Role of PI4-kinases and PI4P lipids in picornavirus replication

Cristina Dorobantu

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Role of PI4-kinases and PI4P lipids in picornavirus replication

Rol van PI4-kinases en PI4P lipiden in picornavirus replicatie

(met een samenvatting in het Nederlands)

Proefschrift

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Fat(al) attraction: picornaviruses usurp lipid transfer at membrane contact sites to create replication organelles

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INTRODUCTION

As obligate intracellular pathogens, viruses heavily depend on the host machinery to fulfill virtually every step of their life cycle. Notably, positive-strand RNA (+RNA) viruses such as picornaviruses, hepatitis C virus and SARS- and MERS coronavirus, pirate the host lipid metabolism to develop unique membranous structures called "replication organelles", where viral genome amplification takes place. This work provides important insights into the molecular interactions and mechanisms underlying the formation and functioning of replication organelles by distinct members of the +RNA virus family *Picornaviridae*.

PICORNAVIRUSES

More than a century ago, Friedrich Loeffler and Paul Frosch achieved one of the milestones in virus research by discovering the first animal virus, namely the picornavirus foot-and-mouth disease virus (FMDV) [1]. Since, the *Picornaviridae* family grew into one of the largest and most diverse virus families, comprising numerous relevant human and animal pathogens. Today, the family consists of 29 genera (http://www.ictvonline.org), and continues to rapidly expand (Fig. 1). Discussed below are some of the most important picornavirus members and their associated diseases.

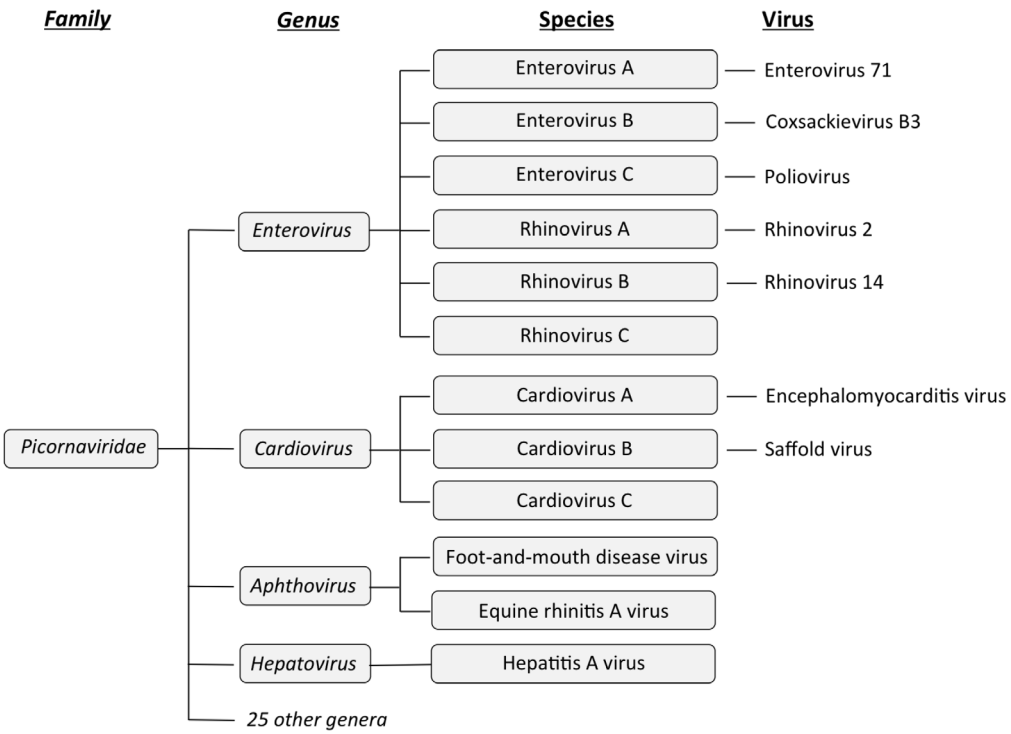

Figure 1. Classification of picornaviruses. Depicted are the genera and members most relevant to this thesis.

Enteroviruses

The genus *Enterovirus* comprises many important human pathogens that can cause a wide array of medical conditions, ranging from mild to severe. Enteroviruses are subdivided into 12 species, which include human enteroviruses A-D and rhinoviruses A-C, as well as other 5 enterovirus genera that only infect animals (some of these are depicted in Fig. 1). The major transmission route for enteroviruses is the faecal-oral route, except for rhinoviruses and some other enteroviruses (e.g. EV-68), which infect the airways via aerosols. Primary viremia occurs following the crossing of the intestinal tissues into the blood, from where the virus easily can reach various other organs. Curiously, although primary infection by enteroviruses is established in tissues of the gastrointestinal tract, enteroviral infections very rarely result in typical enteric perturbations, such as vomiting or diarrhea. In fact, the majority of enteroviral infections are asymptomatic. However, infection can sometimes progress, and to date, many diseases and syndromes and have been attributed to enteroviral infections (reviewed in [2]).

Surely, the best known enterovirus is poliovirus (PV, *Enterovirus* C species), the etiological agent of poliomyelitis, a paralytic disease that, prior to the introduction of the first anti-polio vaccines in the 1950s, has left many children and adolescents disabled. Nevertheless, the majority of poliovirus infections usually remain asymptomatic in most patients [3]. Thanks to the global polio eradication campaign launched in 1988 by the WHO, PV is nowadays virtually eliminated from the developed countries, however, the virus still poses a threat to some parts of the world, where it remains endemic (Pakistan and Afghanistan) and sometimes causes sporadic outbreaks (http://www.polioeradication.org/).

Other important enterovirus members are coxsackie A and B viruses, echoviruses, the numbered enteroviruses and rhinoviruses. These viruses can induce a diverse array of clinical conditions in infected humans, including encephalitis, pancreatitis, conjunctivitis and respiratory illness [2]. It is estimated that enteroviruses are responsible for more than 90% of the viral meningitis cases [4]. Infections with coxsackie B virus (CVB) and echovirus, both of which members of the *Enterovirus*-B species, have been shown to provoke viral myocarditis and cardiomyopathy [2,5]. *Enterovirus* B infections have also been associated with development of type I diabetes, however, this particular aspect of enterovirus pathogenesis remains controversial and calls for further investigation [6–11]. A serious concern in recent years has been enterovirus 71 (EV71), which caused a number of outbreaks, predominantly in regions of Southeast Asia [12]. EV71 and CVA16 (both of Enterovirus A species) are the most common causative agents of hand, foot and mouth disease (HFMD), typically affecting young children. HFMD is a rather mild condition that is characterized by sore throat and fever, accompanied by blisters in the mouth, hands and feet. More severe conditions caused by EV71 involve neurological disorders such as meningitis and encephalitis [13]. Lately, significant progress has been made towards the development of an effective vaccine against EV71 [14–17].

Rhinoviruses (RVs) account for up to 50% of the common cold cases in adults, exerting a significant economic burden on the society, through the loss of working days [18]. Less frequently, RV infections can lead to more severe respiratory illnesses, like bronchiolitis or pneumonia [19]. In addition, RVs have been associated with exacerbations of asthma and chronic obstructive pulmonary disease (COPD) [20–24]. In a recent outbreak in the United States, another enterovirus has been implicated to cause severe lower tract respiratory illness, namely enterovirus D68 (EV-D68) [25–27]. Importantly, EV-D68 was often detected in patients that manifested acute flaccid paralysis (AFP), which suggested that the virus might also exhibit neurotropic properties [28,29].

Cardioviruses

Picornaviruses belonging to the genus *Cardiovirus*, fairly distantly related to enteroviruses, are classified into three species: *Cardiovirus* A, represented by encephalomyocarditis virus (EMCV), *Cardiovirus* B, which includes Theiler's murine encephalomyelitis virus (TMEV), Vilyuisk human encephalomyelitis virus (VHEV), and Saffold virus (SAFV), and *Cardiovirus* C, comprised of Boone cardiovirus. EMCV currently contains two serotypes (EMCV-1 and EMCV-2) and mainly infects rodents. However, EMCV can also be transmitted from rodents to pigs or various zoological mammals, such as elephants and primates [30–33]. EMCV infections can lead to myocarditis, encephalitis, reproductive failure, abortions and even sudden death, thereby exerting a significant economic impact in the veterinary field [34–36]. Interestingly, EMCV has also been isolated from humans, but the subjects were either asymptomatic or presented very mild symptoms [34]. TMEV, like EMCV, primarily infects rodents, and can cause enteric infection or more severe conditions, like encephalitis or chronic infection of the central nervous system [37,38]. SAFV was the first human cardiovirus discovered, isolated from stool samples collected from a child presenting fever of unknown origin [39]. It then became clear that SAFV infections are ubiquitous in young children, most infections remaining asymptomatic [40]. Although SAFV has been associated with clinical manifestations including gastroenteritis, respiratory and neurological perturbations, the pathogenicity of this virus remains largely unclear [41,42].

Other picornaviruses

Many other important human and animal pathogens are comprised in the *Picornavirus* family, apart from entero- and cardioviruses. Human parechoviruses (HPeVs), part of the genus Parechovirus, constitute an important medical threat for young children, causing gastrointestinal or respiratory perturbations, or more severe conditions such as sepsis-like illness and meningitis, sometimes provoking the sudden death of infected infants [43–48]. Hepatitis A virus (HAV), a human picornavirus of the genus Hepatovirus, is the etiological agent of hepatitis A, an acute form of liver disease that is usually self-limiting, with only occasional mortality [49,50]. Despite the existence of an effective anti-HAV vaccine, many infections still occur annually, posing serious medical problems (recently reviewed in [51]). Another potentially relevant human picornavirus is Aichi virus (AiV), a member of the genus *Kobuvirus* [52]. AiV outbreaks have been associated with cases of acute gastroenteritis, apparently caused by the consumption of contaminated oysters or seafood [53,54]. The genus *Aphthovirus* contains perhaps the most important animal picornavirus, FMDV, which causes foot-and-mouth disease (FMD). FMD is a highly contagious disease, primarily affecting cloven-hooved animals, be it livestock or wild specimens [55,56]. The symptoms of FMD are severe and include (ruptured) blisters in and around the mouth and feet. Outbreaks of FMDV in animal farms, like the one in Great Britain in the early 2000, were subject of billions of euros worth economical loss, due to extensive quarantine and the massive slaughtering of infected animals [57].

Antiviral therapies

Currently, there are no approved antiviral therapies available to cure picornavirus infections, and the only treatment provided at the moment is supportive. While vaccines exist against PV, FMDV and HAV, the development of a much-desired pan-enteroviral vaccine is practically impaired by the huge serotypic diversity of these viruses. Thus, there is a great medical need and potential market for the development of effective antiviral therapies against picornaviral infections. Notably, RNA viruses frequently prove refractory to therapies based on direct acting antivirals (DAA) - compounds that target viral proteins directly - due to the emergence of viral resistance. The identification and characterization of virus-host interactions that are essential for virus replication can be fundamental for the discovery

of novel, alternative host-oriented antiviral therapies that may impose a higher genetic barrier to the development of resistance.

Organization of the picornaviral genome

As their name says (pico-RNA-viruses, pico implying "small"), picornaviruses are small, RNA-containing infectious particles, with an average diameter of 30 nm. Picornavirus virions are non-enveloped or naked, meaning they do not contain an outer lipid membrane. The infectious viral particle is composed of an icosahedral capsid, which is the protein shell enclosing the viral genome, a single-stranded RNA molecule of positive polarity, meaning it serves as mRNA and can be thus directly translated into viral proteins. The picornavirus genome, ranging between 7.5-8.5 kb in length, is bound covalently at the 5′ end to a small viral protein called VPg (viral protein genome-linked). Picornaviruses share similarities in the organization of their genome [58], which contains a single open reading frame (ORF), untranslated regions (UTRs) at both the 5′ and 3′ terminus, and a genome-encoded poly(A) tail at the 3′ far end (Fig. 2). In contrast to most cellular mRNAs, the 5′ end of the picornavirus genome is not capped, but instead it contains within the UTR an internal ribosome entry site (IRES) that is involved in cap-independent initiation of protein synthesis. Apart from the IRES, the viral RNA contains several additional highly structured features that are indispensable for virus replication. These include secondary structures termed stem loops, located in both 5′ and 3′ UTRs, as well as a cis-replicating element (CRE) that is essential for the initiation of RNA replication by uridylylating VPg, the primer for viral RNA synthesis. The CRE is found in all picornavirus genomes, but it maps to different regions of the genome in different picornaviruses.

Replication cycle of picornaviruses

One of the fascinating aspects surrounding picornavirus biology is their short life cycle, which is often completed within 6-8 hours post-infection. In a matter of few hours, picornaviruses transform the intracellular landscape into a viral "factory" for the production of progeny virions, events leading to the loss of cell integrity and cell death through lysis. This section provides a description of the different steps in the picornaviral replication cycle, which is schematically illustrated in Fig. 3.

Virus entry

Picornaviruses replicate in the cytoplasm of infected cells. In order to reach the cytoplasm, picornaviruses, just like other viruses, must penetrate the host plasma membrane. These initial events in the virus life cycle are considered the bottlenecks of virus replication, the step where most viruses fail to progress towards productive infection. Entry of picornaviruses into target host cells starts with the binding of the virus capsid to specific receptors present on the cell surface. This triggers internalization of the virus particle via endocytosis, followed by the penetration of the internalized vesicle membrane and expulsion of the viral RNA into the cytosol, where a cellular enzyme called VPg unlinkase removes the VPg [59].

Translation and polyprotein processing

Via the IRES element, the viral RNA genome is then translated by the cellular machinery into a single, large polyprotein [60]. At the same time, picornaviruses shutoff the cap-dependent translation of the host cell through diverse mechanisms, thereby funneling cellular resources towards viral translation only. The polyprotein is cleaved by viral proteinases to yield individual mature viral proteins and stable precursors (Fig. 2). The viral protease $3C^{pro}$ and its precursor $3CD^{pro}$ perform most of the cleavages, which produce the capsid proteins (from P1 region) and the non-structural proteins (from P2-P3 region). Some

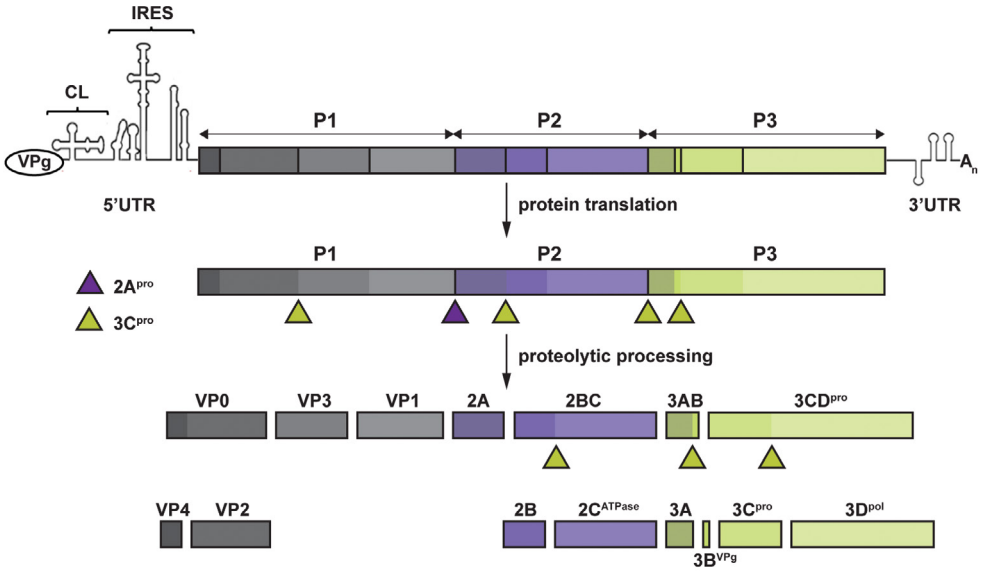

Figure. 2. Enterovirus genome and encoded proteins. Shown is a schematic illustration of the enterovirus genome organization at scale. The genome, covalently linked to the viral protein VPg, encodes a single ORF comprising regions P1, P2 and P3, which are flanked at the 5′ and 3′ ends by highly structured untranslated regions (UTRs), including the IRES and cloverleaf at the 5′ UTR. The IRES mediates cap-dependent translation of the viral polyprotein which is cleaved intramolecularly into stable precursors and individual proteins by the viral proteases $2A^{pro}$ and $3C(D)^{pro}$.

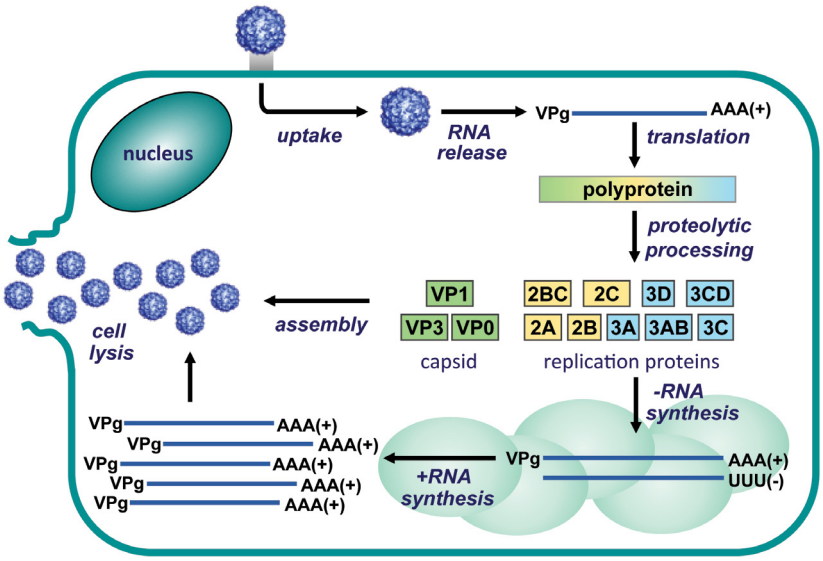

Figure 3. Schematic overview of the picornavirus replication cycle. The life cycle of a picornavirus starts with virion binding to the cell-surface receptor, followed by virus internalization by endocytosis. Next, the genome is released into the cytoplasm where it is immediately translated into a large polyprotein that is processed by viral proteases into stable precursors and individual proteins. Viral non-structural proteins then replicate the RNA genome on modified intracellular membranes. Newly-synthesized viral genomes either enter subsequent rounds of translation and replication or are encapsidated and released from the cell in a lytic (depicted) or non-lytic manner (not depicted).

picornaviruses (e.g. EVs) also encode a second protease, 2Apro, which releases the P1 region from P2. The viral proteinases not only cleave the viral protein, but also a myriad of host proteins involved in different cellular processes, for instance, factors involved in transcription and translation [61–63], or factors acting in the innate immune pathway [64–67]. Altogether, this ensures the establishment of a cellular environment favorable to virus replication.

Viral RNA replication and morphogenesis

Picornaviruses replicate their genomes in tight association with specialized, virus-induced membranes called "replication organelles" (ROs), which are formed through the actions of viral non-structural proteins and hijacked host factors. These aspects of virus replication will be discussed in more detail in the next sections of this thesis. RNA synthesis initiates when the protein primer 3B/VPg is uridylylated to VPgpUpU by the viral RNA-dependent RNA-polymerase $3D^{pol}$ using the CRE element as template [68]. $3D^{pol}$ then extends the protein primer VPgpUpU using the positive-sense genomic RNA as template and thus generates the complementary, full-length negative-sense RNA strand. Using this -RNA strand as template, $3D^{pol}$ transcribes many copies of full-length +RNA molecules. The newly synthesized +RNA strands can either undergo translation or enter another round of replication. This process is repeated until sufficient amounts of genomic viral RNA and capsid proteins accumulate in the cell to trigger encapsidation and virion maturation. Until recently, it was thought that progeny virions are released into the extracellular milieu exclusively via cell lysis, but accumulating evidence suggests they can also be released from infected cells in a non-lytic manner [69–71]. A recent study demonstrated that clusters of progeny enteroviral particles are packaged in autophagy-like, double-membrane vesicles (DMV) enriched in phosphatidylserine (PS) that are subsequently released and transmitted to neighboring cells as single-membrane vesicles [72]. By enabling en bloc transmission of multiple distinct viral genomes, the virion-containing PS vesicles were suggested to enhance the infection efficiency and virus replication compared to single-particle infection.

BIOGENESIS OF PICORNAVIRUS ROs: ROLE OF VIRAL AND HOST FACTORS

Like all +RNA viruses, picornaviruses induce an extensive remodeling of intracellular membranes in order to generate ROs that support viral RNA genome synthesis. ROs are specialized membranous compartments believed to provide a structural cytoplasmic platform that concentrates key viral and host factors required for efficient genome replication [73,74]. Additionally, ROs may support virus replication by shielding viral dsRNA intermediate products from the activity of cellular RNases and/or detection by RNA sensors involved in the activation of host defense antiviral responses [75,76]. How picornaviruses, given their limited genetic capacity, manage to confiscate the cellular machineries and completely redesign the intracellular membrane landscape remains a fascinating issue that is not yet completely understood. Membrane alterations in the cytoplasm of infected cells have been studied for multiple picornaviruses, however, the bulk of information accumulated to date surrounding picornavirus-induced membrane modifications arose predominantly from studies performed using PV and CVB3.

Formation of ROs by enteroviruses

Morphology of enterovirus ROs

Enterovirus ROs have long been visualized as either single- or double-membrane vesicles by conventional electron microscopy [77–79]. This view of the RO architecture was profoundly changed

with the development of more modern EM techniques like electron tomography, which enabled a detailed inspection of the three-dimensional structure of PV and CVB3 ROs (Fig. 4) [80,81]. At earlier stages of infection, the first detected structures are represented by (convoluted) single-membrane tubules, but, as infection progresses, the tubules seem to be converted into DMVs. Multiple tubules may subsequently wrap around DMVs and form multilamellar structures, which accumulate at a late stage of infection. Thus, the enterovirus ROs likely consists of a dynamic network of structures that undergo morphological transformations during the course of infection. The exponential phase of viral RNA replication coincides with the appearance of tubules [81]. However, DMVs have also been shown to be associated with components of the viral replication complex, such as viral proteins or replicating viral RNA [80]. Although DMVs may also support viral genome replication, these structures seem to play a more important role in virion maturation and non-lytic release of progeny virions (recently reviewed in [82]). The time-dependent transformation of enterovirus RO structures might reflect a strategy these viruses have evolved to control the different steps in their life cycle, i.e. genome replication versus assembly and release.

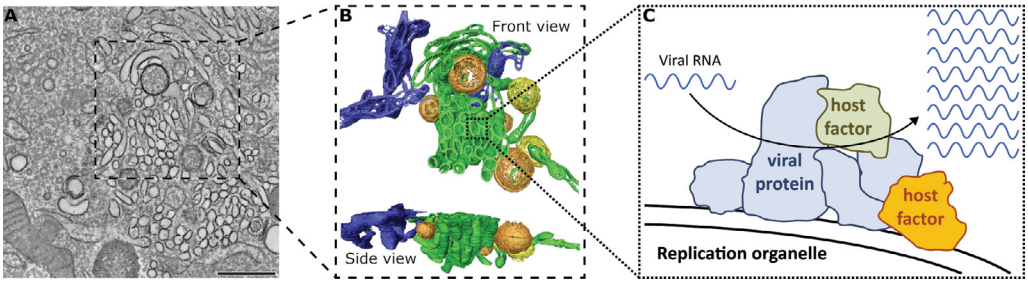

Figure 4. Ultrastructure of enterovirus replication organelles (ROs). (A) Tomographic slice from a tomogram of a CVB3-infected cell at 5 hours post-infection. Scale bar represents 500 nm. (B) Front and side views of a surface-rendered model of the region boxed in panel A showing single-membrane tubules (green), open (orange) and closed (yellow) double-membrane vesicles (DMVs), and ER membranes (blue). (C) Schematic depiction of a RO displaying viral RNA genome replication by viral and host proteins. Adapted from van der Schaar et al, Trends in Microbiology 2016, with permission.

Origin of ROs

Despite tremendous efforts invested in experimental investigations, the origin of enterovirus ROs is not yet understood. Biochemical fractionation analysis showed that PV replication complexes were associated with a wide variety of cellular organelle markers, suggesting a rather ubiquitous source of membranes for the biogenesis of ROs [83]. However, these early results should be interpreted with caution, since the technique of fractionation is often prone to contaminations and the analysis was done at a late time point in infection, when the cellular membrane system is heavily distorted and when exponential RNA replication has subsided. Nevertheless, obtaining a clear picture of the exact origin of ROs became a very arduous task in the field.

Multiple lines of evidence argued that enterovirus ROs might be derived from membranes of the secretory pathway. First, enterovirus replication is completely blocked by brefeldin A (BFA) [84–87], a fungal metabolite that inhibits ER-to-Golgi transport. Second, several components of the secretory pathway (some of which are targeted by BFA) relocalize to ROs and are essential for replication [88–90]. Third, very early in infection, viral infection markers colocalize with some Golgi-resident factors [80,87,90], suggesting that viral replication centers might be initiated at this organelle. Moreover, the emergence of tubular ROs is concomitant with the disappearance of the Golgi apparatus [81], implying

that the early replication structures might indeed originate from Golgi membranes. Fourth, components of the COPI and COPII coat machinery, which mediate vesicular trafficking at the interface of the ER and the Golgi, have been suggested to play a role in RO biogenesis, but the functional significance of these observations is arguable and requires further investigation (reviewed in [91]).

Formation of DMVs is a hallmark of autophagy processes, advancing the assumption that the autophagy pathway may be involved in the formation of ROs. This hypothesis was supported by findings that LC3-phosphatidylethanolamine conjugate (LC3-II), a marker of autophagy, and the lysosomal-associated membrane protein 1 (LAMP-1), colocalized with viral non-structural proteins at ROs in enterovirus-infected cells [71,92,93]. The fact that inhibition of autophagy inhibits virus replication only modestly corroborates with the observation that DMVs accumulate only late in infection, after the point of maximal viral RNA replication, indicating a role of autophagy rather in late steps of the viral life cycle. Indeed, more recent studies confirmed this by highlighting an important role of autophagy in virion maturation and non-lytic release [69–72,94].

Role of viral proteins

Enteroviruses encode seven non-structural proteins, which play important roles in replication. Among these, 2B, 2C, and 3A (and their stable precursors 2BC and 3AB) contain hydrophobic domains that determine their tight association with cellular membranes. Membrane association of 2B and 2C occurs via an amphipathic α-helix, which is distinct in the two proteins [95–98]. 3A contains a hydrophobic domain in its C-terminal region by which it anchors in membranes [99]. 2B targets ER and Golgi membranes where it acts as a viroporin, modulating membrane permeability and causing an efflux of Ca2+ into the cytoplasm, which is thought to promote inhibition of protein transport through the Golgi [100–103]. Whether any of these observed 2B activities contribute to RO formation is not known. 2C of EV71 has been reported to interact with reticulons, cellular proteins involved in shaping ER tubules, which were also suggested to be important for virus replication [104]. Whether reticulons also participate in shaping the tubules (and vesicles) of viral ROs in infected cells remains to be determined. Overexpression of 2C and 2BC results in massive membrane rearrangements [105–107], but only when 2BC is co-expressed with 3A, the structures formed resemble those observed during infection [108], arguing that RO formation requires at least the concerted actions of 2BC and 3A. The critical role of 3A in membrane reorganization and RO formation is described in the following sections, which will focus on 3A-interacting host factors that are essential for virus genome replication.

RO-associated essential host factors of enteroviruses

GBF1

The observation that enteroviruses are sensitive to BFA treatment eventually lead to the identification of the Golgi-specific BFA-resistance factor 1 (GBF1) as an essential host factor for enterovirus genome replication and RO formation [89,109]. BFA inhibits the activation of ADP-ribosylation factor (Arf) [110–113], a small cellular GTPase of the Ras superfamily. Six highly conserved members of the Arf family have been identified in mammalian cells. Arf1 cycles between an inactive, cytoplasmic GDP-bound form, and an active, membrane-associated GTP-bound form. The exchange from GDP to GTP triggers conformational changes in Arf1, enabling its binding to membranes. Membrane-bound, activated Arf1 plays a major role in the regulation of trafficking in the secretory pathway by promoting downstream recruitment of effector proteins, such as the coatomer complex [114–117]. The transition between Arf-GDP and Arf-GTP depends on the activities of guanine nucleotide exchange factors (GEFs). BFA blocks

Arf activation by stabilizing the otherwise transient Arf-GDP-GEF complex. In human cells, BFA targets three GEFs, namely GBF1, BIG1 and BIG2. GBF1 is involved in assembly of COPI vesicles at the interface between ER and Golgi, while BIG1 and BIG2 regulate formation of AP/clathrin- and GGA/clathrin-coated vesicles at the *trans*-Golgi network (TGN) [118].

GBF1 is a large, multi-domain protein whose GEF activity is catalyzed by the Sec7 domain. Two independent mutations located close to the BFA binding site in the Sec7 domain, namely A795E and M832L, were shown to render GBF1 insensitive to BFA and to thereby alleviate enterovirus replication from BFA inhibition in the cell lines where they specifically occur [89,109]. Furthermore, overexpression of the BFA-resistant GBF1 mutants or wild-type GBF1 restored enterovirus replication in the presence of BFA or Golcicide A (GCA) [89,109,119], the latter a specific inhibitor of GBF1 that does not affect BIG1/2, indicating that GBF1, and not BIG1/2, is the critical GEF required for virus replication. The importance of GBF1 for virus RNA replication was also confirmed by siRNA-mediated knockdown of GBF1 expression, which impaired enterovirus replication similar as BFA treatment [89,109]. GBF1 localizes at the ROs during infection, suggesting a direct implication in virus replication [89,90,109]. While BIG1/2 were also shown to be specifically recruited to membranes by the viral precursor 3CD [88], their functional involvement in virus replication is currently unknown.

Localization of GBF1 at viral replication sites is presumably determined by its interaction with 3A. 3As of PV and CVB3 were shown to directly interact with GBF1 and specifically recruit it to membranes [88,120,121]. Enterovirus 3A is a small protein of 86-89 aa, with the C-terminus comprising the hydrophobic domain (aa 61-82) acting as a membrane anchor and the N-terminus containing residues important for homodimerization, as revealed by the NMR structure of the soluble region of PV 3A [122]. Molecular determinants of the 3A-GBF1 interaction include 3A homodimerization, as well as conserved residues in the far N-terminus (amino acids 6-10). The N-terminus of GBF1, including the homology upstream of Sec7 (HUS) domain, was identified to be important for 3A interaction [123]. The functional importance of the 3A-GBF1 interaction is not yet understood, but the observed inhibitory effects of CVB3 3A on protein secretion were attributed to its interaction with GBF1 [120]. Prior to the start of this project, it has been proposed that 3A recruits GBF1 and indirectly Arf1 to CVB3 ROs, which, by a yet unknown mechanism, results in a gradual depletion of COPI from the membranes and concomitant enrichment of the ROs in another Arf1 effector, namely the phosphatidylinositol 4-kinase III β (PI4KB) (Fig. 5) [90]. Thus, enterovirus 3A seems to modulate GBF1/Arf1 activity in infected cells in such a

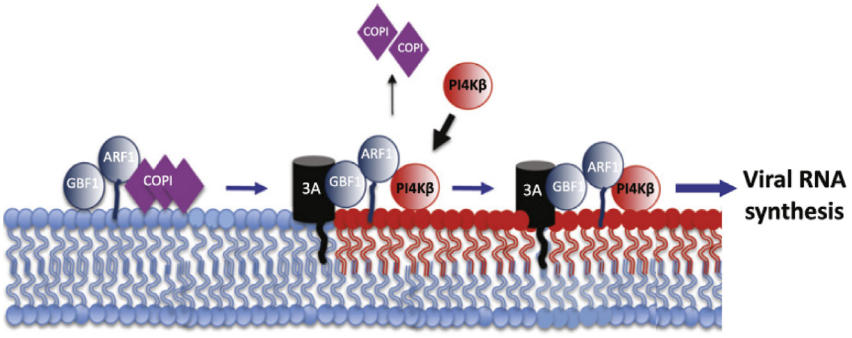

Figure 5. Proposed model of PI4KB recruitment to enterovirus ROs. Membrane-anchored enteroviral protein 3A binds GBF1 and modulates GBF1-dependent Arf1 activity by promoting the selective recruitment of PI4KB over COPI. PI4KB recruitment to the ROs results in a local enrichment of membranes in PI4P (depicted in red) which promotes viral RNA synthesis. Adapted from Hsu et al, Cell 2010, with permission.

manner that it promotes selective recruitment to the ROs of some downstream effectors (i.e. PI4KB), but not others (i.e. COPI).

PI4KB and PI4P

Phosphoinositides are major determinants of organelle identity and functioning, despite accounting for only a minor fraction of the total cellular phospholipids [124]. One of the most abundant and important phosphoinositide in the cell is phosphatidylinositol 4-phosphate (PI4P), in spite of accounting for less than 1% of the cellular phospholipid species. Recently, PI4P emerged from simply being a precursor of phosphatidylinositol 4,5-bisphosphate to key regulator of membrane trafficking and metabolism [125]. PI4P plays essential roles in various cellular processes including TGN-to-PM and TGN-to-endosome membrane trafficking, sphingolipid metabolism, as well as more recently described roles in autophagy and actin dynamics [126].

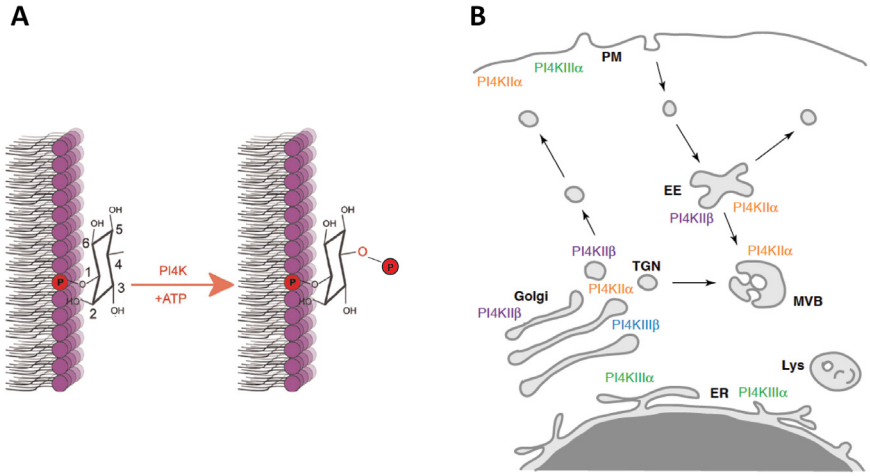

Figure 6. PI4K activity and subcellular distribution. A) Schematic representation of the phosphorylation reaction catalyzed by PI4Ks, which attach a phospho-group at position 4 of the inositol ring. Adapted from Boura et al., Experimental Cell Research 2015 with permission. B) Depicted are the subcellular localizations of PI4Ks and the major vesicular transport routes. Adapted from Balla et al., Trends in Cell Biology 2006, with permission.

