the serum-free DMEM. Viral absorption was performed by adding 400 µL of each dilution to the HeLa cell monolayer, and the plate was incubated on a shaker at room temperature for 30 minutes. In the meantime, the agarose cover was prepared by dissolving 500 mg agarose powder in 75 mL distilled water, followed by adding 20 mL 5X DMEM, 6 mL FBS, and 1 mL penicillin-streptomycin. After aspirating the absorption medium, 4 mL of the prepared overlay solution was added to each well, and the plate was kept at room temperature until the overlay solidified. The plate was incubated at 37°C for 48 hours and subsequently stained with crystal violet. The viral titer was calculated based on the number of plaques that appeared in specific dilutions.

3.6 DNA transfection.

HeLa cells were seeded into a 6-well or 12-well plate with or without coverslips, depending on the experiment’s purpose, and transfected according to the manufacturer's direction. Shortly, for a 12-well plate, the transfection mix for each well was made by adding 100 µL Opti-MEM Reduced-Serum Medium, 1 µg DNA plasmid, and 3 µL Trans-IT-2020 reagent and keeping the

mixture at room temperature for 20 minutes. The monolayer was then transfected with the mixture, and the cells were incubated at 37°C overnight in DMEM supplemented with 10% FBS. The next day, transfected cells were either fixed with paraformaldehyde 4% or collected in the lysis buffer for Western Blot analysis.

3.7 RNA replicon transfection.

HeLa cells were seeded into a 96-well plate and transfected according to the manufacturer's direction. The transfection mix was prepared for the whole plate by adding 1350 µL Opti-MEM Reduced-Serum Medium, 1500 µg RNA replicon stock, and 9 µL of each Trans-IT mRNA and mRNA Boost reagent and keeping the transfection mix at room temperature for 5 minutes. The transfection complex was then mixed with 10% FBS DMEM in the presence of 5µM of EnduRen cell-permeable substrate, and the cells were kept for 18 hours in a plate reader at 37°C for poliovirus replicon replication assay.

3.8 Poliovirus replicon replication assay.

A poliovirus replicon replication assay was performed essentially as described by Viktorova *et al.* (302). Briefly, HeLa cells were transfected in suspension with a GBF1-expressing plasmid and then plated on a 96-well plate so that the cells in all corresponding wells are transfected similarly. The next day, the cells were transfected with a purified poliovirus replicon RNA using Mirus mRNA transfection reagent (Mirus Bio). The cells were incubated after replicon transfection in the medium containing 5µM of EnduRen cell-permeable *Renilla* luciferase substrate in the presence or absence of 1 µg/ml BFA. DMSO was used as a control. *Renilla* luciferase signal was monitored in live cells every hour with Tecan M1000 or Molecular Devices iD5 multifunctional plate readers equipped with a thermostatic incubation chamber. Each data point on the replication graph is an average signal from at least 12 wells.

3.9 Secretion assay.

A secretion assay was performed as described in (303). Briefly, HeLa cells were co-transfected in a suspension with a 9:1 mass ratio of a GBF1-expressing plasmid and pCMV-GLuc and then plated on a 96-well plate, so that the cells in all corresponding wells are transfected similarly. The next day, the cells were washed three times with a serum-free medium and then incubated in a fresh medium supplemented with the indicated amount of BFA for 4 hours. At this time point, 20 μL of the medium was used to monitor the amount of secreted *Gaussia* luciferase using a *Gaussia* luciferase assay kit (New England BioLabs). Secretion data were normalized to the signal obtained from the cells incubated without BFA for each sample. Each data point is an average of the signal from 8 wells.

3.10 Arf1-GST-GBF1 pulldown assay.

For the preparation of recombinant Δ17Arf1, GSTΔ17Arf1 was expressed in BL21(DE3)/pLysS *Escherichia coli* (Promega, Madison, WI) and purified on Pierce glutathione agarose (Thermo Scientific, IL, USA), according to the manufacturer's directions.

For Arf binding, HeLa cells transfected with GFP-tagged GBF1/795, GBF1/795/794, or GBF1/795/7A were lysed in 50 mM Bis-Tris (pH 7.2), 6 N HCl, 50 mM NaCl, 10% (wt/vol) glycerol, 0.001% Ponceau S, and 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) containing a complete protease inhibitor mixture tablet, EDTA-free (Santa Cruz Biotechnology, Santa Cruz, CA). Lysates were precleared by centrifugation at 15,000 rpm for 30 minutes at 4°C. The supernatants (100 μL) were incubated with GST-17-residue NH2-terminal deletion mutant (Δ17Arf1) prebound to glutathione-Sepharose 4B beads for 1 hour at room temperature and processed for SDS-PAGE. The starting material (SM) was loaded as 10 μL of the original supernatant.

3.11 Co-immunoprecipitation (co-IP) assay.

HeLa cells grown on 35-mm petri dishes were co-transfected with pCI-3A-FLAG and a GBF1 expression plasmid at the mass ratio of 1:1, and the next day, co-IP was performed using mouse monoclonal anti-FLAG agarose beads (Sigma-Aldrich), according to the manufacturer's protocol. After a final wash, the proteins were released by heating the beads in the Laemmli SDS protein sample buffer for 5 minutes at 95°C. The proteins were resolved on a 4 to 15% Tris-glycine gel gradient (Bio-Rad), and 3A and GBF1 constructs were detected in Western blots with anti-FLAG and anti-GFP antibodies, respectively. Digital images of Western blots developed with ECL Select luminescent substrate (GE Healthcare) were obtained with a C500 imager (Azure Biosystems) and analyzed using the ImageStudio software (Li-Cor). Relative recruitment is calculated by normalizing the GBF1-to-3A signal ratio in the pulldown material of the mutants to that of the positive-control (wt) sample. Each bar is an average of at least three independent experiments.

3.12 Generation of stable cell lines using a retroviral gene transduction system.

Retroviral virions were generated by transfecting the packaging cell line GP2-293 according to the manufacturer's directions. Briefly, GP2-293 cells seeded into a 6-well plate were co-transfected with pLNCX2 vector expressing Arf1-FLAG-APEX2, and the plasmid coding the vesicular stomatitis virus (VSV) envelope glycoprotein (TaKaRa) at the mass ration of 1:1. Eighteen hours after transfection, the medium was replaced with 1 mL of fresh complete growth medium, and cells were kept in the incubator overnight. The infectious retroviral virions were collected in the culture supernatant 48 hours after the start of transfection. HeLa cells seeded into a 6-well plate were transduced with the freshly harvested supernatant containing the retroviral virions supplemented with 10 μg/mL Polybrene (Sigma-Aldrich). The plate was centrifuged at

1,200Xg for 1 hour at 32°C and then incubated at 37°C for 18 hours. The next day, the medium was replaced with a fresh complete growth medium, and cells were kept in the incubator overnight. Forty-eight hours after the start of transduction, cells were transferred into a T-25 flask, and the drug-resistant colonies were selected with 400 μg/mL of G418 (VWR Life Science) for 10 days. The resistant colonies were pooled, and the stable cell lines were maintained in the complete growth medium supplemented with 400 μg/ml G418. After a two-week course of antibiotic selection, 20% of the cells showed the expression of the transgene indicated by FLAG staining. So, a clonal selection of the transduced cells was performed to generate Arf1-FLAG-APEX2 expressing cells with a uniformly high level of transgene expression. Shortly, the stable cells were cultured in three 96-well plates; each contained about 30 cells so that a single cell, whether expressing the transgene or not, was plated into a separate well and incubated in the growth medium containing 400μg/mL of the selection antibiotic G418. After expanding one of the selected clones for about three weeks, we obtained a culture with about 50% of cells showing strong transgene expression. The cells were subjected to a second round of selection, and the final culture had about 90% of the cells showing strong Arf1-FLAG-APEX2 expression, indicated by FLAG staining. This stable cell line was used for the rest of the study.

3.13 APEX2-based proximity biotinylation.

Depending on the future analysis, HeLa cells stably expressing Arf1-FLAG-APEX2 were seeded in a 12-well plate with or without coverslips and infected (or mock-infected) with 50 PFU/cell of poliovirus and incubated for specified time points post-infection. The biotinylation process was initiated 30 minutes before each time point by replacing the medium with DMEM containing 500 μM biotin phenol (Chemodex) and incubating the cells at 37°C for 30 minutes. Next, the medium was replaced with PBS containing 1 mM hydrogen peroxide (Sigma Millipore),

and the reaction occurred for 1 minute at 37°C. Cells were washed three times with PBS and either fixed with paraformaldehyde 4% to visualize biotinylated proteins with fluorescence microscopy, or lysed in RIPA lysis buffer supplemented with a 1X proteinase inhibitor cocktail (Sigma-Aldrich), followed by a 3-min sonication for SDS-PAGE and blotting analyses. Controls were made by omitting either biotin phenol, hydrogen peroxide, or both to confirm the specificity of the reaction.

For the biotinylation of permeabilized replication organelles, the reaction was initiated at 4 hours post-infection. The cells were treated with a mix of 500 µM of biotin phenol, 1mM of hydrogen peroxide, and 25 µg/mL of digitonin (Calbiochem) in PBS for 1 minute at 37°C, and immediately fixed with 4% paraformaldehyde to visualize the biotinylated proteins.

3.14 Streptavidin pulldown assay.

Streptavidin magnetic beads (Pierce) were equilibrated with RIPA lysis buffer. The whole-cell lysates were mixed with the equilibrated streptavidin magnetic beads and incubated at room temperature on a rotator for 1 hour. Next, the beads containing the bound biotinylated proteins were precipitated in a magnetic rack, and the supernatant was collected as the flow-through containing the non-biotinylated proteins. After washing the beads with RIPA lysis buffer three times, the biotin-labeled proteins were eluted from the beads by boiling the samples at 95°C in 50 µL of 3X sample buffer supplemented with 2 mM biotin and 20 mM dithiothreitol (DTT) for 10 minutes. The streptavidin-enriched eluates were separated from the beads on the magnetic rack and kept at -80°C for further analysis.

3.15 Immunofluorescence assay and microscopy imaging.

HeLa cells grown on coverslips were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) diluted in phosphate-buffered saline (PBS) for 20 minutes and washed three

times with PBS. Fixed cells were then permeabilized with either 0.2% triton X-100, 0.02% saponin, or 25 µg/mL of digitonin for 5 minutes depending on the experiment, with subsequent washing with PBS three times. After blocking the samples with a 3% blocking reagent (Amersham ECL Blocking Agent) dissolved in PBS, cells were incubated with primary antibody for 1 hour, followed by three times washing with PBS. Cells were then incubated with appropriate anti-mouse, or anti-rabbit secondary Alexa Fluor Conjugate or the streptavidin Alexa Fluor conjugate, and Hoechst 33342 for 1 hour, then washed with PBS three times and mounted using Fluoromount-G mounting medium (Electron Microscopy Sciences). Confocal images were taken with a Zeiss LSM800 confocal microscope. The Structural Illuminated Microscopy (SIM) super-resolution images were taken with a Nikon A1R microscope. Digital images were converted to TIFF format by the corresponding microscope software and processed with Adobe Photoshop for illustrations.

3.16 Metabolic RNA labeling with 5-ethynyl-uridine.

HeLa cells grown on coverslips were infected or mock-infected with 50 PFU/cell of poliovirus. At the indicated time post-infection, the cells were incubated with 1 mM of 5-ethynyl-uridine (Cayman Chemical) for 30 minutes. Then, cells were immediately fixed with 4% paraformaldehyde for 20 minutes, washed 3 times with PBS, and processed for the click-chemistry reaction using the Click-&-Go Plus 568 Imaging kit (Vector Laboratories) following the manufacturer's recommendations. Briefly, the cells were incubated for 30 minutes protected from light with the click-chemistry reaction mix containing 1X reaction buffer, 5 µM Alexa Fluor 568 Azide Plus, 4 mM $CuSO_4$, and 1X reducing agent solution. Then, cells were washed with the washing buffer from the kit, one time with PBS and the coverslips were mounted. Depending on the experiment, the click-chemistry reaction was carried out with no permeabilization, or in the

presence of 50 µg/mL of digitonin. The click-chemistry reaction and all washing steps were done in the presence of 10 units of RNasin Ribonuclease Inhibitor (Promega).

3.17 Western blot protocol.

Cells lysed in mild lysis buffer (100 mM Tris-HCl pH=7.8, Triton-100X 0.5%) or RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH=7.5, Triton-100X 1%, sodium dodecyl sulfate 1%, sodium deoxycholate 0.5%) supplemented with 1X proteinase inhibitor were boiled in 2X denaturing Laemmli sample buffer at 95°C for 5 minutes. The boiled lysates were separated in the SDS-PAGE gel (either 12% or a gradient gel 4-15% (Bio-Rad)). Proteins were then transferred to PVDF membranes (Bio-Rad) and immersed in the 2% blocking solution in washing buffer (2 L distilled water, 120 mM NaCl, 20 mM Tris-HCl pH= 7.5, and 0.2% Tween-20) for 30 minutes. The membranes were then incubated with primary antibodies for 1 hour, followed by three times washing with washing buffer and incubation with appropriate secondary antibodies for 1 hour. After washing three times, the membrane was developed using ECL Select western blotting detection reagent (Bio Health) and imaged by Azure Biosystems C500 chemiluminescence.

3.18 Statistical analysis.

Data presentation and unpaired two-tailed *t*-test statistical analysis were performed using the GraphPad Prism software package. Plots show mean values and standard deviation. Statistical significance is indicated as follows: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, nonsignificant.

Chapter 4: Poliovirus Replication Critically Depends on the Functional Sec7 Domain and an N-terminal Sequence of the Cellular ArfGEF GBF1

4.1. Introduction.

Poliovirus, the representative member of the *Enterovirus* genus of the *Picornaviridae* family, is a small, positive, single-stranded RNA virus that replicates its genome in association with remodeled cellular membranes called replication organelles. Viral membrane structures are associated with the cis-Golgi marker GM130 during early poliovirus infection, and the related coxsackievirus B3 uses both ER and then Golgi membranes to initiate the formation of replication organelles (197, 208). These observations suggest that at least some cellular proteins involved in membrane metabolism should participate in the development of infection. Indeed, poliovirus infection is sensitive to brefeldin A (BFA), a fungal metabolite that inhibits the secretory pathway by targeting three large guanine nucleotide exchange factors for the small GTPases Arf (ArfGEFs), GBF1, BIG1, and BIG2 (214, 304, 305). GEFs help exchange GDP for GTP to produce activated GTP-bound Arfs. These activated Arfs then bind to cellular membranes and control the recruitment of Arf-interacting proteins that help with various aspects of membrane metabolism. When GTP is hydrolyzed, the inactive Arf-GDP dissociates from the membranes. Arf cycling regulates Golgi homeostasis and membrane trafficking in the secretory pathway and helps maintain the identity of membranous organelles (222, 223). BFA inhibits GEF catalytic activity by stabilizing a transient intermediate formed by Arf-GDP and the catalytic Sec7 domains of BIG1, BIG2, or GBF1 (but not other cellular ArfGEFs), locking the GEF molecules in a nonfunctional conformation and inhibiting Arf activation (228, 306).

It was previously determined that poliovirus and other enteroviruses are sensitive to BFA because they require the ArfGEF GBF1 for RNA replication (247, 307). Moreover, GBF1 is an important cellular factor for a wide range (but not all) of RNA viruses such as hepatitis C virus (308-310), dengue virus (311), chikungunya virus (312), classical swine fever virus (313), coronaviruses (314), hepatitis E virus (315), and rotavirus (316). However, our understanding of the mechanistic role of GBF1 during enteroviral RNA replication is very limited. It has been suggested that GBF1-mediated Arf activation may play a direct role in the functioning of replication complexes or facilitate the recruitment of other necessary effector proteins to support replication. In the case of poliovirus, it has been proposed that the activated Arf may recruit phosphatidylinositol-4-kinase III beta (PI4KIIIβ), an essential Arf effector for the replication of many enteroviruses (80, 90, 295, 317). However, experimental testing of whether Arf activation by GBF1 is required for viral replication generated controversial results. The detailed investigation of the recruitment of PI4KIIIβ to the replication complexes of multiple enteroviruses demonstrated that it is independent of GBF1 and relies on the interaction of the viral protein 3A with another host factor, ACBD3, which directly interacts with PI4KIIIβ (283, 284, 288, 318). Additionally, the inhibition of viral replication by BFA can be rescued only by GBF1 overexpression but not by the overexpression of constantly activated Arf mutants, and the depletion or knockdown of expression of individual Arfs, or pairs thereof, was tolerated by diverse enteroviruses remarkably well (247, 307, 319). Moreover, it was reported that some GBF1 mutants with an inactivated Sec7 domain could support at least some level of poliovirus replication (250). The multidomain organization of GBF1 allows this protein to interact with many cellular partners, but the evidence shows that many of the interactions that occur under normal cellular metabolism are dispensable for enterovirus

replication (250), suggesting that GBF1 may function in enterovirus replication in a way that differs from its normal cellular functions.

The N-terminus of the nonstructural protein 3A from poliovirus and coxsackievirus B3 strongly interacts with the N-terminus of GBF1, resulting in its recruitment to viral replication complexes and its stabilization in the replication organelle membranes (247, 251, 252), but even the requirement for a strong 3A-GBF1 binding is not absolute, since enterovirus mutants severely compromised in this interaction show only minor growth defects in cell culture (252, 320, 321).

Here, we aimed to revisit these controversial findings and identify the elements/functions of GBF1 that are essential to support poliovirus replication, as well as to understand if they can be separated from those mediating GBF1 function in cellular metabolism. To this end, we generated a library of GBF1 mutants using mutagenesis of conserved motifs, domain swapping, and alanine scanning. Our data demonstrate a much higher tolerance of GBF1 mutations by the viral replication machinery than cellular metabolism, indicating that the virus utilizes only a small subset of GBF1 functions. However, none of the several GBF1 constructs defective in Arf activation could support poliovirus replication. Surprisingly, the cognate Sec7 domain of GBF1 could be substituted with that from a distantly related GEF, indicating that virus replication depends on the Arf activation property but not the amino acid sequence of the Sec7 domain. Our results confirm and extend the previous data that the very N-terminal sequence of GBF1 holds important determinants of the protein functioning in the replication complexes and that all the C-terminal domains of GBF1 downstream of the Sec7 domain are fully dispensable for replication of the wild-type poliovirus. However, the C-terminal HDS2 and HDS3 domains of GBF1 become important for the replication of a poliovirus mutant with impaired 3A-GBF1 interaction. This discrepancy suggests a redundant mechanism of GBF1 recruitment to viral replication complexes. Such a complementary

mechanism likely facilitates the access of the replication machinery to GBF1 and may underlie the resilience of poliovirus replication in diverse cell-specific environments encountered by the virus during the development of infection in a natural host.

4.2 Results.

4.2.1 Poliovirus replication requires the N-terminal domain of GBF1 and does not strictly correlate with GBF1-3A interaction.

4.2.1.1 Alanine substitutions within the N terminal region of GBF1 do not significantly interfere with its contribution to poliovirus replication but affect GBF1 function in cellular secretion.

GBF1 is recruited to enterovirus replication organelles by the nonstructural protein 3A (322). The yeast two-hybrid, pull-down, and co-immunoprecipitation experiments show that the N-terminal domain of GBF1, including the DCB and HUS domains interacts with a conserved region in the N-terminus of the viral protein 3A of poliovirus and coxsackievirus B (251, 322). Additionally, the deletion of the first 37 amino acids of GBF1 (GBF1Δ37) blocks poliovirus 3A-mediated recruitment of GBF1 to membranes and the ability of the protein to rescue poliovirus replication from BFA inhibition (247). These data led us to the hypothesis that the very N-terminal amino acid sequence of GBF1 contains determinants critical to support the replication of the virus. Thus, we performed an extensive mutagenesis of the N-terminal part of GBF1 to identify amino acids important for poliovirus replication. First, we substituted the authentic N-terminal amino acids with blocks of 10 Ala residues, generating mutants GBF1/1-10A, 11-20A, 21-30A, and 31-40A (Figure 4.1A). All the mutants were created in the background of the A795E mutation in the Sec7 domain, making GBF1 resistant to BFA (247, 307). This allowed us to inactivate the endogenous GBF1 in the presence of BFA so that the activity of only the mutant of interest could

be studied. HeLa cells were transfected with plasmids expressing GBF1 mutants and subsequently transfected with a poliovirus replicon RNA with a *Renilla* luciferase gene substituting for the capsid protein-coding region. As a positive control, cells were transfected with a plasmid coding for GBF1 with only the BFA resistance mutation A795E, and as a negative control, cells were transfected with an empty vector. Surprisingly, in the replication assay, all the Ala scanning mutants of GBF1 were just as functional as the positive control (Figure 4.1B, replication).