PI4P is synthesized from phosphatidylinositol (PI) through phosphorylation at position 4 of the inositol ring (Fig. 6A). This reaction is catalyzed by specific cellular enzymes called phosphatidylinositol 4-kinases (PI4Ks) (recently reviewed in [127]). Mammalian cells contain four distinct PI4K isoforms, type II and type III that each consists of an α- and a β-isoform, which reside at different subcellular locations (Fig. 6B) [128]. PI4KIIα/β (PI4K2A/PI4K2B) associate with membranes of the TGN, early and late endosomes, where they localize upon palmitoylation. They are responsible for the synthesis of approximately 50% of the total PI4P in the cell. PI4KIIIα (PI4KA) mainly resides in the ER and is responsible for the production of PI4P at the plasma membrane, where it anchors by interacting with EFR3 and TTC7 [129,130]. PI4KB generates PI4P at the Golgi membranes, where it localizes [117]. Apart from their highly conserved C-terminal catalytic domain, type II and type III PI4Ks differ significantly in structure/sequence and size. To produce PI4P at the Golgi, PI4KB itself must first be recruited to Golgi membranes. PI4KB recruitment to the Golgi complex was shown to be regulated by activated Arf1 [117]. In enterovirus-infected cells, PI4KB accumulates at enterovirus ROs, where it is recruited by the viral protein 3A [90]. Genetic

depletion of PI4KB was detrimental to virus replication, and chemical inhibition of PI4KB by the small-molecule inhibitors inhibited viral genome replication, suggesting that enteroviruses require PI4KB activity [90,131,132]. Indeed, infected cells contained elevated levels of PI4P lipids at the ROs. The viral polymerase $3D^{pol}$ specifically bound *in vitro* to PI4P, but not other phosphorylated PI species [90], which argued that PI4P might function in virus replication to recruit essential components to the RO membranes, such as the viral polymerase. However, this finding awaits future validation in infected cells. PI4P lipids could also serve in infection to recruit other factors, such as cellular proteins that contain a pleckstrin-homology domain (PH), which are known to recognize and bind to PI4P [133,134]. Since PI4KB is an effector of Arf1 and 3A alone is sufficient to accumulate GBF1/Arf1 and PI4KB at membranes, it was proposed that in infection, enterovirus 3A recruits PI4KB to the ROs via GBF1/Arf1 (Fig. 5) [90], but a validation of this recruitment mechanism required further investigation, which is addressed in this thesis.

The dependence of viral RNA replication on PI4K and PI4P lipids is not restricted to enteroviruses. The +RNA virus hepatitis C virus (HCV), which belongs to the distantly-related *Flaviviridae* family, was also shown to hijack the PI4P metabolism, but via the ER-localized PI4KA [135–139]. In addition, some HCV genotypes were shown to also require PI4KB activity [135,137]. HCV derives its ROs, designated the membranous web (MW), from ER membranes and was shown to recruit PI4KA to the MW via its non-structural protein NS5A [135]. NS5A directly interacts with PI4KA and stimulates its activity *in vitro*, which results in an increase in the local PI4P concentration in infected cells [135]. Moreover, PI4KA activity was shown to be critical for the morphological integrity of the MW, arguing for a structural role of PI4P in shaping the viral replication structures [135,136]. These findings indicated that distantly related +RNA viruses have evolved common mechanisms to manipulate the host membrane metabolism towards formation of viral ROs.

ACBD3

While the studies presented in this thesis were in progress, it came to light that another picornavirus, namely Aichi virus (AiV) of the genus *Kobuvirus*, also requires PI4KB for efficient genome replication [140–142]. Similar as in enterovirus-infected cells, PI4KB and PI4P were found to redistribute at AiV ROs during infection, where they colocalized with viral nonstructural proteins 2B, 2C and 3A. Recruitment of PI4KB by AiV was found to be dependent on ACBD3. ACBD3 is a multi-domain protein that under normal conditions localizes to the Golgi by binding to the large Golgi membrane protein giantin [143]. At the same time, ACBD3 also interacts with PI4KB and by this contributes to the association of the kinase with Golgi membranes [140,144]. AiV proteins 2B, 2C and 3A interact with ACBD3 and, by gradually displacing giantin during infection, form a viral protein-ACDB3-PI4KB complex that brings both ACBD3 and PI4KB to the ROs. Based on the important role of ACBD3 in PI4KB recruitment to *kobuvirus* ROs, this thesis investigated whether ACBD3 is also involved in recruiting the kinase to enterovirus ROs.

Formation of ROs by cardioviruses

In contrast to the enterovirus field, information regarding host factor requirements for the RO formation by other picornaviruses is scarce. Membrane alterations in the cytoplasm of cardiovirus-infected cells were visualized long ago by electron microscopy as perinuclear clusters of heterogeneous single- and double- membrane vesicles (DMVs) [145–147]. Zhang *et al*. proposed that EMCV subverts the autophagy pathway to promote virus replication and RO formation [148]. The authors observed induction of autophagy by lipidation of LC3 and accumulation of cytoplasmic double-membrane vesicles (DMVs) in

EMCV-infected cells. However, like with enteroviruses, inhibition of autophagy had stronger effects on extracellular than intracellular virus yields, which pointed towards a role of autophagy rather in virus release. Members of the *Cardiovirus* genus are not sensitive to GBF1 depletion by siRNA or treatment with BFA [84,87,109,119], indicating that EMCV might originate its ROs from ER rather than from the Golgi. These observations suggested that cardioviruses employ replication strategies significantly distinct from those of enteroviruses.

AIM AND OUTLINE OF THIS THESIS

Generation of viral replication organelles is indispensable for picornavirus genome replication. The goal of this thesis was to unravel the molecular mechanisms by which distinct picornaviruses reorganize intracellular membranes and lipid metabolism to develop their own ROs. In particular, we aimed at identifying and characterizing virus-host interactions that engage cellular pathways in the biogenesis and functioning of viral ROs. A deeper understanding of the processes underlying RO formation may promote the development of novel, host-targeted antiviral therapies with potential pan-viral properties.

The Golgi-localized PI4KB is an essential enterovirus host factor, which together with its product PI4P, accumulates at viral RO in infected cells. The small non-structural viral protein 3A was shown to actively recruit PI4KB, but the underlying mechanism has remained unknown. In **Chapters 2** and **3** we investigate how distinct enteroviruses recruit the kinase to their ROs. In non-infected cells, PI4KB is recruited to Golgi membranes by the GBF1/Arf1 machinery and by ACBD3, both of which interact with CVB3 3A. In **Chapter 2** we examined the mechanism of PI4KB recruitment to CVB3 ROs. Using genetic depletion and chemical inhibitors, we investigated the role of GBF1/Arf1 and ACBD3 in PI4KB recruitment to membranes by CVB3 3A. We also studied whether ACBD3 is important for PI4KB recruitment and virus replication. Additionally, direct interactions between 3A, ACBD3 and PI4KB were tested. **Chapter 3** investigates how PI4KB is recruited to ROs by rhinoviruses. In contrast to 3A of CVB3 or PV, RV 3A proteins establish only little, if any, interaction with GBF1. In this chapter, we addressed the contributions of GBF1/Arf1 and ACBD3 to PI4KB recruitment and evaluated the importance of GBF1, ACBD3 and PI4KB as RV host factors.

In **Chapters 4-6** we studied the role of the ER-localized PI4KA in the replication of picornaviruses from the genus *Cardiovirus.* Prior to the start of this project, very little was known about the host factors involved in cardiovirus RO formation, which was proposed to rely on the autophagy pathway. **Chapter 4** provides the first detailed investigation of the mechanism by which cardioviruses develop their ROs. In this chapter, we reveal that the cardiovirus EMCV develops its ROs in a manner strikingly similar to that of the distantly-related +RNA virus HCV of the family *Flaviviridae*, by hijacking PI4KA. Here, we also investigated which viral proteins interact with PI4KA and characterized the changes in PI4P levels and distribution in infected cells. Furthermore, the role of PI4P in the recruitment of downstream host factors and lipids to ROs is studied.

Chapter 5 reports the isolation and characterization of EMCV 3A mutants that, unlike wt EMCV, can replicate largely independent of high levels of PI4P and cholesterol. We investigated to which extent the mutants are resistant to PI4KA/OSBP chemical inhibition or depletion. Furthermore, the appearance and composition of their ROs in the presence of PI4KA inhibitors was examined.

Tyrphostin AG1478 is a well-established, potent inhibitor of the EGFR that has been widely explored as an anti-cancer agent. In **Chapter 6**, we explore the antiviral properties of AG1478 towards EMCV and identify the cellular target responsible for its antiviral activity.

Chapter 7 provides a summary and discussion of the main findings described in this thesis.

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Recruitment of PI4KIIIβ to coxsackievirus B3 replication organelles is independent of ACBD3, GBF1, and Arf1

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ABSTRACT

Members of the *Enterovirus* (poliovirus, coxsackieviruses, human rhinoviruses) and *Kobuvirus* (Aichi virus) genera in the *Picornaviridae* family rely on PI4KIIIβ (phosphatidylinositol-4-kinase IIIβ) for efficient replication. The small membrane-anchored enteroviral protein 3A recruits PI4KIIIβ to replication organelles, yet the underlying mechanism has remained elusive. Recently, it was shown that kobuviruses recruit PI4KIIIβ through the interaction with ACBD3 (acyl-CoA-binding protein domain 3), a novel interaction partner of PI4KIIIβ. Therefore, we here investigated a possible role of ACBD3 in recruiting PI4KIIIβ to enterovirus replication organelles. Although ACBD3 interacted directly with coxsackievirus B3 (CVB3) 3A, its depletion from cells by RNA interference did not affect PI4KIIIβ recruitment to replication organelles and did not impair CVB3 RNA replication. Enterovirus 3A was previously also proposed to recruit PI4KIIIβ via GBF1/Arf1, based on the known interaction of 3A with GBF1, an important regulator of secretory pathway transport and a GEF of Arf1. However, our results demonstrate that inhibition of GBF1 or Arf1 either by pharmacological inhibition or depletion with siRNA-treatment did not affect the ability of 3A to recruit PI4KIIIβ. Furthermore, we show that a 3A mutant that no longer binds GBF1 was capable of recruiting PI4KIIIβ even in ACBD3-depleted cells. Together, our findings indicate that, unlike originally envisaged, coxsackievirus recruits PI4KIIIβ to replication organelles independently of ACBD3 and GBF1/Arf1.

IMPORTANCE

A hallmark of enteroviral infection is the generation of new membranous structures to support viral RNA replication. The functionality of these so-called "replication organelles" depends on the concerted actions of both viral non-structural proteins and co-opted host factors. It is thus essential to understand how these structures are formed and which cellular components are key players in this process. GBF1/Arf1 and ACBD3 have been proposed to contribute to the recruitment of the essential lipid-modifying enzyme PI4KIIIβ to enterovirus replication organelles. Here we describe that the enterovirus coxsackievirus B3 (CVB3) recruits PI4KIIIβ by a mechanism independent of both GBF1/Arf1 and ACBD3. This study shows that the strategy employed by coxsackievirus to recruit PI4KIIIβ to replication organelles is far more complex than initially anticipated.

INTRODUCTION

All positive-strand RNA viruses reorganize intracellular membranes into "replication organelles" in order to amplify their genome [1–3]. These replication organelles are decorated with complexes of viral non-structural proteins and co-opted host factors that mediate viral RNA replication. Yet, the donor organelle of which each virus hijacks host factors and membranes varies among different viruses. Flaviviruses for instance develop a "membranous web" originated from the ER [4–6], while Enteroviruses (family *Picornaviridae*) generate new tubular-vesicular organelles by exploiting Golgi membranes (7–10).

PI4KIIIβ (phosphatidylinositol-4-kinase IIIβ) is a membrane-modifying host factor essential for enterovirus replication [10,11]. In uninfected cells, PI4KIIIβ is mainly localized at the Golgi complex where it catalyzes the synthesis of PI4P (phosphatidylinositol-4-phosphate), lipids with important roles in signaling and vesicle transport [12–14]. Replication organelles of enteroviruses contain elevated levels of PI4KIIIβ and its product PI4P. The sole expression of CVB3 non-structural protein 3A leads to a strong membrane enrichment in PI4KIIIβ and PI4P [10], suggesting that 3A is responsible for the active recruitment of the kinase to replication sites. However, the mechanism by which PI4KIIIβ is recruited to enterovirus replication organelles has remained unknown. PI4KIIIβ is normally recruited to the Golgi by Arf1, a key regulator of transport in the early secretory pathway [12]. Arf1 is a small cellular GTPase cycling between a cytosolic, inactive GDP-bound form and a membrane-associated, active GTP-bound form [15–17]. Activation of Arf1 by the large guanine nucleotide exchange factor (GEF) GBF1 triggers downstream recruitment of effectors to the Golgi membranes, such as PI4KIIIβ or the COPI (coat protein I) complex, which mediates vesicle formation and trafficking in the early secretory pathway [12,18–21]. GBF1 was previously demonstrated to localize to replication organelles and to be crucial for enterovirus RNA replication [22–25]. Based on the observation that enterovirus 3A is able to directly bind GBF1 [26], it has been proposed that 3A recruits PI4KIIIβ via its interaction with GBF1/Arf1 [10].

PI4KIIIβ is also important for the replication of Aichi virus, a member of the genus *Kobuvirus* within the *Picornaviridae* family [27,28]. Recruitment of PI4KIIIβ to Aichi virus replication sites occurs through the interaction of viral non-structural proteins with the cellular protein ACBD3 (Acyl-Co-A Binding Domain containing 3). In uninfected cells, ACBD3 localizes to the Golgi by binding to giantin, a Golgi integral protein [29]. ACBD3 was recently shown to also interact with PI4KIIIβ and thereby contribute to the kinase localization to Golgi membranes in Vero cells [27]. Depleting cells of ACBD3 inhibits Aichi virus replication, highlighting ACBD3 as a novel essential kobuvirus host factor. Interestingly, poliovirus replication was also shown to be reduced upon ACBD3 knockdown [28]. Extensive affinity purification/proteomics studies revealed association of kobuviral, but also enteroviral 3A proteins with ACBD3 and PI4KIIIβ [28]. Based on these findings, ACBD3 was suggested to be important for the recruitment of PI4KIIIβ not only to kobuvirus, but also enterovirus replication organelles.

This work addresses the role of ACBD3 in CVB3 replication and investigates the strategy employed by enteroviruses to recruit the lipid kinase PI4KIIIβ to replication organelles. We reveal that ACDB3 is not required for the kinase recruitment to replication organelles, despite its direct interaction with CVB3 3A and PI4KIIIβ. Depleting cells of ACBD3 rather enhances CVB3 replication, arguing against its previously suggested role as a host factor essential for enteroviral RNA replication. Furthermore, we show that GBF1/Arf1 are dispensable for PI4KIIIβ recruitment to CVB3 replication organelles.

MATERIALS AND METHODS

Cells and reagents. HeLa R19, BGM (buffalo green monkey), Hek 293, and COS-1 cells were grown in Dulbecco's minimal essential medium (DMEM, Lonza) supplemented with 10% fetal bovine serum at 37oC and 5% CO2. BFA and AG1478 were purchased from Sigma-Aldrich and dissolved in methanol and DMSO, respectively.

Plasmids. p3A-wt-myc, p3A-REIKI(6-10)-myc, p3A-Ins16S-myc, p3A-PPP(17-19)A-myc, p3A-VDSE(29-32)A-myc, p3A-VREY(34-37)A-myc, and pBIND-GBF1 constructs were described previously [30]. pArf1-EGFP was received from dr. Eric Fluharty, University of Pittsburgh, Pittsburgh, PA, USA. pEGFP-N1 (for GFP overexpression) was from Clontech. pACT- and pBIND-CVB3 3A were also previously described [31]. cDNA encoding PV, HRV2, HRV14, or Mengovirus 3A was cloned into pACT/pBIND vectors using restriction sites SalI and NotI, while BamHI and XbaI were used for Enterovirus 71 3A, CVB3 3A deletion mutants, and PI4KIIIβ. Plasmid pGEX-6P3-ACDB3 for expression of GST-ACBD3 in *E.Coli* and mammalian two-hybrid constructs for ACBD3 were a kind gift from dr. J. Sasaki (Fujita Health University School of Medicine, Aichi, Japan) [27]. Plasmid pcDNA4/TO-ACBD3-Strep used for mammalian expression and purification of streptavidin (Strep)-ACBD3 was a kind gift from dr. J. deRisi (University of California at San Francisco, San Francisco, USA) [28]. pGEX4T1 (Pharmacia) was used for expression of GST in *E.Coli*. CVB3 3A(1-60) cDNA was cloned into the pET23a vector using NdeI and XhoI restriction sites to construct the plasmid pCVB3 3A(1-60). pACT, pBIND, and pG5Luc vectors were from Promega.

Virus infection. CVB3, CVB3-RLuc, and CVB3-RLuc 3A-H57Y viruses were described previously [32]. Virus infections were carried out by incubating subconfluent BGM or HeLa R19 cells for 30 min with virus. Following virus removal, cells were washed once with PBS, and fresh (compound-containing) medium was added to the cells. Cells were either fixed for immunolabeling at 5 h post-infection (p.i) or lysed at 8 h p.i. to determine replication by measuring the intracellular *Renilla* luciferase activity using the *Renilla* Luciferase Assay System (Promega).

Mammalian two-hybrid assay. Subconfluent COS-1 cells, seeded the previous day in 24-well plates, were transfected with 350 ng of each pACT, pBIND, and pG5Luc plasmids using Fugene6 (Promega) according to the manufacturer's protocol. At 48 h post-transfection (p.t.) cells were lysed and *Renilla* and Firefly luciferase levels were measured using the Dual-Luciferase assay kit (Promega) following the manufacturer's protocol. Values were converted to Firefly/*Renilla* signal ratio's to correct for transfection efficiencies and were normalized to corresponding background signals of the negative controls to express the level of interaction (plotted as fold FLuc/RLuc).

Immunofluorescence microscopy. BGM or HeLa R19 cells were grown to subconfluency on coverslips in 24-well plates. Where indicated, cells were transfected with 200 ng of plasmid using Fugene6 according to the manufacturer's protocol or infected with CVB3 at an MOI of 10. At 24 h p.t. or 5 h p.i., cells were fixed with 4% paraformaldehyde for 15 min at room temperature, followed by permeabilization with PBS-0.1% Triton X-100 for 5 min. Cells were then incubated sequentially with primary and secondary antibodies diluted in PBS containing 2% NGS (normal goat serum). Cellular proteins were detected with antibodies against PI4KIIIβ (rabbit polyclonal, Millipore), GBF1 (mouse monoclonal, BD Biosciences), Arf1 (rabbit polyclonal, from dr. F. Wieland, Biochemie-Zentrum, Heidelberg, Germany), ACBD3 (mouse monoclonal, Sigma). The overexpressed CVB3 3A-myc proteins were detected with the anti-C-myc antibody (mouse monoclonal, Sigma). Viral CVB3 3A was detected using either a rabbit polyclonal

antibody described before (26) or a mouse monoclonal antibody (mAb). The anti-3A mAb was obtained by immunizing mice with His-tagged CVB3 3A(1-60) recombinant protein. Conjugated goat anti-rabbit and goat anti-mouse Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes) were used as secondary antibodies. Nuclei were stained using DAPI. Coverslips were mounted with FluorSave (Calbiochem) and images were acquired with an Olympus BX60 fluorescence microscope.

siRNA treatment. HeLa R19 cells were reverse-transfected with 2 pmoles of siRNA per well of a 96-well plate (2x103 cells/well) or with 14 pmoles siRNA per well of a 24-well plate (14x103 cells/well) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's indications. Scramble siRNA (AllStars Neg. Control, Qiagen) was used as a control. SiRNA against hPI4KIIIβ (target sequence: 5'-UGUUGGGGCUUCCCUGCCCTT-3') and siRNA against hGBF1 (target sequence: 5'-CAGGAGCATGTACATATGGAA-3') were from Qiagen, siRNA against hArf1 was from Sigma (target sequence: 5'-ACGATCCTCTACAAGCTTA-3'), and siRNA against hACBD3 [27] (smart pool of 4, #1: 5'-GGAUGCAGAUUCCGUGAUU-3', #2: 5'- GCAACUGUACCAAGUAAUA-3', #3: 5'-GCAUAUGGGAAGUAACAUU-3', #4: 5'- GUAUAGAAACCAUGGAGUU-3') was from Dharmacon. After 48 h, cells were either infected with virus, transfected with plasmid DNA, or harvested to evaluate the knockdown efficiency by Western Blot analysis.

***In vitro* HeLa S10 cell extract assay.** The HeLa S10 cell extract assay was performed as previously described [22]. Briefly, all translation reactions included 2.5 µg of RNA transcripts and were incubated for 3.5 h at 34oC, followed by centrifugation for 20 min at 16,000 x g at 4oC. Pelleted membranes were subjected to SDS-PAGE and Western Blot analysis. An aliquot of each translation reaction was incubated with 1 µl of 35S Easy-Tag Express protein labeling mix (Perkin Elmer) and further resolved by SDS-PAGE to visualize translation products.

Separation of total cellular membranes from cytosol. HeLa R19 cells were harvested and resuspended in ice-cold PBS supplemented with protease inhibitors cocktail (Roche). Cells were disrupted by three rapid freeze-thaw cycles, followed by centrifugation at 4oC for 30 min at 13000 rpm. Supernatant (cytosol fraction) and pellet (membrane fraction) samples were boiled in sample buffer (SB) for 5 min at 95oC, and further subjected to SDS-PAGE and Western Blotting analysis.

Western Blot analysis. Samples were run on SDS-PAGE gels followed by transfer to nitrocellulose membranes (Bio-Rad). Membranes were incubated with primary antibodies as follows: rabbit polyclonal anti-GBF1 (gift from dr. N. Altan-Bonnet, Rutgers University, New Jersey, USA), mouse monoclonal anti-GBF1 (BD Biosciences), anti-Arf1 (Affinity Bioreagents), anti-αCOPI (gift from dr F. Wieland, Biochemie-Zentrum, Heidelberg, Germany), rabbit polyclonal anti-PI4KIIIβ (Millipore), mouse monoclonal anti-ACBD3 (Sigma), rabbit polyclonal anti-GFP (kind gift from dr. J. Fransen, NCMLS, Nijmegen, the Netherlands), mouse monoclonal anti-β-actin (Sigma), or rabbit polyclonal anti-calnexin (Sigma). Secondary antibody detection included IRDye goat anti-mouse or goat anti-rabbit (LI-COR) or HRP-conjugated antibodies (Amersham). Tagged recombinant proteins were visualized using the ECL kit (GE Healthcare) and the following HRP-conjugated primary antibodies: mouse monoclonal anti-His-HRP (Abcam), mouse monoclonal anti-StrepMAB-classic-HRP (IBA Lifesciences), mouse monoclonal anti-GST-HRP (Millipore). Images of blots were acquired with the Odyssey Imaging System (LI-COR).

Recombinant protein purification and GST-pulldown assay. Recombinant GST, GST-ACBD3, and CVB3 3A(1-60)-His were expressed in *E.Coli* BL21 bacteria under IPTG induction. GST-tagged proteins

were purified with glutathione sepharose 4B beads (GE Healthcare) and CVB3 3A(1-60)-His was purified using Ni-NTA agarose beads (Qiagen). Strep-ACBD3 was expressed in Hek293 cells and purified from lysates using Strep-Tactin sepharose beads (IBA Lifesciences). All purifications were performed according to the manufacturer's protocol. GST-PI4KIIIβ was purchased from Invitrogen. Pulldown experiments were performed under rapid kinetic conditions [28]. Briefly, combinations of 2 µg of each recombinant protein (for GST-ACBD3, 5 and 10 µg were also used) were incubated in PBS-0.02% Tween20 with Pierce glutathione magnetic beads (Thermo Scientific) for 2 h at 4oC. Beads were then washed three times for 5 minutes (total time) with PBS-0.1% Tween20. Bound proteins were eluted by boiling in sample buffer. Samples were further subjected to SDS-PAGE and Western Blot analysis.

RESULTS

Binding of enteroviral 3A proteins to ACBD3. Recent studies put ACBD3 forward as a new picornavirus host factor, responsible for the recruitment of PI4KIIIβ to sites of kobuvirus replication [27,28]. Since the 3A proteins of both entero- and kobuviruses were found to co-purify with ACBD3 and PI4KIIIβ [28], we set out to investigate whether enteroviruses recruit PI4KIIIβ through ACBD3. First, we investigated which picornaviral 3A proteins are able to interact with ACBD3. To this end, several enterovirus 3A proteins - namely those of CVB3, PV, human rhinovirus 2 (HRV2) and 14 (HRV14) – and the 3A protein of mengovirus (a strain of encephalomyocarditis virus, which belongs to the genus *Cardiovirus* within the *Picornaviridae* family) were tested in the mammalian two-hybrid system. All enteroviral 3A proteins potently interacted with ACBD3, while mengovirus 3A did not (Fig. 1A).

To identify which region of ACBD3 is important for 3A interaction, we made use of a number of previously established mutants with domain deletions in the N-terminus, the central part or the C-terminus of ACBD3 [27]. Of the seven ACBD3 deletion mutants tested, only mutants 3 and 5, containing an intact C-terminal domain, were still able to interact with CVB3 3A (Fig. 1B), indicating that the region of ACBD3 required for 3A interaction involves the C-terminal GOLD (Golgi Dynamics) domain. The central part of ACBD3 was shown to be important for PI4KIIIβ interaction [27], thus our data suggest that ACBD3 could bind simultaneously to 3A and PI4KIIIβ, thereby bringing the kinase to replication organelles.

CVB3 3A is a small protein of 89 amino acids (aa) containing a hydrophobic C-terminal domain (aa 61-82) and a conserved, unstructured N-terminal domain. To examine which region in 3A is responsible for the interaction with ACBD3, we generated a series of both N- and C-terminal deletion mutants of CVB3 3A and determined their ability to interact with ACBD3 in the mammalian two-hybrid system. Mutant 3A(1-60), which lacks the C-terminal hydrophobic domain, was still able to interact with ACBD3 (Fig. 1C). Deletion mutants 3A(1-30), 3A(1-40) and 3A(1-50) failed to interact with ACBD3, implying that aa 50-60 are important for the binding of 3A to ACBD3. Consistently, mutant 3A(61-89) lacking the entire 1-60 region showed a markedly reduced interaction with ACBD3, whereas mutant 3A(41-89) efficiently interacted with ACBD3. To conclude, our results indicate that a region in CVB3 3A involving aa 50-60 is important for ACBD3 interaction. Our data are in line with the recent findings of Greninger *et al.* (33) and Téoulé *et al.* (34) that the central part of PV 3A is involved in binding to ACBD3.

ACBD3 interacts directly with CVB3 3A and PI4KIIIβ. To investigate whether ACBD3 and CVB3 3A directly interact with each other, GST-pulldown experiments were performed using recombinant proteins. CVB3 3A-His co-purified with ACBD3-GST in a dose-dependent manner (Fig. 2A), while no binding was detected to GST alone, which shows that CVB3 3A and ACBD3 interact directly with

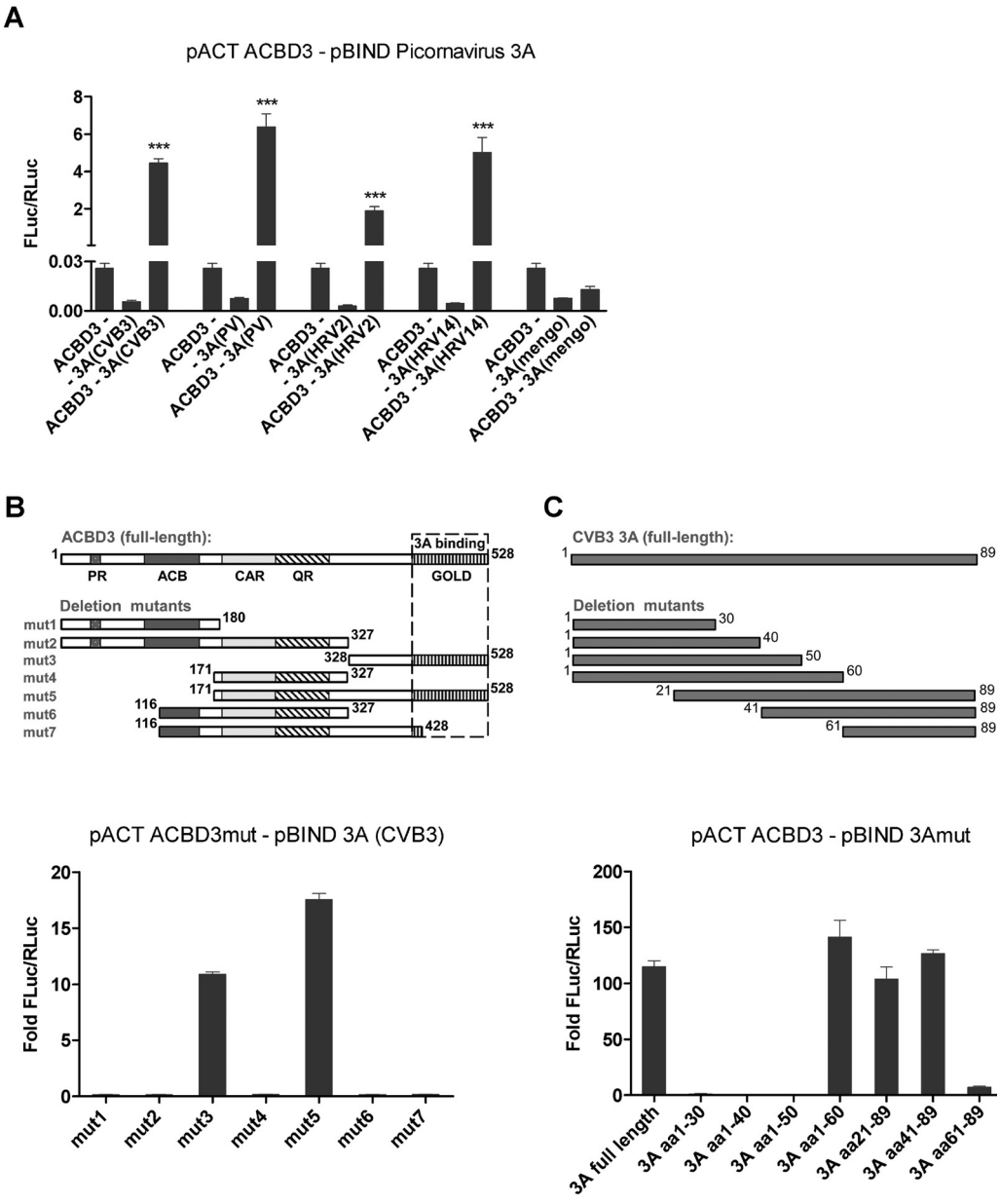


Figure 1. The GOLD domain of ACBD3 and aa 50-60 of CVB3 3A are crucial for interaction. (A) The ability of several picornavirus 3A proteins to interact with ACBD3 was determined using the mammalian two-hybrid system. Error bars represent mean values ± SD. Significant differences were calculated over the highest control, by paired t-test: ***, $p < 0.001$. (B, C) Mapping of interaction domains of ACBD3 and CVB3 3A. Lower panels show interactions of CVB3 3A with truncated ACBD3 (B) and of ACBD3 with truncated CVB3 3A (C) in mammalian two-hybrid. Bars represent mean ± SD. Schematic representations of full-length and truncated variants of ACBD3 (B) and CVB3 3A (C) are depicted in the upper panels.

each other. In addition, we studied the possibility that CVB3 3A and PI4KIIIβ interact directly without the involvement of ACBD3. Rapid capture and washing conditions were applied, as it was previously shown that enterovirus 3A only co-purifies PI4KIIIβ from cell lysates under rapid kinetic conditions due to the transient nature of the interaction [28]. We were unable to show a direct interaction between 3A-His and PI4KIIIβ-GST under the experimental conditions tested (Fig. 2A). However, PI4KIIIβ-GST bound to ACBD3-Strep, which demonstrates that PI4KIIIβ was able to establish a direct interaction. The interaction between these proteins was further characterized in the mammalian two-hybrid system. Interactions were detected between 3A and ACBD3 as well as ACBD3 and PI4KIIIβ in both orientations, validating the functionality of the proteins in this system (Fig. 2B). Co-expression of 3A and PI4KIIIβ only resulted in a minor increase in the luciferase signal in one orientation, while no signal was detected in the second orientation. Taken together, our results indicate that ACBD3 can interact directly with CVB3 3A and PI4KIIIβ, advancing the idea that ACBD3 could be utilized by enteroviruses to recruit PI4KIIIβ to

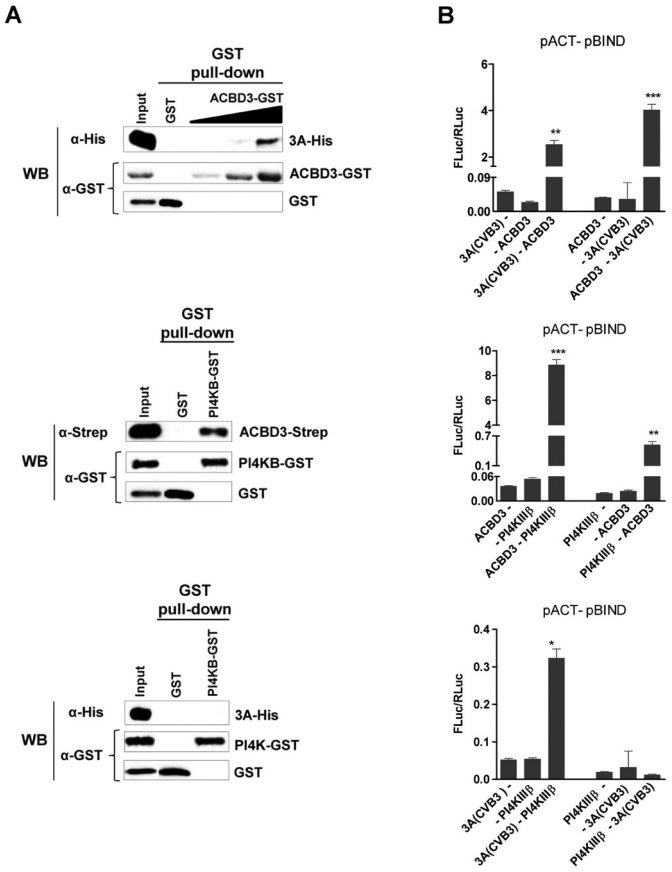


Figure 1. ACBD3 directly interacts with CVB3 3A and PI4KIIIβ. (A) Rapid kinetic GST-pulldown assay to study direct interaction between GST-ACBD3 and CVB3 3A(1-60)-His, Strep-ACBD3 and GST-PI4KIIIβ or GST-PI4KIIIβ and 3A(1-60)-His. Unless otherwise specified, 2 µg of recombinant GST/GST-tagged protein was incubated for 2 h with Pierce Glutathione magnetic beads and 2 µg of the other recombinant proteins. GST was used as a negative control. Following rapid washing, bound-proteins were eluted off the beads and subjected to Western Blot analysis. Proteins were detected with antibodies against the GST-, Strep-, and His-tag. Purification of recombinant proteins is described in Materials and Methods. (B) Mammalian two-hybrid assay testing interactions between 3A, ACBD3, and PI4KIIIβ. Error bars represent mean values ± SD. Significant differences were calculated over the highest control, by paired t-test: *, $p < 0.1$, **, $p < 0.01$; ***, $p < 0.001$.

replication organelles.