The capacity of the same GBF1 mutants to function in cellular metabolism was evaluated by measuring their ability to support cellular secretion. To this end, cells were co-transfected with a plasmid coding for a GBF1 mutant and a plasmid coding for *Gaussia* luciferase. This enzyme has a natural sequence targeting it to the secretory pathway (323). The next day, the medium was replaced with a medium containing BFA, and the amount of secreted *Gaussia* luciferase was determined after 4 h. The 1-10A, 11-20A, and 21-30A mutants were partially compromised in supporting secretion, while the 31-40A mutant supported an even somewhat higher level of secretion than the wild-type GBF1 (Figure 4.1B, secretion). Accordingly, poliovirus replication can tolerate significant modification of the N-terminal sequence of GBF1, while these modifications compromise GBF1 function in cellular secretion.

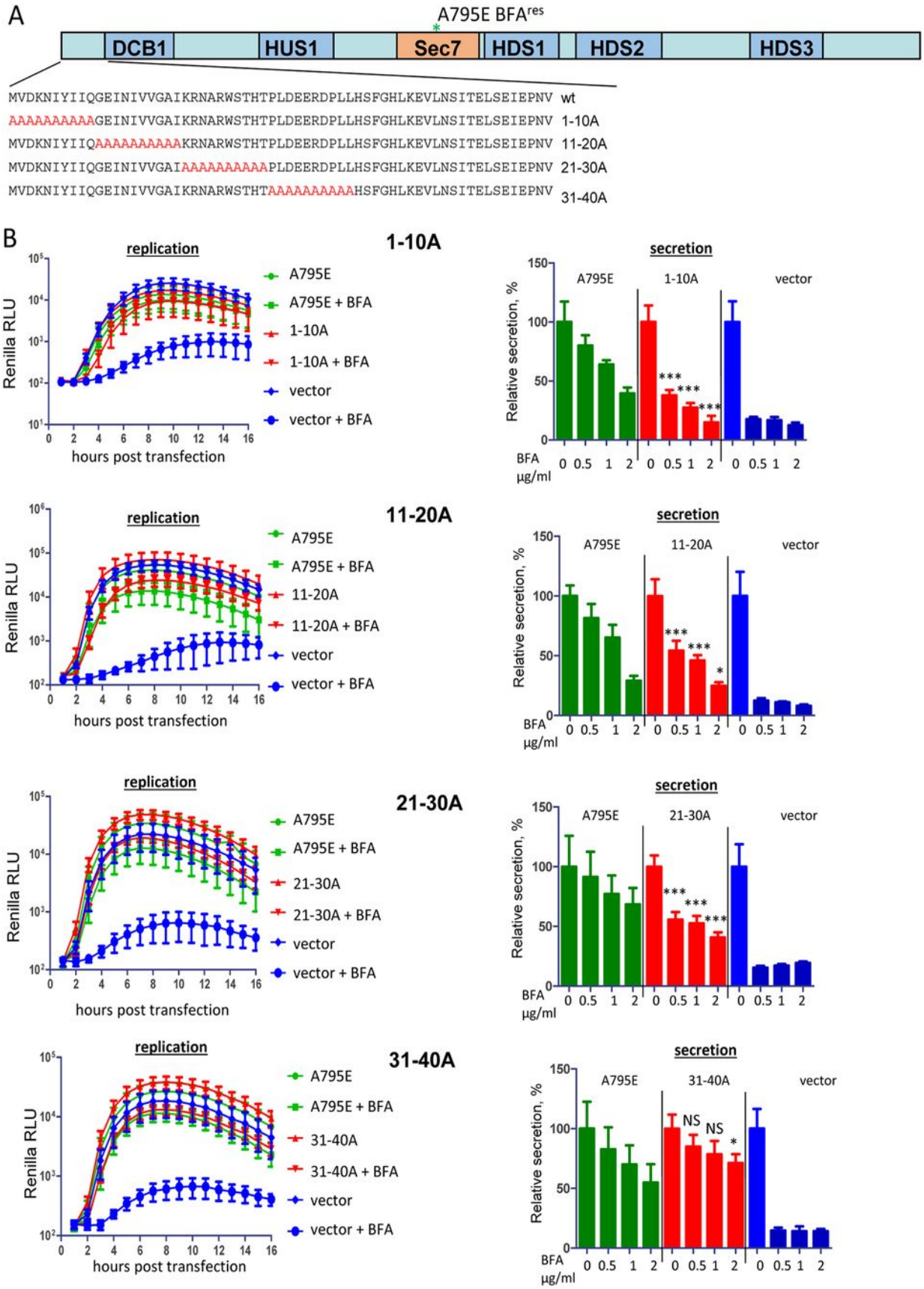

A
A795E BFAres
DCB1
HUS1
Sec7
HDS1
HDS2
HDS3
MVDKNIYIIQGEINIVVGAIKRNARWSTHTPLDEERDPLLHSFGHLKEVLNSITELSEIEPNV wt
AAAAAAAAAAGEINIVVGAIKRNARWSTHTPLDEERDPLLHSFGHLKEVLNSITELSEIEPNV 1-10A
MVDKNIYIIQAAAAAAAAAAKRNARWSTHTPLDEERDPLLHSFGHLKEVLNSITELSEIEPNV 11-20A
MVDKNIYIIQGEINIVVGAIAAAAAAAAAPLDEERDPLLHSFGHLKEVLNSITELSEIEPNV 21-30A
MVDKNIYIIQGEINIVVGAIKRNARWSTHTAAAAAAAAAAHSFGHLKEVLNSITELSEIEPNV 31-40A
B
1-10A
replication
Renilla RLU
hours post transfection
A795E
A795E + BFA
1-10A
1-10A + BFA
vector
vector + BFA
secretion
Relative secretion, %
A795E
1-10A
vector
BFA µg/ml
11-20A
11-20A + BFA
21-30A
21-30A + BFA
31-40A
31-40A + BFA
NS

Figure 4.1: Functional analysis of alanine scanning mutants of the N terminus of GBF1 in replication and secretion. (A) Schematic representation of the alanine substitutions in the GBF1 sequence. All GBF1 expression constructs are GFP tagged and contain the A795E BFA resistance mutation in the Sec7 domain. (B) Performance of the indicated mutants in the poliovirus replicon replication and cellular secretion assays. For the replication assay, cells were transfected with the plasmids expressing a corresponding GBF1 mutant, a full-length GBF1 A795E (positive control), or an empty vector (negative control). The next day, the cells were transfected with a poliovirus replicon RNA expressing *Renilla* luciferase and incubated in the presence or absence of 1 μg/ml BFA. For the secretion assay, the cells were co-transfected with plasmids coding for a corresponding GBF1 mutant, a full-length GBF1 A795E (positive control), or an empty vector (negative control) and a plasmid coding for a secreted *Gaussia* luciferase. The next day, they were washed and incubated in the medium with the indicated amount of BFA, and the amount of secreted luciferase was determined after 4 h. Secretion data were normalized to the signal obtained without BFA for each construct. Statistical significance of the difference between the signal in the positive control and the sample expressing a mutant GBF1 for corresponding concentrations of BFA is indicated. RLU: Relative Light Units.

4.2.1.2 Substitutions of the N-terminal regions of GBF1 with those from BIG2 affect the function of GBF1 in poliovirus replication and cellular secretion.

To introduce more extreme perturbations within the N-terminal domain of GBF1, we used a functional replacement approach to swap domains between the GBF1 and the related ArfGEF BIG2. GBF1 and BIG2 are part of the same family of large ArfGEFs that share a similar domain organization and structure. BIG2 localizes to the trans-Golgi network (TGN) and endosomes in cells and is incapable of rescuing poliovirus and coxsackievirus B3 replication from BFA inhibition (247, 307, 324, 325). Moreover, this domain-swapping method has significant advantages over conventional approaches, such as deletion or extensive mutagenesis, which can lead to protein misfolding and other artifacts.

First, we replaced the entire N-terminal part of GBF1 up to the DCB domain with the corresponding 68-amino-acid-long fragment from BIG2, generating the 2-68/BIG2 construct (the first methionine is the same in both proteins) (Figure 4.2A). The chimeric protein was severely compromised in poliovirus replicon replication and nonfunctional in cellular secretion assay (Figure 4.2B).

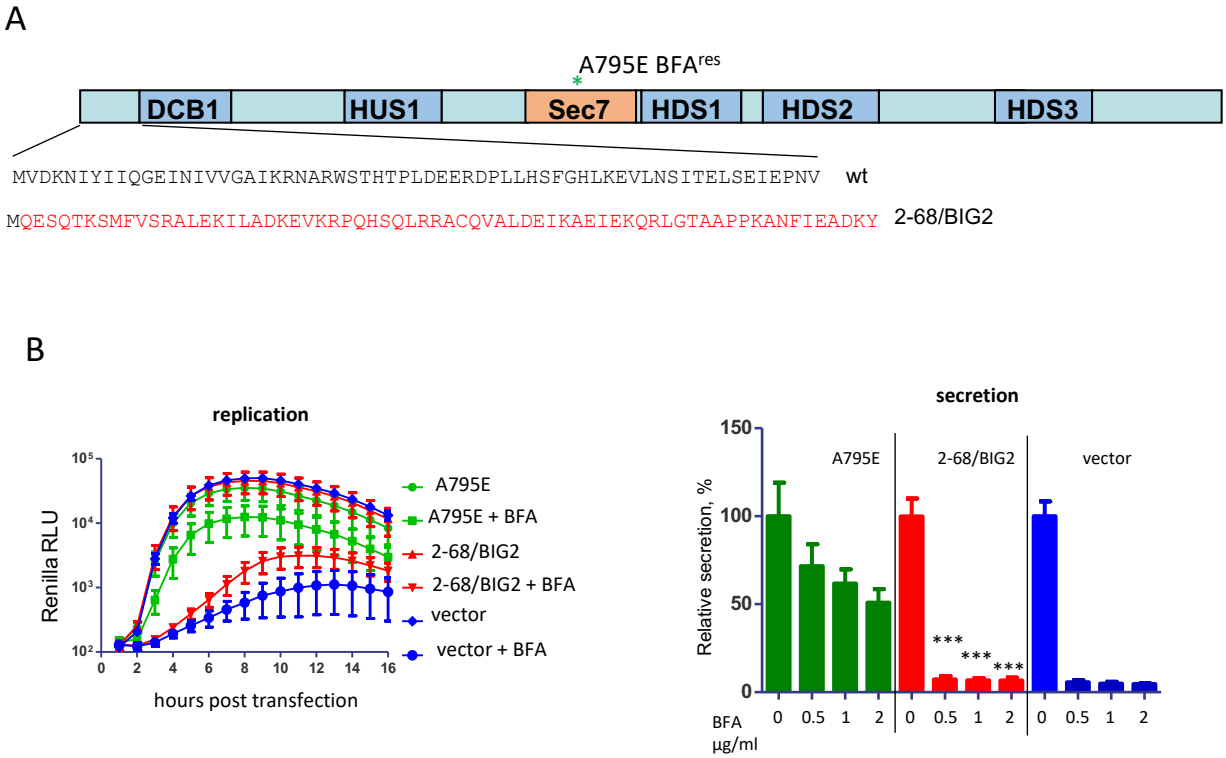
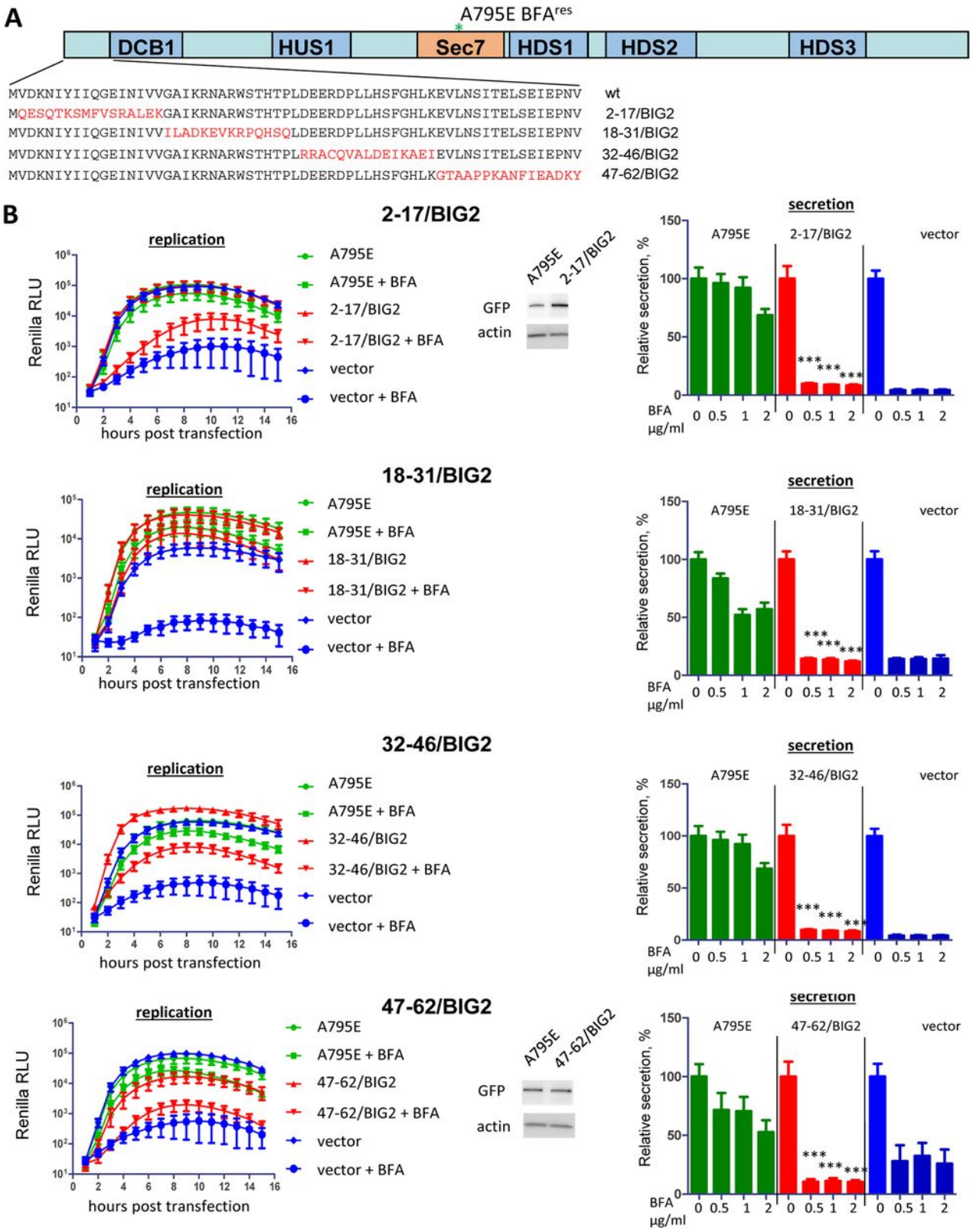


Figure 4.2: Functional analysis of the 2-68/BIG2/GBF1 N-terminal chimera in replication and secretion. (A) Scheme of the BIG2-derived substitutions in the GBF1 sequence. All GBF1 expression constructs are GFP tagged and contain the A795E BFA resistance mutation in the Sec7 domain. (B) Performance of the indicated mutant in the poliovirus replicon replication and cellular secretion assays. For the replication assay, cells were transfected with the plasmids expressing the 2-68/BIG2-GBF1 chimera, a full-length GBF1 A795E (positive control), or an empty vector (negative control). The next day, the cells were transfected with a poliovirus replicon RNA expressing *Renilla* luciferase and incubated in the presence or absence of 1 µg/ml BFA. For the secretion assay, the cells were co-transfected with plasmids coding for the 2-68/BIG2-GBF1 chimera, a full-length GBF1 A795E (positive control), or an empty vector (negative control) and a plasmid coding for a secreted *Gaussia* luciferase. The next day, they were washed and incubated in the medium with the indicated amount of BFA, and the amount of secreted luciferase was determined after 4 h. Secretion data were normalized to the signal obtained without BFA for each construct. The statistical significance of the difference between the signal in the positive control and that in the sample expressing a mutant GBF1 for corresponding concentrations of BFA is indicated.

The 68-amino-acid BIG2 insert was further split into shorter stretches of 15 amino acids to generate the following GBF1 constructs with the corresponding segments from BIG2: 2-17/BIG2, 18-31/BIG2, 32-46/BIG2, and 47-62/BIG2 (Figure 4.3A). Two constructs with the BIG2-derived substitutions in the middle of the N-terminal fragment of GBF1 demonstrated either complete (18-

31/BIG2) or partial (32-46/BIG2) ability to support poliovirus replication, while the ones with the most N-terminal (2-17/BIG2) and the most C-terminal (47-62/BIG2) substitutions were severely impaired in the replication assay. This suggests that two distinct regions (positions 2 to 17 and 47 to 62) within the N terminus facilitate GBF1 function in poliovirus replication or that the two domains may interact to form a single functional interface. In contrast to their performance in viral replication, all four constructs were defective in cellular secretion (Figure 4.3B). This supports the data in Figure 4.1, showing that poliovirus replication is more resilient to N-terminal modification of GBF1.


A
A795E BFA^res
DCB1
HUS1
Sec7
HDS1
HDS2
HDS3
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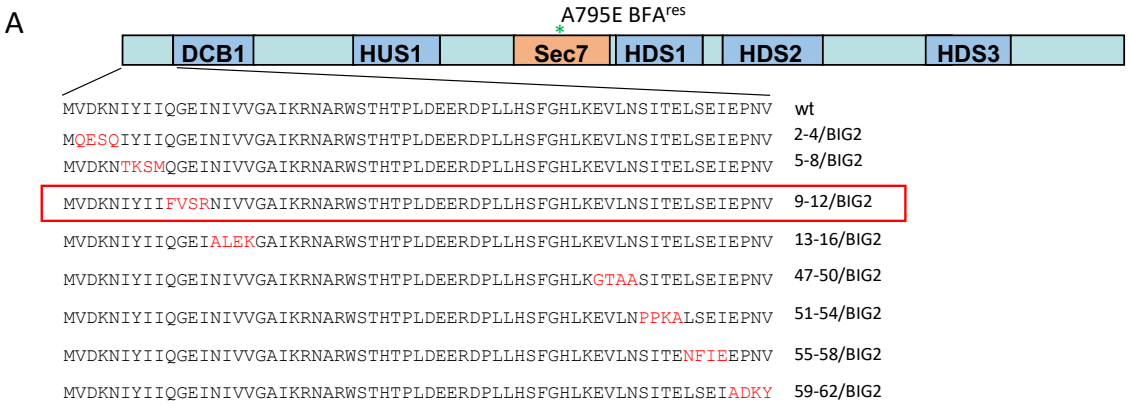
MQESQTKSMFVSRALEKGAIKRNARWSTHTPLDEERDPLLHSFGHLKEVLNSITELSEIEPNV 2-17/BIG2
MVDKNIYIIQGEINIVVILADKEVKRPQHSQLDEERDPLLHSFGHLKEVLNSITELSEIEPNV 18-31/BIG2
MVDKNIYIIQGEINIVVGAIKRNARWSTHTPLRRACQVALDEIKAEIEVLNSITELSEIEPNV 32-46/BIG2
MVDKNIYIIQGEINIVVGAIKRNARWSTHTPLDEERDPLLHSFGHLKGTAAPPKANFIEADKY 47-62/BIG2
B
2-17/BIG2
replication
Renilla RLU
hours post transfection
A795E
A795E + BFA
2-17/BIG2
2-17/BIG2 + BFA
vector
vector + BFA
A795E
2-17/BIG2
GFP
actin
secretion
A795E
2-17/BIG2
vector
Relative secretion, %
BFA µg/ml
18-31/BIG2
18-31/BIG2 + BFA
32-46/BIG2
32-46/BIG2 + BFA
47-62/BIG2
47-62/BIG2 + BFA

Figure 4.3: Functional analysis of the 15-amino acids GBF1/BIG2 N-terminal chimeras in replication and secretion. (A) Scheme of the BIG2-derived substitutions in the GBF1 sequence. All GBF1 expression constructs are GFP tagged and contain the A795E BFA resistance mutation in the Sec7 domain. (B) Performance of the indicated mutants in the poliovirus replicon replication and cellular secretion assays. For the replication assay, cells were transfected with the plasmids expressing a corresponding GBF1 mutant, a full-length GBF1 A795E (positive control), or an empty vector (negative control). The next day, the cells were transfected with a poliovirus replicon RNA expressing *Renilla* luciferase and incubated in the presence or absence of 1 μg/ml BFA. Expression of the 2-17/BIG2 and 47-62/BIG2 constructs, the most compromised in the replication assay, is additionally verified by Western blotting in the samples from the corresponding experiments. For the secretion assay, the cells were co-transfected with plasmids coding for a corresponding GBF1 mutant, a full-length GBF1 A795E (positive control), or an empty vector (negative control) and a plasmid coding for a secreted *Gaussia* luciferase. The next day, they were washed and incubated in the medium with the indicated amount of BFA, and the amount of secreted luciferase was determined after 4 h. Secretion data are normalized to the signal obtained without BFA for each construct. The statistical significance of the difference between the signal in the positive control and that in the sample expressing a mutant GBF1 for corresponding concentrations of BFA is indicated.