ACBD3 is dispensable for PI4KIIIβ recruitment to CVB3 replication organelles. Based on our 3A-ACBD3 interaction studies, we set out to elucidate if CVB3 indeed recruits PI4KIIIβ in an ACBD3-dependent fashion. To achieve this, HeLa R19 cells were depleted of ACBD3 by RNA interference and examined by immunofluorescence for the presence of PI4KIIIβ at CVB3 replication organelles. In mock-infected cells, ACBD3 silencing diminished PI4KIIIβ from the Golgi comparable to silencing PI4KIIIβ itself (Fig. 3A). Similar effects were observed in Vero cells (data not shown), which is in line with a previous report demonstrating that ACBD3 plays a critical role in PI4KIIIβ localization in these cells [27]. As expected, in infected cells treated with scramble siRNA, both ACBD3 and PI4KIIIβ localized with 3A at replication organelles (Fig. 3B, scramble siRNA panels). Surprisingly, ACBD3 depletion did not affect the levels of PI4KIIIβ present at replication organelles in CVB3-infected cells (Fig. 3B, ACBD3 siRNA panels). These results suggest that CVB3 does not rely on ACBD3 to recruit PI4KIIIβ to replication organelles, despite the observed contribution of ACBD3 to the membrane-association of PI4KIIIβ in non-infected cells. While our manuscript was in preparation, Téoulé *et al*. showed that ACBD3 depletion does not affect recruitment of PI4KIIIβ to PV replication organelles (34), which is in line with our observations.

ACBD3 is not required for CVB3 RNA replication. The finding that ACBD3 is not involved in recruiting PI4KIIIβ to CVB3 replication organelles prompted us to question whether ACBD3 plays another important role in replication. Therefore, we analyzed the effects of ACBD3 depletion on replication using *Renilla* luciferase-encoding viruses CVB3-RLuc wt or CVB3-RLuc 3A-H57Y, a mutant that is resistant to treatment with PI4KIIIβ inhibitors as well as to PI4KIIIβ knockdown (11). Viral RNA replication was assessed by measuring the *Renilla* luciferase activity at 8 h post-infection. Efficient knockdown of ACBD3 and PI4KIIIβ was confirmed by Western Blot analysis (Fig. 4B). As we previously reported, PI4KIIIβ knockdown severely inhibited replication of CVB3-RLuc wt, while CVB3-RLuc 3A-H57Y was far less affected by the depletion (6-fold compared to wt, Fig. 4A). Remarkably, ACBD3 silencing had an enhancing effect on CVB3-RLuc wt replication with an increase up to 200% compared to the scramble control. As opposed to CVB3-RLuc wt, replication of CVB3-RLuc 3A-H57Y was not influenced by ACBD3 depletion, suggesting that the effects observed on CVB3-RLuc wt replication are specific, and providing further evidence on the link between 3A, ACBD3, and PI4KIIIβ/PI4P.

Additionally, we confirmed by immunofluorescence that ACBD3 knockdown does not inhibit replication of CVB3 wt, as evidenced by a similar amount of infected cells in both scramble and ACBD3-depleted cells as well as similar levels of 3A expression (Fig. 4C). In contrast, knockdown of PI4KIIIβ dramatically impaired replication, as reflected by the strong reduction in the number of infected cells.

To evaluate a possible role of ACBD3 in other steps of the CVB3 life cycle, we analyzed whether ACBD3 depletion had any impact on virus assembly/release by measuring the amounts of intracellular and secreted virus. However, we found no notable differences between control and ACBD3-depleted cells (data not shown). Collectively, our data strongly indicate that ACBD3 is not required for CVB3 RNA replication.

GBF1 is not essential for the recruitment of PI4KIIIβ by CVB3 3A. Having established that ACBD3 does not play an important role in PI4KIIIβ recruitment by CVB3, we set out to investigate another proposed recruitment mechanism. Previously, it was suggested that enteroviruses recruit PI4KIIIβ to replication organelles through GBF1/Arf1 [10], since the enteroviral protein 3A is able to directly bind GBF1 and

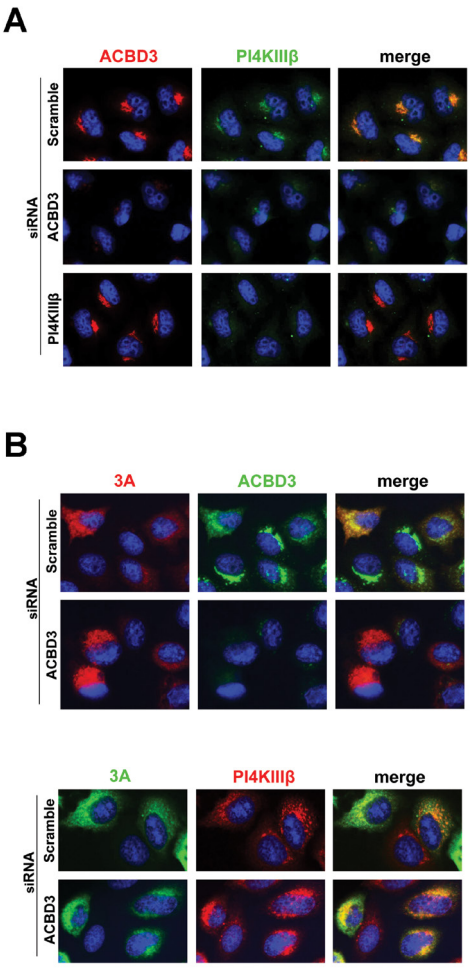


Figure 3. CVB3 recruits PI4KIIIβ to replication organelles independently of ACBD3. (A,B) HeLa R19 cells were reverse-transfected with siRNA against PI4KIIIβ, ACBD3, or scramble siRNA as control. At 48 h post-transfection (p.t.), cells were mock infected (A) or infected with CVB3 wt at an MOI of 10. At 5 h post-infection (p.i.), cells were fixed and stained with antibodies against PI4KIIIβ and ACBD3 (A), or PI4KIIIβ, ACBD3 and CVB3 3A (B).

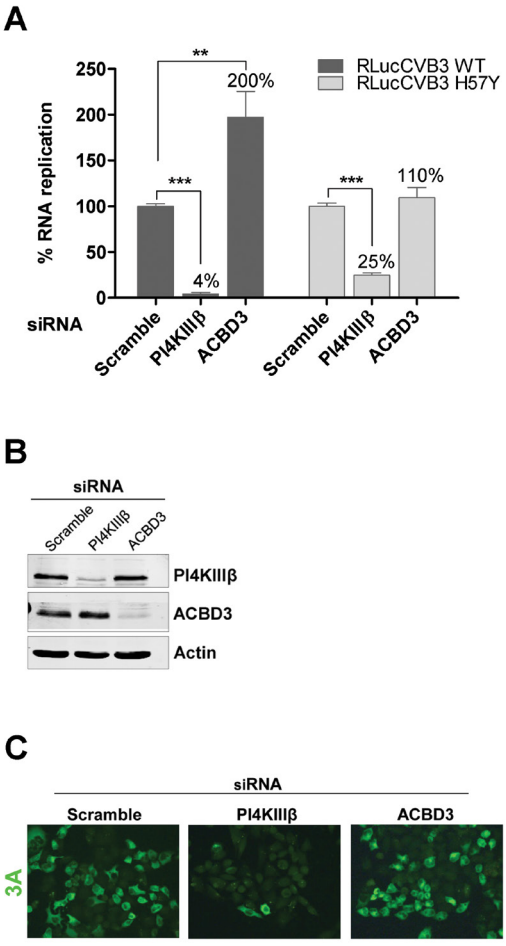


Figure 4. ACBD3 is not essential for CVB3 RNA replication. HeLa R19 cells were reverse-transfected with siRNA against PI4KIIIβ, ACBD3 or scramble siRNA as control. At 48 h p.t., cells were infected with CVB3-Rluc wt or CVB3-RLuc-3A H57Y at an MOI of 0.1 (A), or with CVB3 wt at an MOI of 10 (C). (A) At 8 h p.i. with the luciferase-encoding viruses, cells were lysed and *Renilla* luciferase activity was determined as a measure of viral RNA replication. Error bars represent mean values ± SD. Significant differences were calculated by paired t-test: **, $p < 0.01$; ***, $p < 0.001$. (B) Cells were harvested at 48 h p.t. and subjected to Western Blot analysis to evaluate the knockdown efficiency. Actin was used as a loading control. (C) At 5 h p.i. with CVB3 wt, cells were fixed and stained with antibodies against CVB3 3A.

manipulate Arf1-mediated effector recruitment (26). To investigate the importance of GBF1 activity for PI4KIIIβ recruitment, we utilized an system with HeLa S10 cell extracts [22]. RNA coding for CVB3 3A was translated in these cell extracts in the absence or presence of brefeldin A (BFA), a well-known inhibitor of GBF1 (35). Subsequently, the membranes were isolated by centrifugation and the membrane-associated proteins were analyzed by Western Blot. In line with previous results, 3A expression induced a significant accumulation of GBF1 and Arf1 [22], but also PI4KIIIβ on membranes (Fig. 5A). BFA treatment alone also induced a massive accumulation of GBF1 on membranes, yet in an inactive form, since these samples did not exhibit increased levels of Arf1 or its effector αCOPI. Remarkably, BFA treatment did not affect

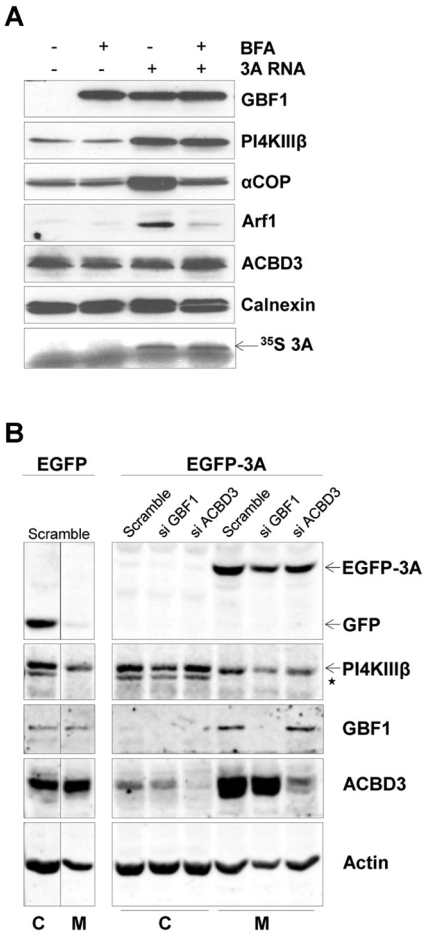


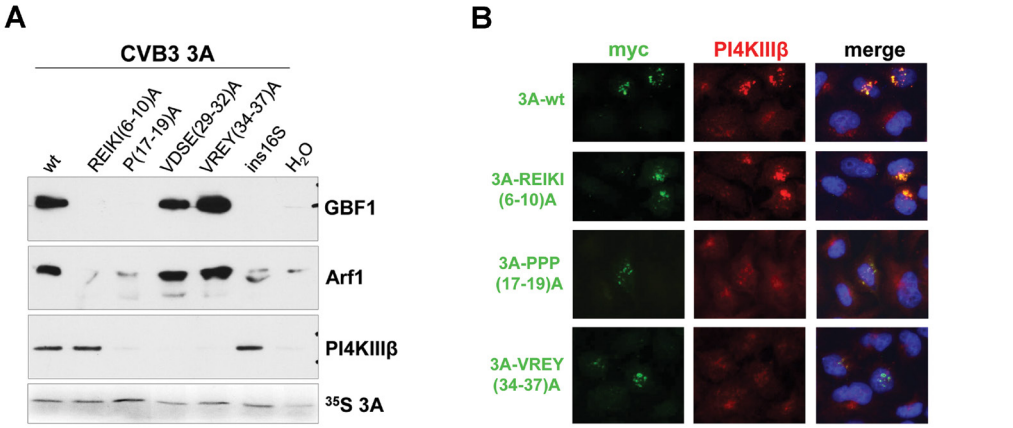
Figure 5. GBF1 is not essential for PI4KIIIβ recruitment by CVB3 3A. (A) *In vitro* HeLa S10 extract assay. RNA coding for CVB3 3A was translated in HeLa S10 cell extracts. Where indicated, the samples contained 80 µg/ml BFA. Membranes were isolated by centrifugation and subjected to Western Blot analysis to detect the membrane-associated proteins using antibodies against GBF1, PI4KIIIβ, αCOPI, and Arf1. Calnexin was used as a loading control. Efficiency of the translation reaction was assessed by 35S methionine labeling. The arrow indicates specific signal for 3A (lowest panel). (B) Effect of GBF1 depletion on PI4KIIIβ recruitment to membranes by 3A. HeLa R19 cells were reverse-transfected with siRNA against GBF1, ACBD3 or scramble siRNA as control. At 48 h p.t., cells were transfected with CVB3 3A-EGFP or GFP as control. At 24 h after DNA transfection, cells were harvested, resuspended in PBS to separate membranes (M) from cytosol (C) by three freeze-thaw cycles and subjected to Western Blot analysis using antibodies against GBF1, ACBD3, PI4KIIIβ, and GFP. Actin was used as a loading control. The star indicates a non-specific band.

the ability of 3A to recruit PI4KIIIβ to membranes, despite its inhibitory effects on GBF1 and Arf1 as evidenced by dissociation of αCOPI from membranes. Altogether, these results demonstrate that 3A is able to recruit PI4KIIIβ regardless of GBF1 activity and accumulation of Arf1 on membranes.

In order to evaluate whether GBF1 provides a physical, non-enzymatic link between PI4KIIIβ and 3A, we performed siRNA knockdown experiments to deplete GBF1 from cells and assess the effects on PI4KIIIβ recruitment. Since GBF1 knockdown inhibits enterovirus replication [22,23], we studied this in cells expressing 3A alone. Unfortunately, the detection of overexpressed 3A by immunofluorescence was severely impaired in cells depleted of GBF1, most probably due to the great overall impact of GBF1 knockdown on Golgi integrity, as reflected by the perturbed pattern of both cis- (GM130) and trans-(TGN46) Golgi markers in immunofluorescence staining (data not shown). An approach to overcome this technical limitation is the detection of proteins by Western Blot analysis. CVB3 3A-EGFP was expressed in HeLaR19 cells treated with siRNA against GBF1, ACBD3, or scramble siRNA. At 24 h following protein expression, cells were harvested, membranes were separated from cytosol by freeze-thawing, and subjected to Western Blotting analysis to evaluate the presence of GBF1, ACBD3 and PI4KIIIβ on membranes. GFP, used as a control, was detected almost exclusively in the cytosol (C) fraction, while 3A-EGFP was found almost exclusively in the membranes (M) fraction (Fig. 5B), thus reflecting a successful separation of cytosol from membranes. In GFP-expressing cells, GBF1, ACBD3, and PI4KIIIβ were present both in the cytosol and on membranes. In cells expressing 3A, nearly all GBF1 and ACBD3 was present in the membrane fraction. This phenomenon was not detected for PI4KIIIβ, where a large proportion was still present in the cytosol fraction. Previous co-IP assays from cell lysates revealed that CVB3 3A formed stable complexes with GBF1 and ACBD3, whereas PI4KIIIβ only transiently interacted with 3A [28]. This could explain why we only detected massive recruitment to membranes by 3A in the case of GBF1 and ACBD3, and not for PI4KIIIβ, since the 3A-PI4KIIIβ interaction might be sensitive to our membrane isolation method. Nevertheless, the presence of PI4KIIIβ on 3A-containing membranes remained largely unaffected in cells lacking either GBF1 or ACBD3, despite efficient depletion of both proteins (Fig. 5B), indicating that neither the enzymatic activity nor the physical structure of GBF1 is required for PI4KIIIβ recruitment by 3A.

To further corroborate that PI4KIIIβ recruitment is not dependent on GBF1, we made use of a series of previously established CVB3 3A mutants (summarized in Fig. 6C). Except for mutant 3A-Ins16S, which contains a serine insertion at position 16, all other mutants were generated by alanine substitution of conserved aa residues in the N-terminus of 3A [30]. The ability of these 3A mutants to recruit GBF1, Arf1, and PI4KIIIβ to membranes was tested in the HeLa S10 cell extract assay. 3A mutants REIKI(6-10)A, Ins16S, and PPP(17-19)A no longer bound to GBF1, while the GBF1 interaction was retained in mutants 3A-VDSE(29-32)A and 3A-VREY(34-37)A (Fig. 6A), in line with our previous observations [30]. CVB3 3A wt, as well as mutants REIKI(6-10)A and Ins16S, were able to recruit PI4KIIIβ to membranes in the absence of elevated levels of GBF1 and Arf1, confirming that 3A does not rely on GBF1/Arf1 for PI4KIIIβ recruitment (Fig. 6A). Mutant PPP(17-19)A, unable to recruit GBF1/Arf1, was severely impaired in recruiting PI4KIIIβ. Mutants VDSE(29-32)A and VREY(34-37)A induced a significant accumulation of GBF1 and Arf1 on membranes, but not PI4KIIIβ. These data demonstrate that elevated levels of GBF1/Arf1 not necessarily lead to elevated levels of PI4KIIIβ. Furthermore, this result identifies residues in the region 29-37 of CVB3 3A as essential for PI4KIIIβ recruitment.

Next, we sought to validate in intact cells our finding that GBF1 is dispensable for PI4KIIIβ recruitment by CVB3 3A. To this end, 3A mutants summarized in Fig. 6C were separately expressed in cells and



C

GPPVYREIKISVAPETPPPPAIADLLKSVDSEAVREYCKEKGWLVPEINSTLQIEKHVSRAFICLQALTTFVSVAGIIYIIYKLFAGFQ

Ins16S

Hydrophobic domain

Construct	GBF1 interaction (30) (M2H)	Active in PI4K recruitment
CVB3 3A-wt	+	+
REIKI(6-10)A	-	+
Ins 16S	-	+
PPP(17-19)A	-	+/-
VDSE(29-32)A	+	-
VREY(34-37)A	+	-

Figure 6. CVB3 3A mutants recruit PI4KIIIβ in the absence of GBF1. (A) *In vitro* translation assay. RNA coding for wt or mutant CVB3 3A was translated in HeLa S10 extracts. Isolated membranes were analyzed by Western Blot to detect the membrane-associated proteins, using antibodies against GBF1, Arf1, and PI4KIIIβ. Efficiency of the translation reaction was assessed by 35S methionine labeling (the faint signal in the H2O sample corresponds to the 35S methionine front). (B) To study PI4KIIIβ recruitment in intact cells, HeLa R19 cells were transfected with myc-tagged CVB3 3A-wt or the indicated mutants. The next day, cells were fixed and stained with antibodies against the myc-tag or endogenous PI4KIIIβ. Shown are representative immunofluorescence example images of proteins listed in the table in panel C with recruitment of PI4KIIIβ by different 3A proteins. (C) CVB3 3A amino acid (aa) sequence. Residues substituted by alanine are indicated by stars. The Ser insertion at position 16 and the C-terminal hydrophobic domain are also depicted. The table outlines the ability of the different 3A mutants to interact with GBF1 in the mammalian two-hybrid system (30) and to recruit PI4KIIIβ.

stained by immunofluorescence for their ability to actively recruit PI4KIIIβ (representative images in Fig. 6B). 3A mutants REIKI(6-10)A and Ins16S, both of which no longer interact with GBF1, recruited PI4KIIIβ to membranes similar to wild-type (wt) 3A, indicating that this event is not mediated by GBF1. Mutant PPP(17-19)A was severely impaired in recruiting PI4KIIIβ, as the level of the kinase on membranes was only slightly higher than the basal level. Mutations VDSE(29-32)A and VREY(34-37)A, which do not impair the interaction with GBF1, markedly abolished PI4KIIIβ recruitment to 3A-containing membranes, demonstrating that binding of 3A to GBF1 is not a prerequisite for PI4KIIIβ recruitment.

CVB3 3A recruits PI4KIIIβ independently of Arf1. Next, we further studied the role of Arf1 in PI4KIIIβ recruitment by 3A. To this end, HeLaR19 cells were depleted of Arf1 by RNA interference and examined by immunofluorescence for the presence of PI4KIIIβ at 3A-positive membranes upon 3A overexpression.

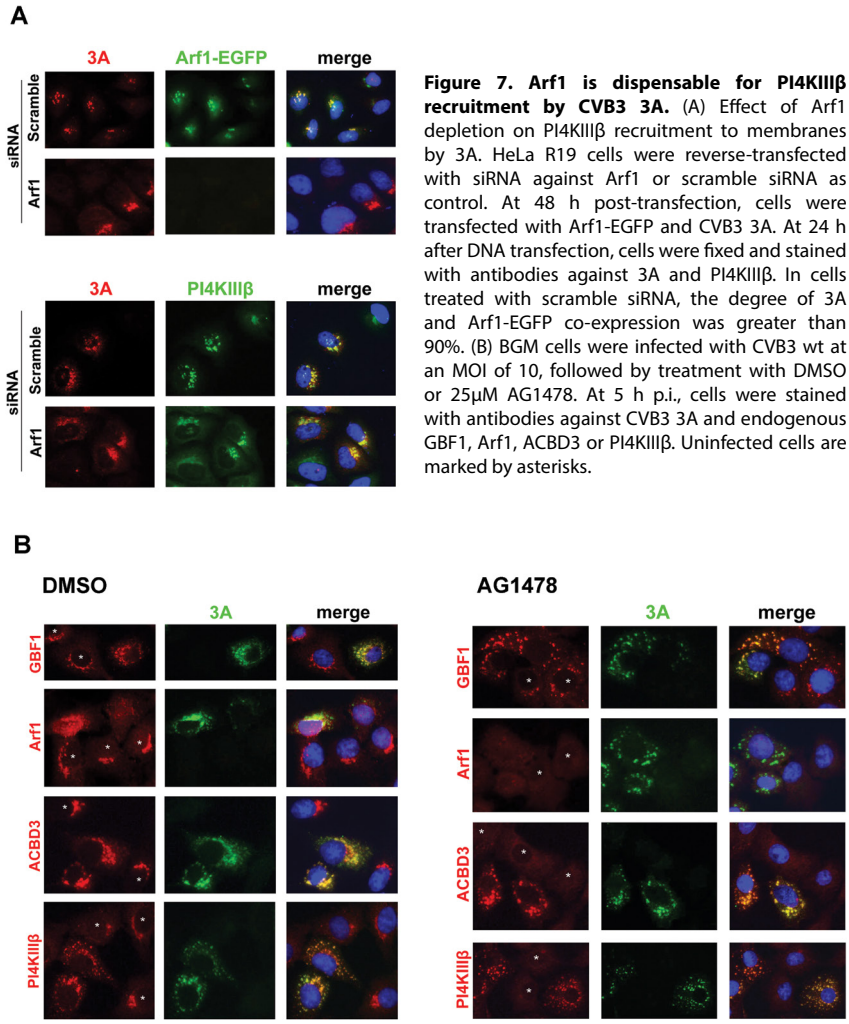


Figure 7. Arf1 is dispensable for PI4KIIIβ recruitment by CVB3 3A. (A) Effect of Arf1 depletion on PI4KIIIβ recruitment to membranes by 3A. HeLa R19 cells were reverse-transfected with siRNA against Arf1 or scramble siRNA as control. At 48 h post-transfection, cells were transfected with Arf1-EGFP and CVB3 3A. At 24 h after DNA transfection, cells were fixed and stained with antibodies against 3A and PI4KIIIβ. In cells treated with scramble siRNA, the degree of 3A and Arf1-EGFP co-expression was greater than 90%. (B) BGM cells were infected with CVB3 wt at an MOI of 10, followed by treatment with DMSO or 25μM AG1478. At 5 h p.i., cells were stained with antibodies against CVB3 3A and endogenous GBF1, Arf1, ACBD3 or PI4KIIIβ. Uninfected cells are marked by asterisks.

Evaluation of the knockdown efficiency was performed by monitoring the levels of ectopically expressed Arf1-EGFP. 3A was equally capable of recruiting PI4KIIIβ in both control and Arf1 depleted cells (Fig. 7A), suggesting that Arf1 does not play a crucial role in recruitment of the kinase by 3A.

Additionally, we addressed the role of Arf1 in PI4KIIIβ recruitment in the context of CVB3 infection. AG1478 is a compound that causes Arf1 to dissociate from Golgi membranes, resulting in Golgi dispersal, without affecting CVB3 replication (36, 37). Here, we used AG1478 as a tool to block Arf1 association with membranes and evaluate the effects on PI4KIIIβ recruitment to enterovirus replication organelles. To this end, BGM cells were infected with CVB3 wt in the presence or absence of AG1478, and stained at 5 h p.i. for the presence of 3A, GBF1, Arf1, ACBD3, and PI4KIIIβ on replication organelles. In untreated cells, GBF1, Arf1, ACBD3, and PI4KIIIβ were all present at the replication organelles of infected cells, as revealed by their overlap with 3A (Fig. 7B). In non-infected cells (indicated by asterisks), AG1478 treatment caused dispersal of GBF1 to discrete punctae throughout the cell and a diffuse, cytosolic

pattern of Arf1, ACBD3 and PI4KIIIβ. However, in infected cells, GBF1, ACBD3, and PI4KIIIβ, but not Arf1, were still detected at 3A-containing replication organelles upon AG1478 treatment. This observation not only reveals an active recruitment of GBF1, ACBD3, and PI4KIIIβ to replication organelles, but also confirms that Arf1 is not involved in PI4KIIIβ recruitment by 3A during infection.

PI4KIIIβ recruitment by CVB3 3A is simultaneously independent of ACBD3 and GBF1/Arf1. Possibly, 3A could recruit PI4KIIIβ via both the ACBD3 and GBF1/Arf1, in which case blocking one of these routes would leave 3A the possibility to recruit the kinase via the other route. To verify this scenario, we tested by immunofluorescence if the CVB3 3A-REIKI(6-10)A mutant, which is unable to interact with GBF1, is still capable of recruiting PI4KIIIβ in ACBD3-depleted cells. Expression of mutant 3A-REIKI(6-10)A, as well as 3A-wt, led to active recruitment of PI4KIIIβ to membranes in both control and ACBD3-depleted cells (Fig. 8). In parallel, efficient knockdown was evaluated by separately staining ACBD3 (data not shown). These results demonstrate that CVB3 3A is able to actively recruit PI4KIIIβ to membranes in the absence of both GBF1/Arf1 and ACBD3.

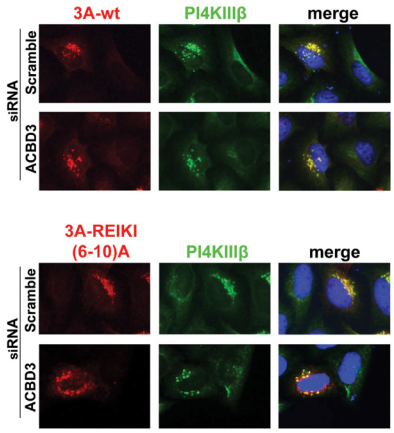


Figure 8. GBF1, Arf1, and ACBD3 are simultaneously dispensable for PI4KIIIβ recruitment by CVB3 3A. HeLa R19 cells were reverse-transfected with scramble siRNA or siRNA against ACBD3. At 48 h post-transfection (p.t.), cells were transfected with myc-tagged CVB3 3A-wt or the REIKI(6-10)A mutant. At 24 h p.t., cells were fixed and stained with antibodies against PI4KIIIβ or the myc-tag to detect 3A.

DISCUSSION

As obligate intracellular pathogens, viruses have evolved diverse mechanisms to subvert cellular pathways for their own advantage. For the purpose of genome replication, positive-strand RNA viruses generate specialized membranous structures with unique lipid and protein composition. Both enteroviruses and kobuviruses of the *Picornaviridae* family manipulate PI4KIIIβ to generate a lipid microenvironment enriched in PI4P for viral RNA replication. PI4KIIIβ is recruited to enterovirus replication sites by the viral protein 3A [10], but the underlying mechanism has remained elusive. Recently, ACBD3 was identified to mediate PI4KIIIβ recruitment to Golgi membranes [27]. The kobuvirus Aichi virus was shown to rely on ACBD3 to attract PI4KIIIβ to replication sites [27,28]. This finding prompted us to investigate whether

ACBD3 is also responsible for the recruitment of PI4KIIIβ by enteroviruses. ACBD3 was able to interact with 3A of CVB3 and other enteroviruses, but not with cardiovirus 3A. Using deletion mutants, we mapped the regions of interaction on both ACBD3 and CVB3 3A. The C-terminal GOLD domain of ACBD3 and residues 50-60 of CVB3 3A were identified to be crucial for the interaction. Our results are in line with the recent finding that residues in the central region of PV 3A are important for ACBD3 interaction (33, 34). However, we observed that CVB3-infected cells depleted of ACBD3 by siRNA treatment still developed replication organelles enriched in PI4KIIIβ, demonstrating that enteroviruses recruit PI4KIIIβ to replication sites by a mechanism that does not depend on ACBD3. Interestingly, ACBD3 depletion had an enhancing effect on the replication of CVB3 wt, but not CVB3 3A-H57Y, a virus carrying a mutation in the 3A protein that renders it resistant to PI4KIIIβ inhibitors or PI4KIIIβ knockdown.

In non-infected cells, Arf activity is important for the recruitment of PI4KIIIβ to Golgi membranes [12]. Knowing that 3A is able to directly interact with GBF1 and thereby modulate Arf1 activity (26), we assessed if the enrichment in PI4KIIIβ at replication organelles by 3A is achieved through hijacking of GBF1/Arf1 [10]. We showed here that PI4KIIIβ was still actively recruited to membranes by CVB3 3A mutants that could no longer interact with GBF1. Furthermore, 3A was able to recruit PI4KIIIβ in the presence of BFA, a well-known inhibitor of GBF1, indicating that the activities of both GBF1 and Arf1 are dispensable for the recruitment process. Accordingly, PI4KIIIβ was present at the replication sites in infected cells treated with AG1478, a compound that dissociates Arf1 from membranes, confirming that active Arf1 is dispensable for PI4KIIIβ recruitment by 3A. Lastly, genetic depletion of either GBF1 or Arf1 by RNA interference did not interfere with the recruitment of PI4KIIIβ by 3A. Together, these results indicated that 3A does not interact with GBF1 in order to recruit PI4KIIIβ via the effector function of Arf1. Whether enteroviral 3A recruits PI4KIIIβ to replication organelles by a direct interaction or with the help of other host factors remains an open issue. Using purified recombinant proteins in pulldown assays, we showed that CVB3 3A is able to interact directly with ACBD3 and that ACBD3 directly interacts with PI4KIIIβ. However, we were unable to detect a direct interaction between the kinase and 3A under our experimental conditions. The reported transient nature of the interaction [28] could impair the detection of a direct binding of the two proteins. Furthermore, we cannot exclude that our result is a consequence of using a recombinant 3A protein that for efficient production in *E. Coli* lacks the C-terminal hydrophobic domain. Anchoring of 3A in membranes might induce topological modifications that may be required for the interaction with PI4KIIIβ.

Our finding that ACBD3 depletion from cells enhanced CVB3 RNA replication contrasts the data of Greninger *et al*. which showed that PV RNA replication is reduced upon ACBD3 silencing, albeit modestly [28]. However, our data are now supported by a new study by Téoulé *et al*. in which PV RNA replication is also found to be significantly increased upon ACBD3 depletion (34). How could ACBD3 negatively influence enterovirus RNA replication? And what is the purpose of the 3A-ACBD3 interaction? The Golgi-localized TBC1D22A/B is a putative Rab33 GTPase-activating protein (GAP) recently identified as a new interacting partner for ACBD3 (33). PI4KIIIβ and TBC1D22A/B bind in a competitive and mutually exclusive manner to the same domain of ACBD3. It is unknown which factors determine whether PI4KIIIβ or TBC1D22A/B interacts with ACBD3. Remarkably, although both enterovirus and kobuvirus 3As interact with the GOLD domain of ACBD3, this seems to distinctly affect the binding of TBC1D22A/B to ACBD3 (33). Affinity purifications using TBC1D22A/B as a bait resulted in pulldown of ACBD3 together with enterovirus 3A, but not with kobuvirus 3A. Thus, kobuviral 3A seems unable to interact with a TBC1D22A/B-bound ACBD3, suggesting that kobuviral 3A can only interact with PI4KIIIβ-bound ACBD3. In contrast, enteroviral 3A was capable of interacting with TBC1D22A/B-bound ACBD3, which might

implicate that enteroviral 3A does not promote association of PI4KIIIβ with ACBD3. Based on the above findings and our data, we speculate that the interaction of PI4KIIIβ with 3A-ACBD3 might be detrimental for enterovirus replication by hampering an optimal engagement of the kinase in RNA replication. Our speculation is supported by our finding that ACBD3 depletion only enhanced replication of CVB3 wt, and not that of CVB3 3A-H57Y, the replication of which is far less sensitive to inhibition or depletion of PI4KIIIβ [11,32].