The BIG2-derived N-terminal inserts in the constructs 2-17/BIG2 and 47-62/BIG2, the most severely compromised in the poliovirus replication assay, were further split into 4 amino acid blocks (Figure 4.4A). All these constructs except for one with the substitution of the GBF1 amino acids in positions 9 to 12 (QGEI) for those derived from BIG2 (FVSR) could rescue poliovirus replication as well as did the positive control (Figure 4.4B). Since we are interested in finding GBF1 mutants that might be compromised in supporting viral replication but still functional in the cellular metabolism, the construct 9-12/BIG2 was also analyzed in a cellular secretion assay. However, 9-12/BIG2 proved to be completely nonfunctional in supporting secretion (Figure 4.4C). Collectively, our data demonstrate that the very N-terminal segment of GBF1 holds important determinants mediating protein functioning in poliovirus replication and that poliovirus replication requirements for the N terminus of GBF1 are much less stringent than those of the secretory pathway.

A


A795E BFA^res
DCB1
HUS1
Sec7
HDS1
HDS2
HDS3
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MQESQIYIIQGEINIVVGAIKRNARWSTHTPLDEERDPLLHSFGHLKEVLNSITELSEIEPNV 2-4/BIG2
MVDKNTKSMQGEINIVVGAIKRNARWSTHTPLDEERDPLLHSFGHLKEVLNSITELSEIEPNV 5-8/BIG2
MVDKNIYIIFVSRNIVVGAIKRNARWSTHTPLDEERDPLLHSFGHLKEVLNSITELSEIEPNV 9-12/BIG2
MVDKNIYIIQGEIALEKGAIKRNARWSTHTPLDEERDPLLHSFGHLKEVLNSITELSEIEPNV 13-16/BIG2
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MVDKNIYIIQGEINIVVGAIKRNARWSTHTPLDEERDPLLHSFGHLKEVLNPPKALSEIEPNV 51-54/BIG2
MVDKNIYIIQGEINIVVGAIKRNARWSTHTPLDEERDPLLHSFGHLKEVLNSITENFIEEPNV 55-58/BIG2
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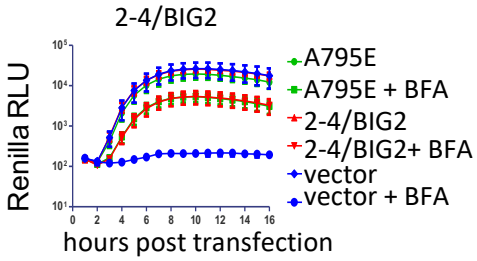
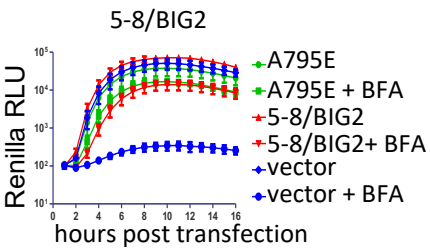
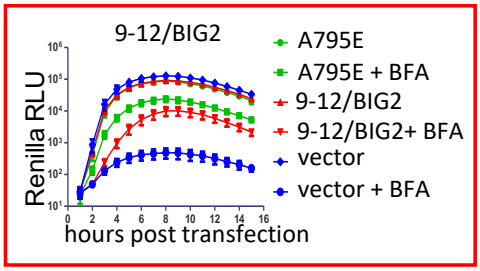
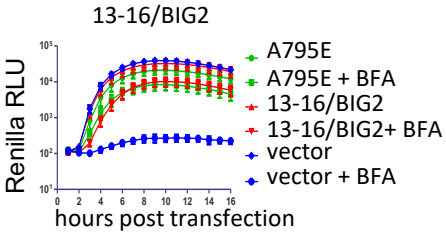
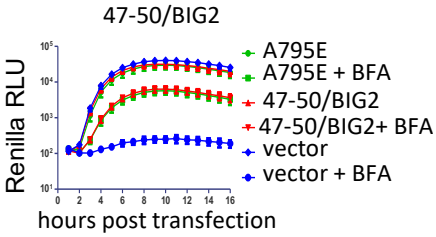
B

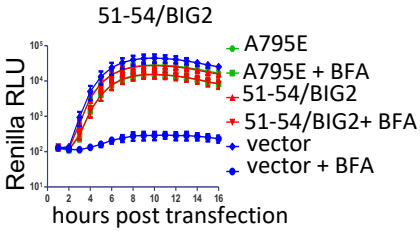

2-4/BIG2
Renilla RLU
hours post transfection
A795E
A795E + BFA
2-4/BIG2
2-4/BIG2+ BFA
vector
vector + BFA

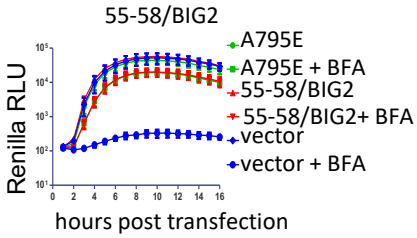

5-8/BIG2
Renilla RLU
hours post transfection
A795E
A795E + BFA
5-8/BIG2
5-8/BIG2+ BFA
vector
vector + BFA

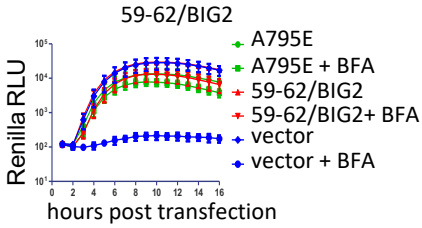

9-12/BIG2
Renilla RLU
hours post transfection
A795E
A795E + BFA
9-12/BIG2
9-12/BIG2+ BFA
vector
vector + BFA


13-16/BIG2
Renilla RLU
hours post transfection
A795E
A795E + BFA
13-16/BIG2
13-16/BIG2+ BFA
vector
vector + BFA


47-50/BIG2
Renilla RLU
hours post transfection
A795E
A795E + BFA
47-50/BIG2
47-50/BIG2+ BFA
vector
vector + BFA


51-54/BIG2
Renilla RLU
hours post transfection
A795E
A795E + BFA
51-54/BIG2
51-54/BIG2+ BFA
vector
vector + BFA


55-58/BIG2
Renilla RLU
hours post transfection
A795E
A795E + BFA
55-58/BIG2
55-58/BIG2+ BFA
vector
vector + BFA


59-62/BIG2
Renilla RLU
hours post transfection
A795E
A795E + BFA
59-62/BIG2
59-62/BIG2+ BFA
vector
vector + BFA

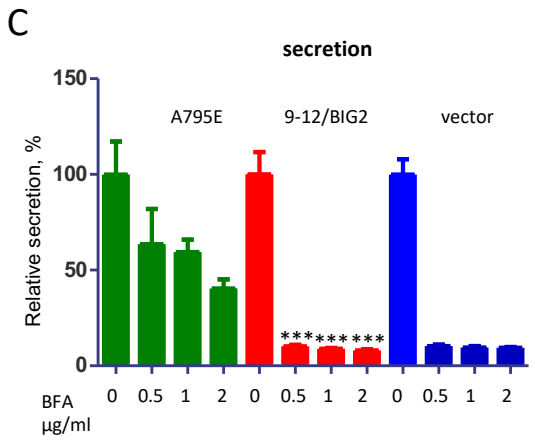


Figure 4.4: Functional analysis of the 4-amino acids GBF1/BIG2 N-terminal chimeras in replication and secretion. (A) Scheme of the BIG2-derived substitutions in the GBF1 sequence. All GBF1 expression constructs are GFP tagged and contain the A795E BFA resistance mutation in the Sec7 domain. (B) Performance of the indicated mutants in the poliovirus replicon replication. For the replication assay, cells were transfected with the plasmids expressing a corresponding GBF1 mutant, a full-length GBF1 A795E (positive control), or an empty vector (negative control). The next day, the cells were transfected with a poliovirus replicon RNA expressing Renilla luciferase and incubated in the presence or absence of 1 µg/ml BFA. (C) Performance of the 9-12/BIG2 construct in a cellular secretion assay. The cells were co-transfected with plasmids coding for the 9-12/BIG2 mutant, a full-length GBF1 A795E (positive control), or an empty vector (negative control) and a plasmid coding for a secreted *Gaussia* luciferase. The next day, they were washed and incubated in the medium with the indicated amount of BFA, and the amount of secreted luciferase was determined after 4 h. Secretion data were normalized to the signal obtained without BFA for each construct. The statistical significance of the difference between the signal in the positive control and that in the sample expressing the 9-12/BIG2 GBF1 for corresponding concentrations of BFA is indicated.

4.2.1.3 Poliovirus replication does not critically depend on a GBF1-3A interaction.

The nonstructural protein 3A from poliovirus and a related coxsackievirus B3 strongly interact with GBF1, resulting in its recruitment to the viral replication organelles (247, 251, 252). Yeast two-hybrid and co-immunoprecipitation experiments show that the first 37 amino acids of the N-terminal domain of GBF1 directly interact with a conserved region in the N-terminus of the viral protein 3A. However, this interaction's functional relevance needs to be clarified since enterovirus mutants severely defective in such interaction demonstrate only minor growth defects, at least in cell culture (252).

We performed co-immunoprecipitation assays to assess if the mutations in the N-terminus of GBF1 that did not support viral replication could have interfered with the interaction with 3A. HeLa cells were co-transfected with a plasmid expressing poliovirus protein 3A with a FLAG-Y insert after the sixth amino acid in the N-terminus of the protein (3A-FLAG-Y), and a plasmid expressing either the GBF1 mutant of interest, GBF1-A795E as a positive control, or the GBF1Δ37 mutant lacking 37 amino acids at the N-terminus as a negative control. The 3A-FLAG-Y insert was previously described as fully functional in poliovirus replication (300, 326).

Surprisingly, all the GBF1 mutants containing ten amino-acid-long Ala substitutions in the N terminus, which were positive in the replication assay, had a noticeably decreased interaction with 3A compared to the positive control (Figure 4.5A). All the N-terminal GBF1 mutants containing 15-aminoacid-long inserts derived from BIG2 were also severely compromised in the interaction with 3A, regardless of whether they supported replication (Figure 4.3B). However, the lowest level of GBF1-3A interaction was observed with the constructs 2-17/BIG2 and 47-62/BIG2, which were almost negative in the replication assay (Figure 4.5B). Altogether, these data indicate that although the 3A-GBF1 interaction generally correlates with the ability of GBF1 to support replication, poliovirus replication can tolerate significant variability in the level of GBF1-3A interaction without compromising infection in cell culture, which is consistent with previous observations (326).

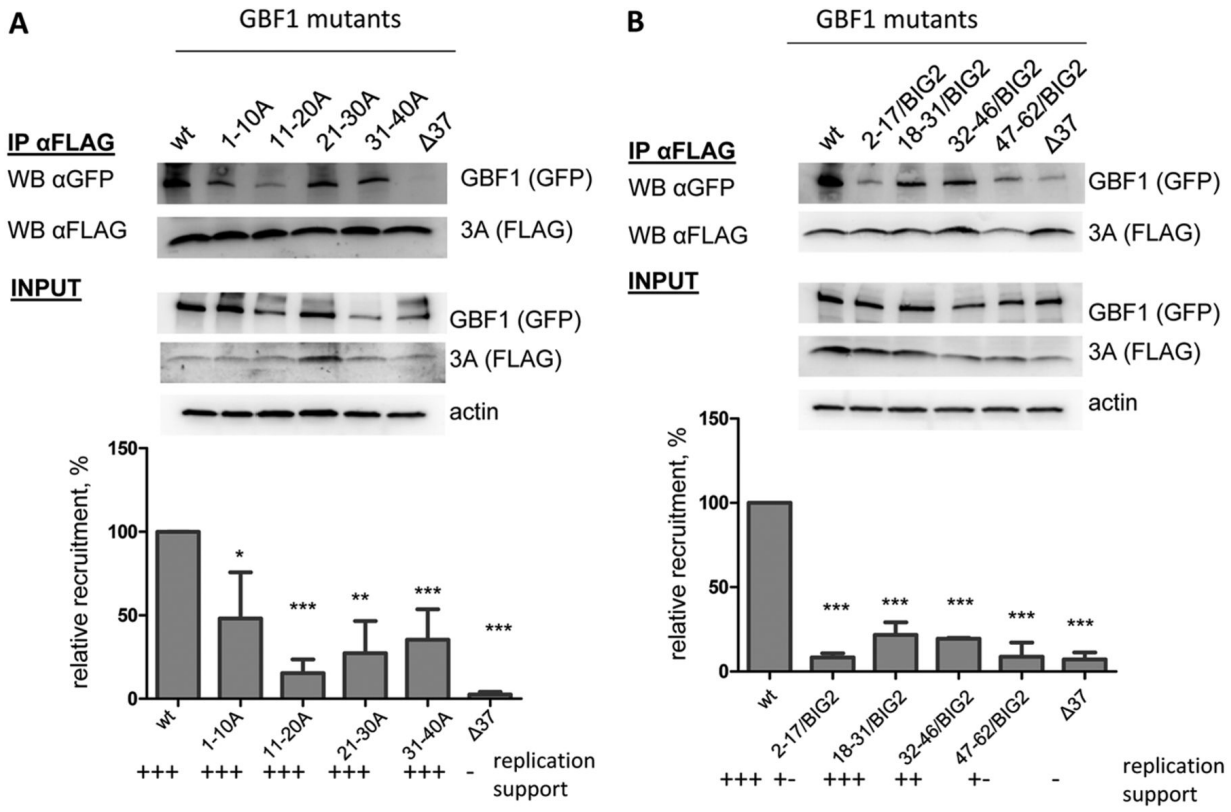


Figure 4.5: Effects of mutations in the N terminus of GBF1 on interaction with the viral protein 3A. (A) Co-IP of 3A-FLAG with GFP fusions of alanine scanning mutants of GBF1. (B) Co-IP of 3A-FLAG with GFP fusions of GBF1/BIG2 chimeras. Cells were co-transfected with plasmids coding for corresponding GFP-tagged GBF1 mutants and a plasmid coding for 3A-FLAG-Y. IP was performed with anti-FLAG resin. GBF1s were detected with anti-GFP antibodies, and 3A was detected with anti-FLAG antibodies. Actin in the lysates is shown as a loading control. Relative recruitment is calculated by normalizing the GBF1-to-3A signal ratio in the pulldown material of the mutants to that of the positive-control (wt) sample. Each bar is an average of the results from at least three independent experiments. The performance of the corresponding GBF1 constructs in the replication assay is indicated.

4.2.2 Poliovirus replication requires the functional Sec7 domain of GBF1.

Experiments testing if GBF1-mediated Arf activation is required for enterovirus infection have generated conflicting results. It was previously reported that a GBF1 mutant truncated just before the Sec7 domain could partially rescue poliovirus replication from inhibition by BFA (250). On the other hand, GBF1 with the mutation E794K that inactivates the nucleotide exchange function of the Sec7 domain could not support the replication of poliovirus (247). However, these results need to be interpreted with caution. The E794K mutation functions similarly to BFA,

locking the Sec7d-Arf-GDP complex in an inactive conformation with increased membrane stability (327). One can argue that the reason why the E794K mutant is unable to support replication is the inability to be recruited to replication complexes rather than the lack of Arf activation capacity. To overcome these experimental limitations, a GBF1 mutant with substitutions of amino acids 883 to 889 with alanine (GBF1/7A) in the loop after the J helix of the Sec7 domain was used in our studies. This mutant is catalytically inactive since it is predicted to not bind to Arfs but has the membrane association properties of the wt GBF1 (226). This allows us to clarify whether the Arf activation property of GBF1 is essential for poliovirus replication.

We generated a full-length GBF1 and two truncated constructs containing the 7A mutation within the context of the E795A mutation conferring BFA resistance (Figure 4.6A). To confirm that the 7A mutation prevents the interaction of GBF1 with Arf, we performed a GBF1 pulldown assay with purified Arf1-glutathione S-transferase (Arf1-GST) that demonstrated that Arf1-GST interacts with wt GBF1 and the GBF1/A794K mutant but does not interact with the GBF1/7A mutant, as predicted (Figure 4.6B). We then tested in a replication assay the full-length GBF1/7A construct, as well as 1060 and 894 truncated 7A constructs, similar to those previously shown to be partially functional in poliovirus replication in the contexts of the A794K Sec7-inactivating mutation (250). None of the GBF1 constructs with the 7A mutation could support poliovirus replication, even though they were expressed at a somewhat higher level than the positive control GBF1/A795E (Figure 4.6C). Thus, we conclude that the Arf-activating function of GBF1 is essential for viral replication.

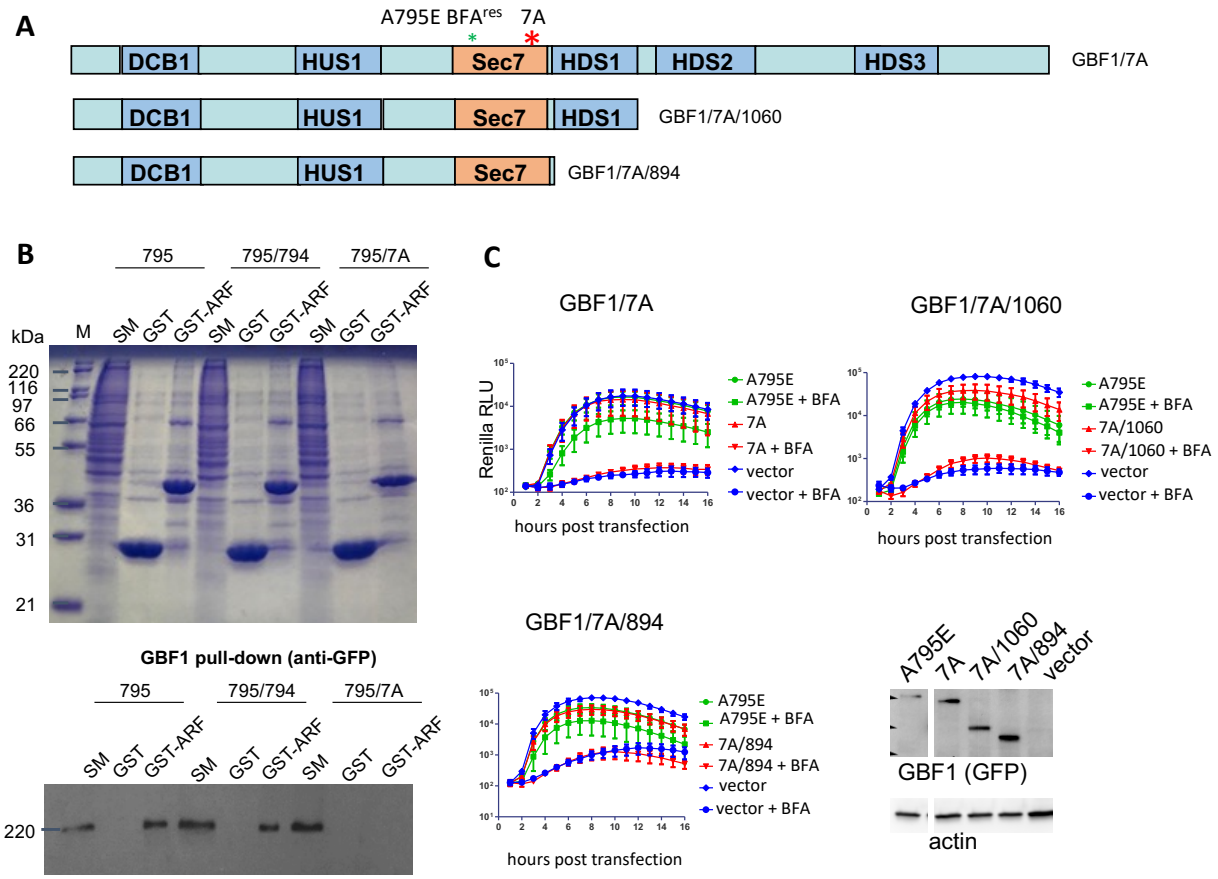


Figure 4.6: GBF1 mutants unable to activate Arf are defective in replication. (A) Schematic of GBF1 constructs containing the inactivating 7A mutation. All GBF1 expression constructs are GFP tagged. (B) GST-Arf1-GBF1 pulldown assay. GST or GST-Δ17 ARF1 was immobilized on glutathione beads and incubated with lysate from cells (designated SM [starting material]) expressing GFP-tagged GBF1/A795E, GBF1/A795E/A794K, or GBF1/A795E/7A. The bound material was analyzed by SDS-PAGE and the gel either stained with Coomassie blue (top) or transferred to nitrocellulose (NC) and immunoblotted with anti-GFP antibodies (bottom). GBF1/795/7A does not bind the ARF substrate, whereas GBF1/795 and GBF1/795/794 bind ARF. (C) Performance of the corresponding mutants in a poliovirus replication assay. Cells were transfected with the plasmids expressing a corresponding GBF1 mutant, a full-length GBF1 A795E (positive control), or an empty vector (negative control). The next day, the cells were transfected with a poliovirus replicon RNA expressing *Renilla* luciferase and incubated in the presence or absence of 1 μg/ml BFA. Expression of the 7A constructs in the samples from the corresponding replication experiments is additionally verified by Western blotting. Representative positive-control (A795E) and negative-control (vector) samples are shown.