Another possible link between ACBD3 and enterovirus replication could reside in the regulation of lipid homeostasis. Subversion of host lipid metabolism is central to many positive-strand RNA viruses, which utilize particular lipid species as building blocks for replication organelles. Flaviviruses generate their replication factories by exploiting either PI4Ks (10, 38) or other enzymes involved in *de novo* lipid synthesis like FASN (fatty acid synthase) (39, 40). In addition to hijacking PI4Ks for increased PI4P synthesis, picornaviruses have also recently been shown to divert the host fatty acid homeostasis towards formation of replication organelles with an altered species of phosphatidylcholines (41). Recently, ACBD3 was suggested to play a role in lipid homeostasis by contributing to the regulation of ceramide transfer from ER to Golgi membranes (42). Since we observed that depletion of ACBD3 enhanced CVB3 RNA replication, it is conceivable that the normal role of ACBD3 in lipid homeostasis could be hampering viral RNA replication and formation of replication organelles. The interaction of CVB3 3A with ACBD3 may diverge ACBD3 from this harming role and thereby favor the process of viral RNA replication.

In conclusion, we demonstrate here that recruitment of PI4KIIIβ to CVB3 replication organelles by 3A is independent of both ACBD3 and GBF1/Arf1. Furthermore, we establish that ACBD3 is dispensable, and even disadvantageous for CVB3 RNA replication. Ultimately, our study reveals that the strategy employed by enteroviruses to recruit PI4KIIIβ to replication organelles is not as simple as initially believed, thus highlighting the complexity of the mechanisms governing the host-pathogen relationship.

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GBF1- and ACBD3-independent recruitment of PI4KIIIβ to replication sites by rhinovirus 3A proteins

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ABSTRACT

PI4KIIIβ recruitment to Golgi membranes relies on GBF1/Arf and ACBD3. Enteroviruses like poliovirus and coxsackievirus recruit PI4KIIIβ to their replication sites via their 3A proteins. Here, we show that human rhinovirus (HRV) 3A also recruited PI4KIIIβ to replication sites. Unlike other enterovirus 3A proteins, HRV 3A failed to bind GBF1. Although previously HRV 3A was shown to interact with ACBD3, our data suggest that PI4KIIIβ recruitment occurred independently of both GBF1 and ACBD3.

Enteroviruses (family *Picornaviridae*), such as poliovirus (PV) and coxsackievirus B3 (CVB3), rely on host factor phosphatidylinositol-4-kinase IIIβ (PI4KIIIβ) for genome replication [1,2]. The recruitment of PI4KIIIβ to the replication sites is mediated by the viral non-structural protein 3A [1]. PI4KIIIβ is normally recruited to the Golgi by the small GTPase Arf1 [3] and the Arf1 activator guanine nucleotide exchange factor (GEF) GBF1 [4]. ACBD3 (acyl-CoA-binding protein domain 3) also interacts with PI4KIIIβ to recruit it to the Golgi [5,6]. CVB3 3A and PV 3A both interact with the N-terminus of GBF1 (7, 8) as well as ACBD3 (9). However, we and others recently showed that the PI4KIIIβ recruitment by 3A of PV and CVB3 seems to occur independently of both GBF1/Arf1 and ACBD3 [7,8]. We studied this by individually expressing mutant 3A proteins of CVB3 that no longer interact with GBF1 [7]. Unfortunately, these 3A mutations render CVB3 unviable (unpublished data FJMvK), thus we have not been able yet to study the role of GBF1 in PI4KIIIβ recruitment in infected cells. Yeast two-hybrid analysis suggests that the 3A proteins of Human Rhinoviruses (HRV), which also belong to the *Enterovirus* genus, do not interact with the N-terminus of GBF1 [9]. In this study, we set out to investigate the consequences of the inability of HRV 3A proteins to interact with GBF1, focusing on the possible role of GBF1 and ACBD3 in PI4KIIIβ recruitment and virus replication.

First, we studied the interaction between the N-terminus of GBF1 and the 3A proteins of HRV2 and HRV14, which are members of group A and B rhinoviruses respectively, in a mammalian environment employing the mammalian two-hybrid (M2H) assay (Promega) as described previously [7]. We used the 3As of CVB3, PV, and mengovirus, belonging to the *Cardiovirus* genus of the Picornaviridae, as controls. Expression of all 3A proteins was verified by Western Blot analysis (Figure 1A, lower half). Importantly, all enterovirus 3A proteins were previously tested and validated to be competent in interacting with other proteins in the M2H assay [7]. CVB3 and PV 3As both interacted with the N-terminus of GBF1, while no interaction was detected for mengovirus 3A (Figure 1A, upper panel). HRV2 3A did not bind the N-terminus of GBF1, while HRV14 3A only interacted to some extent. We considered the possibility that the HRV 3A proteins bind to the C-terminus of GBF1. Therefore, we studied whether 3A expression induced recruitment of full-length GBF1 to membranes in HeLa S10 cell extracts as described elsewhere [10]. In line with previous results [7,10], PV and CVB3 3A expression induced accumulation of GBF1, Arf1, and the Arf1 effector COP-I on membranes (Figure 1B). HRV14 3A showed a low level of GBF1/Arf1 recruitment and no COP-I recruitment, whereas HRV2 3A accumulated little, if any GBF1/Arf1 and COP-I on membranes. Taken together, these results demonstrate that the 3A proteins of HRVs poorly interact with GBF1 compared to those of PV and CVB3.

Next, we investigated whether GBF1 is present at replication sites in HRV2-infected cells. BGM cells were infected with HRV2, fixed after 8 hours, and stained with antibodies against GBF1 (provided by K. Nakayama, Kyoto University, Japan) and COP-I (provided by F. Wieland, Biochemie-Zentrum, Heidelberg, Germany). In uninfected cells, both GBF1 and COP-I displayed a perinuclear Golgi pattern (Figure 1D). In HRV2-infected cells, GBF1 and COP-I showed a dispersed pattern, similar as in PV- and CVB3-infected cells (1, 12). Moreover, the signal of GBF1, but not that of COP-I, overlapped with that of dsRNA, a marker of viral replication (Figure 1D), as also observed in PV- and CVB3-infected cells (1, 12). Thus, GBF1 localizes at replication sites in HRV2-infected cells despite the lack of a direct interaction with the 3A protein.

Localization of GBF1 to the HRV replication sites implies, but does not prove, that it is essential for replication. HRV replication is sensitive to brefeldin A (BFA) (13), an inhibitor of the ArfGEFs GBF1, BIG1, and BIG2, but it remained to be determined which of these ArfGEFs is important for HRV. To test whether GBF1 is indeed crucial to HRV replication, we determined the sensitivity of HRV2 and HRV14 to the

specific GBF1 inhibitor Golgicide A (GCA). Figure 2A shows that both HRV2 and HRV14 were sensitive to GCA. HRV replication was largely insensitive to treatment with AG1478, a compound that disperses the Golgi without directly targeting GBF1 (14), showing that mere disruption of the secretory pathway is not detrimental to HRV replication. Together, these results suggest that inhibition of GBF1 impairs HRV replication. To corroborate this finding, we examined whether expression of a BFA-resistant GBF1 (11, 15) could restore HRV replication in the presence of BFA as described elsewhere (15). Figure 2B shows that

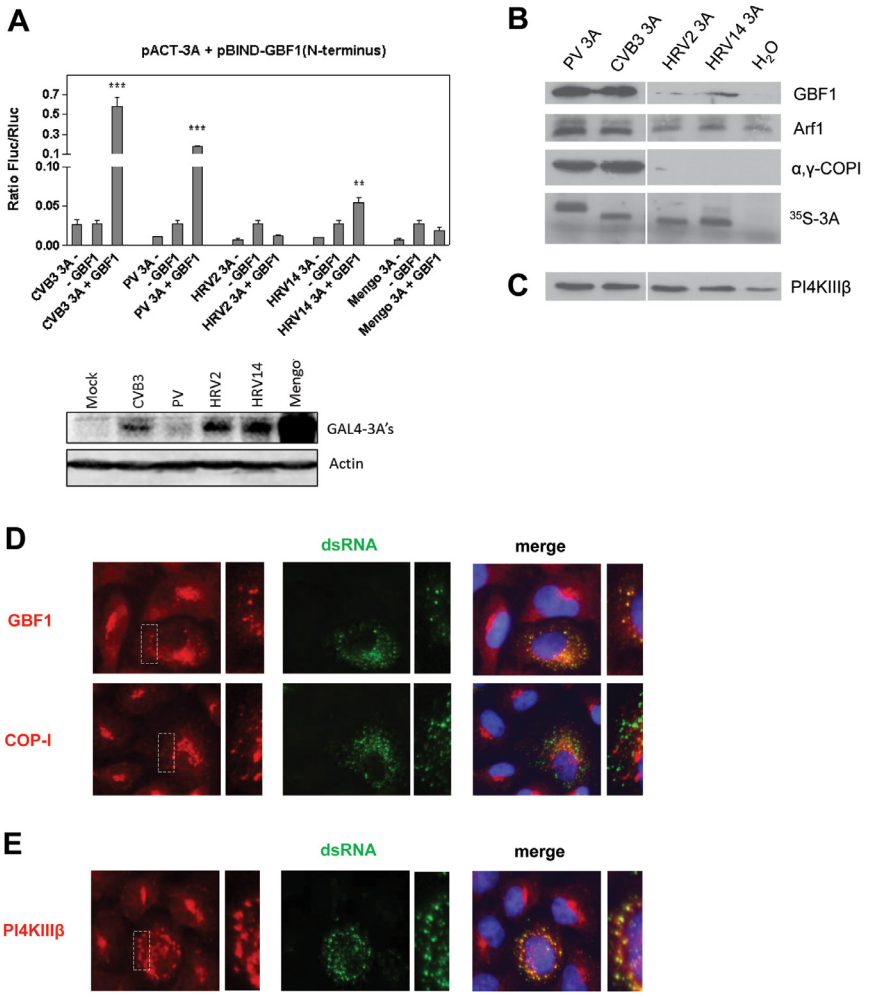

Figure 1. Interaction of HRV 3A with GBF1. A) Upper half: Interaction of different picornavirus 3A proteins with the N-terminus of GBF1 in the mammalian two-hybrid system (M2H). Bars show the means of the results from three samples with standard deviations (SD). Significant differences compared to the highest control determined with a Student's T-test are indicated as follows: **= P<0.01, ***= P<0.001. Lower half: Western Blotting of picornaviral GAL4-3A proteins tested in M2H. B and C) *In vitro* HeLa S10 cell extract assay. RNA coding for various enterovirus 3A proteins was translated in HeLa S10 cell extracts. Membranes were isolated by centrifugation and subjected to Western Blot analysis to detect the membrane-associated proteins using antibodies against GBF1, Arf1, and α,γ-COPI (B), or PI4KIIIβ (C). Efficiency of the translation reaction was assessed by 35S-methionine labeling. D and E) BGM cells were infected with HRV2 for 8 hours, followed by staining of dsRNA, an infection marker, together with GBF1 or COP-I (D) or PI4KIIIβ (E). Nuclei were visualized with DAPI. The dashed areas are enlarged on the right to show overlap between signals.

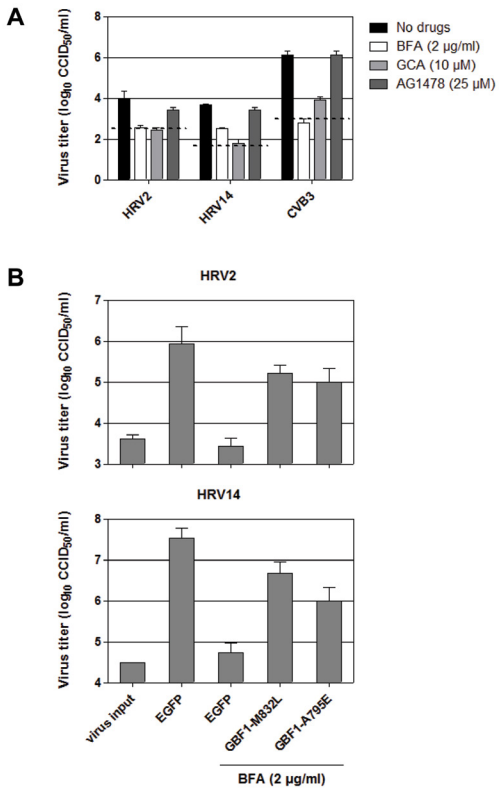

Figure 2. HRV replication depends on GBF1. A) Sensitivity of HRV2 and HRV14 to GBF1 inhibitors. HeLa R19 cells were infected with virus at low MOI for 30 min. After removal of the inoculum, fresh compound-containing medium was added to the cells. After 8 hours, cells were freeze-thawed to release intracellular viruses. Samples were titrated on HeLa R19 cells. CVB3 was used as a positive control. Virus input levels (determined at t = 0.5 hours) are indicated with a dashed line. B) Replication rescue experiment. HeLa R19 cells were transfected with plasmids encoding EGFP, BFA-resistant GBF1-M832L, or BFA-resistant GBF1-A795E. One day later, cells were infected with virus in the presence of BFA. After 24 hours, cells were freeze-thawed and samples were titrated on HeLa R19 cells.

the BFA-resistant GBF1-M832L and GBF1-A795E markedly protected HRV replication against the inhibitory effects of BFA. This result confirms that GBF1, and not BIG1 or BIG2, is important for HRV replication. Importantly, this result also indicates that a direct interaction between 3A and GBF1 is not essential for rescue of virus replication, as was previously suggested [10].

For PV and CVB3, PI4KIIIβ recruitment to replication sites is mediated by their 3A protein (1). Recently, we and others found that replication of all enteroviruses, including HRV, is abolished in the presence of PI4KIIIβ inhibitors (2, 16). Although this indicates that the kinase is also a crucial host factor for HRV, it is yet unknown whether HRV also actively recruits PI4KIIIβ to replication sites and whether this is mediated via its 3A protein, since the absence of a 3A-GBF1 interaction for HRV could affect recruitment of downstream factor PI4KIIIβ. Immunofluorescence analysis showed that PI4KIIIβ localized to replication sites in HRV-2 infected cells (Figure 1E), similar as observed for GBF1 (Figure 1D). To test whether PI4KIIIβ is recruited by 3A, the HeLa S10 cell extracts expressing HRV 3A proteins were also analyzed for the presence of PI4KIIIβ on membranes. Figure 1C shows that the 3A proteins of both HRVs induce an accumulation of PI4KIIIβ on membranes, similar to that of CVB3 and PV 3As. We corroborated this finding in intact cells by immunofluorescence. HeLa cells expressing EGFP-tagged 3A of CVB3, HRV2, and HRV14 were stained for endogenous GBF1 and PI4KIIIβ (Millipore). Basal levels of GBF1 were localized to 3A-containing membranes, but we did not observe an active recruitment (data not shown). The presence of GBF1 on 3A-containing membranes is most likely a consequence of HRV 3A localizing to and modifying Golgi membranes, where GBF1 (and also other Golgi proteins) normally resides. In contrast, the signal of PI4KIIIβ was substantially increased on 3A-containing membranes compared to the Golgi membranes in untransfected cells, indicating that all 3A proteins induced an active recruitment of PI4KIIIβ to membranes (Figure 3).

PI4KIIIβ was recently shown to be recruited to replication sites of Aichivirus (family *Picornaviridae*, genus *Kobuvirus*) via ACBD3 [5]. PV and CVB3 also interact with ACBD3, but this interaction seems dispensable for kinase recruitment (9, 10, 17). Moreover, ACBD3 depletion does not inhibit PV and CVB3 replication [7,8]. Since HRV 3A proteins lack the ability to bind GBF1, we considered the possibility that they recruit

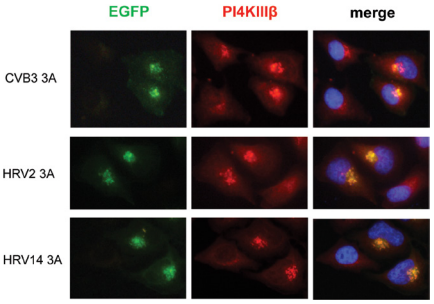

Figure 3. HRV 3A recruits PI4KIIIβ to membranes. EGFP-tagged 3A proteins were expressed in HeLa R19 cells one day prior to fixation. Cells were stained for endogenous PI4KIIIβ. Nuclei were visualized with DAPI.

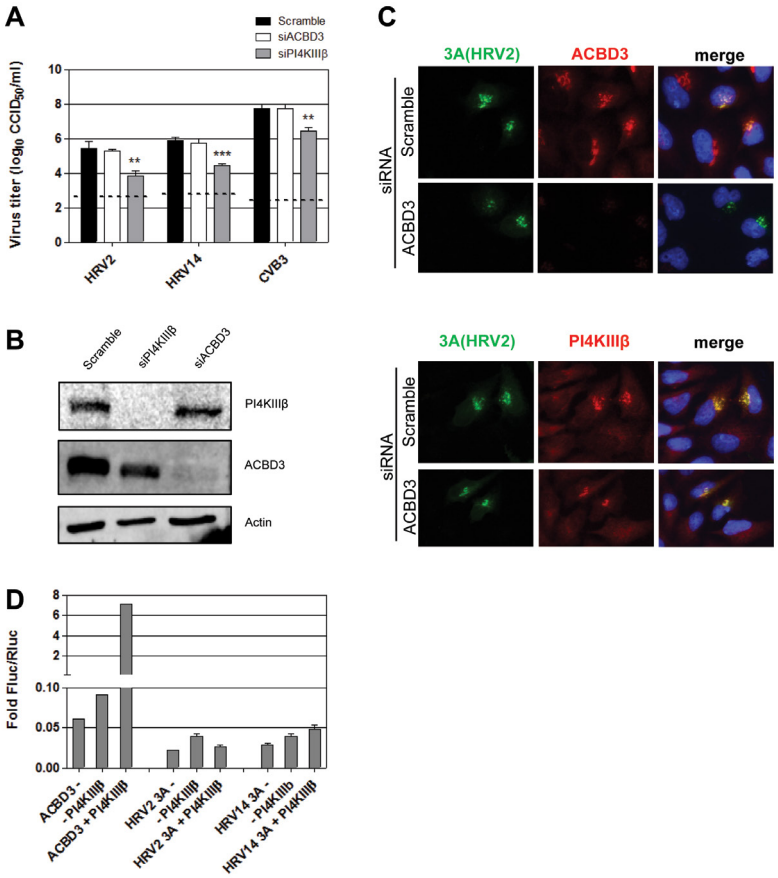

Figure 4. GBF1-independent recruitment of PI4KIIIβ by HRV2 3A does not rely on ACBD3. A) Sensitivity of HRV2 and HRV14 to depletion of ACBD3 or PI4KIIIβ by siRNA treatment. HeLa R19 cells (2000 cells) were reversely transfected with 2 pmol siRNA and 0.1 µl lipofectamine2000 mixed in 20 µl OptiMEM in a 96 well format. Two days post transfection, cells were infected with virus for 30 min, after which the medium was replaced with fresh medium. Samples were either immediately frozen to determine virus input levels (indicated with a dashed line) or incubated for 24 hours. Significant differences compared to the treatment with Scramble siRNA are determined with a Student's T-test (**= P<0.01, ***= P<0.001). B) Western Blot analysis of cell lysates confirming efficient knock-down by siRNA treatments. C) HRV2 3A recruits PI4KIIIβ in ACBD3-depleted cells. HeLa R19 cells were reverse-transfected with Scramble siRNA or siRNA against ACBD3 as described in A. After 48 h, cells were transfected with EGFP-tagged HRV2 3A. At 24 h after DNA transfection, cells were fixed and stained with antibodies against endogenous ACBD3 or PI4KIIIβ. D) Interaction of HRV 3A proteins with PI4KIIIβ in the mammalian two-hybrid system. Bars show the mean of three samples with SD.

PI4KIIIβ via ACBD3. Recently, we showed that HRV 3A proteins interact with ACBD3 in a M2H assay [7]. Here, we investigated whether ACBD3 is essential for HRV replication and kinase recruitment. To this end, HeLa cells were treated for three days with scramble siRNA (9), siRNA targeting hACBD3 (5, 9), or siRNA targeting PI4KIIIβ (9). Efficient knock-down was verified with Western Blot analysis (Figure 4B). Depletion of PI4KIIIβ significantly reduced HRV2 and HRV14 replication similar as for CVB3, whereas knock-down of ACBD3 had no effect on replication (Figure 4A). ACBD3 depletion also did not affect the ability of HRV2 3A to recruit PI4KIIIβ (Figure 4C). Thus, ACBD3 is not a crucial host factor for HRV replication and does not contribute to PI4KIIIβ recruitment.

Having found that GBF1 and ACBD3 are dispensable for PI4KIIIβ recruitment by HRV 3A, we tested the interaction between 3A and PI4KIIIβ in the M2H assay. In line with our previous results, PI4KIIIβ was interaction competent in this assay as shown by the signal detected together with ACBD3 (Figure 4D). No signal was detected for both HRV 3A proteins with PI4KIIIβ, which suggests that there is no direct interaction between HRV 3A and PI4KIIIβ. Together, these results indicate that HRV 3A proteins indirectly induce PI4KIIIβ recruitment.

In conclusion, we have shown that HRV replication depends on GBF1 and PI4KIIIβ, but not on ACBD3. The recruitment of the lipid kinase is mediated by the 3A protein in a GBF1- and ACBD3-independent manner. These findings are in agreement with our recent observations with ectopic expression of mutant CVB3 3As that were unable to bind GBF1, but still recruited the kinase in ACBD3-depleted cells (9). It remains to be elucidated by which viral protein GBF1 is recruited to HRV replication sites. PI4KIIIβ recruitment is mediated indirectly by HRV 3A, but possibly also by other viral proteins. For PV it was recently shown that, besides 3A, 2BC also recruits the kinase (18), which may also be the case for HRVs. Together, these results show that the replication requirements and recruitment strategies of rhinoviruses are more similar to other enteroviruses than initially believed based on the lack of a 3A-GBF1 interaction. Furthermore, these findings suggest that GBF1 binding, ACBD3 interaction, and PI4KIIIβ recruitment are three separate functions of enterovirus 3A proteins.

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Modulation of the host lipid landscape to promote RNA virus replication: the picornavirus encephalomyocarditis virus converges on the pathway used by hepatitis C virus

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ABSTRACT

Cardioviruses, including encephalomyocarditis virus (EMCV) and the human Saffold virus, are small non-enveloped viruses belonging to the *Picornaviridae*, a large family of positive-sense RNA [(+)RNA] viruses. All (+)RNA viruses remodel intracellular membranes into unique structures for viral genome replication. Accumulating evidence suggests that picornaviruses from different genera use different strategies to generate viral replication organelles (ROs). For instance, enteroviruses (e.g. poliovirus, coxsackievirus, rhinovirus) rely on the Golgi-localized phosphatidylinositol 4-kinase III beta (PI4KB), while cardioviruses replicate independently of the kinase. By which mechanisms cardioviruses develop their ROs is currently unknown. Here we show that cardioviruses manipulate another PI4K, namely the ER-localized phosphatidylinositol 4-kinase III alpha (PI4KA), to generate PI4P-enriched ROs. By siRNA-mediated knockdown and pharmacological inhibition, we demonstrate that PI4KA is an essential host factor for EMCV genome replication. We reveal that the EMCV nonstructural protein 3A interacts with and is responsible for PI4KA recruitment to viral ROs. The ensuing phosphatidylinositol 4-phosphate (PI4P) proved important for the recruitment of oxysterol-binding protein (OSBP), which delivers cholesterol to EMCV ROs in a PI4P-dependent manner. PI4P lipids and cholesterol are shown to be required for the global organization of the ROs and viral genome replication. Consistently, inhibition of OSBP expression or function efficiently blocked EMCV RNA replication. In conclusion, we describe for the first time a cellular pathway involved in the biogenesis of cardiovirus ROs. Remarkably, the same pathway was reported to promote formation of the replication sites of hepatitis C virus, a member of the *Flaviviridae* family, but not other picornaviruses or flaviviruses. Thus, our results highlight the convergent recruitment by distantly related (+)RNA viruses of a host lipid-modifying pathway underlying formation of viral replication sites.

AUTHOR SUMMARY

All positive-sense RNA viruses [(+)RNA viruses] replicate their viral genomes in tight association with reorganized membranous structures. Viruses generate these unique structures, often termed "replication organelles" (ROs), by efficiently manipulating the host lipid metabolism. While the molecular mechanisms underlying RO formation by enteroviruses (e.g. poliovirus) of the family *Picornaviridae* have been extensively investigated, little is known about other members belonging to this large family. This study provides the first detailed insight into the RO biogenesis of encephalomyocarditis virus (EMCV), a picornavirus from the genus *Cardiovirus*. We reveal that EMCV hijacks the lipid kinase phosphatidylinositol-4 kinase IIIα (PI4KA) to generate viral ROs enriched in phosphatidylinositol 4-phosphate (PI4P). In EMCV-infected cells, PI4P lipids play an essential role in virus replication by recruiting another cellular protein, oxysterol-binding protein (OSBP), to the ROs. OSBP further impacts the lipid composition of the RO membranes, by mediating the exchange of PI4P with cholesterol. This membrane-modification mechanism of EMCV is remarkably similar to that of the distantly related flavivirus hepatitis C virus (HCV), while distinct from that of the closely related enteroviruses, which recruit OSBP via another PI4K, namely PI4K IIIβ (PI4KB). Thus, EMCV and HCV represent a striking case of functional convergence in (+)RNA virus evolution.

INTRODUCTION

Picornaviridae is a large family of positive sense (+)RNA viruses comprising many clinically relevant human and animal pathogens. Members of the genus *Enterovirus* include important human viruses like poliovirus (PV), the causative agents of poliomyelitis, coxsackieviruses (CV), causing meningitis and myocarditis, and rhinoviruses (RV), responsible for the common cold and exacerbations of asthma and chronic obstructive pulmonary disease. Perhaps the best known non-human picornavirus is foot-and-mouth-disease virus (FMDV, genus *Aphthovirus*), which can cause devastating outbreaks in cattle leading to severe economic loss. Closely related to the *Aphthovirus* genus is the genus *Cardiovirus*, composed of three species: Theilovirus (TV), encephalomyocarditis virus (EMCV) and the more recently discovered Boone cardiovirus. The species Theilovirus includes, among others, Theiler's murine encephalomyocarditis virus (TMEV) and Saffold virus (SAFV), a human cardiovirus. While TMEV is known to cause enteric infections and sometimes more severe encephalitis or chronic infection of the central nervous system [1], as yet, SAFV has not been firmly associated with a clinical disease [2]. EMCV can infect a wide range of animals, of which rodents are considered the natural reservoir. Of all domesticated animals, pigs are most prone to EMCV infection, which can lead to fatal myocarditis [3], reproductive failure in sows or sudden death of piglets [4–6].

Like other (+)RNA viruses - such as hepatitis C virus (HCV), dengue virus (DENV), chikungunya virus (ChikV) and coronavirus (CoV) - picornaviruses replicate their genomic RNA on specialized, virus-modified intracellular membranes. These remodeled membranes termed replication organelles (ROs) arise from the concerted actions of both viral nonstructural proteins and co-opted host factors. Enteroviruses, for instance, hijack members of the secretory pathway for replication and formation of ROs [7,8]. Among the viral nonstructural proteins, 2B, 2C, 3A as well as their precursors 2BC and 3AB contain hydrophobic domains which confer them membrane-modifying properties [9–11]. Considerable interest has been given to the study of the small viral protein 3A, which is the key viral player involved in membrane rearrangements. 3A interacts with and recruits secretory pathway components GBF1 (Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1) and PI4KB (phosphatidylinositol-4 kinase type III isoform β) to ROs [12–16]. Despite intensive investigation, the role of GBF1 in enterovirus replication is not yet elucidated (reviewed in [8]). Recruitment of PI4KB to ROs leads to a significant local increase of membranes in its enzymatic product, PI4P [15]. This PI4P-rich environment serves to further recruit other essential viral and host factors to replication sites, such as the viral polymerase $3D^{pol}$, which is able to specifically bind PI4P *in vitro*. Recently, we and others revealed that PI4P plays a central role in enterovirus replication by recruiting the oxysterol-binding protein (OSBP) to ROs [17–19]. In uninfected cells, OSBP bridges the ER and Golgi membranes by binding to the ER integral membrane protein VAP-A and to PI4P and Arf1-GTP at the *trans*-Golgi [20]. Through its sterol-binding domain, OSBP shuttles cholesterol from ER to the Golgi and PI4P from the Golgi to the ER, thereby generating a lipid counterflow at ER-Golgi membrane contact sites (MCSs). In enterovirus infection, OSBP exchanges PI4P for cholesterol most likely at ER-RO MCSs [18]. The unique lipid and protein composition of enterovirus ROs determines their particular 3D architecture, which consists of a complex tubulo-vesicular network, as shown in cells infected with PV and coxsackievirus B3 (CVB3) [21,22].

The lipid transfer function of OSBP at membrane contact sites is not only vital for enteroviruses, but also for HCV [23]. HCV genome replication occurs in association with an ER-derived network of specialized membrane vesicles called the membranous web (MW). Like enterovirus ROs, the HCV MW is enriched in PI4P lipids and cholesterol [23–25]. In the case of HCV, PI4P are generated through recruitment and

activation of the ER-localized enzyme PI4KA (phosphatidylinositol-4-phopshate kinase type III isoform α) by the viral protein NS5A [24,26].

Thus far, information regarding virus-host interactions that govern the formation of cardiovirus ROs remains scarce. In a report by Zhang *et al*., it was suggested that autophagy supports EMCV replication [27]. The study showed that EMCV infection triggered an accumulation of autophagosome-like vesicles in the cytoplasm and that EMCV 3A colocalized with the autophagy marker LC3. However, inhibition of autophagy exerted only minor effects on virus replication [27], which argues against a strong implication of the autophagy pathway in cardiovirus genome replication and/or formation of ROs. Evidence for a role of autophagy in virus replication also exists for enteroviruses and flaviviruses, but rather related to non-lytic virus release or modulation of host innate immune responses than viral genome replication [28–31].

Based on observations that cardioviruses do not require GBF1 or PI4KB for replication [32–34], it is generally believed that cardiovirus replication strategies are distinct from those of enteroviruses. Here, we set out to elucidate whether cardiovirus replication depends on another PI4K isoform. By siRNA-mediated knockdown, we identified PI4KA as a key player in the replication of EMCV. EMCV 3A interacts with and recruits PI4KA to ROs, which increases local PI4P synthesis, eventually leading to downstream recruitment of OSBP. We show that the cholesterol-PI4P shuttling activity of OSBP is important for the global distribution of the ROs and virus genome replication. Our data reveal that, by exploiting the same cellular pathway, the cardiovirus replication strategy profoundly resembles that of the distantly related HCV and is dissimilar to those of other characterized picornaviruses and flaviviruses in this critical aspect. Thus, the similarity between EMCV and HCV is a striking case of functional convergence in virus-host interactions, indicating that diverse RNA viruses might have a limited choice of pathways in the remodeling of host membrane network for virus replication.

RESULTS

Cardiovirus replication requires PI4KA. Unlike enteroviruses, cardioviruses do not require PI4KB for replication [34]. To investigate whether other PI4Ks might be involved in cardiovirus replication, we depleted each of the four distinct cellular PI4Ks by siRNA-mediated gene knockdown using a set of siRNA sequences (Ambion) which we previously tested for efficiency and toxicity [35], and monitored the subsequent effects on replication of EMCV. We observed inhibitory effects on EMCV replication when silencing PI4KA, but not upon silencing of the other PI4Ks (Fig. 1A). To confirm the importance of PI4KA for EMCV replication, we performed another series of knockdown experiments using another set of siRNA sequences (Qiagen). Depletion of PI4KA, but not PI4KB, significantly reduced EMCV infection, measured by end-point titration of progeny virus production (Fig. 1B).