4.2.3 Mutations in the conserved elements of C-terminal domains of GBF1 do not affect viral replication.

GBF1 orthologs regulate membrane trafficking in all eukaryotes, and they all contain several domains showing a high level of sequence and structure conservation among diverse taxa, separated by less-conserved linkers (328). Previously, we generated a series of mutations in the highly conserved regions of the HDS1, HDS2, and HDS3 domains of GBF1 targeting the most conserved residues. The HDS1 domain was recently found to target GBF1 to phosphoinositide phosphate (PIP)-enriched membranes, and the LF926AA mutation (number corresponds to the position of the first amino acid changed in the GBF1 sequence) disrupted this property and inhibited GBF1 function in secretion (329). Similarly, the HDS2 mutants L1246R, LF1266AA, and RDR1168AAA were severely compromised in supporting the cellular secretory pathway and targeting to the Golgi, while the HDS2 mutant LMK1135AAA behaved like a positive control in a secretion assay (303). HDS3 mutants FPL1594AAA and PLL1544AAA also inhibit GBF1 association with Golgi membranes and do not support secretion (330). Here, we tested these GBF1 mutants (Figure 4.7A) for their ability to support poliovirus replication. As shown in Figure 4.7B, all the GBF1 mutants tested, including those previously shown to be severely compromised in cellular metabolism, supported poliovirus replication as the positive control. These data are consistent with the previously reported results that truncation of the whole C-terminal part of GBF1 downstream of the HDS1 domain does not interfere with GBF1 function in poliovirus replication complexes (250) and confirm that virus-specific function(s) of GBF1 require only a subset of the interactions/activities of this protein essential for cellular metabolism.

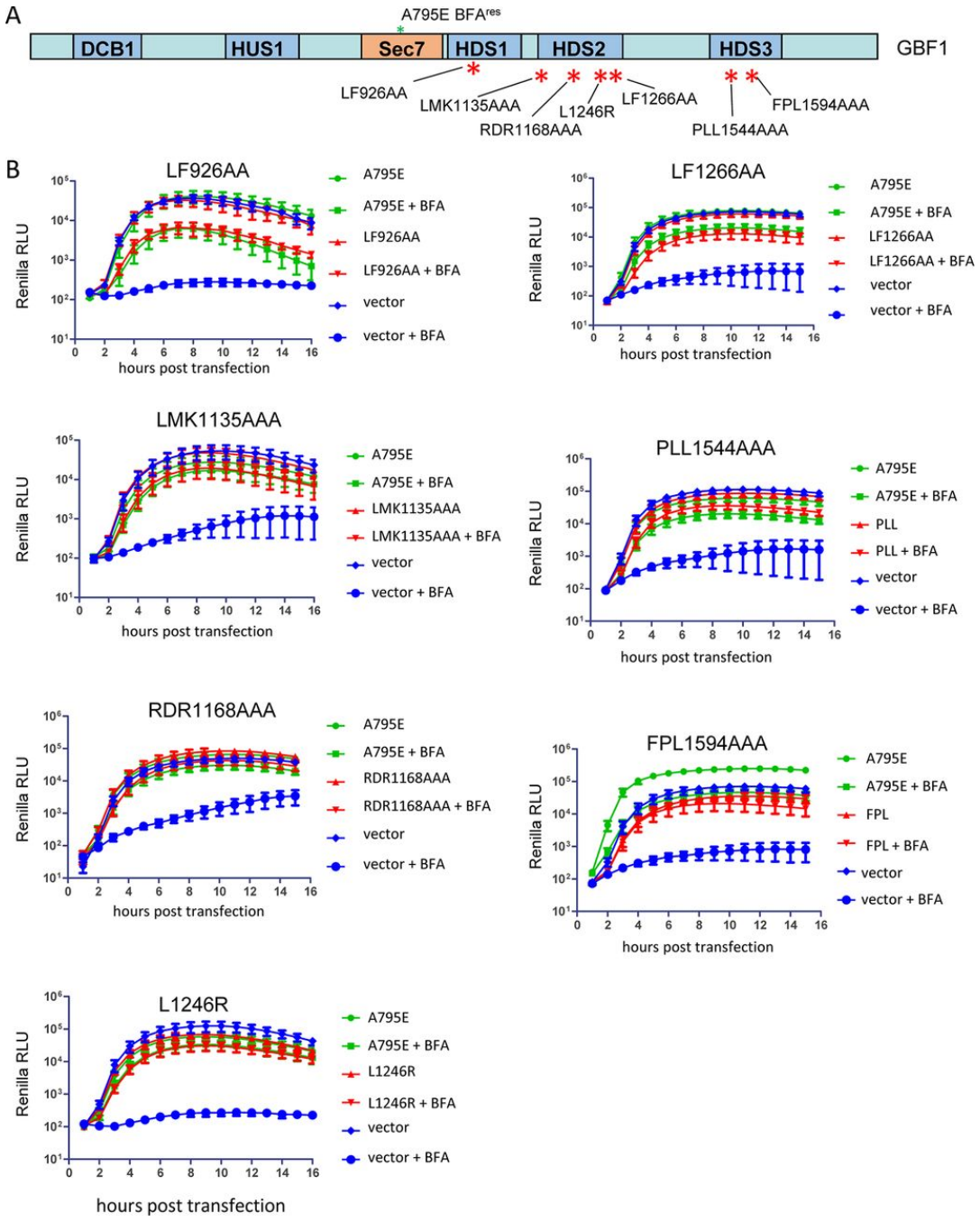


Figure 4.7: Functional analysis of GBF1 mutants targeting conserved elements in the C-terminal noncatalytic domains in poliovirus replicon replication. (A) Scheme of the mutations in the GBF1 sequence. All GBF1 expression constructs are GFP tagged and contain the A795E BFA resistance mutation in the Sec7 domain. (B) Performance of the corresponding mutants in a poliovirus replicon replication assay. Cells were transfected with the plasmids expressing a corresponding GBF1 mutant, a full-length GBF1 A795E (positive control), or an empty vector (negative control). The next day, the cells were transfected with a poliovirus replicon RNA expressing Renilla luciferase and incubated in the presence or absence of 1 μg/ml BFA.

4.2.4 GBF1 constructs containing the Sec7 domain from the ArfGEF ARNO are fully functional in poliovirus replication.

To determine whether viral replication requires the cognate Sec7 domain of GBF1 or whether any active Sec7 domain would work, we generated chimeric constructs where the Sec7 domain of GBF1 was replaced with the Sec7 domain of another ArfGEF, ARNO. ARNO is a guanine nucleotide exchange factor for Arf6 with a tripartite structure consisting of an N-terminal coiled-coil domain, a central Sec7, and a C-terminal pleckstrin homology domain (223). It localizes at the plasma membrane, modulating actin remodeling, endocytosis, and cellular migration (331-333). Thus, ARNO engages in a completely different set of interactions than GBF1, which functions at the ER-Golgi interface. Importantly, the amino acid sequence conservation between ARNO and GBF1 Sec7 domains is only 44%, indicating that substituting the Sec7 domains can be used to test the requirement for the GBF1 sequences in poliovirus replication. Due to the different amino acid sequences, ARNO is intrinsically resistant to BFA. We generated a construct (GARG, from GBF1-ARNO-GBF1), where the GBF1 Sec7 domain was substituted with that from ARNO (Figure 4.8A). We have shown previously that GARG targets to the Golgi and supports secretion as well as wild-type GBF1 (334). Importantly, the GARG chimeric protein also was fully functional in supporting poliovirus replication (Figure 4.8B). Thus, poliovirus replication has no specific requirement for the Sec7 domain of GBF1 but rather can be supported by any Sec7 domain capable of Arf activation.

Next, we tested if the GBF1-ARNO Sec7 substitution can function in the context of the 1060 truncation, i.e., when GARG is terminated after the HDS1 domain (GARG/1060). The truncated protein retained the full capacity to support poliovirus replication (Figure 4.8B).

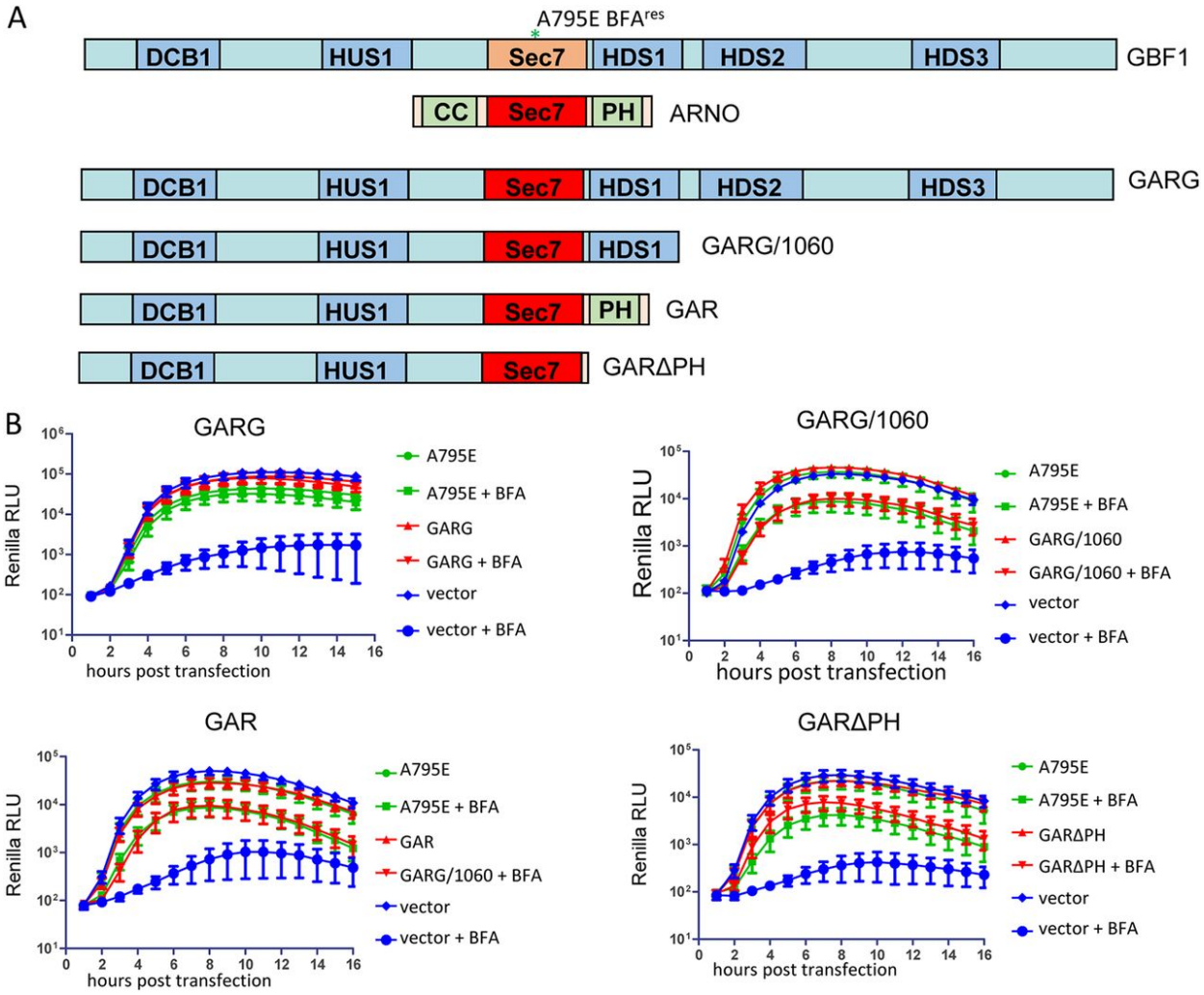


Figure 4.8: GBF1 constructs containing a Sec7d from another GEF support poliovirus replication. (A) Scheme of the GBF1/ARNO chimeras. All GBF1 expression constructs are GFP tagged. (B) Analysis of the corresponding constructs in a poliovirus replicon replication assay. Cells were transfected with the plasmids expressing a corresponding GBF1 mutant, a full-length GBF1 A795E (positive control), or an empty vector (negative control). The next day, the cells were transfected with a poliovirus replicon RNA expressing Renilla luciferase and incubated in the presence or absence of 1 µg/ml BFA.

It was previously observed that a GBF1 construct truncated just after the Sec7 domain (GBF1/894) is much less efficient in supporting poliovirus replicon replication than the GBF1/1060 construct truncated after the HDS1 domain (250). This phenomenon suggests two non-mutually exclusive possibilities. Either the HDS1 domain contains important elements

necessary for proper functioning of GBF1 in replication, or HDS1 presence at the C terminus of the protein stabilizes the functionally important Sec7 domain. To see if the HDS1 domain of GBF1 provides any specific contribution to poliovirus replication, we generated a chimeric construct where the Sec7-HDS1 fragment of GBF1 was substituted with Sec7-PH domains of ARNO (GAR, from GBF1-ARNO) (Figure 4.8A). GAR supported poliovirus replication like the GBF1 positive control (Figure 4.8B), thus ruling out the need for HDS1-specific interactions for the functioning of the replication complexes. To assess if the PH domain in this construct is important, we generated a truncated mutant, GARΔPH, with just 10 amino acids of PH after the end of the ARNO Sec7 domain (Figure 4.8A). This protein was also fully functional in the replication assay, confirming the HDS1-independent role of GBF1 in poliovirus replication (Figure 4.8B), at least under the conditions of HeLa cells and a wt poliovirus RNA.

4.2.5 The C-terminal HDS2 and HDS3 domains of GBF1 are important for viral replication under conditions of limited 3A-GBF1 interaction.

The surprising tolerance of poliovirus replication to varied levels of 3A-GBF1 interaction suggests that there may be an additional mechanism facilitating GBF1 recruitment to the replication complexes. The intrinsic propensity of GBF1 to bind membranes with specific lipid and protein compositions could be an important component of such a mechanism, positioning the protein where it can be easily accessed by the viral replication machinery. To test this possibility and to identify the GBF1 domains responsible, we used a poliovirus replicon containing the 3A-2 mutation, in which an additional serine is inserted at position 16 within the 3A sequence (Figure 4.9A). The 3A-2 virus demonstrates a cold-sensitive phenotype, much higher sensitivity to BFA inhibition, a slightly reduced replication level, and a delay in the development of CPE compared

to the WT virus (247, 320, 335). Importantly, the 3A-2 mutant and similar coxsackievirus B3 constructs are severely compromised in interactions with GBF1 (247, 307, 336).

We compared the abilities of different GBF1 mutants to support the replication of the wild-type and 3A-2 replicon. As shown in Figure 4.9B, the replication of a wild-type replicon was efficiently supported by both the full-length GBF1 and a construct truncated after the HDS1 domain (A795E/1060), consistent with data in this study (Figure 4.8B) and reported elsewhere (250). In contrast, the replication of the 3A-2 poliovirus mutant could be efficiently supported by the full-length GBF1, although the level of rescue was lower than with the wt poliovirus as was previously reported (247, 336), but not by the A795E/1060 truncated GBF1 construct (Figure 4.9B). Thus, the C-terminal domains of GBF1 are dispensable for the replication of wild-type virus but become important when GBF1-3A interaction is compromised.

To identify the domains within the C terminus of GBF1 important for the replication of 3A-2, we examined the ability of C-terminal GBF1 mutants, which were fully functional in the replication of the wild-type replicon (Figure 4.7), to support 3A-2 replication. GBF1 with the mutation LF926AA in the HDS1 domain consistently rescued replication of the 3A-2 replicon somewhat better than the positive control (Figure 4.9C). GBF1 with the mutation LMK1135AAA or RDR1168AAA in the HDS2 domain and mutation PLL1544AAA in the HDS3 domain rescued replication of the 3A-2 replicon just like the positive control (Figure 4.9C). However, the mutation FPL1594AAA in HDS3 and the mutations LF1266AA and especially L1246R in HDS2 severely impaired the capacity of GBF1 to rescue replication of the 3A-2 replicon (Figure 4.9C). Thus, the C-terminal HDS2 and HDS3 domains of GBF1, while dispensable for the replication of the wild-type replicon, hold important determinants facilitating the recruitment/function of the protein in

replication complexes under conditions of reduced interaction between GBF1 and the viral protein 3A.