We next wondered which step in the virus life cycle is dependent on PI4KA. To omit the step of virus attachment and cell entry, EMCV RNA was *in vitro* transcribed and subsequently transfected in cells depleted of PI4KA by siRNAs. Virus replication was strongly inhibited upon PI4KA silencing, as measured by end-point titration of progeny virions (Fig. 1C). This indicated that PI4KA is involved in a post-entry step in the virus life cycle. To elucidate whether EMCV requires PI4KA for viral genome amplification, we infected cells with a *Renilla* luciferase-encoding EMCV (RLuc-EMCV) and quantified the luciferase activity as a measure of viral RNA replication. EMCV RNA replication was severely impaired in cells lacking PI4KA, but not PI4KB (Fig. 1D). We excluded that inhibition of EMCV replication by PI4KA silencing was due

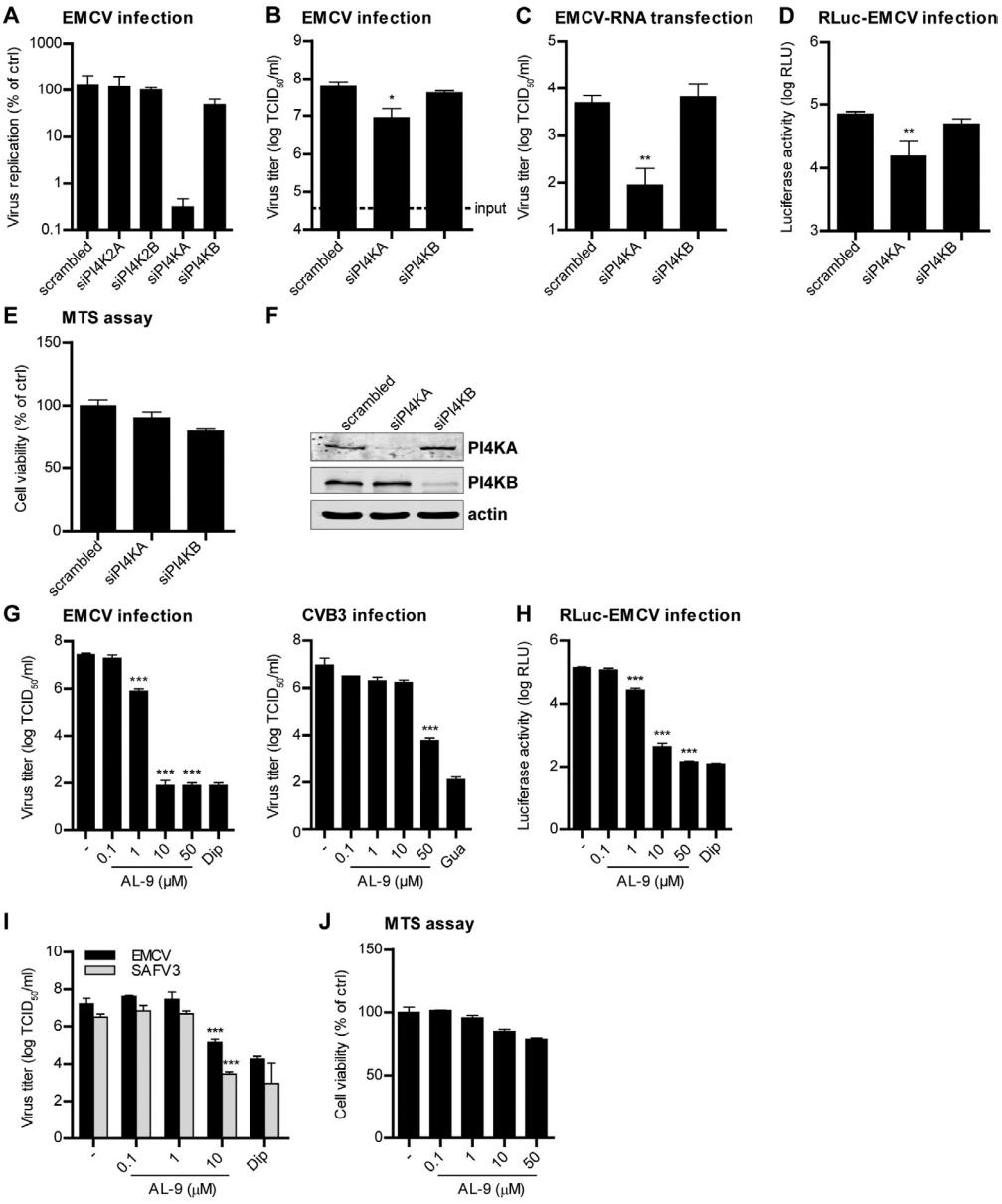

Figure 1. PI4KA is essential for cardiovirus replication. (A-D) Effects of PI4KA knockdown on EMCV infection. HeLa R19 cells were reverse transfected with siRNA against PI4K2A, PI4K2B, PI4KA, PI4KB or scrambled siRNA as a control (A) or siRNA against PI4KA, PI4KB or scrambled siRNA (B-D). At 48 h p.t., cells were infected with EMCV (A-C) or RLuc-EMCV (D) at an MOI of 0.1 or transfected with full-length infectious EMCV *in vitro*-transcribed RNA (C). After 8 h, cells were freeze-thawed to release intracellular virus particles and the total virus titers were determined by endpoint titration (A-C). Alternatively, cells were lysed and *Renilla* luciferase activity was determined as a measure of viral RNA replication (D). In parallel, a cell viability assay was performed to evaluate the cytotoxicity of the siRNA treatment (E). (F) Western blot analysis showing efficient knockdown of PI4KA and PI4KB. Actin was used as loading control. (G-I) AL-9 treatment inhibits cardiovirus replication. HeLa R19 cells were infected with virus at an MOI of 0.1 (G and H) or 1 (I), followed by AL-9 treatment for 8 h, after which cells were lysed and virus replication was measured by endpoint titration (G and I) or by determining the *Renilla* luciferase activity (H). Cytotoxicity of AL-9 was determined in a cell viability assay run in parallel (J). Bars represent mean values of triplicates ± standard error of the means (SEM). Means were statistically compared using unpaired t tests. *P < 0.05, **P<0.01; ***P<0.001.

to cytotoxic effects by a cell viability assay (Fig. 1E) and verified the knockdown efficiency by western blot analysis (Fig. 1F). Altogether, these results showed that PI4KA plays a key role in EMCV genome RNA replication.

Next, we investigated whether EMCV required the enzymatic activity of PI4KA using AL-9, a PI4K inhibitor that also blocks PI4KB, but at 5-fold higher concentration [36]. Cells were infected with EMCV or RLuc-EMCV at MOI 0.1 and treated with increasing concentrations of AL-9 for 8 h. Coxsackievirus B3 (CVB3), as well as all other enteroviruses, has been previously shown to hijack the Golgi-localized PI4KB for replication [15,34] and was included as a control. As measured by end-point titration and analysis of the luciferase activity (Fig. 1G and H), EMCV replication was efficiently inhibited by AL-9 in a dose-dependent manner with complete inhibition detected at 10 µM, while CVB3 replication was hampered only at 50 µM (Fig. 1G), which is in line with the 5-fold preference of AL-9 for PI4KA over PI4KB. Dipyridamole, a well-established inhibitor of EMCV RNA replication, was included here as positive control. Importantly, AL-9 inhibited EMCV replication also when infection was performed at high MOI (Fig. S1A, MOI 10). To corroborate that PI4KA activity is required for the step of viral genome replication, we performed a time of addition experiment in which AL-9 was added to the cells at different time points after infection with RLuc-EMCV. Similar as dipyridamole, AL-9 strongly inhibited replication when added up to 3h after infection (Fig. S1B), indicating that not entry but rather a step during genome replication was blocked by AL-9.

Next, we tested whether other members of the *Cardiovirus* genus also depended on PI4KA for replication. Similar to EMCV, replication of the human cardiovirus Saffold virus 3 (SAFV3) (species *Theilovirus*) was also sensitive to AL-9 treatment (Fig. 1I). The cell viability assay demonstrated that AL-9 treatment only exerted

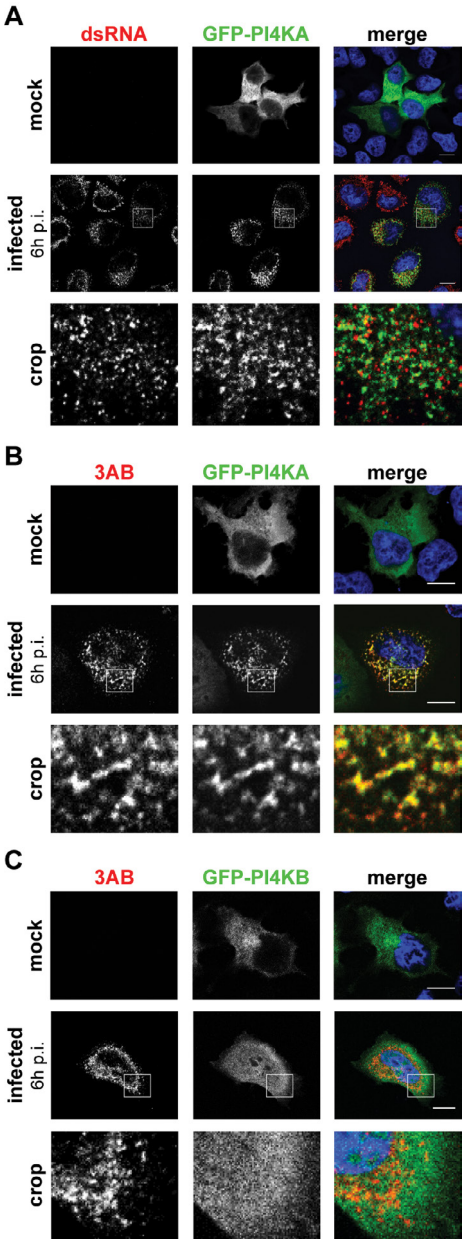

Figure 2. PI4KA is recruited to EMCV replication sites. HeLa R19 cells were transfected with plasmids encoding GFP-PI4KA (A and B) or GFP-PI4KB (C). The next day, cells were mock-infected or with infected EMCV at an MOI of 250. At 6 h p.i., cells were fixed and stained with antibodies against dsRNA as a marker of infection (A) or 3AB as a RO marker (B and C). Nuclei were stained with DAPI (blue). The crop panels at the bottom depict enlargements of boxed areas. Scale bars represent 10 µm.

slight cytotoxic effects at the highest concentration tested (Fig. 1J). These results indicated that different cardiovirus species required the enzymatic activity of PI4KA for genome replication.

PI4KA localizes to EMCV replication sites. Soon after infection, the cytoplasm of EMCV-infected cells accumulates an impressive amount of vesicular membranous structures [37,38]. As yet, there is little information available regarding which viral proteins and host factors are associated with these new virus-induced organelles [27,39]. We set out to investigate whether PI4KA was present at EMCV ROs. Despite repeated efforts, we were unable to detect the endogenous kinase by immunofluorescence staining in any of the cell lines tested. As an alternative, we chose to analyze possible changes in the subcellular distribution of ectopically expressed PI4KA upon EMCV infection. In mock-infected cells (Fig. 2A, upper panel), GFP-PI4KA was distributed diffusely throughout the entire cytoplasm, as previously reported by others [40,41]. In infected cells visualized by dsRNA staining, we instead observed a clear difference in the localization of the kinase, which was redistributed to discrete cytoplasmic punctae in a perinuclear region (Fig. 2A, lower panel).

We next aimed to elucidate whether these PI4KA punctae coincided with the viral ROs. The small picornaviral protein 3A and its precursor 3AB are membrane-associated and play key roles in viral RNA replication and recruitment of essential host factors [15,42–45]. Hence, we considered 3AB as a suitable marker for EMCV ROs and compared the staining of PI4KA to that of 3AB in infected cells. We observed a striking overlap of GFP-PI4KA with 3AB-positive structures (Fig. 2B, lower panels) and could confirm this phenotype when analyzing the localization of ectopically expressed PI4KA bearing an HA-tag (Fig. S2). By contrast, the signal for GFP-PI4KB, which was mainly localized at the Golgi in non-infected cells (Fig. 2C, top panel), failed to overlap with 3AB (Fig. 2C, lower panels). Interestingly, although in close proximity to dsRNA signals (Fig. 2A, lower panel), PI4KA did not clearly overlap with dsRNA (Fig. 2A, insets). Taken together, these data demonstrated that PI4KA is selectively recruited to EMCV ROs.

Interestingly, we noticed a loss of the typical Golgi localization of PI4KB in EMCV-infected cells (Fig. 2C, lower panel), suggesting that Golgi integrity might be affected upon EMCV infection. Prompted by this and our finding that EMCV utilizes the ER-localized PI4KA for replication, we set out to elucidate whether other ER or Golgi components are present at EMCV ROs. In order to use more antibody combinations in immunofluorescence, we constructed a recombinant EMCV bearing an HA-tag in the nonstructural protein 2C. The tag was introduced after the second amino acid, leaving the 2B-2C cleavage site intact (Fig. S3A), and did not impair virus replication (Fig. S3B). First, we checked whether 2C-HA and 3AB are present on the same membranes by immunofluorescence microscopy. Indeed, 2C and 3AB signals greatly overlapped (Fig. S3C), supporting the idea that these proteins occupy the same membranes of the ROs. Using this tagged EMCV, we noticed that the Golgi structure was indeed altered in infected cells, from 4 h p.i. onwards, as revealed by the dispersed pattern of both *cis*- and *trans*-Golgi markers GM130 (Fig. 3A) and TGN46, respectively (Fig. 3B). However, neither TGN46 nor GM130 were present at 2C-HA-positive structures, suggesting that EMCV ROs are not Golgi-derived. ERGIC53, a marker of the ER-Golgi intermediate compartment also appeared scattered throughout the cytoplasm in infected cells, but without overlapping 2C-HA (Fig. 3C). We next compared the localization of 3AB with Sec13 (COPII-coatomer complex component), an ER exit site (ERES) marker, and the ER marker calreticulin. While in non-infected cells, Sec13 displayed mainly a typical perinuclear localization, in EMCV-infected cells it appeared dispersed, but without significantly colocalizing with 3AB (Fig. 3D, Mander's colocalization coefficient M2 = 0.14 ± 0.01, fraction of Sec13 overlapping 3AB). We observed a greater degree of overlap between 3AB and calreticulin (Fig. 3E, M2 = 0.4 ± 0.02, fraction of calreticulin overlapping 3AB). Images

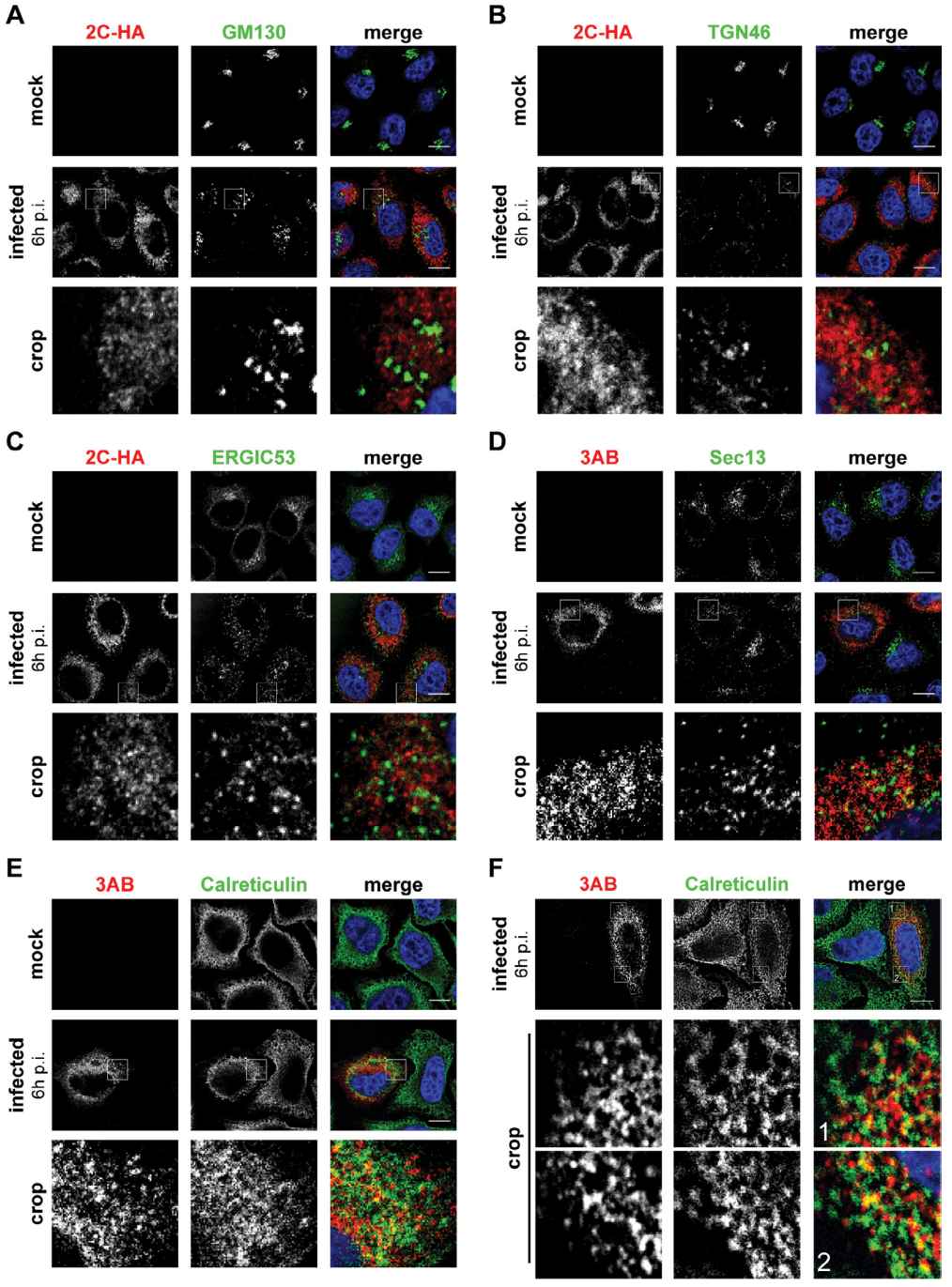

Figure 3. Localization of early secretory membranes in EMCV-infected cells. HeLa R19 cells were mock-infected or infected with EMCV-2C-HA (A-C) or EMCV (D-F) at MOI 10. 6 h later, cells were fixed and co-stained with antibodies against viral 3AB or HA as RO markers and antibodies against endogenous GM130 (cis-Golgi marker; A), TGN46 (trans-Golgi marker, B), ERGIC53 (ERGIC marker, C), Sec13 (ER exit site marker, D) or calreticulin (ER marker, E and F). Nuclei were stained with DAPI (blue). The crop panels at the bottom depict enlargements of boxed areas. Scale bars represent 10µm.

acquired with higher magnification revealed that most of 3AB was in close contact with ER tubules (Fig. 3F). Taken together, these data suggested that EMCV possibly replicates on ER-derived membranes.

EMCV 3A interacts with PI4KA. Based on the great overlap between PI4KA and 3AB and the drastic change in PI4KA pattern in infected cells, we hypothesized that PI4KA might be recruited to replication sites by interacting (directly or indirectly) with one or more of the viral nonstructural proteins. To investigate this, we used the stable cell line Huh7-Lunet/T7 that allows ectopic protein expression under the control of a T7 promoter and has been previously optimized and validated as a reliable and reproducible cellular system to study PI4KA-protein interactions by radioactive Co-IP assays [40,46]. Myc-tagged EMCV nonstructural proteins 2A, 2B, 2C, 3A, 3AB, 3C and 3D were individually co-expressed together with HA-PI4KA in Huh7-Lunet/T7 cells, radioactively labeled, and affinity purified from cell lysates using anti-myc specific antibodies. Autoradiography analysis showed that HA-PI4KA was specifically co-purified by 3A and 3AB, but not by the other viral proteins (Fig. 4A). To confirm this interaction by co-immunoprecipitation (co-IP) coupled with western blot analysis, myc-tagged EMCV 3A was co-expressed with HA-PI4KA and subjected to affinity purification using either monoclonal or polyclonal anti-myc antibodies. As shown in Fig. 4B, HA-PI4KA only interacted with EMCV 3A, but not with CVB3 3A, which interacts with PI4KB [15,16,45] and was included here as a negative control. These data implied that EMCV nonstructural protein 3A is responsible for PI4KA recruitment to ROs. Interestingly, a diffuse band just below 17 KDa appears to co-purify with EMCV 3C when HA-PI4KA is co-expressed (Fig. 4A, indicated by *). We reasoned this could be indicative of a temporal regulation of the PI4KA activity during infection via 3C-dependent degradation. To explore this possibility, we performed western blot analysis of endogenous PI4KA during the time course of infection, but did not detect any bands indicative of degradation, neither in Huh7-Lunet/T7 or HeLa R19 cells (Fig. S4). To test if 3A alone can recruit PI4KA, we examined by immunofluorescence the subcellular localization of HA-PI4KA when co-expressed with 3A, 3AB or 2B, which we considered as a negative control. When expressed alone, HA-PI4KA localized throughout the cell in a diffuse pattern (Fig. 4C, top panel), as previously described [40]. EMCV 3A- and 3AB-myc were both localized throughout the cytoplasm and at discrete punctate structures, of which a subset was also positive for PI4KA (Fig. 4C). 2B-myc was also distributed in punctae throughout the cytoplasm, but failed to recruit PI4KA (Fig. 4C). Collectively, these results indicated that EMCV 3A is the viral protein responsible for engaging PI4KA in replication.

EMCV alters PI4P homeostasis. While PI4KB produces PI4P at Golgi membranes, PI4KA is responsible for the synthesis of the PI4P pool at the plasma membrane, where it dynamically localizes [41,47–49]. Our finding that PI4KA activity was critical for EMCV RNA replication prompted us to investigate whether PI4P metabolism is altered during virus replication. Given that EMCV replicates on intracellular membranes, we monitored potential changes in the subcellular distribution of both plasma membrane (PM) and intracellular (IC) pools of PI4P in Huh7Lunet/T7 cells following EMCV infection. The two pools of PI4P can be selectively visualized using two different immunocytochemistry protocols previously established by Hammond et al [50]. While the plasma membrane pool of PI4P appeared unaffected in EMCV-infected cells (Fig. 5A), the intracellular PI4P distribution changed from a perinuclear, Golgi-like pattern in mock-infected cells to dispersed throughout the cytoplasm in EMCV-infected cells (Fig. 5A). We observed similar PI4P phenotypes in HeLa cells (Fig. S5), indicating that the observed effects were not cell line-specific. Notably, quantitative analysis of the fluorescent PI4P signals revealed a marked increase in the level of intracellular PI4P in infected cells (Fig. 5B).

To rule out a possible involvement of PI4KB in establishing the elevated PI4P levels observed in EMCV infected cells, we treated cells with the PI4KB inhibitor BF738735 (Compound 1) [34]. For simultaneous detection of PI4P and viral ROs by immunofluorescence, we infected cells with EMCV-2C-HA. Short treatment with BF738735 severely depleted the Golgi PI4P pool in non-infected cells (Fig. 5C), thus reflecting an effective inhibition of PI4KB activity. However, the PI4P phenotype remained unaltered in infected cells (Fig. 5C), demonstrating that the EMCV-induced accumulation of intracellular PI4P was not mediated by PI4KB. Most PI4P localized in the vicinity of 2C-HA, with at least a small subset of PI4P overlapping with 2C-HA (Fig. 5C). These data together with the finding that EMCV requires PI4KA

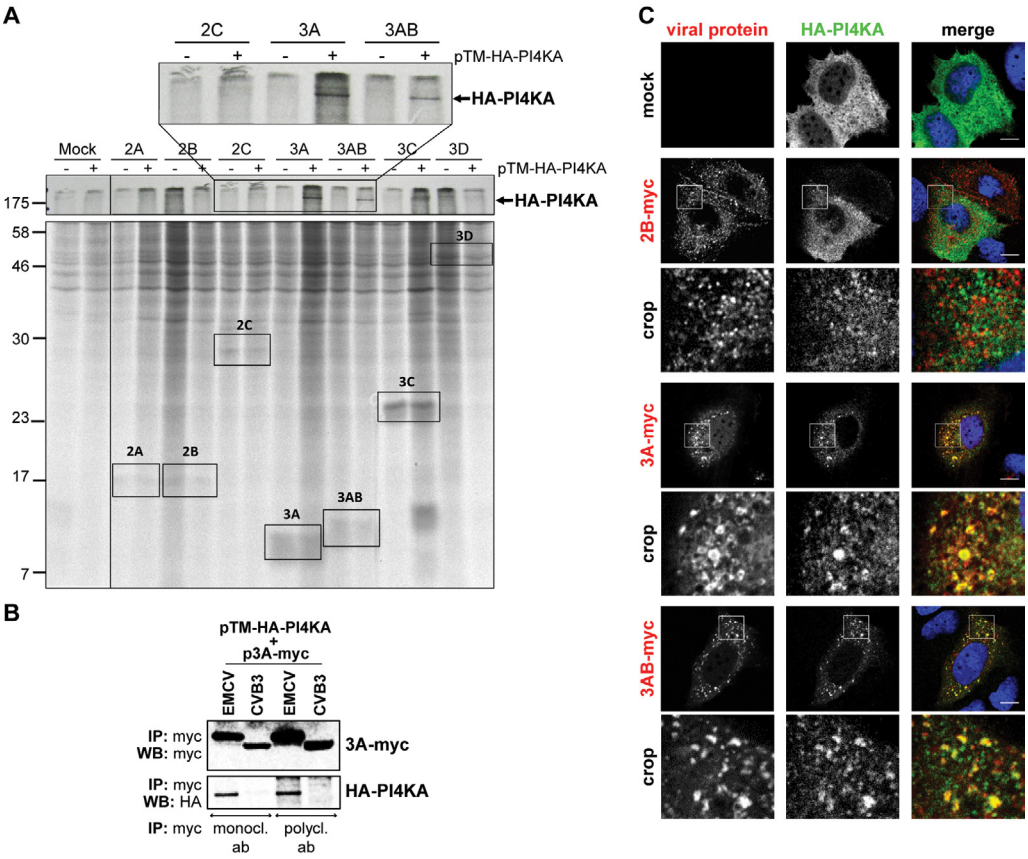

Figure 4. EMCV 3A interacts with PI4KA. (A) Only EMCV 3A and 3AB can interact with PI4KA. Huh7-Lunet/T7 cells were transfected with empty vector or plasmids encoding myc-tagged EMCV nonstructural proteins and co-transfected with pTM-HA-PI4KA where indicated. 7 h later, cells were radiolabeled for 16 h with [35S] methionine/cysteine-containing medium, lysed and subjected to immunoprecipitation using anti-myc antibodies. Samples were analyzed by SDS-PAGE and visualized by autoradiography. An enlargement of the boxed area at the top shows co-precipitation of HA-PI4KA with 3A- and 3AB-myc, but not with 2C-myc. (B) Huh7-Lunet/T7 cells were transfected with plasmids encoding HA-PI4KA and either myc-tagged EMCV 3A or CVB3 3A proteins. One day later, cells were lysed and subjected to immunoprecipitation using two different anti-myc antibodies. Captured complexes were separated by SDS-PAGE and subjected to western blot analysis using specific antibodies against the myc- or HA-tags. (C) Specific recruitment of PI4KA to 3A-positive membranes. Huh7-Lunet/T7 cells were cotransfected with the HA-PI4KA expression construct and either empty vector or plasmids encoding myc-tagged EMCV 2B, 3A or 3AB. The next day, cells were fixed and co-stained with antibodies against myc and HA to detect the overexpressed proteins. The crop panels at the bottom depict an enlargement of the boxed areas. Scale bars represent 10 µm.

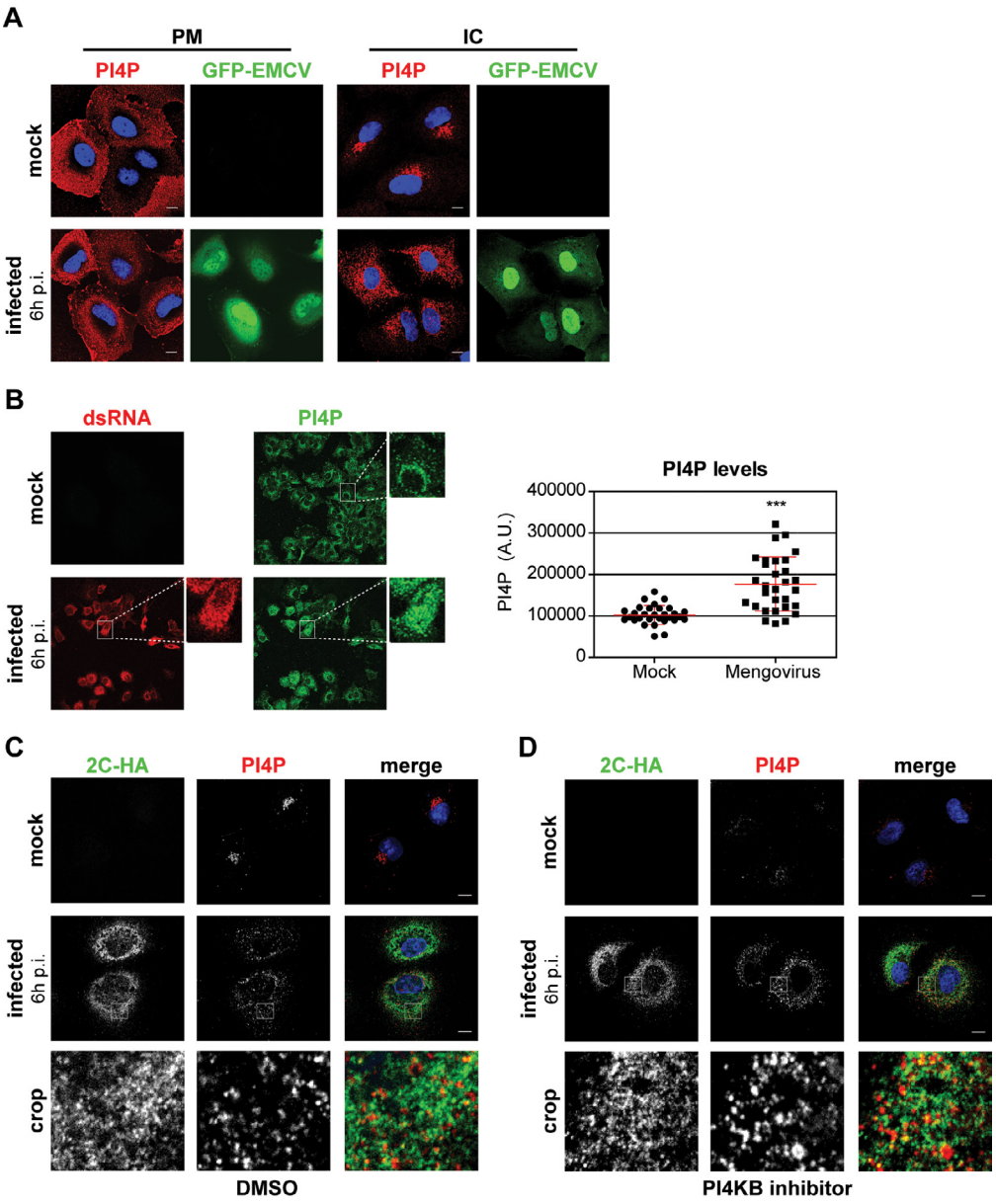

Figure 5. PI4P homeostasis is affected upon EMCV infection. (A) EMCV alters the distribution of intracellular PI4P lipids. Huh7-Lunet/T7 cells were mock-infected or infected with GFP-EMCV at an MOI of 10. At 6 h p.i., cells were fixed and stained with antibodies against PI4P using specific protocols for detection of plasma membrane and intracellular PI4P pools, as described previously [50]. Nuclei were stained with DAPI (blue). (B) Quantification of PI4P levels by immunofluorescence analysis. Huh7-Lunet/T7 cells were mock-infected or infected with EMCV at an MOI of 10. At 6 h p.i., cells were fixed and stained for intracellular PI4P and viral dsRNA. The intensities of fluorescent PI4P signals from whole-cell z-stacks were quantified using ImageJ. Shown are mean values ± SEM of 30 cells per condition. Means of mock and infected cells were statistically analyzed using the Mann–Whitney test. ***P<0.001. (C) EMCV ROs contain PI4P. Huh7-Lunet/T7 cells were mock-infected or infected with EMCV-2C-HA at an MOI of 10. At 5.5 h p.i., cells were treated with DMSO or 1μM BF738735 (PI4KB inhibitor) for 30 min, then fixed and stained for intracellular PI4P and HA using specific antibodies. Nuclei were stained with DAPI (blue). The crop panels at the bottom depict an enlargement of the boxed areas. Scale bars represent 10 μm.

activity suggested that PI4KA-derived PI4P lipids play a central role in EMCV genome replication.

OSBP supports EMCV genome replication downstream of PI4KA. Various cellular proteins carrying a PI4P-binding domain called the pleckstrin-homology domain (PH), such as the ceramide-transfer protein (CERT), four-phosphate-adaptor protein 1 (FAPP1), or the oxysterol-binding protein (OSBP) can sense and specifically bind PI4P lipids [47,51–53]. Recently, we and others showed that enteroviruses generate PI4P-enriched membranes to recruit OSBP, which in turn exchanges PI4P for cholesterol at ROs [17,18,54]. Moreover, we showed that EMCV is sensitive to itraconazole, which we identified to be an OSBP inhibitor [17], and that cholesterol shuttling is important for EMCV replication [54]. We therefore reasoned that in EMCV-infected cells one purpose of PI4P lipids might be to recruit OSBP at replication membranes to support viral RNA replication. To test if OSBP is required for EMCV replication, we efficiently reduced OSBP expression in HeLa cells by siRNA gene silencing (Fig. 6A) and evaluated the subsequent effects on EMCV replication by end-point titration analysis. Replication of EMCV was significantly reduced in cells in which OSBP was depleted compared to control-treated cells (Fig. 6A), indicating that OSBP is required for efficient replication. We further used OSW-1, an OSBP ligand that interferes with normal OSBP functioning [55], to pharmacologically inhibit OSBP and analyze if its lipid transfer function is linked to EMCV infection. Using luciferase-encoding EMCV, we observed a complete inhibition of genome RNA replication after 7 h of treatment with OSW-1 at nanomolar concentrations, with no cytotoxicity present (Fig. 6B). A similar inhibition by OSW-1 was observed when infection was performed at high MOI (Fig. S6, MOI 10). Furthermore, by performing OSW-1 time of addition experiments, we excluded the possibility that OSBP was involved in early steps in the virus life cycle (Fig. 6C). Similar results were obtained when using 25-hydroxycholesterol (25-HC, Fig. 6C), another established OSBP ligand [20,56].

Next, we wondered whether endogenous OSBP was present at EMCV ROs and if so, whether this localization was dependent on the PI4P pool generated by PI4KA. To this end, cells were infected with EMCV for 5.5 h and then treated with DMSO or AL-9 for 30 min to acutely deplete PI4P, prior to immunofluorescence analysis. While in non-infected cells OSBP localized throughout the cytoplasm and at the Golgi, OSBP was mainly found at ROs in infected cells, where it largely colocalized with 3AB (Fig. 6D, Pearson's correlation coefficient = 0.71). Since other Golgi proteins were not present at the ROs (Fig. 3A and B), these results suggested that OSBP is specifically recruited by EMCV. Following inhibition of PI4KA by short treatment with AL-9, we observed a strong and significant reduction of OSBP and 3AB colocalization (Fig. 6D, Pearson's coefficient = 0.58). Importantly, the subcellular localization of OSBP in non-infected cells was not affected by AL-9 treatment (Fig. 6D), demonstrating that the presence of OSBP at EMCV replication structures is conditioned by PI4KA-produced PI4P.

Given the colocalization of OSBP with 3AB, we sought to verify whether EMCV 3A was responsible for OSBP recruitment. To this end, myc-tagged EMCV 3A-, 3AB- or 2B-myc were ectopically expressed in Huh7-Lunet/T7 cells and recruitment of endogenous OSBP was analyzed by immunofluorescence analysis. In cells expressing 3A and 3AB, OSBP was redistributed in punctate structures throughout the cytoplasm, with some of these punctae colocalizing with 3A/3AB (Fig. 6E). By contrast, OSBP remained localized at the Golgi and did not localize at 2C-positive punctae (Fig. 6E). These data indicated that during EMCV infection, OSBP is recruited to ROs via 3A.