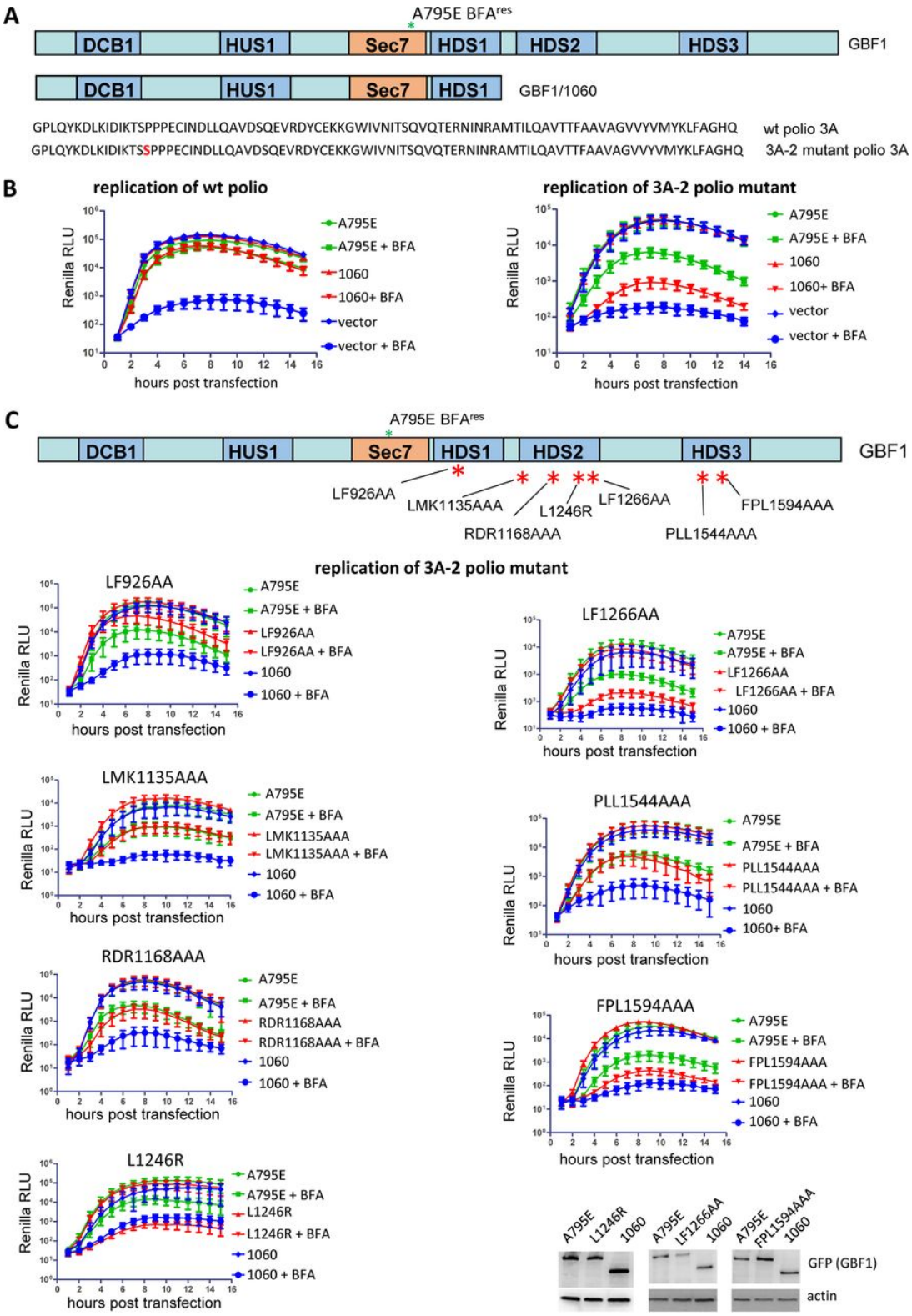


Figure 4.9: C-terminal GBF1 mutants show differential capacity to support replication of a wild-type versus the 3A-2 mutant replicon. (A) Top, scheme of the full-length and 1060 truncated GBF1 constructs; bottom, amino acid sequence of 3As from the wt poliovirus and 3A-2 mutant. All GBF1 expression constructs are GFP tagged and contain the A795E BFA resistance mutation in the Sec7 domain. (B) Performance of the full-length and 1060 truncated GBF1 constructs in a replication assay with a wt replicon and a replicon bearing the 3A-2 mutation, severely inhibiting 3A-GBF1 interaction. Cells were transfected with the plasmids expressing a full-length GBF1 A795E, a 1060 GBF1 A795E truncated construct, or an empty vector (negative control). The next day, the cells were transfected with a wt or 3A-2 poliovirus replicon RNAs expressing Renilla luciferase and incubated in the presence or absence of 1 μg/ml BFA. (C) Performance of the GBF1 mutants in the HDS1, HDS2, and HDS3 domains in a replication assay with a replicon containing 3A-2 mutation. Scheme of the mutation positions in the GBF1 domains is shown. All GBF1 expression constructs are GFP tagged and contain the A795E BFA resistance mutation in the Sec7 domain. Cells transfected with the full-length GBF1 A795E serve as a positive control, and those transfected with the 1060 truncated GBF1 A795 serve as a negative control. Expression of constructs L1246R, LF1266AA, and FPL1594AAA, severely impaired in rescuing the replication of the 3A-2 poliovirus replicon, is additionally verified by Western blotting in the samples from the corresponding experiments.

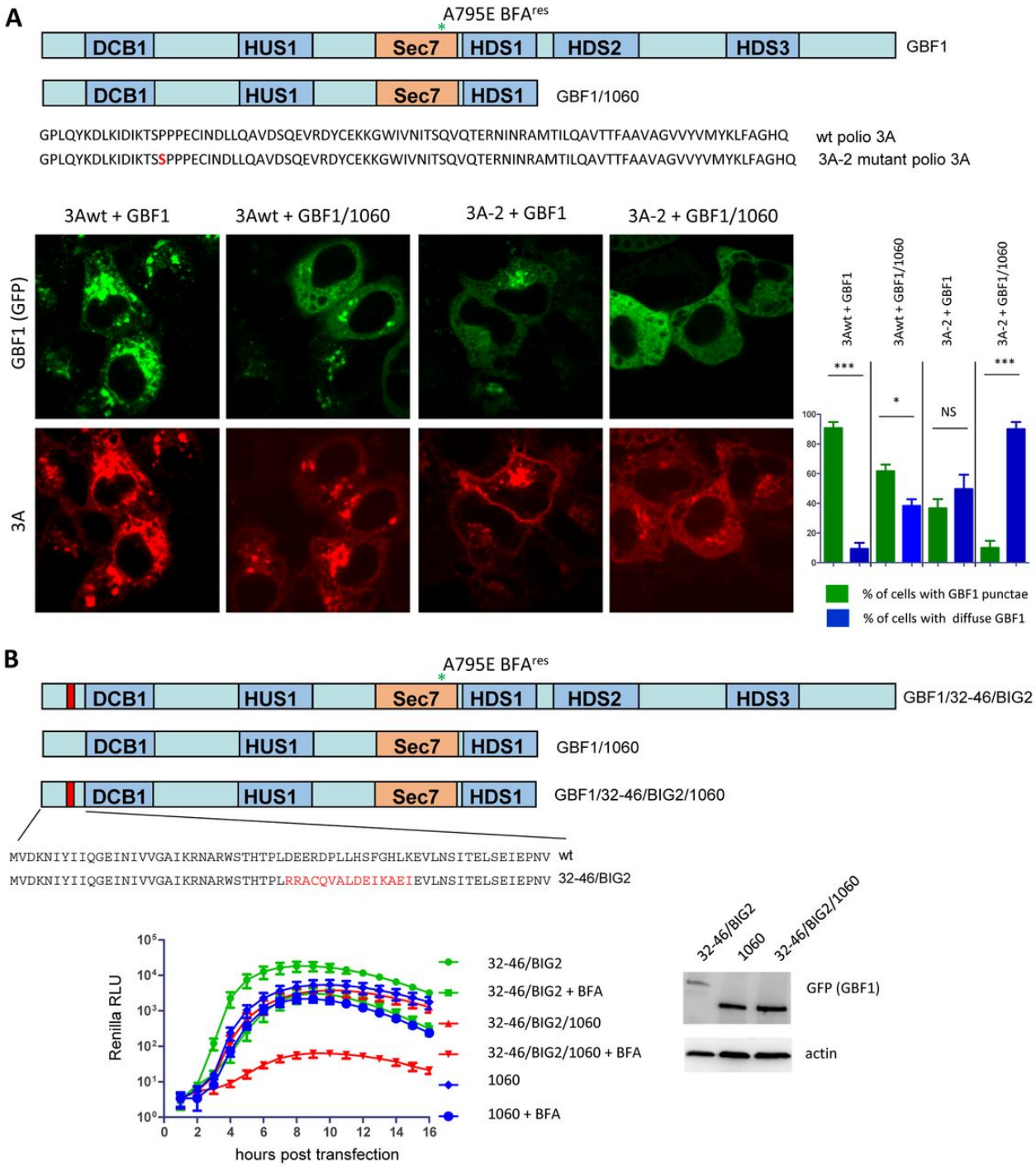
4.2.6 The C-terminal domains of GBF1 functionally alleviate the defects of GBF1-3A interaction in poliovirus replication.

To further investigate the role of C-terminal domains of GBF1 in its recruitment by 3A, we co-transfected HeLa cells with plasmids expressing either wt or 3A-2 mutant poliovirus 3A proteins and plasmids expressing either a full-length GBF1 or a truncated GBF1/1060 construct lacking HDS2 and HDS3 (Figure 4.10A). In cells co-expressing wt 3A and a full-length GBF1 construct, the GBF1 signal was concentrated in bright agglomerates in virtually every cell, and such a distribution almost perfectly mirrored the 3A signal. In cells co-expressing wt 3A and the truncated version of GBF1, the GBF1 punctate became smaller, and they were present in only about half of the cells. A significant amount of 3A and GBF1 signals were distributed in an ER-like reticular pattern. A similar distribution of 3A and GBF1 signals was observed in cells co-expressing 3A-2 mutant and a full-length GBF1, although both the GBF1 and the 3A agglomerates were noticeably smaller than those observed in cells expressing wt 3A. However, removing the GBF1 C-terminal domains resulted in an almost complete loss of the punctate GBF1 distribution in cells co-expressing the 3A-2 mutant protein (Figure 4.10A). Thus, combinations of 3A and

GBF1 corresponding to those found in functional replication complexes (wt 3A plus full-length or truncated GBF1, or 3A-2 plus full-length GBF1) demonstrated a significant amount of colocalization of GBF1 and 3A signals, while in cells co-expressing a nonfunctional combination (3A-2 plus truncated GBF1), such punctate co-localization was completely lost.

Our data support the double targeting model of GBF1 recruitment to the replication complexes where the C-terminal domains of GBF1 can compensate for deficiencies in 3A-GBF1 interactions. To further test this model, we made a truncated GBF1 construct lacking the C-terminal domains and incorporated the substitution of amino acids 32 to 46 for those derived from BIG2 (GBF1/32-46/BIG2/1060), and we tested its ability to support replication of the wt poliovirus replicon (Figure 4.10B). The full-length GBF1 construct with 32-46/BIG2 substitution could support poliovirus replication but was strongly compromised in interaction with 3A (see Figures 4.3 and 4.5). Consistent with the previous data, both the 1060 construct with the wt N terminus and the full-length construct with the 32-46/BIG2 substitution could efficiently support poliovirus replication. However, when the 32-46/BIG2 substitution was introduced into the 1060 truncated context, such a construct was completely nonfunctional in the replication assay (Figure 4.10B).

Thus, the recruitment of GBF1 to the replication complexes relies on both 3A-GBF1 interaction and membrane targeting mediated by the C-terminal domains. Functional replication complexes may be formed if either of these mechanisms is compromised but not when both are inactivated at the same time.


A
A795E BFA^res
DCB1
HUS1
Sec7
HDS1
HDS2
HDS3
GBF1
GBF1/1060
GPLQYKDLKIDIKTSPPPECINDLLQAVDSQEVRDYCEKKGWIVNITSQVQTERNINRAMTILQAVTTFAAVAGVVYVMYKLFAGHQ wt polio 3A
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3Awt + GBF1
3Awt + GBF1/1060
3A-2 + GBF1
3A-2 + GBF1/1060
GBF1 (GFP)
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% of cells with GBF1 punctae
% of cells with diffuse GBF1
B
A795E BFA^res
DCB1
HUS1
Sec7
HDS1
HDS2
HDS3
GBF1/32-46/BIG2
GBF1/1060
GBF1/32-46/BIG2/1060
MVDKNIYIIQGEINIVVGAIKRNARWSTHTPLDEERDPLLHSFGHLKEVLNSITELSEIEPNV wt
MVDKNIYIIQGEINIVVGAIKRNARWSTHTPLRRACQVALDEIKAEIEVLNSITELSEIEPNV 32-46/BIG2
Renilla RLU
hours post transfection
32-46/BIG2
32-46/BIG2 + BFA
32-46/BIG2/1060
32-46/BIG2/1060 + BFA
1060
1060 + BFA
32-46/BIG2
1060
32-46/BIG2/1060
GFP (GBF1)
actin

Figure 4.10: C-terminal part of GBF1 can compensate for the defects in 3A-GBF1 interaction. (A) The C-terminal part of GBF1 facilitates its recruitment by 3A. Top, scheme of the full-length and 1060 truncated GBF1 constructs and amino acid sequence of 3As from the wt poliovirus and 3A-2 mutant. HeLa cells were co-transfected with plasmids expressing wt 3A or 3A-2 mutant proteins together with plasmids expressing either full-length or 1060 truncated GBF1 constructs. The next day, the cells were fixed and stained for 3A upon mild permeabilization, and the distribution of GBF1 signal in 3A-expressing cells was evaluated. Graph shows quantification of three independent experiments, with at least 200 cells counted for each sample. Statistical significance is indicated. (B) The C-terminal part of GBF1 can functionally compensate for the defect of GBF1-3A interaction in poliovirus replication. Top, scheme of a full-length GBF1 construct with substitution of amino acids 32 to 46 for those derived from a corresponding segment of BIG2; 1060 truncated GBF1 construct with wt N-terminal sequence; and 1060 truncated GBF1 construct with BIG2-derived substitution of amino acids 32 to 46. HeLa cells were transfected with the plasmids expressing indicated GBF1 constructs. The next day, the cells were transfected with a wt poliovirus replicon RNA coding for the Renilla luciferase gene and incubated in the presence or absence of 1 μg/ml of BFA. Expression of the GBF1 constructs was verified by Western blot.

4.3 Discussion.

The cellular protein GBF1 that supports the functioning of the secretory pathway has long been known to be recruited to the replication complexes of poliovirus and related viruses and to mediate the sensitivity of infection to the fungal metabolite BFA (214, 247, 304, 305, 322). The replication-independent expression of poliovirus polyprotein in HeLa cells in the presence of BFA as well as the experiments in an *in vitro* system that allows separation of individual steps in the viral replication cycle revealed that GBF1 is required for proper assembly and/or functioning of the membrane-associated viral replication complexes (247, 337). However, the exact mechanism by which GBF1 supports the viral replication machinery remains unknown. GBF1 is a large protein of ~200 kDa, containing six conserved domains, and in this chapter, we aimed to identify GBF1 elements within each domain necessary to sustain viral replication.

Previous co-IP and yeast two-hybrid assays established that the N-terminal ~60-kDa fragment of GBF1 is sufficient for direct interaction with the 3A protein of coxsackievirus B3, a close relative of poliovirus (307, 322) and that the deletion of the first 37 amino acids of GBF1 blocks such 3A-GBF1 interaction (247). Here, we report that the N terminus of GBF1 can tolerate

significant modifications, such as replacements of long stretches of the protein sequence with alanine residues, without a noticeable effect on its function in poliovirus replication. Interestingly, GBF1 function in the cellular secretory pathway was more sensitive to those modifications in a cellular secretion assay, although some modifications like the 31-40A mutant could be tolerated. Substitutions of N-terminal fragments of GBF1 with segments derived from a related ArfGEF BIG2, which has a similar domain architecture, proved to be more detrimental. None of the four tested 16-amino-acid-long substitutions were tolerated in the secretion assay, but only two of them, the one substituting amino acids 2 to 17 and the one replacing amino acids 47 to 62, severely inhibited the replication-supporting function of GBF1. Further dissection of the BIG2-derived segments revealed that the substitution of only four GBF1 amino acids in positions 9 to 12 significantly inhibits viral replication.

The most studied function of GBF1 is its guanidine nucleotide exchange activity required for activation of the small GTPase Arf. This activity is mediated by the Sec7 domain of GBF1 and is the target of BFA and similar molecules that inhibit the cellular secretory pathway (224, 233, 328). GBF1 with the A794K mutation in the Sec7 domain locking GBF1 and Arf-GDP in a nonfunctional conformation does not support the replication of either poliovirus or coxsackievirus B3 (247, 250, 307). Here, we used a novel mutation in the Sec7 domain that prevents the binding of Arf-GDP to GBF1 and found that similarly to the inactive A794K mutant, GBF1 constructs containing the 7A mutation could not support poliovirus replication. Collectively, these data strongly suggest that activated Arf is required for viral replication, although its mechanistic contribution to the replication process is unknown. The importance of Arf activation for poliovirus replication is also supported by the fact that a chimeric protein consisting of only the N-terminal half of GBF1 and a Sec7 domain from the Arf GEF ARNO was fully functional in the replication

assay. These data prompt the hypothesis that activated Arf molecules on the replication membranes may serve to recruit other cellular proteins, or that the presence of activated Arf may be necessary to keep cellular or even viral proteins in a functional conformation. These data seem to contradict previous reports that demonstrated at least partial functionality of certain truncated GBF1 species, either containing the A794K mutation or completely lacking the Sec7 domain, in poliovirus replication (250). Such a discrepancy may reflect the existence of some Arf activation-independent role of GBF1 in poliovirus replication, which may to a certain extent be sustained in the context of A794K (or in the constructs lacking the entire Sec7 domain) but not in the context of a 7A mutation. It is also possible that different modes of inactivating Sec7 domains, i.e., trapping of Arf-GDP in an inactive conformation like in the case of the A794K mutation, or preventing Arf-GDP binding like in the case of 7A mutation, result in different conformation and/or cellular localization of GBF1 molecules.

It has been shown that the specificity of GEFs, including GBF1, toward activation of certain Arfs, is mediated by the cellular localization of GEFs rather than by specificity of their Sec7 domains (334). It is likely that in infected cells such targeting signals are no longer functional, and the spectrum of Arfs activated by GBF1 may be different than that in noninfected cells, thus probably explaining the resilience of enterovirus replication to knockout or knockdown of expression of Arfs normally activated by GBF1 (307, 319).

The protein 3A of picornaviruses has increasingly been recognized as an important player in organizing the membranous environment suitable for viral replication. In poliovirus and related viruses, the interaction of 3A with GBF1 appears to be required for the recruitment of GBF1 to the replication complexes (247, 251, 252, 307). In addition, 3A proteins of diverse picornaviruses directly interact with another cellular protein, ACBD3, and this interaction is important for

recruitment to the replication complexes of a phosphatidylinositol kinase, PI4KIIIβ (283, 338-341). The data available so far, such as the accumulation of distinct resistance mutations upon propagation of picornaviruses in the presence of inhibitors of GBF1 or PI4KIIIβ, as well as GBF1-independent recruitment of PI4KIIIβ and vice versa (248, 249, 293), indicate that GBF1- and PI4KIIIβ-controlled processes in the replication complexes are mutually independent, despite being coordinated by the same viral protein. These findings lead to two contrasting hypotheses of whether the same molecule of protein 3A, which in poliovirus is only 87 amino acids long, simultaneously interacts with both ACBD3 and GBF1 or if there are different populations of 3As in the replication complexes that participate in different sets of interactions with cellular and/or viral proteins.

While the 3A-GBF1 interaction appears to be important for poliovirus replication, numerous findings indicate that the strength of such an interaction can vary significantly without a dramatic effect on virus replication. This suggests that there may be a significant excess of GBF1 available in cells, at least in the conventional cell culture models used for the study of picornaviruses, or that there is an auxiliary mechanism targeting GBF1 to the replication membranes that may compensate for the weakened 3A-GBF1 interaction. We previously found that mutations in the conserved regions of GBF1 located in the C-terminal noncatalytic domains of the protein prevent its targeting to Golgi membranes and inhibit GBF1 function in the secretory pathway (303, 329). When we tested such mutants in poliovirus replication, all fully supported replication of the wild-type replicon, but several C-terminal GBF1 mutants were severely compromised in supporting the replication of a 3A-2 replicon carrying a mutation that strongly diminishes the 3A-GBF1 interaction (247, 307, 336). The mechanism of how the C-terminal

domains of GBF1 facilitate recruitment of GBF1 to the replication complexes is, however, far from obvious and requires further investigation.

It is known that replication organelles of picornaviruses, including those of poliovirus, are highly enriched in PI4P (168). Yet, the mutation LF926AA in the HDS1 domain of GBF1, previously found to impair the targeting of GBF1 to PIP-containing membranes (329), did not decrease the replication of the 3A-2 mutant and even somewhat outperformed the positive control. The most severe negative effect on the replication of the 3A-2 replicon was observed with the L1246R mutation of a conserved amino acid in the HDS2 domain of GBF1. This mutation was originally identified in zebrafish embryos with severe vasculature development abnormality (342). It was later established that such a mutation in the human GBF1 makes the protein incapable of supporting the secretory pathway and results in dramatic differences in the localization of the mutant protein compared to the normal GBF1 (303). Thus, a mislocalized GBF1 with the L1246R mutation was fully functional in the replication of a wt replicon but completely incapable of supporting replication under conditions when GBF1 interaction with the viral protein 3A was impaired. This suggests that the membrane-targeting determinants of the GBF1 C terminus provide an additional mechanism facilitating GBF1 recruitment/function in the replication complexes. Supporting the existence of a redundant mechanism of GBF1 recruitment to poliovirus replication complexes is the incompatibility of a substitution of 16 amino acids in the N-terminal region of GBF1 reducing its interaction with 3A and truncation of the C-terminal domains, neither of which by itself is detrimental to replication. Thus, poliovirus can replicate when either the 3A-GBF1 interaction or the proper cellular targeting of GBF1 is inhibited but not when both are compromised at the same time (Figure 4.11). This likely explains the surprising tolerance of at least some picornaviruses, including poliovirus, to the variability of the level of direct 3A-GBF1 interaction

(247, 251). Whether specific membrane targeting can compensate for a complete loss of GBF1-3A interaction, or if at least some level of such direct interaction is essential for replication remains to be established.