OSBP transfers cholesterol to EMCV ROs in a PI4P-dependent manner. To test whether OSBP is involved in transferring cholesterol to ROs in a PI4P-dependent manner, cells were infected with

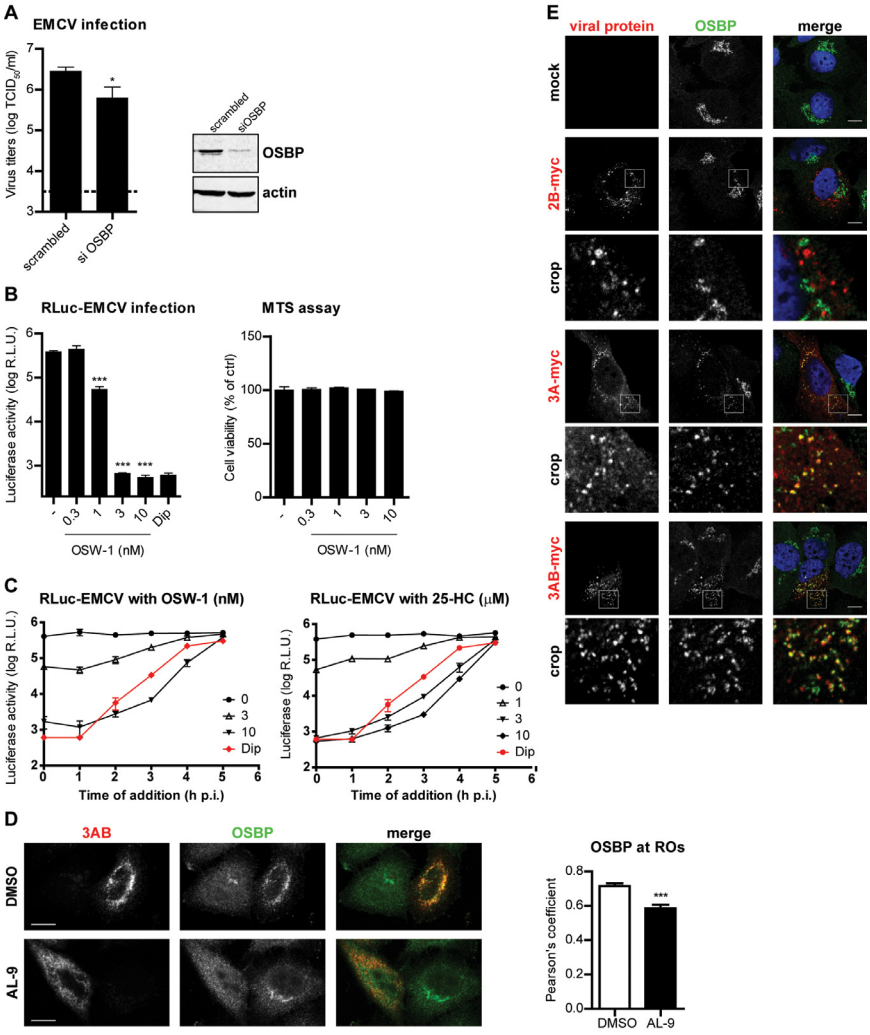

Figure 6. OSBP is a PI4KA effector important for EMCV replication. (A) OSBP knockdown reduces EMCV replication. HeLa R19 cells were reverse transfected with siRNA against OSBP or scrambled siRNA as a control. At 48 h p.t., cells were infected with EMCV at MOI 0.1. After 8 h, cells were freeze-thawed to release intracellular virus particles and total virus titers were determined by endpoint titration. OSBP knockdown efficiency was verified by western blot analysis. Actin was used as loading control. (B) Antiviral effects of the OSBP inhibitor OSW-1 against EMCV. HeLa R19 cells were infected with RLuc-EMCV at MOI 0.1, followed by treatment with OSW-1 at the indicated concentrations. *Renilla* luciferase levels at 8 h p.i were determined as a measure of virus genome replication. Cell viability was measured in parallel. (C) HeLa R19 cells were infected with RLuc-EMCV at an MOI of 1 and treated at the indicated time points after infection with DMSO, OSW-1 or 25-HC at the specified concentrations. At 7 h p.i., cells were lysed and *Renilla* luciferase activity was measured. (D) OSBP localization at EMCV ROs is PI4P-dependent. HeLa R19 cells were infected with EMCV at MOI 10. At 5.5 h p.i., cells were treated for 30 min with DMSO or 10 μM AL-9 to inhibit PI4KA activity, then fixed and subjected to immunofluorescence analysis using antibodies against OSBP and viral 3AB. Colocalization of OSBP with 3AB was determined by calculating the Pearson's correlation coefficients for at least 15 cells for each condition. (E) Specific recruitment of OSBP to 3A-positive membranes. Huh7-Lunet/T7 cells were transfected with empty vector or plasmids encoding myc-tagged EMCV 2B, 3A or 3AB. One day later, cells were fixed and co-stained with antibodies against myc and endogenous OSBP. The crop panels at the bottom depict enlargements of boxed areas. Scale bars represent 10 μm. Shown are mean values ± SEM. Means were statistically compared using either unpaired t tests (A and B) or the Mann–Whitney test (D). *P < 0.05, ***P<0.001.

EMCV for 4 h, treated with AL-9 or OSW-1 for 2 h to block PI4KA activity or OSBP function respectively, and subjected to immunofluorescence analysis. In non-infected cells, cholesterol mainly localizes at endosomes in the perinuclear area and at the plasma membrane, as visualized by filipin staining [54]. In infected cells treated with DMSO, we detected cholesterol primarily colocalizing with 3AB-positive structures (Pearson's coefficient = 0.62), while in drug-treated cells this colocalization was markedly reduced (Fig. 7, Pearson's coefficient = 0.32 for AL-9 and 0.39 for OSW-1). This result confirmed that EMCV ROs acquire cholesterol through the actions of both PI4KA and OSBP.

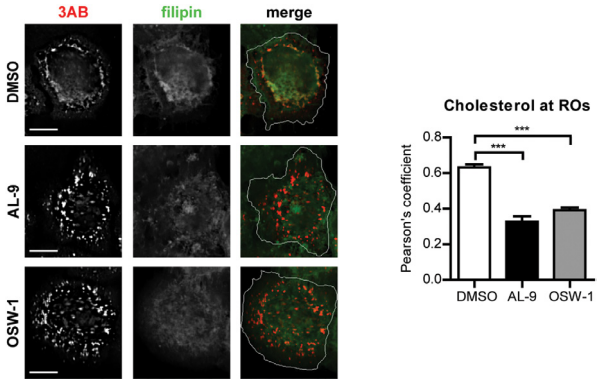

Figure 7. Cholesterol delivery to EMCV ROs depends on PI4KA and OSBP activities. (A) HeLa R19 cells were infected with EMCV at MOI 10. At 4 h p.i., cells were treated with DMSO, 10 µM AL-9 or 3nM OSW-1. After 2 h, cells were fixed, followed by staining with antibodies against 3AB or filipin for cholesterol detection. The merged panels also depict the outline of the cell (white line). Scale bars represent 10 µm. Pearson's correlation coefficients of colocalization between filipin and 3AB were calculated for at least 15 cells for each condition. Shown are mean values ± SEM. Means were statistically compared using the Mann–Whitney test. ***P<0.001.

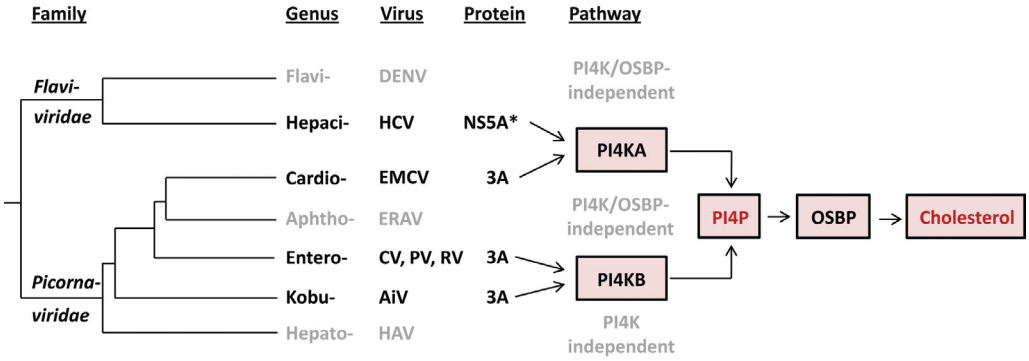

Figure 8. Evolution of the recruitment of lipid-modulating pathways by picorna- and flaviviruses. Our current understanding of the major elements of lipid-modulating pathways targeted by proteins of picorna- and flaviviruses in the context of the virus phylogeny is presented. Only viruses characterized so far with respect to this function are depicted. The tree branches are of arbitrary scale, while the branching of picornaviruses reflects the topology of maximum-likelihood RdRp-based tree according to Gorbalenya & Lauber, 2010 [80], and the entire tree is rooted according to Gibrat et al., 2013 [81]. *In addition to NS5A, NS5B has also been suggested to be involved in the interaction with PI4KA [24,46]. Abbreviation of virus names are as follows: DENV, dengue virus; HCV, hepatitis C virus; EMCV, encephalomyocarditis virus; ERAV, equine rhinitis A virus; CV, coxsackievirus; PV, poliovirus; RV, rhinovirus; AiV, Aichivirus; HAV, hepatitis A virus.

DISCUSSION

(+)RNA viruses display immense genetic diversity, yet they all rely on remodeled membranes for viral genome replication. A diverse array of cellular organelles can be remodeled into viral replication structures. For instance, picornaviruses from the genera *Enterovirus* and *Kobuvirus* are thought to replicate at modified Golgi membranes [15,45,57], while the flavivirus HCV replicates on a membranous web originated from the ER [58]. To do so, viruses rewire host pathways involved in lipid synthesis and transport to generate replication membranes with unique lipid signatures [59–62]. How picornaviruses from the genus *Cardiovirus* build their ROs is currently unknown. Here, we identified PI4KA and OSBP as essential host factors for genome replication of the cardiovirus EMCV. Our data suggest that EMCV ROs may be derived from the ER and that PI4KA was recruited to ROs by interacting with the viral protein 3A(B). PI4KA recruitment led to a significant increase of intracellular PI4P levels in infected cells, which proved important for the downstream recruitment of OSBP. Finally, data are presented suggesting that the OSBP-mediated exchange of PI4P and cholesterol at RO-MCSs is critical for EMCV genome replication and the global organization of ROs.

Membrane alterations in the cytoplasm of cardiovirus-infected cells were already observed decades ago by electron microscopy [37,38,63]. As also described for enteroviruses, cardiovirus-induced membranes consist of perinuclear clusters of heterogeneous single- and double-membrane vesicles (DMVs). While recent studies greatly contributed to our understanding of the origin and biogenesis of enterovirus ROs [18,19,21,22,64,65], for cardioviruses these details have remained scarce. In a report by Zhang *et al.* it was proposed that EMCV subverts the autophagy pathway to promote virus replication and RO formation [27]. The authors observed induction of autophagy and accumulation of cytoplasmic double-membrane vesicles (DMVs), a hallmark of autophagosomes, upon EMCV infection. However, inhibition of autophagy had stronger effects on extracellular than intracellular virus yields, which pointed towards a role of autophagy in virus release. Indeed, recent studies using enteroviruses and flaviviruses support the hypothesis that autophagy-derived membranes rather serve as means of non-lytic virus release and spread than as a membrane source for the viral ROs [28–31].

As opposed to enteroviruses, members of the *Cardiovirus* genus are insensitive to GBF1 depletion by siRNA [32] or treatment with BFA [33,39,66], a compound that targets GBF1 and subsequently blocks activation of Arf1, a key regulator of membrane trafficking in the secretory pathway. Furthermore, cardiovirus replication does not require the Golgi-localized PI4KB, which is essential for enterovirus replication [34]. Collectively, these data suggested that cardioviruses do not rely on Golgi components for replication. In line with these previous findings, we here present data suggesting that EMCV may derive its ROs from ER membranes. Confocal microscopy analysis revealed that EMCV nonstructural proteins partially overlapped with the ER marker calreticulin, but not with markers of ERES, ERGIC, *cis*- or *trans*-Golgi network, which appeared dispersed in infected cells, even at the earliest stages of infection. Furthermore, PI4KA, which normally resides at the ER network, was redistributed in EMCV-infected cells to discrete cytoplasmic structures that also contained the viral protein 3AB. Interestingly, the majority of PI4KA-positive punctae were detected in close proximity to viral dsRNA, but did not completely overlap with dsRNA, suggesting a spatial segregation of dsRNA from the viral replication membranes, previously also shown for coronaviruses [67,68].

PI4P and cholesterol are key lipid components of EMCV ROs

Using a pharmacological inhibitor of PI4KA, we prove that EMCV and SAFV, which belong to distinct cardiovirus species, both require the lipid kinase activity for replication. In agreement with this result, we observed elevated PI4P levels at intracellular membranes in infected cells, suggesting an important role of PI4P lipids in cardiovirus replication. In non-infected cells, OSBP plays a critical role in lipid homeostasis by exchanging cholesterol for PI4P at the interface of ER and Golgi membranes, to which it localizes under normal conditions [20]. In this process, PI4P lipids also serve as a membrane anchor for OSBP. We hypothesized that in cardiovirus infection, PI4P may serve to recruit OSBP and cholesterol to viral replication sites. Indeed, OSBP was present at EMCV ROs, where it colocalized with the viral protein 3AB. This colocalization was markedly reduced upon AL-9 treatment, demonstrating that OSBP is recruited through PI4KA-produced PI4P. OSBP is an essential cardiovirus host factor, since both genetic depletion by siRNA treatment and pharmacological inhibition by OSW-1 and 25-HC blocked viral genome replication. Cholesterol was redistributed to EMCV ROs upon infection, and treatment with AL-9 or OSW-1 resulted in a significantly reduced colocalization of cholesterol with 3AB, arguing that accumulation of cholesterol at ROs is mediated by both PI4KA and OSBP. These data are in agreement with our recent findings that cholesterol shuttling is important for cardiovirus genome replication [54] and that cardioviruses are sensitive to itraconazole, which we recently discovered to be an. inhibitor of OSBP [65].

Our results indicate that PI4P and cholesterol are vital for the global organization of EMCV ROs. However, as these lipids fulfill multiple functions in various cellular processes [53,69–71], other roles in virus replication should be envisaged. A potential task of PI4P in virus replication may be linked to the PI(4,5)P2 synthesis pathway, since PI4P is the major precursor of PI(4,5)P2 lipids, which were recently attributed an important role in HCV replication [72]. Cholesterol homeostasis was recently shown to play an important role in efficient PV polyprotein processing [73]. Whether cholesterol also ensures a proper microenvironment that supports cardiovirus polyprotein processing remains to be determined. Interestingly and in apparent parallel with the distantly related enteroviruses, exploitation of the PI4K-OSBP pathway by HCV correlates with the induction of membranes of positive curvature [58]. By contrast, the flavivirus DENV, although closely related to HCV, does not require PI4K or OSBP [23] and generates membranes of negative curvature [74]. Hence, the interplay between PI4P and cholesterol may dictate the positive curvature of the membranes at which diverse RNA viruses replicate their genomes.

Through co-IP assays, we identified PI4KA as a novel interaction partner of EMCV proteins 3A and its precursor 3AB. EMCV 3A is a small protein (88 amino acids) of unknown structure, containing a predicted hydrophobic domain in the C-terminus half. Expression of 3A alone was sufficient for PI4KA recruitment in intact cells, arguing that in infection, PI4KA is recruited to ROs by this viral protein. Enteroviruses and kobuviruses recruit PI4KB to ROs also via their 3A protein [15,16,45,57], raising the possibility that diverse picornaviruses might use an evolutionary conserved and 3A-mediated mechanism to generate PI4P-enriched membranes. However, the 3A proteins of entero-, kobu- and cardioviruses do not share any apparent sequence similarity, their name simply reflecting the occupancy of the same locus (3A) in the respective viral genomes. With the exception of their catalytic domain, also the PI4KA and PI4KB isoforms do not share any sequence similarity, [75]. Furthermore, unlike the 3A of most enteroviruses, cardiovirus 3A does not interact with GBF1 nor blocks protein transport in the secretory pathway when expressed alone [76], highlighting the functional diversification associated with these small viral proteins.

EMCV and HCV converged to recruit a common lipid-modifying pathway in building replication sites
Several lines of evidence suggest that the picornavirus EMCV and the distantly related flavivirus HCV have evolved to exploit common host components in assisting virus RNA replication. First, HCV genome replication occurs at the "membranous web" (MW), a network of single and DMVs that, like the EMCV RO, also mainly originates from the ER [58]. Second, both EMCV and HCV express a viral protein dedicated to recruitment of PI4KA, in order to induce a PI4P-rich environment at the replication sites [24,46]. Third, in HCV infection, PI4P lipids were also shown to be important for the recruitment of OSBP, which mediates cholesterol transfer to the MW [23]. Fourth, inhibition of either PI4KA or OSBP induced clear alterations in the global distribution of EMCV ROs, which appeared more "clustered" upon treatment with AL-9 or OSW-1. A similar clustering effect was also observed for replication structures of the HCV MW upon PI4KA or OSBP inhibition [23,24], whereas no obvious disruption of the enterovirus ROs was observed upon PI4KB or OSBP inhibition [18,19,65]. Together, these observations indicate that EMCV and HCV replication structures share critical host components, and possibly also a similar architecture, although the latter still remains to be determined.

Based on at least two lines of emerging evidence in the context of phylogeny of flavi- and picornaviruses, EMCV and HCV have likely converged on rather than retained their functional similarities upon divergence from the common ancestor (Fig. 8). First, the observed commonalities between EMCV and HCV are not shared by other characterized viruses in their respective families, indicating that they are not a manifestation of the properties conserved among the two families. For instance, picornaviruses from different genera exhibit different host factor requirements. Members of the *Cardiovirus* genus hijack the ER-localized PI4KA (this study), whereas members of the *Enterovirus* and *Kobuvirus* genera depend on the Golgi-localized PI4KB [15,34,57]. In contrast, equine rhinitis A virus (ERAV, member of *Aphthovirus* genus, which is prototyped by FMDV) and hepatitis A virus (*Hepatovirus* genus) seem to replicate independent of both PI4KB and PI4KA (Fig. S7 and [34,77]). Likewise, the flaviviruses DENV and WNV, representing a sister genus to that of HCV, do not rely on either PI4KA or PI4KB [23,78]. While DENV was also shown not to require OSBP [23], for WNV this is not known yet. Importantly, cardioviruses targeting PI4KA occupy a lineage that is farther from the root compared to those of entero- and kobuviruses targeting PI4KB (Fig. 8). This phylogenetic pattern is indicative of the relatively recent emergence of the EMCV-specific target properties. Second, EMCV and HCV employ apparently unrelated proteins to mediate the interaction with PI4KA, namely 3A and NS5A (although HCV NS5B may contribute as well [24,46]). Both proteins are membrane-bound, albeit through a hydrophobic domain located at either N-terminus (NS5A) or in the C-terminus-half (3A), and each includes another region which is among the least conserved in the nonstructural proteins of the respective families [79,80].

Our study contributes to the hypothesis that viruses may be confronted with powerful constraints that limit the diversity of host pathways recruited for efficient replication. Thus, a common pathway is used by different RNA viruses which either only moderately diverged (e.g. different species of same genus) or converged on a host target while diverging profoundly (different families – e.g. EMCV and HCV). To date, only a small number of (+)RNA viruses have been studied in terms of host lipid requirements. Identification of the lipid pathways used by other viruses will hopefully provide a deeper insight on the constraints that viruses are confronted with during the endeavor to replicate their genome.

MATERIALS AND METHODS

Cells and reagents. Buffalo green monkey (BGM) kidney cells, baby hamster kidney 21 (BHK-21) and HeLa R19 cells were grown at 37oC and 5% CO2 in Dulbecco's modified Eagle's medium (DMEM, Lonza) supplemented with 10% fetal bovine serum (FBS). Huh7Lunet/T7 cells (provided by R. Bartenschlager, Department of Molecular Virology, University of Heidelberg, Heidelberg, Germany) [82] were grown in DMEM (Lonza) supplemented with 10% FBS and 10 ug/ml Blasticidin (PAA). BGM cells were purchased from ECACC and BHK-21 cells were purchased from ATCC. HeLa R19 cells were obtained from G. Belov (University of Maryland and Virginia-Maryland Regional College of Veterinary Medicine, US) [83]. AL-9 and OSW-1 were a kind gift from J. Neyts (Rega Institute for Medical Research, University of Leuven, Leuven, Belgium) and M.D. Shair (Department of Chemistry and Chemical Biology, Harvard University, Cambridge, USA) respectively. 25-HC was purchased from Santa Cruz. BF738735 [84] was provided by Galapagos NV. Filipin III and dipyridamole were from Sigma.

Plasmids. Constructs pTM-HA-PI4KA [24], pEGFP-PI4KA (provided by G. Hammond, NICHD, National Institutes of Health, Bethesda, USA) [41,85] and p3A(CVB3)-myc [86] were described previously. pGFP-PI4KB was a kind gift from N. Altan-Bonnet (Laboratory of Host-Pathogen Dynamics, National Institutes of Health, Bethesda, USA). To generate C-terminal myc-tagged EMCV proteins, genes encoding EMCV nonstructural proteins 2A, 2B, 2C, 3A, 3AB, 3C and 3D were amplified by PCR using the plasmid pM16.1 [87] and primers introducing restriction sites BamHI and HindIII. pM16.1 contains the full-length infectious cDNA sequence of EMCV, strain mengovirus. The PCR products were then cloned into the p3A(CVB3)-myc backbone from which the CVB3-3A gene was removed using the same restriction enzymes. To allow ectopic expression of PI4KA under a CMV promoter, the gene encoding HA-PI4KA was amplified by PCR using pTM-HA-PI4KA as template and introduced in the pEGFP-N3 backbone using restriction enzymes SalI and NotI. EMCV-2C-HA infectious clone was generated by introducing the HA coding sequence (YPYDVPDYA) in-frame after codon 2 in 2C of pM16.1 using mutagenesis primers and the Q5 Site-Directed Mutagenesis Kit (New England Biolabs).

Viruses and infections. EMCV, EMCV-2C-HA and RLuc-EMCV, which contains the *Renilla* luciferase gene upstream of the capsid coding region [54], were obtained by transfecting BHK-21 cells with RNA transcripts derived from full length infectious clones pM16.1, pM16.1-2C-HA and pRLuc-QG-M16.1, respectively, linearized with BamHI. GFP-EMCV, which contains the EGFP gene upstream the capsid region, was generated similar as RLucEMCV [54]. CVB3 (strain Nancy) was obtained by transfecting BGM cells with RNA transcripts of the full length infectious clone p53CB3/T7 [86] linearized with SalI. Saffold virus (type 3) was described previously [2]. ERAV (NM11/67) was kindly provided by David Rowlands and Toby Tuthill (University of Leeds, United Kingdom). Virus infections were performed by incubating subconfluent cell monolayers for 30 min at 37oC with virus, after which the virus-containing medium was removed and fresh (compound-containing) medium was added to the cells (t=0). In the time-of-addition experiments, medium without compound was added at t=0 and replaced by medium with compound at the indicated time points. At the given time points post-infection, cells were either fixed for immunolabeling, freeze-thawed to determine virus titers or, in the case of RLuc-EMCV, lysed to determine replication by measuring the intracellular *Renilla* luciferase activity using the *Renilla* Luciferase Assay System (Promega). Virus titers were determined by endpoint titration according to the method of Reed and Muench and expressed as 50% tissue culture infective doses (TCID50).

Immunofluorescence microscopy. HeLa R19 or Huh7Lunet/T7 cells were grown to subconfluency

on coverslips in 24-well plates. Where indicated, cells were transfected with 400 ng of plasmids using Lipofectamine2000 according to the manufacturer's protocol and/or infected with EMCV at the specified multiplicity of infection (MOI), followed by compound treatment where specified. At the indicated time points, cells were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature (RT). Permeabilization was done with PBS-0.5% Triton X-100 for 15 min or PBS/0.2% saponin/5% BSA for 5 min, in the case of filipin staining. Cells were incubated sequentially with primary and secondary antibodies diluted in PBS containing 2% normal goat serum (NGS). The following primary antibodies were used for detection: mouse monoclonal anti-GM130 (BD Biosciences), rabbit polyclonal anti-TGN46 (Novus Biologicals), mouse monoclonal anti-ERGIC53 (Enzo Life Sciences), rabbit polyclonal anti-Sec13 (kindly provided by B.L Tang, Department of Biochemistry, The National University of Singapore, Singapore), rabbit polyclonal anti-calreticulin (Sigma), rabbit polyclonal anti-HA (Santa Cruz), mouse monoclonal anti-HA (Abcam), mouse monoclonal anti-C-Myc (Sigma), rabbit polyclonal anti-myc (Thermo Scientific), mouse anti-PI4P IgM (Echelon Biosciences), mouse monoclonal anti-dsRNA (J2, English & Scientific Consulting), mouse monoclonal anti-EMCV 3AB (kind gift from A.G. Aminev) [88] and rabbit polyclonal anti-OSBP (kindly provided by M.A. De Matteis, Telethon Institute of Genetics and Medicine, Naples, Italy) [65]. Alexa Fluor 488-, 594-conjugated IgG and Alexa Fluor 488- or 594-conjugated IgM (Invitrogen, Molecular Probes) were used as secondary antibodies. Cholesterol was stained with 25 µg/ml filipin III (Sigma) for 1 h at room temperature, included during the incubation with the secondary antibody. Nuclei were counterstained with DAPI.

Staining of plasma membrane or intracellular PI4P was performed as described elsewhere [50]. Briefly, for PM staining, cells were fixed at RT in 4% PFA and 0.2% glutaraldehyde. All subsequent steps were performed on ice. Cells were blocked and permeabilized for 45 min in buffer A (20mM Pipes, pH 6.8, 137 mM NaCl, 2.7 mM KCl) containing 5% NGS, 50 mM NH_4Cl and 0.5% saponin. Slides were incubated with primary and secondary antibodies in buffer A containing 5% NGS and 0.1% saponin for 1h. Finally, slides were post-fixed in 2% PFA in PBS for 10 min. The intracellular PI4P staining was performed at RT as follows: cells were fixed with 2% PFA, then permeabilized for 5 min in 20 µM digitonin in buffer A, blocked for 45 min in buffer A with 5% NGS and 50 mM NH_4Cl and then incubated sequentially with primary and secondary antibodies in buffer A with 5% NGS, before post-fixation in 2% PFA. All coverslips were mounted with FluorSave (Calbiochem). Images were acquired with a Leica SPE-II DMI-4000 confocal laser scanning microscope or a Nikon Ti Eclipse microscope equipped with an Andor DU-897 EMCCD-camera.

Image analysis. PI4P quantification was performed for at least 40 cells for each condition, using the ImageJ software as described elsewhere [46]. To determine colocalization of Sec13 or calreticulin with 3AB, images were first deconvoluted using NIS advanced Research 4.3 software (Nikon) (10 iterations) and further processed using Image J as follows. Individual infected cells were outlined and a mask was created, and all signal outside the mask was cropped to exclude it from the calculations. Manders' colocalization coefficient was calculated for at least 10 cells for each condition using the JACoP plugin [89] with a manually set threshold. Colocalization of OSBP with 3A in infected cells was analyzed using ImageJ by determining Pearson's coefficient for at least 15 cells per condition using the Coloc 2 plugin with default settings. To quantify colocalization of filipin with 3AB, images were first deconvoluted using NIS software (20 iterations), then ImageJ was used to select infected cells and the Pearson's coefficient of colocalization for at least 15 cells per condition was calculated using the Coloc 2 plugin with default settings.

siRNA treatment. HeLa R19 cells were reverse-transfected with 2 pmoles of siRNA per well of a 96-well plate (2x103 cells/well) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's indications. Scramble siRNA (AllStars Neg. Control, Qiagen) was used as a control. SiRNA against hPI4KA (cat. no. S102777390) and hPI4KB (target sequence: 5'-UGUUGGGGCUUCCCUGCCCTT-3') were from Qiagen. siRNA against hOSBP (two siRNAs mixed at 1:1 ratio, target sequences: 5'-CGCUAAUGGAAGAAGUUUA[dT][dT]-3' and 5'-CCUUUGAGCUGGACCGAUU[dT][dT]-3')) was from Sigma. 48 h p.t., cells were either infected with virus, transfected with *in vitro* transcribed RNA derived from the full length infectious clone pM16.1 or harvested to evaluate the knockdown efficiency by Western Blot analysis.

Cell viability assay. Cell viability was determined in parallel with virus infection as follows. One day after seeding cells in a 96-well plate, the compounds were added to the cells and incubated for 8 h. Alternatively, cells were transfected with siRNAs and incubated for 48 h. Subsequently, the medium was replaced with CellTiter 96 AQueous One Solution Reagent (Promega) and optical densities were measured at 490 nm. The obtained raw values were converted to percentage of untreated samples or samples transfected with Scramble siRNAs, following correction for background absorbance.

Radioactive labeling and immunoprecipitation. Metabolic labeling of myc-tagged EMCV proteins and HA-PI4KA was performed as described elsewhere [46]. Briefly, Huh7-Lunet/T7 cells seeded in 6-well plates were co-transfected with 2µg of plasmid encoding EMCV nonstructural proteins and 2 µg of either pTM HA-PI4KIIIa or an empty pTM vector (mock) using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. 7 h later, cells were starved in methionine/cysteine-free medium for 1h. Radiolabeling of cells was done by overnight incubation in methionine/cysteine-free medium, supplemented with 10 mM glutamine, 10 mM Hepes, and 100 µCi/ml of Express Protein labeling mix (Perkin Elmer, Boston). Cells were then harvested and lysed in lysis buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40 and protease inhibitors) for 1 h on ice, followed by centrifugation at 14,000 g for 10 min at 4OC. Supernatants were further subjected to immunoprecipitation by a 3 h incubation at 4OC with anti-c-myc rabbit polyclonal antibody (Santa Cruz). Immunocomplexes were then captured with protein G-sepharose beads (Sigma) by an additional 3 h incubation at 4^{O}C. Beads were washed three times in lysis buffer, followed by elution of immunocomplexes by boiling in sample buffer, separation by polyacrylamide-SDS gel electrophoresis and detection by autoradiography. For co-IP followed by western blot, cells were seeded in 55 cm2 dishes and transfected with 3.5 µg of each plasmid using polyethelyneimine (PEI) (Polysciences). Immunoprecipitation was carried out as described above, but using protein A-sepharose beads (GE Healthcare) and mouse monoclonal anti-C-Myc (Sigma) or rabbit polyclonal anti-myc (Thermo Scientific) antibodies.

Western Blot analysis. Samples separated by SDS-PAGE were transferred to nitrocellulose membranes (Bio-Rad). Membranes were incubated with the following primary antibodies: rabbit polyclonal anti-PI4KA (Cell Signaling), rabbit polyclonal anti-PI4KB (Upstate), rabbit polyclonal anti-OSBP (ProteinTech), rabbit polyclonal anti-EMCV capsid (kind gift from Ann Palmenberg) and mouse monoclonal anti-β-actin (Sigma). Secondary antibodies included IRDye 680-conjugated goat anti-mouse or IRDye 800-conjugated goat anti-rabbit (LI-COR). Images of blots were acquired with Odyssey Imaging System LI-COR.

Statistical analysis. Where indicated, unpaired one-tailed Student's t-test or two-tailed Mann–Whitney test were applied as statistical analyses using the GraphPad Prism software.

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SUPPORTING INFORMATION

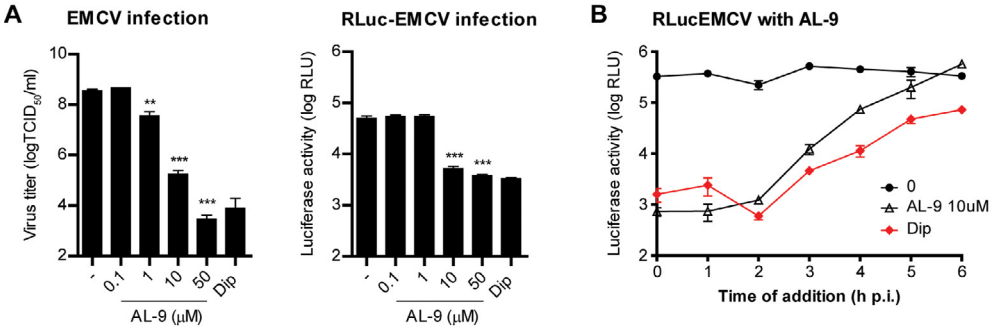

Figure S1. AL-9 inhibits EMCV at the step of genome replication. (A) AL-9 is effective against EMCV also at high MOI. HeLa R19 cells were infected with RLuc-EMCV at an MOI of 10 followed by AL-9 treatment for 8 h, after which cells were lysed and virus replication was measured by determining the *Renilla* luciferase activity. (B) AL-9 blocks EMCV genome replication. HeLa R19 cells were infected with RLuc-EMCV at an MOI of 1. At the indicated time points, DMSO, 10 μM AL-9 or 100 μM Dipyridamole was added to the cells and virus replication was measured by determining the *Renilla* luciferase activity at 8 h p.i. Bars represent mean values of triplicates ± standard error of the means (SEM). Means were statistically compared using unpaired t tests. *P < 0.05, **P<0.01; ***P<0.001.

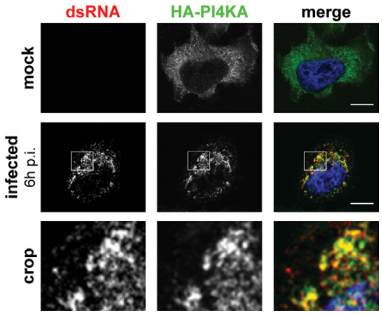

Figure S2. EMCV 3A recruits PI4KA to replication sites. HeLa R19 cells were transfected with a plasmid encoding HA-PI4KA and infected the following day with EMCV at MOI 250. At 6 h p.i., cells were fixed and co-stained with antibodies against HA and viral 3AB. Nuclei were stained with DAPI (blue). The crop panels at the bottom depict enlargements of boxed areas. Scale bars represent 10 μm.

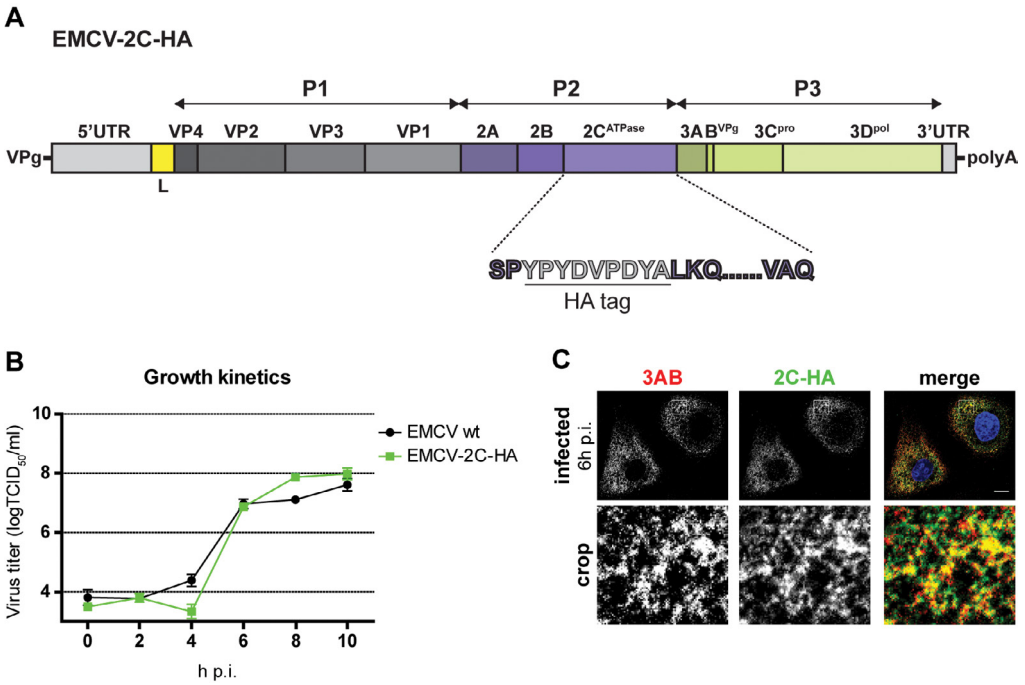

Figure S3. Characterization of EMCV-2C-HA. (A) Schematic representation of EMCV-2C-HA genome organization drawn to scale (nucleotide base length). The HA tag (YPYDVPDYA) was introduced in frame after the second amino acid in the viral protein 2C. (B) Replication kinetics of EMCV-2C-HA compared to wt EMCV. HeLa R19 cells were infected at MOI 1 with EMCV or EMCV-2C-HA. At the indicated time points p.i., cells were freeze-thawed to release intracellular virus particles and the total virus titers were determined by endpoint titration. Shown are mean values ± SEM. (C) 2C-HA is present at 3AB-positive replication membranes. Huh7-Lunet/T7 cells were infected with EMCV-2C-HA at MOI 10. At 6 h p.i., cells were fixed and co-stained with antibodies against HA and viral 3AB. Nuclei were stained with DAPI (blue). The crop panels at the bottom depict enlargements of boxed areas. Scale bars represent 10 µm.

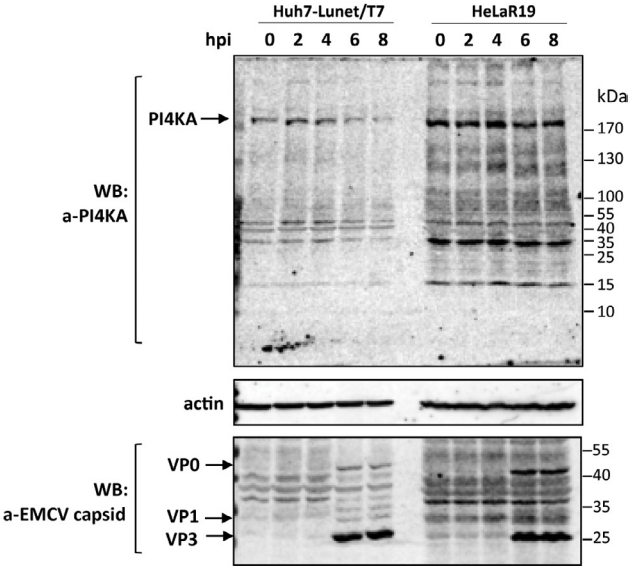

Figure S4. Time-course analysis of endogenous PI4KA in EMCV-infected cells. Huh7-Lunet/T7 cells or HeLa R19 cells were infected with EMCV at MOI 10. At the indicated time-points p.i., cells were lysed and the whole-cell lysates were subjected SDS-PAGE followed by western blot analysis using specific antibodies against actin, EMCV capsid or endogenous PI4KA. For PI4KA visualization, samples were separated in a resolving SDS-PAGE gel composed of two different acrylamide concentrations: 7.5% (for the upper half) and 15% (for the lower half).

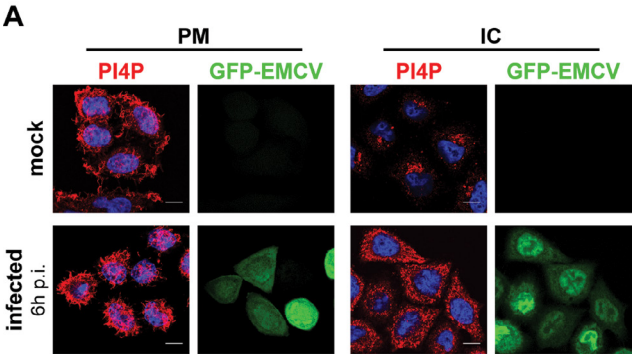

Figure S5. EMCV alters the distribution of intracellular PI4P lipids. HeLa R19 cells were mock-infected or infected with GFP-EMCV at MOI 10. At 6 h p.i., cells were fixed and stained with antibodies against PI4P using specific protocols for detection of plasma membrane and intracellular PI4P pools [50]. Nuclei were stained with DAPI (blue). Scale bars represent 10 µm.

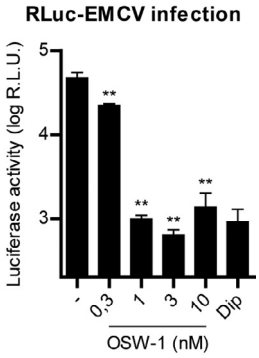

Figure S6. OSW-1 inhibits EMCV replication also at high MOI. HeLa R19 cells were infected with RLuc-EMCV at an MOI of 10 followed by OSW-1 treatment for 8 h, after which cells were lysed and virus replication was measured by determining the *Renilla* luciferase activity. Bars represent mean values of triplicates ± standard error of the means (SEM). Means were statistically compared using unpaired t tests. *P < 0.05, **P<0.01; ***P<0.001.

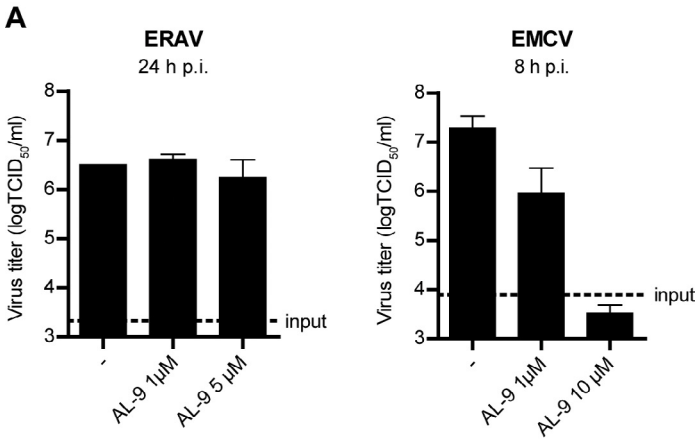

Figure S7. Aphthoviruses are insensitive to AL-9 treatment. HeLa R19 cells were infected with ERAV or EMCV at MOI 1 followed by treatment with AL-9 at the indicated concentrations. Cells were freeze-thawed to release intracellular virus particles and the total virus titers at 8 h p.i. (EMCV) or 24 h p.i. (ERAV) were determined by endpoint titration. For ERAV, the highest concentration of AL-9 used was 5 µM in order to avoid cytotoxicity. Dashed lines represent input levels. Shown are mean values ± SEM.

Mutations in encephalomyocarditis virus 3A protein uncouple the dependency of genome replication on host factors phosphatidylinositol 4-kinase IIIα and oxysterol-binding protein

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ABSTRACT

Positive-strand RNA [(+)RNA] viruses are true masters of reprogramming host lipid trafficking and synthesis to support virus genome replication. Via their membrane-associated 3A protein, picornaviruses of the genus Enterovirus (e.g. poliovirus, coxsackievirus, rhinovirus) subvert the Golgi-localized phosphatidylinositol 4-kinase IIIβ (PI4KB) to generate replication organelles (ROs) enriched in phosphatidylinositol 4-phosphate (PI4P). PI4P lipids serve to accumulate OSBP, which subsequently transfers cholesterol to the ROs in a PI4P-dependent manner. Single point mutations in 3A render enteroviruses resistant to both PI4KB and OSBP inhibition, indicating a coupled dependency on these host factors. Recently, we showed that encephalomyocarditis virus (EMCV), a picornavirus that belongs to the *Cardiovirus* genus, also builds PI4P/cholesterol-enriched ROs. Like the hepatitis C virus (HCV) of the *Flaviviridae* family, it does it by hijacking the ER-localized phosphatidylinositol 4-kinase IIIα (PI4KA). Here we provide genetic evidence for the critical involvement in this process of EMCV protein 3A. Using a genetic screening approach, we selected EMCV mutants with single amino acid substitutions in 3A, which rescued RNA virus replication upon siRNA knockdown or pharmacological inhibition of PI4KA. In the presence of PI4KA inhibitors, the mutants no longer induced PI4P, OSBP or cholesterol accumulation at ROs, which aggregated into large cytoplasmic clusters. In contrast to the enterovirus escape mutants, we observed little, if any cross-resistance of EMCV mutants to OSBP inhibitors, indicating an uncoupled level of dependency of their RNA replication on PI4KA and OSBP activities. This study may contribute to a better understanding of PI4KA and OSBP roles to membrane modifications induced by (+)RNA viruses.

IMPORTANCE

Positive-strand RNA viruses modulate lipid homeostasis to generate unique, membranous "replication organelles" (ROs) where viral genome replication takes place. Hepatitis C virus, encephalomyocarditis virus (EMCV) and enteroviruses have convergently evolved to hijack host phosphatidylinositol 4-kinases (PI4Ks), which produce PI4P lipids, to recruit oxysterol-binding protein (OSBP), a PI4P-binding protein that shuttles cholesterol to ROs. Consistent with the proposed coupling between PI4K and OSBP, enterovirus mutants resistant to PI4KB inhibitors are also resistant to OSBP inhibitors. Here, we show that EMCV can replicate without accumulating PI4P/cholesterol at ROs, by acquiring point mutations in the non-structural protein 3A. Remarkably, the mutations conferred resistance to PI4K but not OSBP inhibitors, thereby uncoupling the level of dependency of EMCV RNA replication on PI4K and OSBP. This work may contribute to a deeper understanding of the roles of PI4K/PI4P and OSBP/cholesterol in membrane modifications induced by positive-strand RNA viruses.

INTRODUCTION

Genome replication of (+)RNA viruses is tightly associated with virus-induced membranous structures that accumulate in the cytoplasm of infected cells. In the case of hepatitis C virus (HCV), a virus of the Flaviridae family, these structures are referred to as "the membranous web" (MW), whereas for viruses belonging to the *Picornaviridae* family, the most commonly term used is "replication organelles" (ROs) [recently reviewed in [1] and [2]]. These virus-induced membranes have been suggested to provide a structural platform that facilitates cooperation between components of the viral replication complex and possibly to also provide shelter from host defense systems [3,4], but their precise function is not yet understood. Viruses build these specialized membranous structures by drastically rewiring essential cellular processes, especially pathways involved in lipid metabolism.

Picornaviruses manage to efficiently manipulate the cellular environment and transform it into a membranous replication factory using only a few viral non-structural proteins [5]. One of the key viral players involved in this process is the small protein 3A. Picornavirus 3A proteins invariably include a hydrophobic domain at the C-terminus, but otherwise share little sequence similarity in viruses from different genera, likely due to profound divergence [6]. The best-studied picornavirus 3A protein is that of enteroviruses [such as poliovirus (PV; *Enterovirus* C species), coxsackievirus B3 (CVB3; *Enterovirus* B)]. The interaction of 3A with a number of essential host factors results in their accumulation at the membranes of ROs to promote viral RNA synthesis [7–12]. Often referred to as "recruitment", the molecular mechanism behind this accumulation remains unknown and may involve both recruitment and/or "retention" of the host factors. Among the factors that are enriched at enterovirus ROs are the guanine exchange factor GBF1 and the Golgi complex-localized lipid kinase PI4KB (phosphatidylinositol 4-kinase type III isoform β). PI4KB is one of the four mammalian kinases that catalyzes the phosphorylation of PI (phosphatidylinositol) to PI4P (phosphatidylinositol 4-phosphate) [13–15]. The accumulation of PI4KB at ROs via 3A interaction results in a local increase of the PI4P concentration [8]. PI4P was suggested to function in enterovirus replication by promoting downstream processes of RO membrane biogenesis involving essential host factors. Indeed, we and others have recently shown that PI4P is important for the accumulation of oxysterol-binding protein (OSBP) at enterovirus ROs [10,16,17]. In non-infected cells, OSBP plays a key role in membrane homeostasis by mediating the non-vesicular transport of cholesterol in exchange for PI4P between the endoplasmic reticulum (ER) and Golgi [18]. OSBP bridges ER and *trans*-Golgi membranes at membrane contact sites (MCSs), and shuttles cholesterol from the ER into the Golgi and PI4P from the Golgi back to the ER, where it is hydrolyzed by the PI4P-phosphatase Sac1. PI4P lipids are critical in this process by both providing the energy required for the counter transport of cholesterol and by serving as a membrane anchor for OSBP. In enterovirus-infected cells, OSBP functions at membrane contact sites (MCSs) between the ROs and ER by shuttling PI4P from the ROs to ER membranes and cholesterol from ER to ROs, thereby enriching the ROs in cholesterol. To date, why enteroviruses promote cholesterol accumulation at their ROs remains poorly understood. The lipid transfer function of OSBP is critical for enterovirus replication, since OSBP inhibitors such as OSW-1 or itraconazole (ITZ), which impair this lipid exchange, also inhibit virus genome replication [16]. We and others have shown that enteroviruses CVB3 and PV can acquire single point mutations in their 3A proteins that render them resistant to PI4KB inhibitors [19–23]. Remarkably, the same mutations also provide cross-resistance to OSBP inhibitors [16,24,25], indicating the coupled dependency of enterovirus replication on PI4KB and OSBP.

Recently, we have also addressed the role of membranes in the replication of another picornavirus, namely the cardiovirus encephalomyocarditis virus (EMCV, *Cardiovirus* A species), which is closely related to the theiler's murine encephalomyelitis virus and the human saffold virus (both of *Cardiovirus* B species) [26]. Similar to what was observed during enterovirus infection, PI4P lipids proved essential for the accumulation of OSBP and cholesterol to the ROs of cardioviruses. We discovered that EMCV induces the formation of PI4P-enriched ROs by hijacking the ER-localized PI4KA (phosphatidylinositol 4-kinase type III isoform α) instead of the Golgi-associated PI4KB. Similar as enteroviruses, and despite little sequence conservation, EMCV seems to employ the viral protein 3A in this process, as revealed by co-immunoprecipitation of PI4KA with 3A from cell lysates and by immunofluorescence studies showing co-localization of 3A with PI4KA. Thus, enteroviruses and cardioviruses, representing two distantly related picornavirus genera, have evolved to employ their sequence-dissimilar 3A proteins to hijack different host kinases that ensure abundant PI4P production at the ROs. Remarkably, the PI4KA-OSBP pathway used by EMCV is also exploited by HCV to develop a cholesterol-enriched MW [27], suggesting a functional convergence of EMCV and HCV. To date, studies with (viable) HCV mutants resistant to PI4KA inhibitors that could bring novel insights into the role of PI4KA and PI4P in virus replication are still lacking.

In this study, we aimed to gain further insight into the molecular mechanism through which picornaviruses remodel host membranes, by combining the power of traditional forward genetics with modern techniques of selective protein targeting, reverse genetics and cell visualization, assisted by bioinformatics. To this end, we report the first isolation of (+)RNA viruses that exhibit a markedly decreased dependence on the essential host factor PI4KA. In contrast to the enterovirus mutants described previously, the EMCV mutants were only minimally cross-resistant to OSBP inhibition or depletion, suggesting a remarkable uncoupling of the level of virus replication dependency on PI4KA and OSBP activities. In conclusion, we here uncover that the phenotype of PI4KA-resistant cardioviruses both resembles and differs from that of PI4KB-resistant enteroviruses, with respect to usage of critical host factors for RO membrane biogenesis. Our findings indicate prospects of novel insights into the roles of PI4P and cholesterol in (+)RNA virus replication.

RESULTS

EMCV acquires single point mutations in the 3A gene to restore replication in cells with low levels of PI4KA. We set out to isolate mutants of the EMCV species (strain Mengovirus), which could replicate in cells with compromised PI4KA activity. To this end, we attempted to propagate wt EMCV in cells in the presence of AL-9, a well-established PI4KA inhibitor [28] that hampers EMCV replication by targeting the enzymatic activity of PI4KA [26]. However, all our attempts proved unsuccessful, most likely due to long-term cytotoxic effects of the compound. We then explored another approach that involved propagation of wt EMCV in the stable cell line Huh7-Lunet/T7-shPI4K, in which levels of endogenous PI4KA have been reduced using short hairpin RNA (shRNA)-expressing lentiviral vectors [29]. First, we checked whether EMCV replication was impaired in these cells compared to the control cell line Huh7-Lunet/T7-shNT, which expresses a non-targeting shRNA vector. Indeed, the EMCV progeny yield was significantly reduced in the shPI4K cell line compared to the control cell line (Fig. 1A). The observed inhibition was genuine, since replication of CVB3, which depends on PI4KB rather than PI4KA [8], was similar in both cell lines (Fig. 1A). Thus, we considered the Huh7-Lunet/T7-shPI4K cell line as a promising system for the selection of EMCV mutants that are less dependent on PI4KA for replication.

To select EMCV escape mutants, we next infected Huh7-Lunet/T7-shPI4K cells with wt EMCV and harvested individual virus cultures from the wells which developed CPE faster than others. From these, we selected two virus cultures, named 3E1 and 4E3 after their position in the plate, which we found to be less sensitive to AL-9 treatment than the wt virus (Fig. 1B). We interpreted this result as indicative of a decreased dependence of the 3E1 and 4E3 mutants on PI4KA. Genomic analysis of the P2P3 region of these mutants identified two independently acquired single point mutations, one in each analyzed virus pool: A32V (alanine at position 32 changed to alanine) in 3E1 and A34V (alanine at position 34 changed to valine) in 4E3. No other mutations were found in the sequenced P2P3 region of the two virus pools.

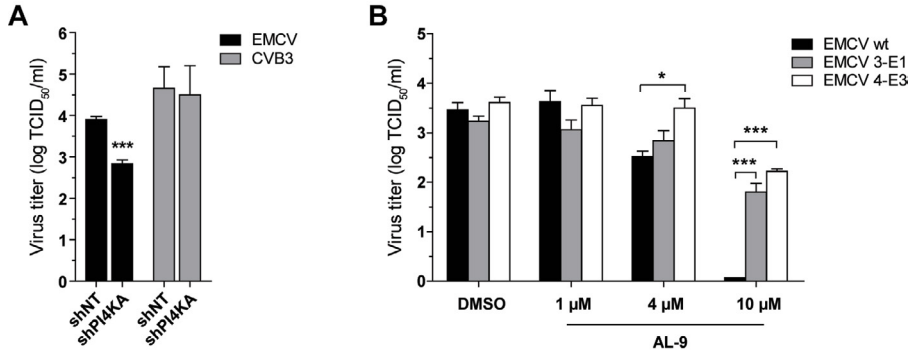

Figure 1. Isolation of EMCV cultures resistant to inhibition of host factor PI4KA. (A) Effects of PI4KA knockdown on EMCV and CVB3 infection. Huh7-Lunet/T7-shNT and Huh7-Lunet/T7-shPI4KA cells were infected with EMCV or CVB3 at MOI 1. After 8 h, cells were freeze-thawed to release intracellular virus particles and the total virus titers were determined by endpoint dilution. (B) Effect of PI4KA inhibitor AL-9 on wt EMCV and EMCV pools 3-E1 and 4-E3. HeLa R19 cells were infected at MOI 1. Total virus titers were determined as in (A). (A, B) Shown are mean values ± SEM, following subtraction of the input levels. Means were statistically compared using unpaired t tests. *, P<0.05; ***, P<0.001.

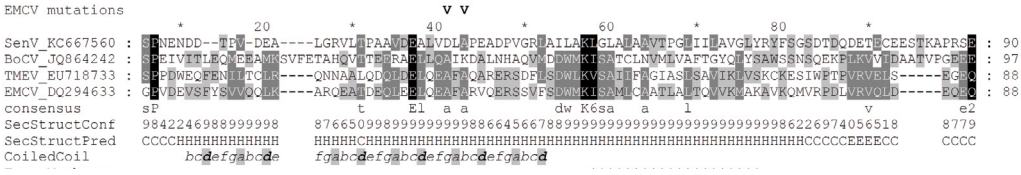

Figure 2. Mutant EMCV viruses harbor single amino acid substitutions in the non-structural protein 3A. Shown are the location of the acquired mutations in 3A in the context of sequence conservation and putative structural features of this protein. Multiple sequence alignment (MSA) of 3A of four picornaviruses forming a monophyletic cluster was obtained as specified in materials and methods. The name of each MSA entry includes the acronym of the virus name and the GenBank accession number of the respective sequence. SenV, Seneca virus; BoCV, boone cardiovirus; TMEV, theiler's murine encephalomyelitis virus; EMCV, encephalomyocarditis virus. Amino acid conservation is highlighted with shadows using conserved panel in default mode, which produces consensus line featuring invariant (capital letters) and most frequent (small letters) residues, as well as 6 groups of physico-chemically similar residues (digits from one to six). SecStructConf and SecStructPred, confidence and state (C, coil; H, helix; E, strand) of predicted secondary structure of EMCV 3A according to PsiPred analysis of the MSA. CoiledCoil, elements of coiled-coil heptads in EMCV 3A protein as assigned by Paircoil2 with window length of 21 under the P-score cutoff of 0.025 (0.0223-0.0041 range). The hydrophobic a and d positions are contrasted with background shading. Transmembrane residues of EMCV 3A that are above TMHMM2 threshold of 0.4 to form transmembrane helix are indicated (*).

3A protein mutations are located in a putative determinant of intermolecular interaction. The fact that both independently acquired substitutions were of the same type, from Ala to Val, and were accepted at the two positions in close proximity, indicated a common mechanism of escape of the mutants. Subsequent bioinformatics analysis of 3As revealed considerable sequence conservation of this protein in viruses of the known three *Cardiovirus* species and one species of the sister *Senecavirus* genus (Fig. 2). According to Psipred v.2.5 [30], the protein may predominantly adopt alpha-helix conformation, with the N-terminal part of EMCV (from 7 to 45 residue) being folded into a left-handed coiled-coil [according to Paircoil2, [31]] and the C-terminal hydrophobic-rich region (from 47 to 65) forming a trans-membrane helix [according to TMHMM2, [32]]. The most conserved amino acid (aa) block is located at the junction between the putative coiled-coil and transmembrane structural elements, indicative of considerable constraints and functional importance of this region. The coiled-coils are alpha-helix oligomers which are distinguished by the presence of several heptad repeats with alternating hydrophilic and hydrophobic residues that are labelled from a to g letters [33]. Residues at the a and d positions are found at the helix interface, while residues at the other positions are solvent-exposed. In line with the canonical coiled-coil organization, hydrophobic and hydrophilic residues dominate at, respectively, the a and d and the remaining five positions in the aligned 3A proteins (Fig. 2). The A32V and A34V substitutions in the 3A protein of the mutants are located in the g and b positions of two adjacent heptads, respectively, being partially conserved and among few exceptional hydrophobic residues at these positions in all heptads. Accordingly, the mutated residues in 3A may be part of the solvent-exposed coiled-coil surface, which would be compatible with the properties of molecular determinants mediating interaction of 3A with PI4KA directly or indirectly.

Introduction of the point mutations into the 3A of wt EMCV rescues virus replication from PI4KA inhibition. Since we had sequenced only a part of the genome of the EMCV mutants, mutations in other regions might in theory have contributed to the observed phenotype. To exclude this possibility, we introduced the acquired 3A single point mutations into the full-length infectious clone of wt EMCV, generating the recombinant viruses carrying the individual substitutions: EMCV-3A-A32V and EMCV-3A-A34V. We have also generated the respective luciferase reporter viruses RLuc-EMCV-3A-A32V and RLuc-EMCV-A34V, which contain additionally the gene encoding for *Renilla* luciferase upstream of the capsid-coding region. To gain insight into the replication kinetics of the mutant viruses compared to that of the wt virus, single-cycle replication assays were performed. Both mutant viruses replicated similar to wt (Fig 3A), demonstrating that the point mutations in 3A are not affecting the kinetics of virus replication under standard conditions. Subsequently, we investigated the replication kinetics of the mutants relative to that of wt virus under conditions of PI4KA depletion by shRNA-mediated knockdown. To this end, Huh7-Lunet/T7-shNT and Huh7-Lunet/T7-shPI4K cells were infected with wt EMCV or each of the two mutants, and at different time points post-infection, cells were lysed to determine the total amount of virus by titration. The amount of wt progeny virus was significantly decreased in the PI4KA-knockdown cell line compared to the control cells particularly at 6 h p.i.and to a lesser extent at 8 h p.i., while no such effect was observed for the mutant viruses (Fig. 3B). These results indicated that the 3A mutations completely rescued the delayed replication kinetics observed for wt EMCV.

Next, we asked whether a similar rescue effect could be observed in a single-cycle assay when PI4KA is inhibited by AL-9 treatment. To this end, HeLa cells were infected with EMCV wt, EMCV-3A-A32V, or EMCV-3A-A34V, treated with DMSO or AL-9, and lysed at different time points post-infection to determine the total amount of virus by titration. In the presence of AL-9, only the wt virus was significantly inhibited, while the mutants replicated very efficiently throughout infection, almost to

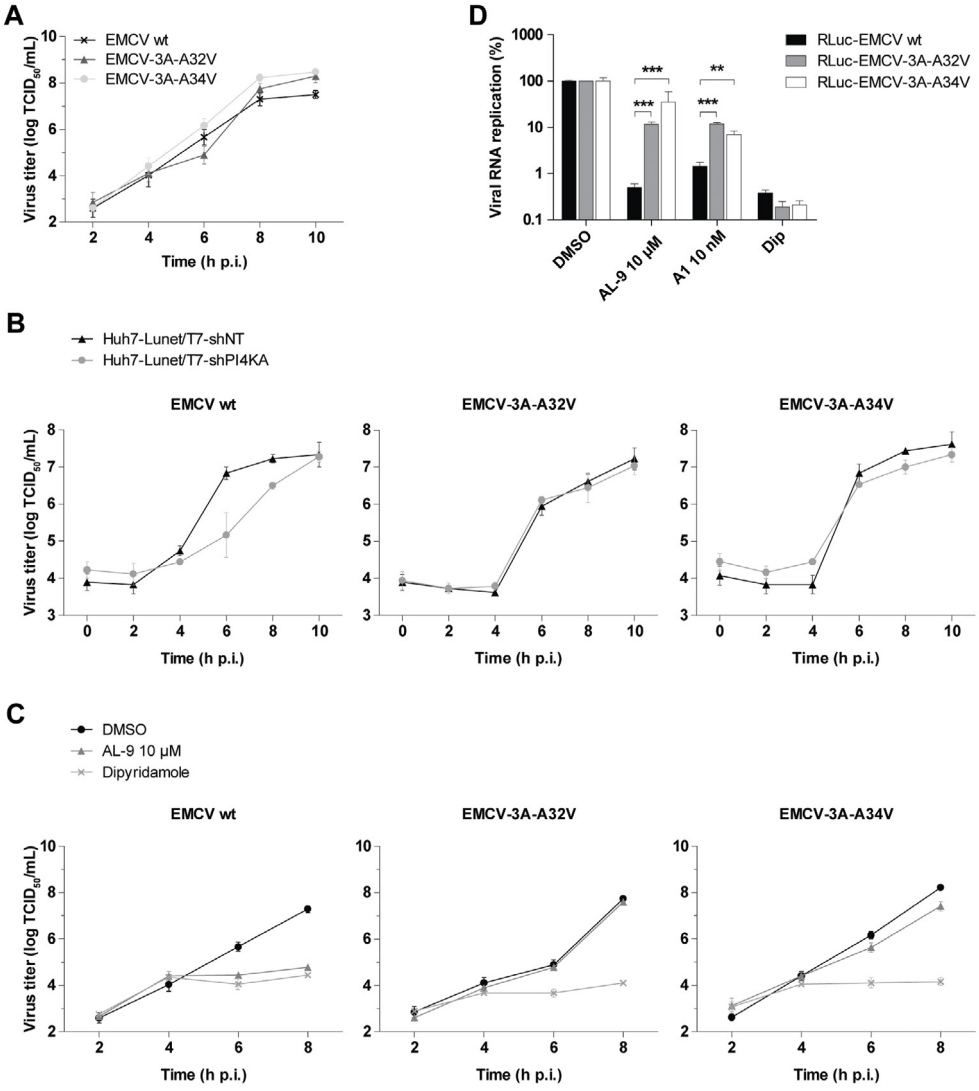

Figure 3. Distinct single point mutations in 3A render EMCV less dependent on PI4KA. (A-C) Growth curve analysis of EMCV wt and mutants. After infection at MOI 1 for 30 min, the inoculum was removed and fresh medium was added to the cells. At the indicated time-points, cells were freeze-thawed to determine the total virus titers by endpoint dilution. (A) Growth curves in the absence of compounds in HeLa R19 cells. (B) Growth curves in Huh7-Lunet/T7-shNT and Huh7-Lunet/T7-shPI4KA cells. (C) Growth curves in HeLa R19 cells in the presence of DMSO, 10 µM AL-9 or dipyridamole (Dip). (D) RNA replication of EMCV mutants is resistant to PI4KA inhibitors. HeLa R19 cells were infected at MOI 1 with wt or mutant EMCV reporter viruses carrying the *Renilla* luciferase gene. After 30 min, virus was removed and compound-containing medium was added to the cells. At 8 hours p.i., cells were lysed to determine the intracellular luciferase activity. DMSO samples were set at 100%. Shown are mean values ± SEM. Means were statistically compared using unpaired t tests. **, P<0.01; ***, P<0.001.

the same extent as in the control treatment (Fig. 3C). Dipyridamole, an established inhibitor of EMCV RNA replication [34], completely inhibited the replication of both wt and mutant viruses (Fig. 3C). These results demonstrated that each of the two point mutations in 3A can render EMCV less dependent on PI4KA enzymatic activity.

Previously, we demonstrated that EMCV requires PI4KA activity for the step of RNA genome replication [26]. To elucidate if the point mutations in 3A rescue EMCV replication by restoring viral RNA synthesis, we measured the genome replication of the luciferase reporter viruses in the presence of PI4KA inhibitors AL-9 and A1, another PI4KA inhibitor that was recently described [35]. Indeed, both substitutions considerably rescued viral RNA replication from the inhibitory activities of both AL-9 and A1 (Fig. 3D).

PI4KA is recruited to ROs of EMCV mutants. Previously, we showed that PI4KA redistributes to EMCV ROs during infection, where it colocalizes with 3A(B) [26]. To investigate whether the introduced mutations interfere with PI4KA recruitment by 3A, we examined by immunofluorescence (IF) whether GFP-PI4KA colocalizes with the mutant 3A proteins, both in the context of virus infection and upon 3A-myc co-expression, using our previously established experimental systems [26]. In cells infected with EMCV wt, as well as EMCV-3A-A32V or EMCV-3A-A34V, PI4KA was concentrated at ROs where it overlapped with 3A(B). By contrast, PI4KA did not overlap with the 3A signal in CVB3-infected cells,

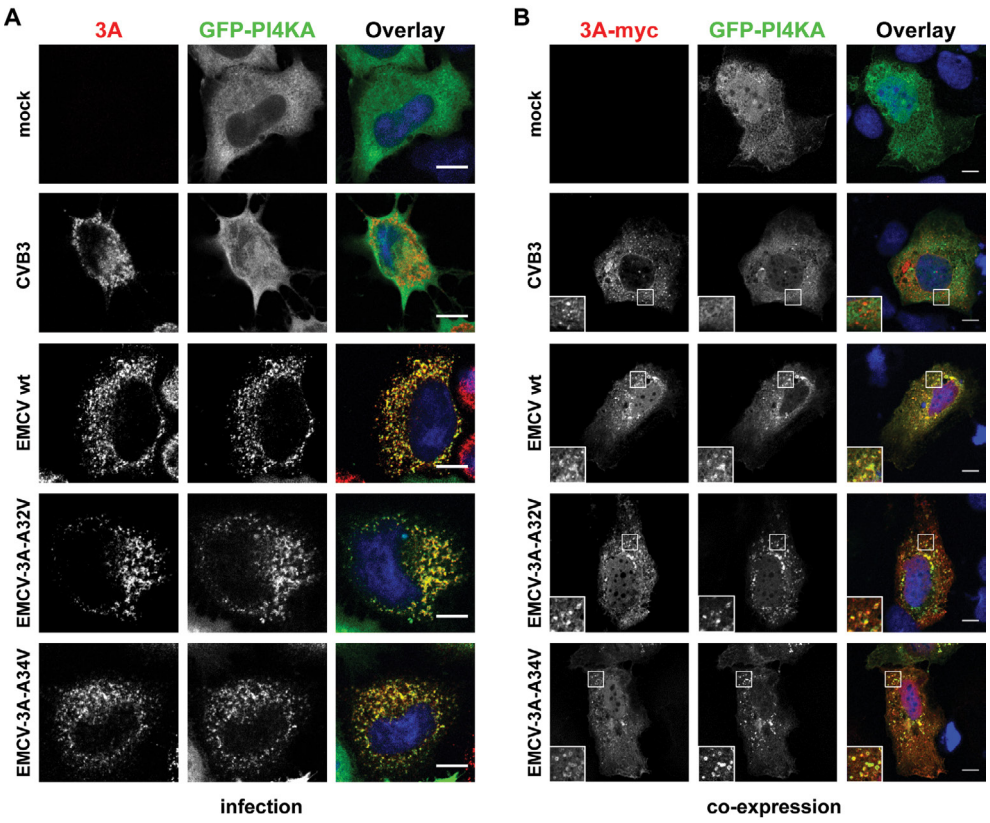

Figure 4. EMCV mutants recruit PI4KA to their ROs. (A) HeLa R19 cells were transfected with a plasmid encoding GFP-PI4KA. The next day, cells were mock-infected or infected with CVB3 (included as negative control) or EMCV wt or mutants at high MOI. At 6 h post infection (p.i.), cells were fixed and stained with antibodies against EMCV 3AB or CVB3 3A as RO markers. (B) 3A-A32V and 3A-A34V alone can recruit PI4KA to membranes. Huh7-Lunet/T7 cells were co-transfected with the GFP-PI4KA construct and either empty vector or plasmids encoding myc-tagged 3A of CVB3 (included as negative control) or 3A of wt or mutant EMCV. The next day, cells were fixed and stained with antibodies against the myc tag to detect the overexpressed 3A proteins. Insets depict enlargements of boxed areas. (A and B) Nuclei were stained with DAPI (blue). Scale bars represent 10 µm.

demonstrating the specificity of the recruitment by EMCV wt and mutants (Fig. 4A). Similarly, upon co-expression, PI4KA was recruited to discrete cytoplasmic 3A-positive punctae by wt or mutant EMCV 3A proteins, but not by the 3A of CVB3 (Fig. 4B). Collectively, these data suggested that the mutations introduced in EMCV 3A do not impair PI4KA recruitment to membranes.