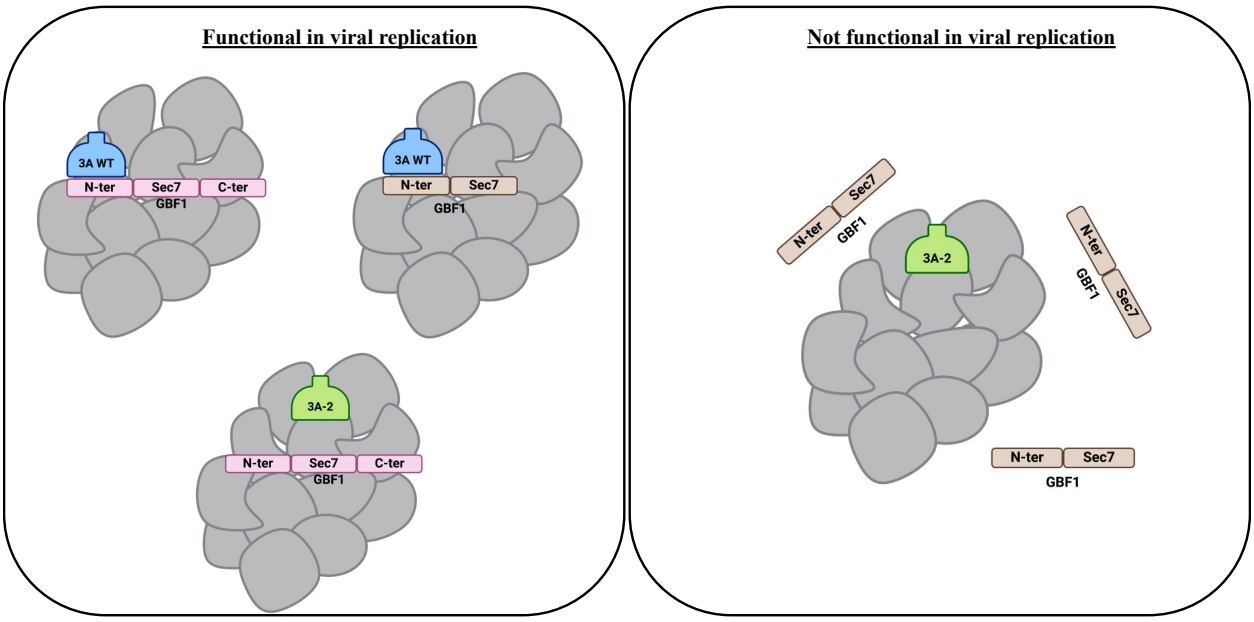

Figure 4.11: Schematic representation of the redundant mechanism of recruitment of GBF1 to enterovirus replication organelles. GBF1 recruitment to enterovirus ROs is mediated by its C-terminal domain that contains the membrane targeting properties and by its interaction with the viral nonstructural protein 3A. Functional replication complexes may form when either the 3A-GBF1 interaction or the proper cellular targeting of GBF1 is inhibited but not when both are compromised at the same time.

In general, these and previous findings demonstrate that poliovirus replication can be supported by GBF1 derivatives that are completely nonfunctional in cellular metabolism, including extensively truncated mutants, indicating that the virus hijacks only a subset of GBF1 activities/interactions that are essential for normal GBF1 function in the cell. Given that GBF1 is an essential factor for the replication of diverse (+)RNA viruses (308, 315, 343), it will be interesting to determine if these diverse viruses rely on the same mechanistic contribution(s) of GBF1 to the replication process. The identification of the molecular processes by which GBF1 supports viral replication may allow interventions to target specific GBF1 activities in infected

cells while retaining those for its normal cellular functions. This may open novel directions in the development of antiviral therapeutics.

Chapter 5: Complex Microdomain Organization of Enterovirus Replication Organelles

5.1. Introduction.

Enteroviruses include many known and emerging pathogens, such as poliovirus, coxsackieviruses, enteroviruses A71 and D68, rhinoviruses, and others. Measures to control enterovirus infections are challenging because of their high genetic diversity and the ease with which they develop resistance to drugs targeted against viral proteins. To date, there are no antiviral drugs approved against enteroviruses, and only poliovirus and enterovirus A71 have licensed vaccines (344, 345). To develop effective anti-viral strategies, we must characterize the virus replication cycle and the cellular proteins supporting infection.

The optimal functioning of the enteroviral replication machinery depends on host factors recruited to the replication organelles, which facilitates the establishment of a composition of proteins and lipids conducive to replication. Indeed, enterovirus RNA replication critically depends on the activation of small cellular Arf GTPases by GBF1. There are five Arf isoforms in primates, Arf1 and Arf3-6, which are divided into three classes. In human cells, class 1 comprises Arf1 and -3, class 2 includes Arf4 and -5, and Arf6 is the only member of class 3. The Arf family of GTPases act as molecular switches by changing between inactive GDP-bound and active GTP-bound states. Guanine nucleotide exchange factors such as GBF1 activate Arfs by catalyzing the GDP to GTP exchange, whereas GTP hydrolysis is mediated by GTPase-activating proteins (GAPs). GTP binding leads to a conformational change that allows Arf association with membranes and increases their interaction with multiple effector proteins, including those engaged in membrane remodeling, lipid synthesis, and trafficking. Activation of different Arf isoforms is subjected to tight spatial and temporal regulation and is important for controlling multiple steps of

membrane metabolism (217). Enterovirus infection is inhibited by brefeldin A (BFA), which prevents Arf activation by forming a stable transient intermediate with Arf-GDP and the catalytic Sec7 domain GBF1, locking the complex in a nonfunctional conformation.

Enteroviruses induce the dynamic recruitment of all five isoforms of Arf GTPases to the replication organelles membranes, and Arf1 is the most important for viral replication. Recruitment of Arf1 to the replication organelles occurs early during infection, and its depletion critically increases viral replication sensitivity to BFA (242). Moreover, studies of synthetic lethal partners also strengthen the importance of Arf1 for enteroviruses. Synthetic lethality is a genetic interaction between two genes that are involved in a process to perform an essential function. Compromising one gene does not affect cell viability. However, compromising both genes results in lethality. Navare *et al*. showed that the viability of Arf1 knockdown cells decreased when GBF1 was compromised by the expression of the enterovirus protein 3A, which directly interacts with and recruits GBF1 to the replication organelle membranes (243).

Importantly, Moghimi *et al.* demonstrated that enteroviral antigens show different patterns of association with Arf1-containing membranes, and the dsRNA is excluded from Arf-enriched regions of the replication organelle membranes (242). This, along with the sequential recruitment of different Arf isoforms during the time course of infection, indicates that the protein composition of the replication organelles is not homogeneous and that they have compositionally, and therefore functionally specific microdomains that create a unique biochemical environment that supports different steps of the viral replication cycle. Nevertheless, further studies are required to elucidate the organization and protein composition of these microdomains in the replication organelles.

Thus, we sought to characterize the microdomain organization of enterovirus replication organelles. We used super-resolution microscopy, peroxidase-based proximity biotinylation, and

metabolic RNA labeling to investigate the distribution of viral proteins and Arf1 on the replication organelles. We observed that Arfs form isolated microdomains and that viral antigens are localized in both Arf1-depleted and Arf1-enriched microdomains, where they show different degrees of colocalization with the cellular protein. Pulse-labeling of viral RNA with 5-ethynyl uridine showed that active RNA replication is always associated with Arf1-enriched membranes. Furthermore, we established a HeLa cell line stably expressing Arf1 fused to the peroxidase APEX2. The viral protein biotinylation was detected as early as 3 h.p.i., and the non-cleaved fragments of the viral polyprotein were overrepresented in the Arf1-enriched domains. Furthermore, we show that after 4 h.p.i. viral proteins could be efficiently biotinylated only upon digitonin permeabilization of the replication organelle membranes, while such permeabilization inhibited the Arf1 biotinylation signal at the Golgi in non-infected cells, highlighting a difference in the membrane architecture and/or composition in infected and non-infected cells.

5.2. Results

5.2.1 Enterovirus replication complexes are highly enriched in Arf1.

Findings from our lab have previously shown that during enterovirus infection, the dsRNA signal, an intermediate of the RNA replication process, is excluded from Arf1-enriched membranes (242). However, the signal from the dsRNA antibody may not reflect the location of active replication complexes but rather the accumulation of long dsRNA dead-end replication by-products. To overcome these limitations, active viral replication complexes can be detected using the alkyne-modified uridine analog 5-ethynyl uridine (5-EU), which is metabolized inside the cell into ethinyl-uridine triphosphate and is incorporated into the newly synthesized RNA. The ethynyl group can be detected by a copper (I)-catalyzed cycloaddition reaction (click-chemistry reaction)

with an azide-tagged fluorophore that allows its visualization using fluorescent microscopy imaging (346, 347).

To test if enterovirus replication complexes can be visualized by the incorporation of the cell-permeable 5-EU during viral RNA replication, we added 5-EU to HeLa cells infected with poliovirus (and mock-infected) at 4 hours post-infection. The click-chemistry reaction was performed with an azide-containing fluorescent dye after 5, 15, and 30 minutes of incubation with 5-EU. The mock-infected cells showed a nuclear 5-EU signal consistent with the 5-EU incorporation into endogenous transcripts. However, in infected cells, the signal was exclusively cytoplasmic, reflecting the inhibition of nuclear transcription upon infection and the incorporation of 5-EU into actively replicating viral RNA (Figure 5.1A). Notably, the labeled RNA was detected after 30 minutes but not after the 5-minute or 15-minute incubation. Thus, the signal reflects the RNA synthesized within a 15-minute interval (from 15 to 30 minutes post-5-EU addition). The period before the signal detection likely reflects the time required for the transport of 5-EU into the cell and its metabolization into EU-triphosphate.

Next, we compared the signals of actively replicating RNA labeled with 5-EU and the one detected with the dsRNA antibody in poliovirus-infected cells at 4 hours post-infection. The signals only partially co-localized (Figure 5.1B), and sometimes cells with a strong dsRNA antibody signal lacked the 5-EU signal for actively replicating RNA (Figure 5.1B, arrow). These data demonstrate the importance of detecting active replication complexes and the limitation of the dsRNA signal.

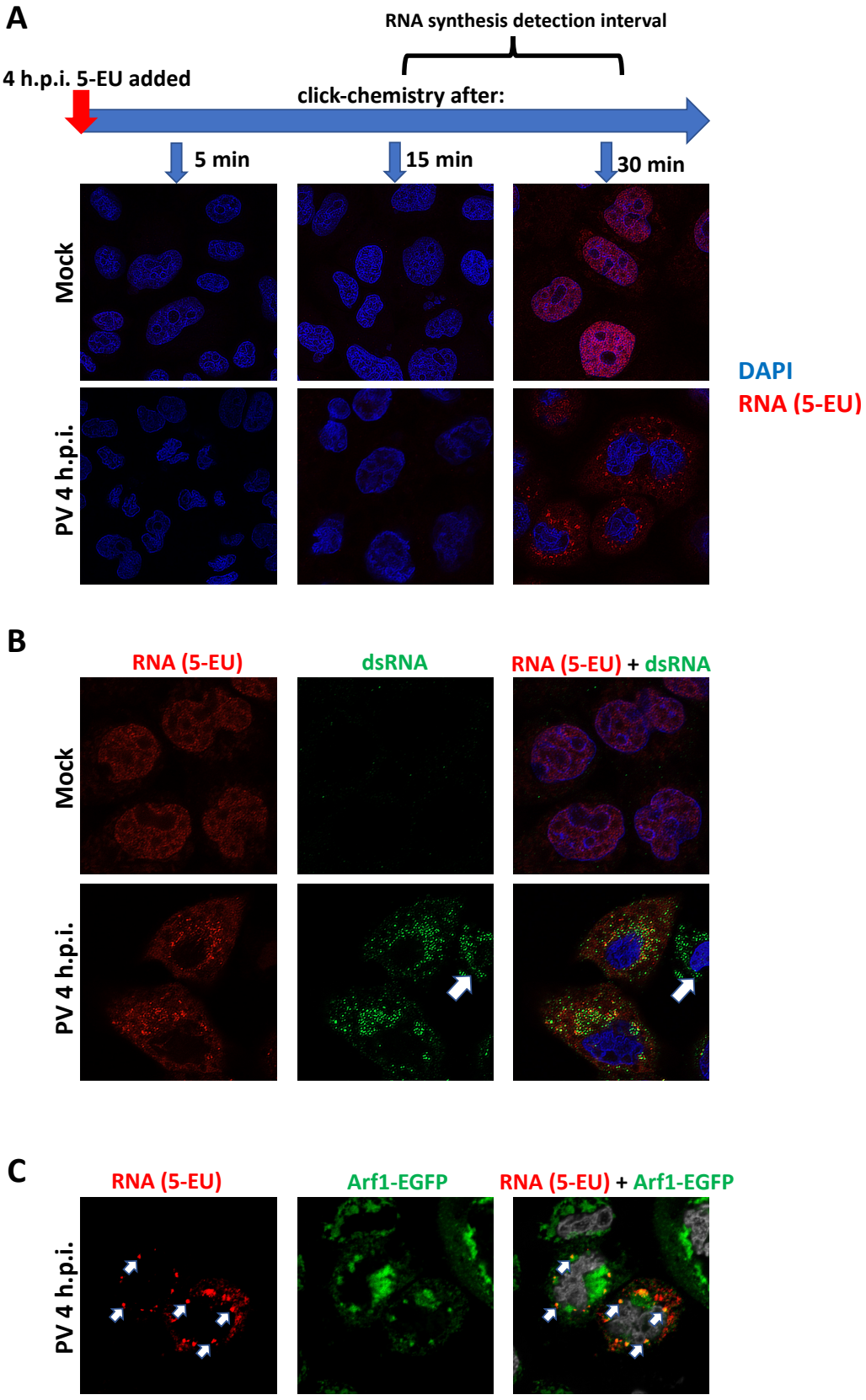
A
RNA synthesis detection interval
4 h.p.i. 5-EU added
click-chemistry after:
5 min
15 min
30 min
Mock
PV 4 h.p.i.
DAPI
RNA (5-EU)
B
RNA (5-EU)
dsRNA
RNA (5-EU) + dsRNA
Mock
PV 4 h.p.i.
C
RNA (5-EU)
Arf1-EGFP
RNA (5-EU) + Arf1-EGFP
PV 4 h.p.i.

Figure 5.1: Enterovirus replication complexes are highly enriched in Arf1. (A) Visualization of enterovirus replication complexes using 5-EU. HeLa cells were infected with poliovirus (and mock-infected) at 50 pfu/cell and the click-chemistry reaction was performed with an azide-containing fluorescent dye after 5, 15, and 30 minutes of incubation with 5-EU at 4 h.p.i. (B) HeLa cells were infected and mock-infected with 50 pfu/cell of poliovirus, treated with 5-EU for 30min at 4 h.p.i, and fixed. The fixed cells were processed for click-chemistry in the presence of 50 µg/mL of digitonin and stained with anti-dsRNA antibody. (C) HeLa cells stably expressing Arf1-EGFP were infected with poliovirus at 50 pfu/cell, treated with 5-EU for 30 minutes at 4hpi, fixed, and processed for click-chemistry without permeabilization. Nuclear DNA was stained by Hoechst 33342 in all samples.

To determine if active replication complexes are associated with Arf1-enriched membranes, we infected a stable HeLa cell line expressing Arf1-EGFP (242) with poliovirus and performed the 5-EU labeling of replicating RNA at 4 hours post-infection. Notably, in this experiment, no membrane permeabilization was performed since the click-chemistry detection of 5-EU relies on small molecules that can penetrate the fixed cells; hence, the structure of the membranous replication organelles was maximally preserved. As shown in Figure 5.1C, active replication complexes are localized in areas enriched in Arf1. These data strongly suggest that Arf1 is essential for the formation and functioning of enterovirus replication complexes.

5.2.2 GBF1 is always associated with 3A, but not 3A with GBF1, in the replication organelles.

GBF1 recruitment to enterovirus replication organelles by the viral protein 3A results in the enrichment of the RO membranes with Arf GTPases. To analyze the detailed distribution of GBF1 and 3A in the ROs, we infected HeLa cells with poliovirus, stained them for both proteins, and used super-resolution structural illumination microscopy (SIM), which allows the imaging of subcellular structures beyond the optical diffraction limit (348, 349). The experiment shows that GBF1 is always associated with a 3A signal (Figure 5.2B); however, not all 3A-positive foci are positive for GBF1. Since the antibodies for enterovirus proteins recognize the mature peptides as well as all the intermediate cleavage products containing the corresponding antigens (Figure 5.2A),

GBF1 may interact with different 3A-containing polyprotein fragments. To check for these interactions, we performed co-immunoprecipitation of HeLa cells expressing EGFP-GBF1 infected with poliovirus for 4 hours, using anti-GFP nanobodies. We observed that the mature 3A, as well as the intermediates 3AB, 2B-3D, and the whole P2P3 fragment, co-precipitated with GBF1, with a slight enrichment of the 2B-3D fragment in the co-IP fraction (Figure 5.2C). These results indicate that functionally different populations of 3A are present on ROs and/or that GBF1 may preferentially interact with different 3A-containing polyprotein fragments.

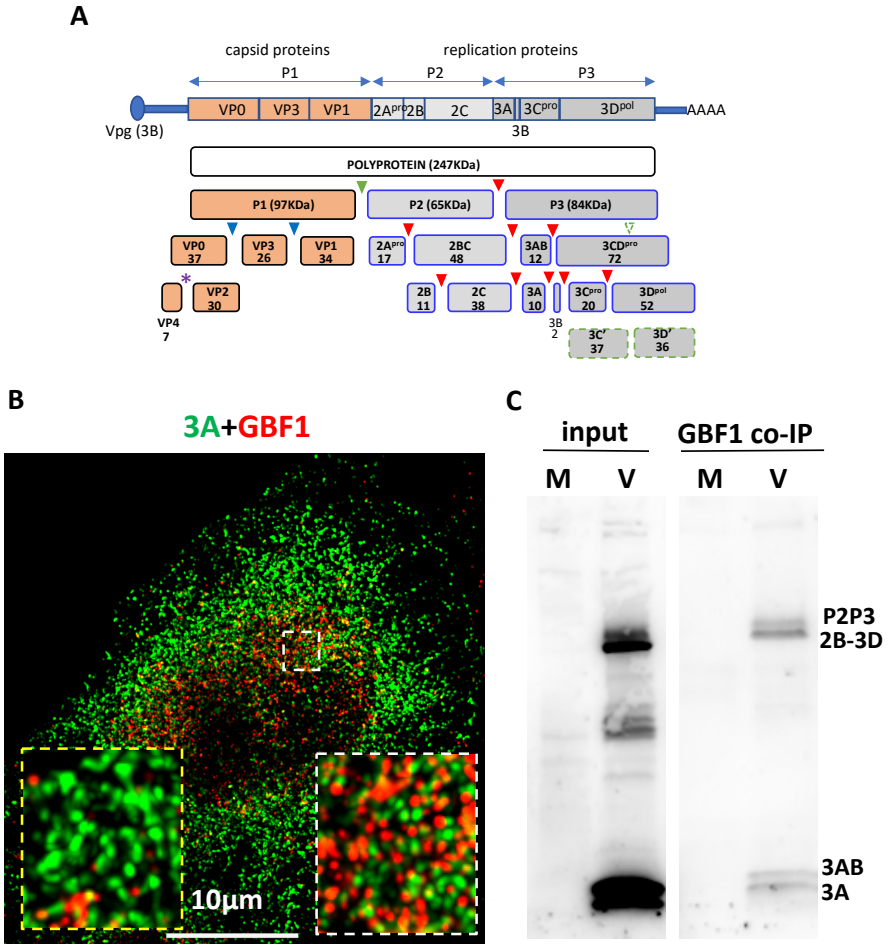

Figure 5.2: Analysis of the detailed distribution of GBF1 and 3A in the ROs. (A) Scheme of the processing of poliovirus polyprotein. (B) HeLa cells were infected with poliovirus at 50 pfu/cell, fixed at 4 h.p.i., permeabilized, and stained with antibodies for GBF1 and 3A. (C) HeLa cells expressing EGFP-GBF1 were infected with PV for 4h and processed for co-IP with anti-GFP nanobody. Western blot with anti-3A antibody is shown.

5.2.3 Viral antigens localize in Arf1-depleted and Arf1-enriched microdomains in enterovirus replication organelles.

Next, we study the distribution of Arf1 in the replication organelles and its association with viral proteins using structural illuminating microscopy imaging. We infected the stable HeLa cell line expressing Arf1-EGFP with poliovirus at 50 pfu/cell and fixed them at 4 h.p.i. Cells were permeabilized, and viral proteins were detected using 2B and 3A antibodies. As can be seen in Figure 5.3, Arf1 forms distinct microdomains in the replication organelles, and the signals from the viral antigens 2B and 3A are localized in both Arf1-depleted and Arf1-enriched microdomains. Moreover, the signal from antigen 2B (Figure 5.3A) shows a higher degree of colocalization with Arf1 than the 3A antigen (Figure 5.3B), which was also observed in previous studies (242). This observation indicates that an infection-specific set of interactions among viral proteins, Arf1, and possibly Arf1 effectors create a specific biochemical environment within each microdomain, likely necessary to regulate and support diverse protein functions during enterovirus replication.

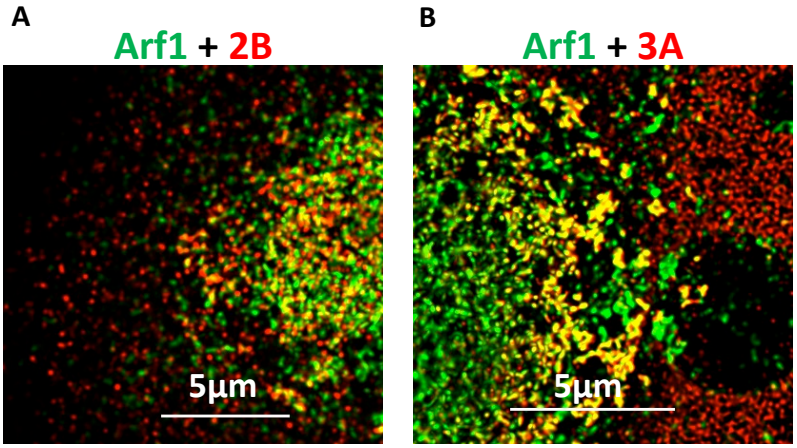

Figure 5.3: Arf1 forms distinct microdomains in the ROs and enteroviral antigens are differentiated in Arf1-depleted and Arf1-enriched microdomains. A stable HeLa cell line expressing Arf1-EGFP was infected with poliovirus type I Mahoney at 50 pfu/cell, fixed at 4 h.p.i., permeabilized, and stained with 2B (A) and 3A (B) antibodies.

5.2.4 Proximity biotinylation investigation of the viral protein composition of Arf1-enriched microdomains of enterovirus replication organelles.

The early recruitment of Arf1 to enterovirus replication organelles implies that the protein integrates into a set of molecular interactions with other viral and host proteins to enable replication. Furthermore, poliovirus proteins are expressed from a single polyprotein that undergoes proteolytic processing. Hence, the antigen signal detected in an immunofluorescence experiment may indicate the localization of the fully processed protein, a stable precursor, or any other higher-level precursor containing the antigen. Thus, we used a proximity biotinylation technique to identify individual viral proteins associated with Arf1-enriched domains. This proximity biotin-labeling approach is based on the engineered ascorbate peroxidase 2 (APEX2) (350). APEX2 initiates the biotinylation reaction by generating short-lived biotin-phenoxyl radicals from biotin phenol in the presence of hydrogen peroxide. These radicals bind the electron-rich amino acids of the proteins in the vicinity of the bait. Key advantages of using APEX2 include the speed of labeling (≤1min) and the labeling radius (about 20nm), which allows the identification and characterization of various proteins in their native cellular environment (351). This approach has been implemented to characterize the interactome in signal transduction pathways (352), endosome-trans Golgi network (353), stress granules (354), ER-Golgi network (355), lipid droplets (356), and most importantly, we successfully applied it to characterize the GBF1-controlled network of host proteins supporting enterovirus replication (245).

5.2.4.1 Establishment and characterization of a stable HeLa cell line expressing Arf1-APEX2 for proximity biotinylation.

We generated a stable HeLa cell line expressing a FLAG-tagged APEX2 C-terminally fused to Arf1 (referred to as Arf1-APEX2 from now on) using a retrovirus-based transduction

system as described by Moghimi *et al*. (242, 245). We performed a clonal selection of transduced cells to generate a culture with a uniformly high level of transgene expression. After two rounds of clonal selection, we obtained a culture in which more than 90% of the cells exhibited high Arf1-APEX2 expression. Next, we evaluated the specificity of the biotinylation reaction by omitting either the substrate biotin-phenol, hydrogen peroxide, or both from the system. After the biotinylation reaction, the cells were fixed with 4% paraformaldehyde, stained with a fluorescent streptavidin conjugate to visualize the biotinylated proteins, and with an antibody against the FLAG tag to detect the Arf1-APEX2 protein. As can be seen in Figure 5.4, only cells treated with both biotin-phenol and hydrogen peroxide show a high signal of biotinylated proteins (streptavidin, red) that colocalize with the transgene Arf1-APEX2 (FLAG, green). In these cells, the biotinylation signal is mainly concentrated in the Golgi, which recapitulates the expected behavior of endogenous Arf1. However, in cells lacking either one of the reagents or both, the Arf1-APEX2 protein localizes in the Golgi with no specific biotinylation signal. Notably, a background level of biotinylated proteins was detected in samples lacking one or both reagents for the reaction, likely representing the mitochondria that are enriched in biotin-containing enzymes (357-360). These data demonstrate that the transgene can efficiently biotinylate proteins in the vicinity of the bait (Arf1), and the biotinylation reaction is specific for the presence of the biotin-phenol substrate and the activator H_2O_2.

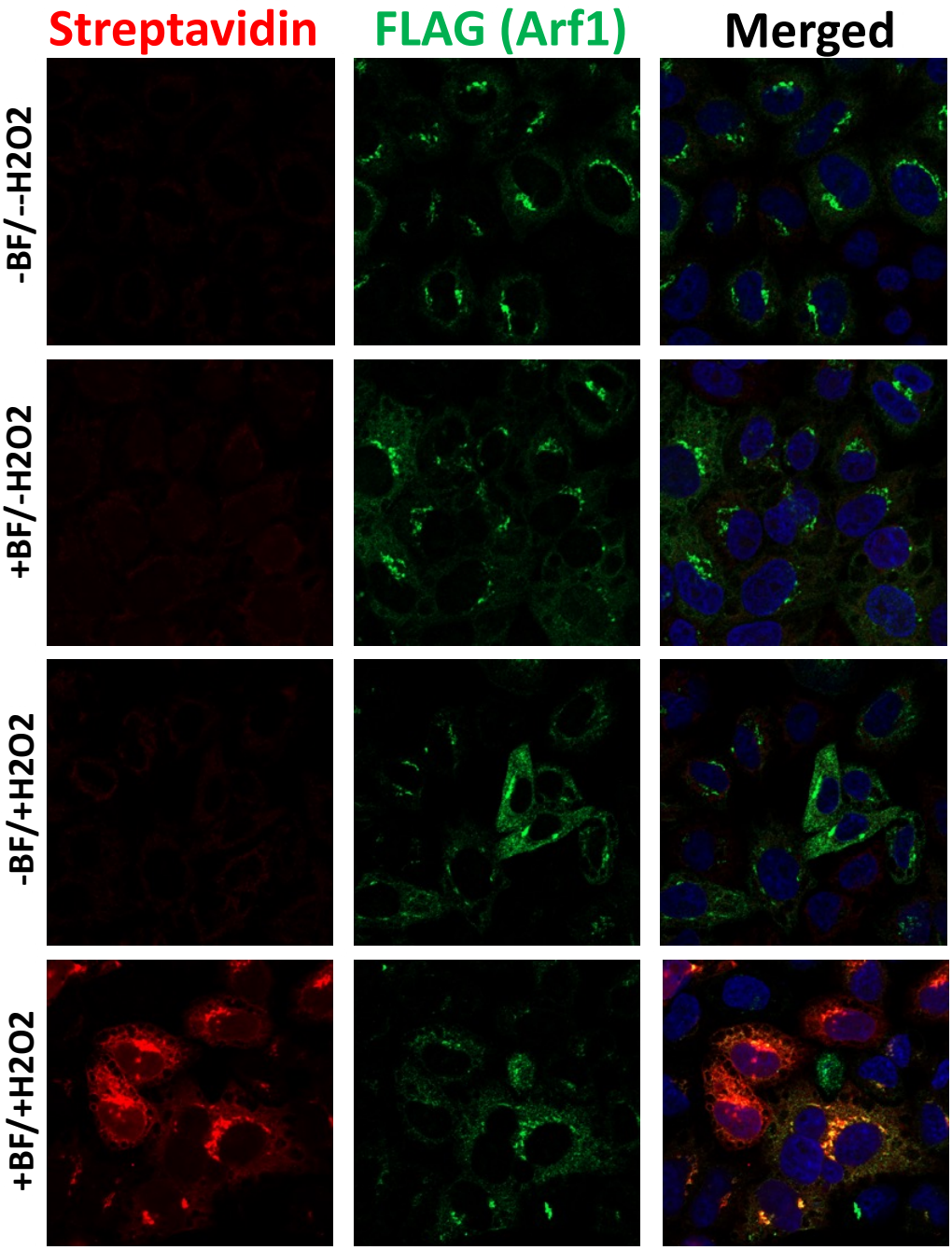

Figure 5.4: Specificity of the biotinylation reaction. HeLa cells stably expressing FLAG-tagged APEX2 C-terminally fused to Arf1 (Arf1-APEX2) were seeded in a 12-well plate with coverslips. The next day, the cells were incubated with 500µM of biotinyl-phenol for 30 minutes, and then treated with 1mM of hydrogen peroxide for 1 minute for the biotinylation reaction. The specificity of the biotinylation reaction was evaluated by omitting either the substrate biotin-phenol, hydrogen peroxide, or both from the reaction. The cells were fixed immediately after the biotinylation reaction, permeabilized, and stained with a fluorescent streptavidin conjugate to detect biotinylated proteins and with FLAG antibody for detecting the Arf1-APEX2 protein. BF = Biotin-phenol.

5.2.4.2 Characterization of the biotinylation reaction in enterovirus replication organelles.

Next, we assessed by confocal microscopy the biotin-labeling process in the stable cells expressing Arf1-APEX2 during poliovirus infection. Since Arf1 is initially recruited to the replication organelles during the early stages of viral replication, we performed the biotinylation reaction at 3 and 4 hours post-infection. Cells were infected (or mock-infected) with poliovirus at an MOI of 50 PFU/cell, and the biotinylation reaction was performed with 1mM of hydrogen peroxide for 1 minute. After the biotinylation reaction, the cells were fixed with 4% paraformaldehyde, stained with a fluorescent streptavidin conjugate to visualize the biotinylated proteins, and with an antibody against the viral non-structural protein 2B. In the mock-infected cells, the signal from biotinylated proteins and the Arf1-APEX2 protein recapitulate the behavior of endogenous Arf1 (Figure 5.5, mock). At 3 h.p.i., the biotinylation signal (streptavidin in red) and the Arf1-APEX2 protein (in green) are associated with the viral antigen 2B (purple), a component of the replication organelle membranes (Figure 5.5, 3 h.p.i.). This indicates that Arf1-APEX2 was recruited to the replication organelles, where it can efficiently biotinylate nearby proteins. Surprisingly, at 4 h.p.i., poliovirus infection affects the specificity of the biotinylation reaction in replication organelles, indicated by the lack of biotinylation signal in infected cells with a high level of Arf1-APEX2 recruited to the replication organelles (Figure 5.5, 4 h.p.i. arrows).

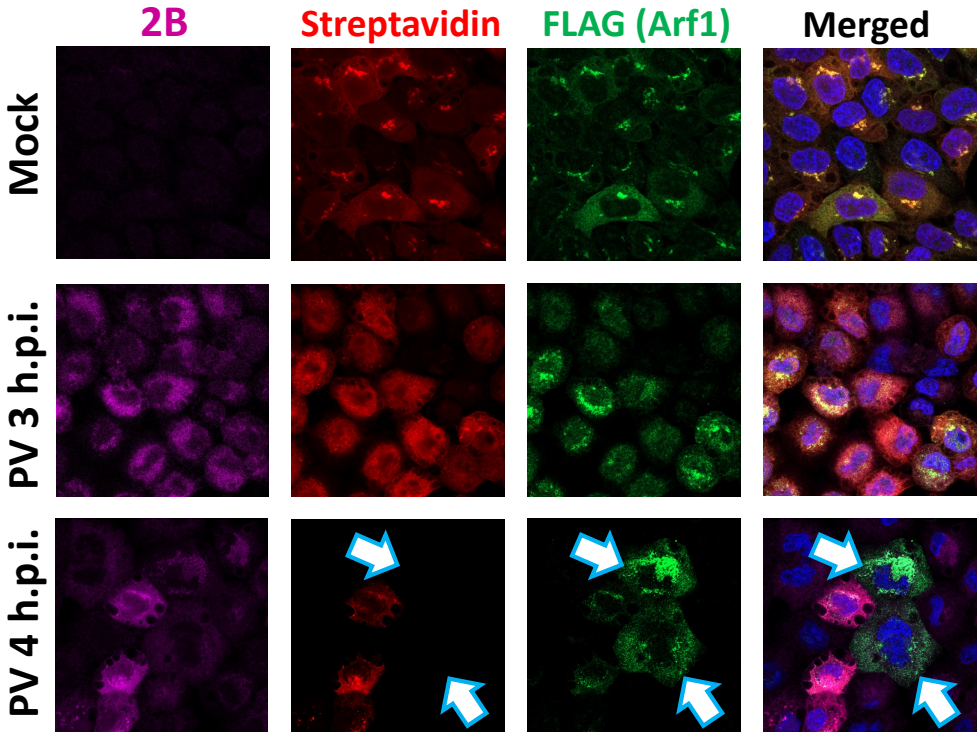

Figure 5.5: Biotinylation of proteins in enterovirus replication organelles. HeLa cells stably expressing Arf1-APEX2 were mock-infected and infected with poliovirus type I Mahoney at 50 pfu/cell. The cells were incubated with 500µM of biotin-phenol for 30 minutes, and the biotinylation reaction was performed with 1mM of hydrogen peroxide for 1 minute at the indicated time point (3 and 4 h.p.i.). The cells were immediately fixed, permeabilized, and stained with a fluorescent streptavidin conjugate to detect biotinylated proteins, FLAG antibody to detect Arf1-APEX2 and 2B for the replication organelles. ROs could be efficiently biotinylated at 3 h.p.i. as indicated by the colocalization of the Arf1-APEX2 protein (FLAG, green), the viral antigen 2B (purple), and biotinylated proteins (streptavidin, red). However, there is a loss of specificity of the reaction at 4 h.p.i., as indicated by the lack of biotinylation signal (streptavidin, red) in cells with high level of expression of Arf1-APEX2 protein (4 h.p.i, arrows)

We wanted to monitor the integrity of the Arf1-APEX2 protein during infection to see if this could have affected the specificity of the biotinylation reaction. We infected the HeLa Arf1-APEX2 cell line with poliovirus at an MOI of 50pfu/cell for 2, 3, and 4 hours, lysed the cells, and analyzed the protein using Western blot. Our findings showed that poliovirus infection did not impact the stability of either the Arf1-APEX2 protein or the endogenous Arf1 at any of the time points (Figure 5.6).

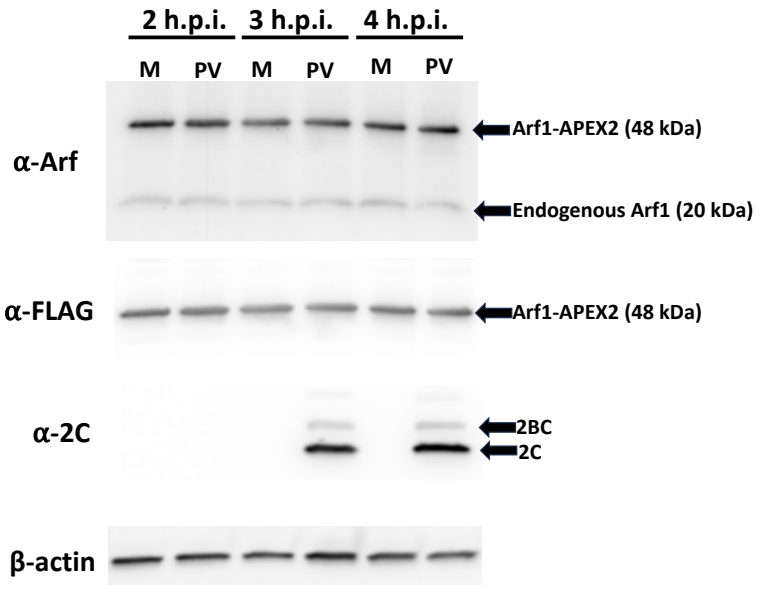

Figure 5.6: Poliovirus infection does not affect the stability of the Arf1-APEX2 protein. HeLa cells stably expressing Arf1-APEX2 were mock-infected and infected with poliovirus type I Mahoney at 50 pfu/cell for 2, 3, and 4 hours. Cells were lysed with mild lysis buffer at the indicated time points and the total lysate was processed for western blot. The Arf1-APEX2 protein was detected using antibodies for Arf and FLAG tag. Infection was monitored by the presence of the viral protein 2C. Actin is shown as a loading control

Since the structural complexity of the replication organelles increases during infection, from single-membrane to double-membrane structures with dynamic recruitment of viral and host proteins, we hypothesized that the lack of specificity of the biotinylation reaction at 4 hours post-infection was caused by the inaccessibility of biotin-phenol and hydrogen peroxide to the Arf1-APEX2 in the replication organelles. To test this hypothesis, we infected the stable cell line expressing Arf1-APEX2 with poliovirus at an MOI of 50 pfu/cell for four hours and performed the biotinylation reaction in the presence of 25μg/mL of digitonin. Digitonin is a mild non-ionic detergent with a high affinity for cholesterol that disrupts protein-lipid and lipid-lipid interactions (361-364). Moreover, enterovirus replication organelles are highly enriched in cholesterol (261, 365, 366). Thus, digitonin can be used to effectively permeabilize the cholesterol-rich membranes of the ROs. As can be seen in Figure 5.7, the permeabilization of replication organelle membranes

with 25µg/mL of digitonin restores the specificity of the biotinylation reaction at 4 h.p.i., as indicated by the colocalization of biotinylated proteins (streptavidin, red), Arf1-APEX2 (green), and the viral antigen 2B (purple). However, the permeabilization inhibited the biotinylation signal at the Golgi in mock-infected cells. This highlights the difference in the membrane architecture/composition and/or the mechanism of Arf1 association with membranes in normal cellular organelles and viral replication organelles.

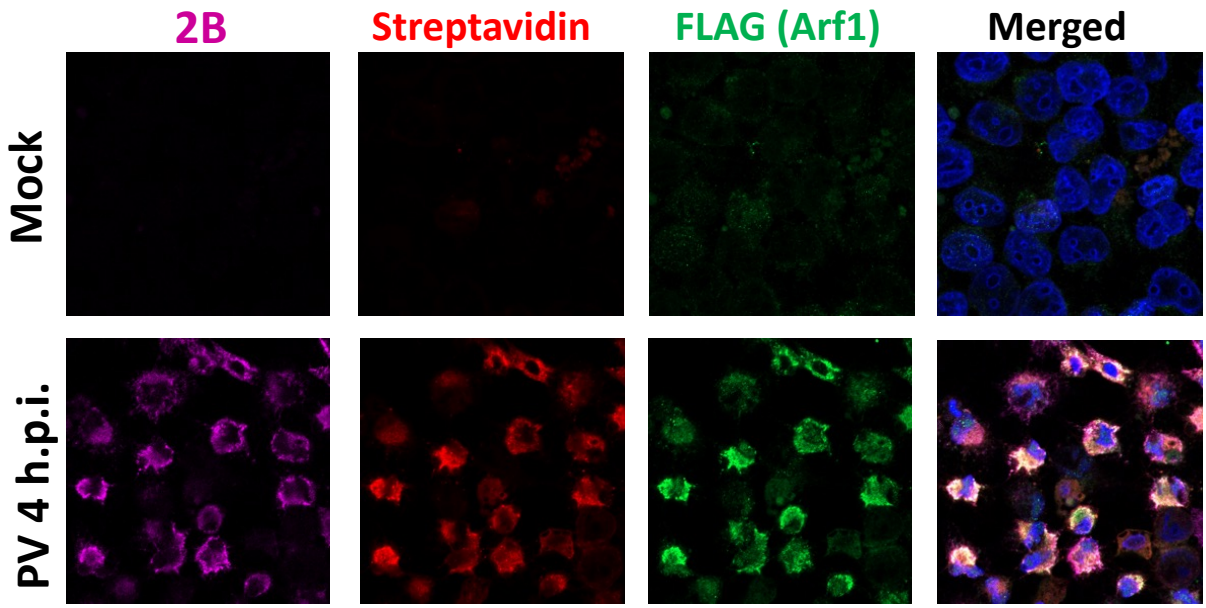

Figure 5.7: The permeabilization of enterovirus replication organelles restores the specificity of the biotinylation reaction at 4 hours post-infection. HeLa cells stably expressing Arf1-APEX2 were infected with poliovirus with 50 pfu/cell. The biotinylation reaction was carried out at 4 h.p.i. for 1 minute by mixing 500µM of biotin-phenol, 1mM of hydrogen peroxide, and 25µg/mL of digitonin in PBS. Cells were fixed and stained with anti-2B, anti-FLAG, and a fluorescent streptavidin conjugate.

5.2.4.3 Non-cleaved fragments of the viral polyprotein are overrepresented in Arf1-enriched domains.

Next, we searched for the viral polyprotein fragments in the Arf1-enriched domains of the replication membranes. HeLa cells stably expressing Arf1-APEX2 were infected with poliovirus at an MOI of 50 pfu/cell, and the biotinylation reaction was performed at 3 hours post-infection

(when the biotinylation reaction in infected cells is still effective without digitonin treatment). Then, the cells were lysed in RIPA lysis buffer supplemented with a proteinase inhibitor cocktail, followed by sonication. The sonicated RIPA lysates were used either directly for SDS-PAGE and western blotting (for the input analysis) or for further purification of biotinylated proteins using streptavidin magnetic beads (streptavidin pull-down). As shown in Figure 5.8, all the tested viral antigens were present in the biotinylated fractions. A higher amount of fully processed viral antigens was observed in the input material. However, in the biotinylated fraction, there was a higher amount of intermediates of polyprotein processing. For example, a higher amount of the precursors P2 and 2BC was observed in the biotinylated fraction with anti-2B antibody at 3 h.p.i., while the signal for the fully processed 2B was weak. We also observed viral antigen-positive fragments whose molecular weight do not correspond to the canonical products of the viral polyprotein processing. Note the red arrow marking a 3A-positive fragment of about 60 and 70 kDa or a 2B-positive fragment of a molecular weight of about 25 kDa. This may indicate that the composition of the Arf1-enriched domains of the replication organelles differs from that of the total replication complexes and that active polyprotein maturation occurs in the vicinity of Arf1-enriched domains. However, a preferential enrichment of larger polyprotein fragments due to a higher degree of biotinylation cannot be excluded.

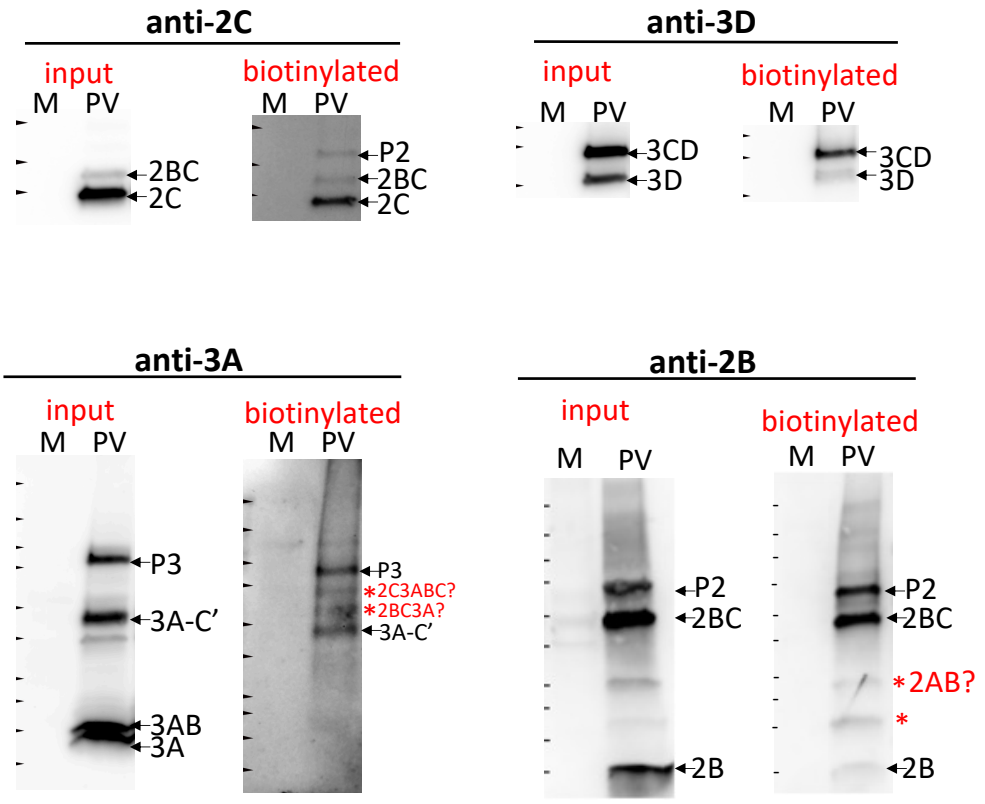

Figure 5.8: Biotinylation of the viral proteins by Arf1-APEX2. The stable HeLa cells expressing Arf1-APEX2 were infected and mock-infected with 50 pfu/cell poliovirus. At 6 h.p.i., the biotinylation reaction was performed, the biotinylated proteins were collected on streptavidin beads, resolved on SDS-PAGE, and analyzed in a western blot with antibodies against indicated viral antigens (2B, 2C, 3A, and 3D). The antibodies recognize the final and intermediate polyprotein cleavage products containing the corresponding antigen. Red stars on anti-3A and anti-2B panels indicate polyprotein fragments that do not match the known stable polyprotein cleavage products. Note the red asterisks marking a 3A-positive fragment in the 60-70 kDa range, or a 2B-positive fragment of a molecular weight of 25 kDa.

Collectively, this data shows the complex biochemical differentiation of replication organelles and supports a model in which GBF1 recruitment generates foci of activated Arfs on the membranes, which further differentiate into specific microdomains through the recruitment of a specific complex of viral proteins and possibly cellular Arf effectors, necessary for establishing an environment conducive to forming active replication complexes.

5.3 Discussion.

The development of replication organelles is an essential hallmark of enterovirus replication. The viral proteins rewire the cellular membrane metabolism to develop novel membranous structures with unique protein and lipid compositions to create a biochemical environment conducive to replication. However, the landscape of the host and viral proteins on the membranes of the replication organelles and their functional associations are understood only superficially. The cellular protein GBF1 is an essential host factor for enterovirus RNA replication. GBF1 functions as a guanine nucleotide exchange factor for small cellular Arf GTPases, and its activity in the replication organelles results in the massive enrichment of all five isoforms of Arf GTPases expressed in humans. While the ArfGEF function of GBF1 is critical for viral replication, the mechanistic role of the Arf GTPases is poorly understood. In this project, we used super-resolution structural illuminating microscopy, metabolic RNA labeling, co-immunoprecipitation, and peroxidase-based proximity biotinylation to investigate the distribution of viral proteins, GBF1, and Arfs on the replication organelles and their biochemical environment.

GBF1 is recruited to the replication organelles by the viral nonstructural protein 3A (251, 252, 322). Detailed analysis of the association of GBF1 and 3A using structural illumination microscopy revealed that while GBF1 is always associated with 3A-positive signals in the replication organelles, not all 3A-positive foci contain signals from GBF1. This observation implies that GBF1 may preferentially interact with different 3A-containing polyprotein fragments. Indeed, the co-immunoprecipitation experiment showed that several 3A-containing fragments co-precipitated with GBF1. Still, whether these peptides bind with the same affinity and the functional significance of their interaction with GBF1 requires further investigation. Nevertheless, the presence of distinct functionally different 3A populations cannot be excluded. Enterovirus 3A is

known to interact with multiple host proteins. For instance, the N-terminal domain of 3A binds to the GOLD domain of the cellular protein ACBD3, which recruits PI4KIIIb to the replication organelles (283, 287, 318). Since the N-terminal domain of 3A mediates the interaction of the protein with ACBD3 and GBF1, different 3A populations in the replication organelles likely engage in interactions with different host proteins. Nevertheless, whether a 3A molecule can interact with GBF1 and ACBD3 simultaneously requires further investigation.

The detailed analysis of the distribution of viral proteins and Arf1 revealed a complex microdomain organization of the ROs. We observed that viral proteins are localized in Arf1-depleted and Arf1-enriched microdomains in the ROs, and in accordance with previously reported observations, viral antigens show different degrees of colocalization with Arf1, with 2B being the most colocalized (242). The observation that viral proteins are distributed in Arf1-depleted and enriched microdomains implies that there is a specific mechanism for the delivery of the complex of viral proteins to these Arf1-enriched microdomains and that functionally different populations of these proteins with functions independent of RNA replication may exist in the Arf1-depleted microdomains. However, how these populations of proteins are established and the interactions they engage in are far from clear and require further investigation.

We used a proximity biotinylation technique based on the ascorbate peroxidase 2 (APEX2) using Arf1 as bait to identify individual viral proteins in the vicinity of Arf1-enriched microdomains. The biotinylated fraction of viral proteins contained an increased proportion of intermediates of polyprotein processing. This was also reported in a proximity biotinylation study of enterovirus infection using GBF1 as bait (245), although some peptides enriched differ between the studies. For instance, the biotinylated fraction obtained using GBF1 as bait contained fully processed 3A and 3A-containing intermediates between 15 and 20 kDa (245). In contrast, fully

processed 3A was never detected in the biotinylated fraction from Arf1-enriched domains, only 3A-containing peptides of 48 kDa and above. This indicates that the composition of enterovirus ROs is not homogenous and that GBF1 and Arf1 may be in different domains of the replication membranes engaging with different populations of viral proteins. Furthermore, this highlights the requirement of the GBF1-Arf1 axis for the functioning of the RNA replication complexes that need to assemble from the same polyprotein molecule (367, 368).

The loss of the specificity of the biotinylation reaction from 4 hours post-infection was quite striking. Poliovirus replication organelles could only be efficiently biotinylated at 4 h.p.i. upon digitonin permeabilization. The structural and compositional complexity of enterovirus replication organelles increases during the time course of infection, and the changes that occur in infected cells from 3 to 4 h.p.i. were enough to shield Arf1 from the substrates of the biotinylation reaction. Remarkably, the digitonin permeabilization of non-infected cells inhibited the biotinylation reaction, most likely due to the loss of the Arf1-APEX2 protein in the process. Arfs are abundant proteins that under normal cellular conditions constantly cycle between activated, GTP-bound, and inactivated GDP-bound states, and only a fraction of the total Arf pool exists in the membrane, GTP-bound form, which would explain why the majority of the Arf-APEX2 population was lost upon permeabilization in non-infected cells. In contrast, Arf1 remains in the GTP-bound state stabilized in membranes during enterovirus infection, and does not require constant GEF activity (242). Overall, this highlights the difference in the membrane architecture and composition of cellular organelles and viral ROs.

Collectively, these data show a complex biochemical differentiation of replication organelles and support a model where recruitment of GBF1 to the replication organelles generates foci of activated Arfs on the membranes, which further differentiate into specific microdomains

through the recruitment of a specific complex of viral proteins and cellular Arf required for the establishment of the environment conducive to the formation of active replication complexes.

Chapter 6: Conclusions and Future Work

In this study, we characterized the contribution of the GBF1-Arf1 axis in enterovirus RNA replication.

Like all (+) ssRNA viruses of eukaryotes, enteroviruses replicate their genome in association with remodeled membranous structures called replication organelles, whose lipid and protein composition are important for the assembly and functioning of viral replication complexes. The cellular protein GBF1 is essential for RNA replication of enteroviruses and some other +RNA viruses. Under normal cellular metabolism, GBF1 functions as a guanine nucleotide exchange factor for Arf GTPases (ArfGEF) that cycle between an inactive, cytosolic GDP-bound state and an active, membrane-associated, GTP-bound state. The GBF1-Arf axis is critical for the ER-Golgi vesicular traffic that supports secretion and maintains Golgi structure. As a large multidomain protein, GBF1 interacts with many cellular proteins but the evidence shows that many of the interactions that occur under normal cellular metabolism are dispensable for enterovirus replication, suggesting that GBF1 may function in enterovirus replication in a way that differs from its normal cellular functions.

This study carried out a detailed functional analysis of the domains/functions of GBF1 that are required to support normal cellular metabolism and enterovirus replication. We demonstrated that multiple GBF1 mutants in the N-terminal and C-terminal non-catalytic domains are inactive in cellular metabolism but could fully support poliovirus replication in cell culture (Figures 4.1 and 4.7), highlighting the remarkable plasticity of the requirement of GBF1 for viral replication. This suggests that enteroviruses require a very limited subset of the normal cellular functions of GBF1 and/or that the environment/interactions of the protein in the replication complexes are infection-specific.

GBF1 is recruited to enterovirus ROs by the viral protein 3A via the interaction of the N-terminal domains of both proteins and deletion of the first 37 amino acids of GBF1 abolishes poliovirus replication (246, 250, 251). Our study revealed that amino acids 2 to 17 and 47 to 62 in the N-terminal domain of GBF1 severely impair viral replication (Figure 4.3), and further dissection of those regions showed that the amino acids 9 to 12 are critical for poliovirus replication (Figure 4.4). How these regions in the N-terminal domain of GBF1 support poliovirus replication requires further investigation. Structural modeling of these mutants could provide insight into functional interfaces that may be required in the context of infection. Furthermore, we showed that the ability of the N-terminal GBF1 mutants to support poliovirus replication does not strictly correlate with the interaction with 3A at least in the context of cell culture and the wild-type virus (Figure 4.5). In line with these observations, we also demonstrate a redundant mechanism of recruitment of GBF1 to the replication sites, which is dependent not only on the direct interaction of the protein with the viral protein 3A but also on amino acids located in the noncatalytic C-terminal domains of GBF1. Such a double-targeting mechanism explains the previous observations (247, 307, 336) about the remarkable tolerance of different levels of GBF1-3A interaction by the virus and likely constitutes an important element of the resilience of viral replication. However, how the C-terminal domain mediates GBF1 recruitment to the replication organelles is not known. Several conserved elements in the C-terminal domains of GBF1 are crucial for its membrane-binding ability and proper cellular localization. Furthermore, when GBF1 binds to membranes, it undergoes conformational changes across various domains, which may help position the protein in the right conformation to interact with Arf and facilitate the GDP/GTP exchange (330). Therefore, the C-terminal domains could not only ensure that the protein is correctly localized within the cell to be accessible by the viral replication machinery, but also play a role in the

conformational adjustments likely required for Arf binding and activation. Nevertheless, whether specific membrane targeting properties can compensate for a complete loss of GBF1-3A interaction, or if at least some level of such direct interaction is essential for replication remains to be established.

Previous attempts to determine if the catalytic Sec7 domain of GBF1, which is responsible for Arf activation, generated controversial results (247, 250). Here, we used a GBF1 mutant that cannot bind to Arfs and therefore is catalytically inactive but has the membrane association properties of the wild-type GBF1 (226). We demonstrated poliovirus replication critically depends on a functional Sec7 domain of GBF1 (Figure 4.6). Additionally, the necessity of Arf activation for poliovirus replication is further evidenced by the observation that a chimeric protein made up of the N-terminal portion of GBF1 and a Sec7 domain from the ArfGEF ARNO was entirely functional in the replication assay (Figure 4.8). This agrees with recent observations about the critical importance of activated Arfs for enterovirus replication (242, 244). Poliovirus can develop resistance against BFA, a drug that inhibits GBF1-mediated Arf activation. The BFA-resistant poliovirus accumulated mutations in the nonstructural protein 2C, which enabled the viral protein to strongly interact with activated Arf1 (244). The 2C mutations provided resistance to BFA in the context of the 3A-2 mutation, which impairs the recruitment of GBF1 to replication organelles; and in cells depleted of GBF1, but not in Arf1-knockout HeLa cells, suggesting a mechanism of replication that relies on Arf1 but not GBF1 (244). Moreover, it has been reported that the 3A proteins of rhinovirus 2 and 14 do not efficiently interact with GBF1 (252). Interestingly, the 2C protein of rhinovirus 1A, which is less sensitive to BFA, interacts with activated Arf1 stronger than the 2Cs of BFA-sensitive enteroviruses such as poliovirus and coxsackievirus B3, suggesting a connection between the ability of the virus to hijack activated Arf1 and the virus's sensitivity to

BFA (244). Hence, a stronger 2C-Arf1 interaction could reduce enterovirus dependence on GBF1 activity for the recruitment of Arf1 to the replication organelles.

While the Arf activation property of GBF1 is essential for viral replication, the mechanistic roles that Arf GTPases play in enterovirus infection are unknown. Previous studies from our lab showed that enterovirus infection induces the recruitment of all isoforms of Arf GTPases expressed in humans to the replication organelles, with Arf1 being the most important for infection (242). Thus, in Chapter 5, we study the association of Arf1 with viral proteins and active RNA replication sites in poliovirus replication organelles.

Our experiments demonstrate that active enterovirus replication complexes localize in regions of the replication organelles enriched in Arf1 (Figure 5.1C), which strongly supports the observation that Arf1 is essential for the formation and/or functioning of enterovirus replication complexes. When we analyzed the distribution of Arf1 and viral proteins in the replication organelles using structure-illuminating super-resolution microscopy we found that Arf1 forms isolated microdomains and that viral antigens are localized in both Arf1-enriched and Arf1-depleted microdomains (Figure 5.3), which indicates that the protein composition of enterovirus replication organelles is not homogenous and that diverse populations of viral proteins exist in the infected cell. This agrees with the observation that there is an extensive localization of viral non-structural proteins outside of the replication organelles, where they can perform replication-independent functions that remain unexplored (242). Indeed, the nonstructural proteins found on membranes outside the replication organelles might disrupt cellular signaling processes, which typically need membrane-based signal transduction platforms to function properly. Moreover, a population of diffusible cytosolic viral proteins that perform functions related to producing virus particles and the modulation of the host gene expression and metabolic pathways is as important

as those associated with membranes. The elucidation of the functions that these distinct populations of viral proteins perform in the infected cell will be important to establish the network of interactions they engage in and find possible therapeutic targets.

Furthermore, we successfully set up a proximity biotinylation system using Arf1 as bait to characterize the viral protein composition of Arf1-enriched domains. We found that viral proteins could be efficiently biotinylated at 3 hours post-infection and that the non-cleaved fragments of polyprotein processing were enriched in the biotinylated fractions (Figures 5.5 and 5.8). However, as the infection progresses and the structural and compositional complexity of the ROs increases, Arf1 becomes strongly associated with and protected by membranes, and is no longer accessible to the biotinylation substrates, as indicated by the lack of specificity of the biotinylation system at 4 hours post-infection (Figure 5.5). In this case, viral proteins could be efficiently biotinylated only upon digitonin permeabilization of the replication organelle membranes, while such permeabilization inhibited the Arf1 biotinylation signal at the Golgi in non-infected cells, which underscores a difference in the membrane architecture and/or composition in infected and non-infected cells and indicates that the Arf-membrane association may differ between the two (Figure 5.7).

The association of Arf-GTPases with the replication organelle membranes likely results in the recruitment of host proteins that contribute to the functioning of the replication complexes. However, the Arf-controlled network of host proteins needed to support infection remains unexplored. The peroxidase-based proximity biotinylation system implemented here could be used to study such network of proteins using Arf1 as bait, or other isoforms of interest such as Arf3 and Arf6. It would be interesting to compare how the complexes of viral proteins and host factors

recruited to specific microdomains of the replication organelles differ between specific Arf isoforms.

Collectively, the results obtained in this study show that during enterovirus infection, the interactions and functions across the GBF1-Arf1 axis are specific for infection and different from the noninfected conditions and that the continuous study of these viral-host interactions is critical to understand those only required for infection to develop antiviral therapies targeting these interactions.

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