EMCV mutants can replicate in the absence of a PI4P- and cholesterol-enriched environment. PI4KA plays an essential role in EMCV replication by inducing a local increase in PI4P, necessary for the downstream accumulation of OSBP and cholesterol at RO membranes [26]. We next investigated whether the EMCV mutants might still use a PI4P-dependent pathway in which the PI4P lipids are synthesized by other PI4K isoforms when PI4KA is inhibited. We reasoned that the mutants might use PI4KA just like the wt virus does in the absence of PI4KA inhibitors, while in the presence of PI4KA inhibitors the mutants might switch to using another PI4K isoform. To explore this possibility, we measured the replication of EMCV-3A-A32V in cells depleted of either PI4K2A, PI4K2B, or PI4KB by siRNA-silencing, using a set of siRNAs previously tested and validated for efficiency in this cell line [9,21]. Two days after the siRNA treatment, cells were infected with either wt or mutant virus in the absence or presence of the PI4KA inhibitor A1. wt EMCV was significantly inhibited only upon PI4KA knockdown, as we showed previously [26], but not upon knockdown of the other PI4K isoforms (Fig. 5A). As expected, EMCV-3A-A32V was resistant to PI4KA knockdown, and was not impaired by knockdown of the other isoforms, regardless of the A1 treatment, indicating that the mutants do not rely on PI4P lipids produced by another PI4K when PI4KA activity is impaired.

To verify by a different method that the EMCV 3A mutants do not rely on PI4P lipids, we examined by IF whether these viruses induce PI4P-rich membranes in the presence of a PI4KA inhibitor. Cells were infected for 30 min with virus, after which the virus-containing medium was replaced with fresh medium containing DMSO or AL-9. At 6 h p.i., cells were fixed and analyzed by IF for the presence of viral proteins and PI4P. In mock-infected cells, AL-9 treatment did not affect the intracellular pool of PI4P lipids (Fig. 5B, upper panels), as these are mainly produced by the Golgi-localized PI4KB [13–15]. In cells infected with the mutants, we detected a strong PI4P signal, distributed throughout the cytoplasm and at 3A-positive structures (Fig. 5B, lower panels). In contrast and despite high levels of viral protein being present, the PI4P signal was hardly detected in cells treated with AL-9 throughout the infection with the mutant, likely due to inhibition of PI4KA activity by AL-9 (Fig. 5B, lower panels). These cells also lacked an apparent Golgi PI4P signal, in line with our previous observations that EMCV induces disruption of Golgi membranes [26]. Under similar conditions, we could not detect cells infected with wt EMCV, since AL-9 blocks its replication (data not shown). Importantly, AL-9 treatment also had a profound impact on the subcellular distribution of the ROs, as revealed by the aggregation of 3A-positive structures into large cytoplasmic clusters that contrast the small 3A-positive punctate structures typically found in control-treated cells (Fig. 5B, see lower 3AB panels of DMSO and AL-9). The EMCV-3A-A34V mutant displayed a similar phenotype (data not shown). A similar clustering effect induced upon PI4KA inhibition was also observed on the cytoplasmic distribution of viral dsRNA, a marker of viral replication sites (FIG 5C). Altogether, these findings argued that, in contrast to the wt virus, the mutants no longer necessitate elevated levels of PI4P to establish infection.

Since PI4P lipids serve to accumulate OSBP and cholesterol at EMCV wt ROs [26], we next addressed the question whether OSBP and cholesterol were present at the ROs of EMCV 3A mutants when PI4KA was inhibited throughout infection. Cells were infected for 30 min with virus, after which the virus-containing medium was replaced with fresh medium containing DMSO or AL-9. At 6 h p.i., cells

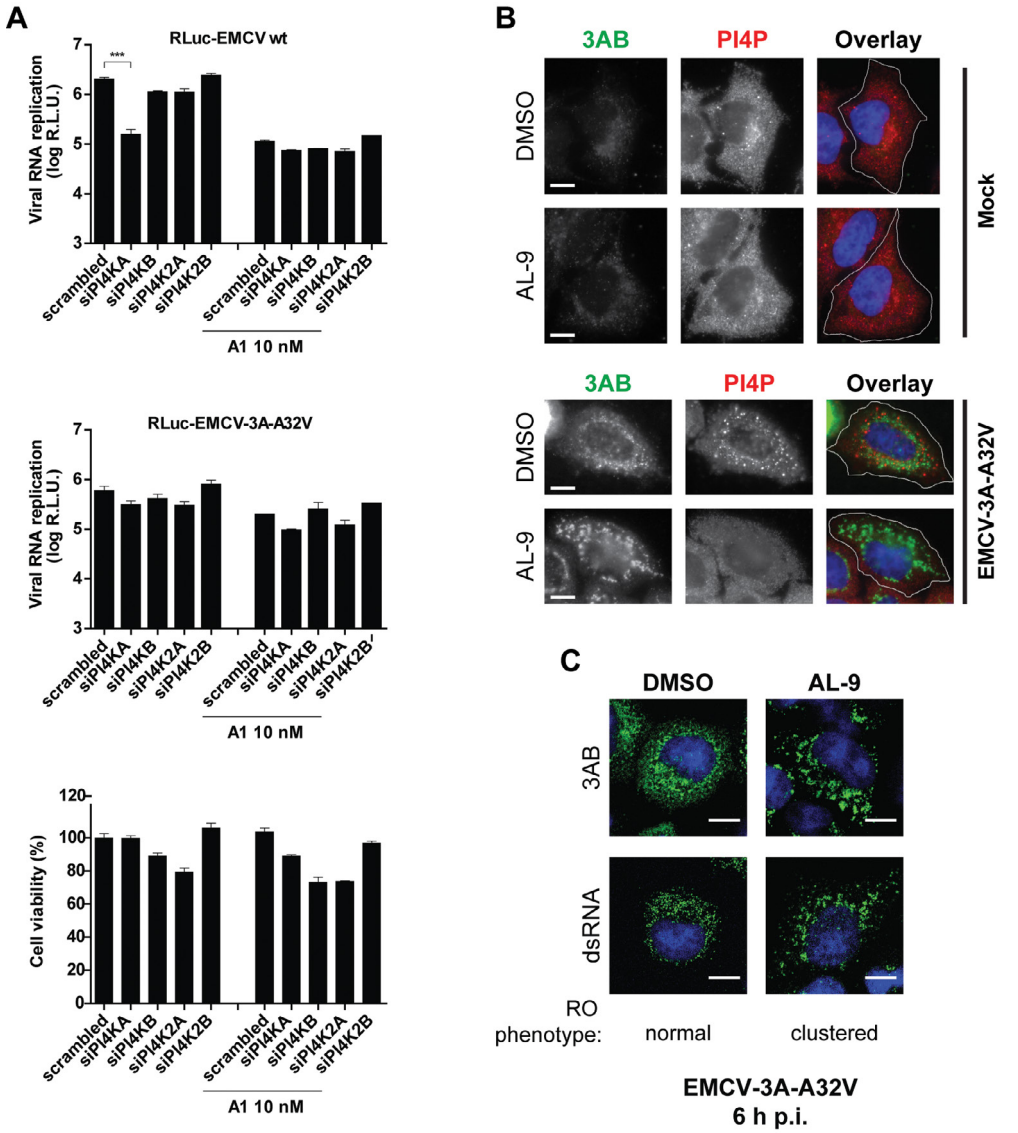

Figure 5. EMCV mutants can replicate in the absence of a PI4P-enriched environment. (A) EMCV-3A-A32V does not rely on other PI4Ks for replication when PI4KA is inhibited. HeLa R19 cells were treated with siRNAs targeting each of the four mammalian PI4Ks. After 48h, cells were infected with RLuc-EMCV wt or RLuc-EMCV-3A-A32V for 30 min at MOI 1. Medium containing the PI4KA inhibitor A1 (10 nM) was added after removing the inoculum. Cells were lysed to determine the intracellular luciferase activity after 8 hours. In parallel, cytotoxicity of the siRNA-treatment and A1 treatment was determined in a cell viability assay. Shown are mean values ± SEM. Means were statistically compared to the corresponding scrambled siRNA sample using one-way ANOVA analysis. (B) HeLa R19 cells were infected with EMCV-3A-A32V for 30 min at MOI 10, followed by treatment with 10 μM AL-9 where indicated. At 6 h p.i., cells were fixed and stained for 3AB and PI4P. For clarity, panels depict the outline of the cells (white line). (C) PI4KA inhibition induces clustering of ROs in mutant EMCV-infected cells. HeLa R19 cells were infected and treated as in (B), followed by staining for either 3AB or dsRNA. (A, B) Nuclei were stained with DAPI (blue). Scale bars represent 10 μm.

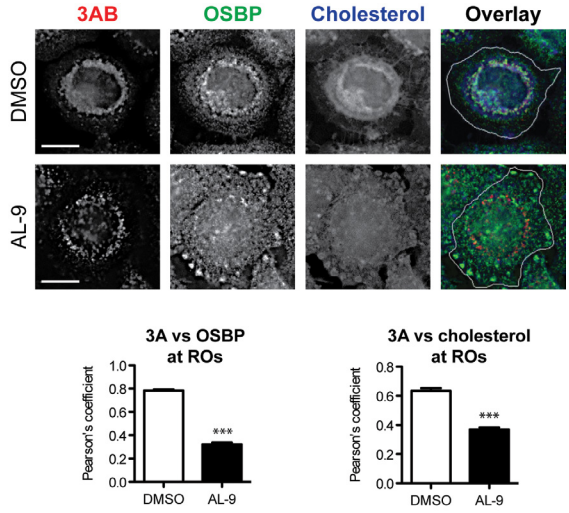

Figure 6. EMCV mutants do not accumulate OSBP and cholesterol at the ROs in the presence of PI4KA inhibitors. HeLa R19 cells were infected with EMCV-3A-A32V for 30 min at MOI 10, followed by treatment with PI4KA inhibitor AL-9 where indicated. At 6 h p.i., cells were fixed and stained for 3AB, OSBP and cholesterol. Cholesterol was visualized using filipin, a fluorescent antibiotic that specifically binds free cholesterol. For clarity, the overlay panels depict the outline of the cell (white line). Colocalization of OSBP or cholesterol with 3AB was determined by calculating the Pearson's correlation coefficients for at least 15 cells for each condition. Shown are mean values ± SEM. Means were statistically compared using the Mann–Whitney test. ***, P<0.001. Scale bars represent 10 µm.

were fixed and subjected to IF analysis for the detection of viral 3AB, OSBP and cholesterol. OSBP and cholesterol colocalized with 3AB at the ROs in infected cells treated with DMSO (Fig. 6, upper panels). The AL-9 treatment significantly reduced the colocalization of 3AB with both OSBP and cholesterol at ROs, which were now also dispersed in patches throughout the cell and close to the plasma membrane (Fig. 6, lower panels). The same phenotypes were observed for EMCV-3A-A34V mutant (data not shown). These data indicated that the EMCV mutants do not require high levels of OSBP or cholesterol at their ROs to establish infection.

The 3A mutations provide only minimal resistance against OSBP inhibition. Since the EMCV mutants can replicate without causing apparent enrichment of PI4P, cholesterol and OSBP at their ROs, we wondered whether they also exhibit a diminished dependence on OSBP. To this end, we first compared the sensitivities of EMCV wt and EMCV-3A-A32V to PI4KA vs OSBP knockdown, using a set of siRNAs previously tested and validated for efficiency in this cell line [16,26]. As expected, the mutant replicated considerably more efficient than the wt in cells depleted of PI4KA. In contrast, OSBP knockdown inhibited both viruses to a similar extent (Fig. 7A, left panel). To exclude potential off-target effects of the OSBP siRNA treatment, we tested in parallel the sensitivity of the mutant enterovirus CVB3-3A-H57Y, previously demonstrated to be resistant to both PI4KB and OSBP inhibition [16,20,21,25]. Replication of the CVB3 mutant was not affected by OSBP knockdown (Fig. 7A, middle panel), thus validating the specificity of the effects observed on EMCV replication. No cytotoxicity of the siRNA treatment was observed in the cell viability assay (Fig 7A, right panel).

Additionally, we measured the effects of different OSBP inhibitors on viral RNA synthesis. To this end, cells were transfected for one hour with *in vitro* transcribed genomic viral RNA encoding for the RLuc-EMCV wt or each of the mutants, followed by treatment with either AL-9 (as control) or increasing

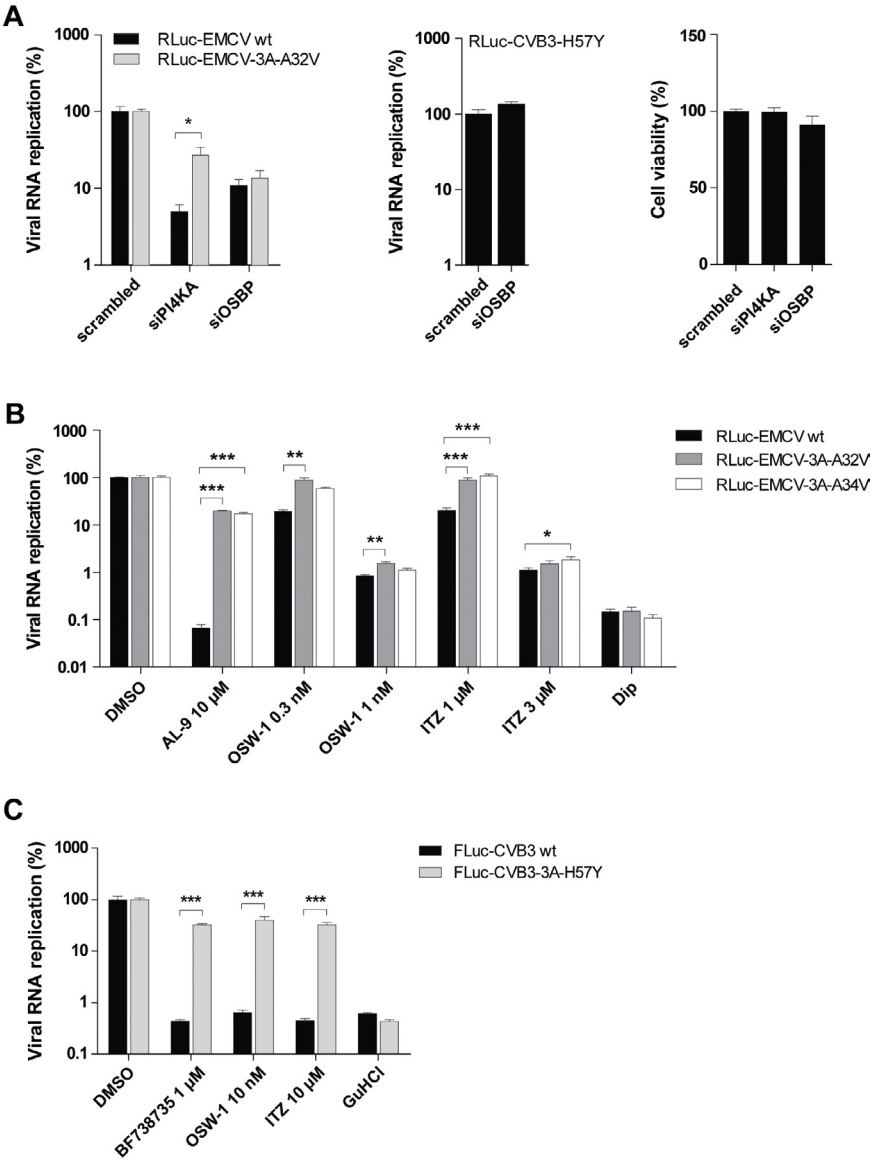

Figure 7. EMCV mutants remain largely sensitive to OSBP depletion or inhibition. (A) Effects of OSBP knockdown on EMCV-3A-A32V. Following treatment with siRNAs for two days, HeLa R19 cells were infected with wt or mutant RLuc-EMCV at MOI 1. After 8 h, cells were lysed to determine the intracellular luciferase activity (left panel). In parallel, off-target effects of the OSBP siRNA treatment were excluded by monitoring replication of the mutant RLuc-CVB3-3A-H57Y (middle panel), previously shown to be resistant to OSBP inhibition [16]. Cytotoxicity of the siRNA-treatment was determined in a cell viability assay (right panel). (B, C) Sensitivity of wt or mutant EMCV to OSBP inhibitors compared to enteroviruses. HeLa R19 cells were transfected with *in vitro* transcribed RNA of the EMCV infectious clones encoding *Renilla* luciferase (B) or with *in vitro* transcribed RNA of the CVB3 subgenomic replicons encoding Firefly luciferase in place of the capsid-coding region (C). After RNA transfection, cells were DMSO-treated or treated with AL-9 or the OSBP inhibitors OSW-1 or itraconazole (ITZ) (B), or the PI4KB inhibitor BF738735 and OSW-1 or ITZ (C). Guanidine hydrochloride (GuHCl), an established inhibitor of enterovirus replication, was included as control in (C). Viral RNA replication was determined as in (A). DMSO samples were set at 100%. (A, B, C) Shown are mean values ± SEM. Means were statistically compared using unpaired t tests. *, $P < 0.05$; **, $P<0.01$; ***, $P<0.001$.

concentrations of the OSBP inhibitors OSW-1 [36] or ITZ [16]. The mutants were strongly resistant to AL-9 treatment, but remained largely sensitive to OSBP inhibition, with only weak resistance observed when low concentrations of OSW-1 or ITZ were used (Fig. 7B). As a comparison, we tested in parallel the sensitivity of the mutant enterovirus CVB3-3A-H57Y. As expected, replication of CVB3-3A-H57Y was similarly resistant to the PI4KB inhibitor BF738735 and either OSW-1 or ITZ treatment (Fig. 7C). Collectively, these data suggested that, unlike the CVB3 3A mutants, the replication of EMCV 3A mutants was still largely dependent on OSBP.

DISCUSSION

Viruses heavily depend on the cellular machinery and resources to accomplish virtually every step of their replication cycle. In particular, (+)RNA viruses redirect the cellular lipid metabolism towards building specialized membranous replication sites for viral RNA genome synthesis, known as replication organelles (ROs) in the case of picornaviruses [1,2]. Recently, we reported that the picornavirus EMCV relies on the activities of PI4KA and OSBP for RNA genome replication and formation of PI4P- and cholesterol-enriched ROs [26]. Here, we describe the selection and characterization of mutant EMCV variants that are less sensitive than the wt to inhibition of PI4KA activity. These EMCV mutants were selected in cells stably depleted of PI4KA by shRNA-mediated knockdown, in which replication of wt virus was significantly delayed. Genomic analysis of two different virus pools with accelerated virus growth revealed two independently acquired single point mutations in the viral 3A protein: A32V and A34V. Introduction of each of these mutations in the wt virus rescued virus replication upon PI4KA knockdown or pharmacological inhibition. In the absence of PI4KA inhibitors, EMCV 3A mutants accumulated PI4P, OSBP and cholesterol at their ROs, similar as we have also shown recently for wt EMCV. The mutants did not resort to using another PI4K isoform nor accumulated PI4P, OSBP or cholesterol at ROs when PI4KA was inhibited. Furthermore, PI4KA inhibition resulted in aggregation of 3A into large cytoplasmic clusters, indicating that accumulation of PI4P and cholesterol is important for the overall organization of the ROs. Notably, the mutants remained largely sensitive to OSBP inhibition or knockdown, indicative of uncoupled level of dependency on PI4KA and OSBP.

The PI4KA-OSBP pathway is likewise exploited by HCV [27], which belongs to the distantly related (+) RNA virus family *Flaviviridae*. A previous study by Bianco et al. disclosed unsuccessful attempts to select HCV replicons resistant to the PI4KA inhibitor AL-9 [28]. This is in agreement with our repeated failure to select for EMCV mutants by resistance culturing in the presence of AL-9, which might reflect that AL-9 is not a suitable compound for resistance culturing. In only one study of this kind, the isolation of a mutant HCV replicon that was slightly resistant (20-fold compared to wt) to PI4KA inhibition was reported [37]. Genetic analysis of the resistant clone identified several mutations distributed throughout the HCV nonstructural region, but a detailed assessment of these mutations with respect to resistance to PI4KA inhibitors was hampered due to impaired replication of the recombinant mutant HCV replicon. Accumulating evidence suggests an additional role of PI4KA in HCV replication through the regulation of NS5A phosphorylation [38–40], a phenomenon that seems to be independent of the need for high levels of PI4P. The complex role of PI4KA in HCV replication likely imposes a high genetic barrier to the development of resistance to PI4KA inhibitors, thereby hampering the generation of viable, PI4KA-independent HCV mutants that could be used to dissect the role of PI4KA in the biogenesis of viral replication membranes. Given the similarities between EMCV and HCV with respect to host requirements for virus genome replication, further characterization of the EMCV mutants described here may be insightful also for understanding the role of PI4P in the context of HCV infection.

Our results indicate that, by acquiring single point mutations in the 3A protein, EMCV can replicate without an apparent accumulation of PI4P lipids at the ROs. A similar phenotype was previously described for 3A-mutant enteroviruses, which also use their 3A protein to hijack the Golgi-localized PI4KB (8). Independent single point mutations in the 3A of CVB3 (e.g V45A, I54F, H57Y) and PV (A70T) render these enteroviruses resistant to various PI4KB inhibitors [19–23]. While the considerable difference between aa sequences of 3As of cardioviruses and enteroviruses is a common knowledge, prior characterization together with our bioinformatics analysis suggest that 3As of the genera *Enterovirus*, *Cardiovirus* and *Senecavirus* may adopt alpha-alpha folds, with the N-term and C-term domains being soluble and transmembrane, respectively. Mutations A32V and A34V in EMCV 3A were predicted to be located at the solvent-exposed surface of the coiled-coil, and could therefore act, directly or indirectly, as putative determinants of intermolecular interactions between 3A and other viral and/or host factors, e.g. PI4KA. Likewise, mutations V45A, I54F, and H57Y in CVB3 3A are located in the soluble domain, and may also, in theory, modulate intermolecular interactions between 3A and either PI4KB or other factors. Another possible mechanism by which these 3A mutations could modulate 3A functionality is by affecting the interaction of 3A with the RO membranes. This latter assumption is partially explained by the PV 3A mutant A70T, which contains the aa change within the 3A hydrophobic membrane anchor (aa 61-82). Whether virus replication is rescued by a mechanism that involves a modified interaction of 3A with PI4K or with membranes remains to be determined.

Recently, we demonstrated that acute treatment with PI4KA or OSBP inhibitors impairs the accumulation of PI4P and cholesterol at EMCV ROs and leads to pronounced alterations in the subcellular distribution of the ROs, as revealed by the formation large of 3A(B)-positive clusters [26]. This "clustered" phenotype of EMCV ROs highly resembles the NS5A-positive clusters previously observed in HCV-replicon cells, also upon PI4KA or OSBP inhibition (possibly reflecting further similarities shared by EMCV and HCV with respect to the organization of viral replication sites) [27,28,38,41]. Here, we observed that prolonged PI4KA inhibition induced a similar RO clustering for the EMCV 3A mutants, as shown by the presence of large, 3A(B)-positive structures in the cytoplasm of infected cells. These 3A(B) clusters also lacked an apparent enrichment in PI4P and cholesterol. Importantly, we observed a similar clustering of the dsRNA signal upon PI4KA inhibition, suggesting that these altered ROs are indeed the sites where viral RNA replication takes place. The clustered structures of wt EMCV and HCV seem to directly correlate with impaired viral RNA replication, but remarkably, they do not interfere with the replication of EMCV 3A mutants. Two studies demonstrated that the NS5A-positive clusters observed by IF correspond to modifications of the HCV MW at the ultrastructural level [27,41]. Whether these observations also apply to the EMCV remains to be determined.

Apart from contributing to the biogenesis and/or integrity of the ROs, increasing evidence from the enterovirus field suggests an additional role of PI4P and cholesterol in modulating the correct processing of the viral polyprotein. Acute cholesterol depletion was shown to impact the processing of the precursor proteins in CVB3-infected cells [42]. Moreover, the A70T mutation in PV 3A was recently suggested to rescue virus replication from PI4KB inhibitors by restoring correct polyprotein processing [43,44]. It remains to be established whether cardioviruses also depend on the lipid microenvironment for efficient polyprotein processing and, if so, whether the single point mutations A32V or A34V can rescue processing in the presence of PI4KA inhibitors.

Cardioviruses and enteroviruses both induce a high-PI4P microenvironment at the ROs to locally concentrate OSBP, which then shuttles cholesterol to ROs in a PI4P-specific manner [10,16,17,26].

The mutations in 3A that render enteroviruses resistant to PI4KB inhibitors also confer strong cross-resistance to OSBP inhibitors [16,24,25], providing genetic evidence of the coupled dependency of enteroviruses on PI4KB and OSBP. In contrast to the enterovirus mutants, the EMCV mutants described in this study remain largely sensitive to OSBP inhibition, thus showing an apparent uncoupled level of dependency of virus replication on PI4KA and OSBP. The strong sensitivity of the EMCV mutants to OSBP inhibition compared to PI4KA inhibition points to a non-canonical function of OSBP in virus replication, likely not related to PI4P/cholesterol homeostasis at the RO-MCSs. Thus, the EMCV mutants described here may represent unique tools to investigate the individual roles of PI4KA and OSBP in (virus-induced) membrane biogenesis.

What could account for the dissimilar sensitivities of the enterovirus versus cardiovirus mutants to OSBP inhibition? The answer to this question awaits further research, but the way mutants were selected could be of relevance. In all studies so far, virus was propagated in cells where PI4K activity was targeted, either by using PI4K(B) inhibitors in the case of enteroviruses [20,22,23] or through stable knockdown of PI4K(A) expression for cardioviruses. Thus, upon selection, EMCV needed to adapt not only to shortage of PI4P but also to low PI4KA protein levels, which may have influenced the nature of the escape mutations. Another explanation for the distinct resistance profile of the mutants could reside in their differential host cell requirements. As shown here and in previous studies [16,26], wt EMCV compared to wt CVB3 seems to be more sensitive to OSBP inhibition, corroborating with the distinct resistance phenotypes of the mutant viruses. Further divergence in host factor usage is also reflected in the fact that enteroviruses mainly target Golgi membranes and factors [45], whereas cardioviruses rather rely on ER-derived membranes [26,46]. Finally, our study proves that genetically and phenotypically divergent picornaviruses can acquire mutations in sequence dissimilar 3A proteins to replicate efficiently without inducing enrichment of PI4P at ROs, highlighting the outstanding adaptability of picornaviruses to changing environmental conditions.

MATERIALS AND METHODS

Cells and reagents. Buffalo green monkey (BGM), baby hamster kidney 21 (BHK-21) and HeLa R19 cells were maintained at 37oC, 5% CO2 in Dulbecco's modified Eagle's medium (DMEM, Lonza) supplemented with 10% fetal bovine serum (FBS). Huh7-Lunet/T7 cells (provided by R. Bartenschlager, Department of Molecular Virology, University of Heidelberg, Heidelberg, Germany) [47] were grown in DMEM (Lonza) supplemented with 10% FBS and 10 ug/ml Blasticidin (PAA). For the stable cell lines Huh7-Lunet/T7-shNT and Huh7-Lunet/T7-shPI4KA [29], 2 µg/ml Puromycin and 5 µg/ml Zeocin (Invitrogen) were added to the culture medium. Huh7-Lunet/T7-shPI4KA cells stably express short hairpin RNA (shRNA) for knockdown of PI4KA, while Huh7-Lunet/T7-shNT is the corresponding control cell line expressing non-targeting short hairpin RNA. OSW-1 was generously provided by M.D. Shair (Department of Chemistry and Chemical Biology, Harvard University, Cambridge, USA). A1 [35] was a kind gift from T. Balla (National Institutes of Child Health and Human Development, National Institutes of Health, Bethesda, USA). BF738735 [20,48] was provided by Galapagos NV. Itraconazole was purchased from Santa Cruz Biotechnology. Filipin III, dipyridamole (Dip), and guanidine hydrochloride (GuHCl) were from Sigma. All compounds were dissolved in DMSO, except for dipyridamole, which was dissolved in ethanol.

Plasmids. Plasmids pEGFP-PI4KA (kindly provided by G. Hammond, NICHD, National Institutes of Health, Bethesda, USA) [49,50], p3A-myc (CVB3) [51] and p3A-myc (EMCV) [26] were described previously. Plasmid pM16.1 contains the full-length infectious cDNA sequence of EMCV, strain mengovirus. Plasmid

pRLuc-QG-M16.1 was obtained by introducing the *Renilla* luciferase gene upstream of the capsid coding region [52] in pM16.1. Infectious clones EMCV-3A-A32V and RLuc-EMCV-3A-A32V were generated by introducing the mutation A32V in the 3A sequence of pM16.1 and pRLuc-QG-M16.1 respectively, using mutagenesis primers designed with the NEBaseChanger tool and the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) according to the manufacturer's instructions. EMCV-3A-A34V and RLuc-EMCV-3A-A34V were generated similarly as the 3A-A32V mutants. Constructs p3A-A32V-myc and p3A-A34V-myc were generated from p3A-myc (EMCV) using the same strategy. Subgenomic replicons pRib-LUC-CB3/T7 and pRib-LUC-CB3/T7-3A-H57Y, encoding Firefly luciferase in place of the capsid-coding region, were described previously [19,53].

Virus infection and replicon assays. All EMCV and RLuc-EMCV viruses were obtained by transfecting BHK-21 cells with RNA transcripts derived from their respective full-length infectious clones linearized with BamHI. CVB3 (strain Nancy) and RLuc-CVB3-3A-H57Y were obtained by transfecting BGM cells with RNA transcripts of the full length infectious clones p53CB3 [51] and pRLuc-53CB3/T7-3A-H57Y [21] respectively, linearized with SalI. Virus infections were performed by incubating subconfluent cell monolayers for 30 min at 37oC with virus, after which the inoculum was removed and fresh (compound-containing) medium was added to the cells (t=0). Alternatively, cells were transfected with *in vitro* transcribed RNA of linearized subgenomic replicons or infectious clones RLuc-EMCV wt or mutant, using Lipofectamine 2000 (Invitrogen). One hour later, transfection medium was replaced with fresh (compound-containing) medium. At the indicated time points post-infection/transfection, cells were either fixed for immunolabeling, freeze-thawed to determine virus titers or lysed to determine intracellular *Renilla* or Firefly luciferase activity using the *Renilla* or Firefly Luciferase Assay System (Promega), when using the luciferase reporter viruses and replicons. Virus titers were determined by endpoint titration according to the method of Reed and Muench and expressed as 50% tissue culture infective doses (TCID50).

Selection of PI4KA-independent EMCV variants. PI4KA-independent EMCV mutants were selected by serial passaging of wild-type (wt) EMCV in Huh7-Lunet/T7-shPI4KA cells. This procedure was started by preparing serial dilutions of a wt EMCV stock in 96-well plates containing Huh7-Lunet/T7-shPI4KA cells. The highest virus dilution that induced CPE (108) was subsequently passaged in Huh7-Lunet/T7-shPI4KA cells in 96-well plates, and virus cultures from individual wells were harvested based on early CPE development. Next, two virus cultures (from wells 3E1 and 4E3) were selected and further passaged four times in Huh7-Lunet/T7-shPI4KA cells at multiplicity of infection (MOI) 1, with supernatants containing mutant viruses harvested each time at 16 hours p.i. (prior to full CPE). The obtained virus cultures were then tested for resistance to AL-9. Finally, the viral genomic RNA was isolated, used for cDNA synthesis by RT-PCR, and subjected to Sanger sequencing analysis.

Immunofluorescence microscopy. HeLa R19 or Huh7-Lunet/T7 cells were grown to subconfluency on coverslips in 24-well plates. Where indicated, cells were transfected with 400 ng of plasmids using Lipofectamine2000 according to the manufacturer's protocol and/or mock infected or infected for 30 min with EMCV or CVB3 at the specified multiplicity of infection (MOI), followed by compound treatment where specified. At the indicated time points, cells were fixed with 4% paraformaldehyde for 20 min at room temperature. For filipin staining, permeabilization was done with PBS/0.2% saponin/5% BSA for 5 min. Staining of intracellular PI4P was performed as described elsewhere [26,54]. Briefly, cells were fixed with 2% PFA, permeabilized for 5 min in 20 µM digitonin in buffer A (20mM Pipes, pH 6.8, 137 mM NaCl, 2.7 mM KCl), blocked for 45 min in buffer A with 5% NGS and 50 mM NH_4Cl and

then incubated sequentially with primary and secondary antibodies in buffer A with 5% NGS, before post-fixation in 2% PFA for 10 min. The following primary antibodies were used for detection: mouse monoclonal anti-PI4P IgM (Echelon Biosciences), mouse monoclonal anti-EMCV 3AB (kind gift from A.G. Aminev) [55], mouse monoclonal anti-dsRNA (J2, English & Scientific Consulting), rabbit polyclonal anti-myc (Thermo Scientific), and rabbit polyclonal anti-OSBP (kindly provided by M.A. De Matteis, Telethon Institute of Genetics and Medicine, Naples, Italy) [16]. Alexa Fluor 488-, 594-conjugated IgG and Alexa Fluor 594-conjugated IgM (Invitrogen, Molecular Probes) were used as secondary antibodies. Cholesterol was stained during the incubation with the secondary antibody with 25 µg/ml filipin III for 1 h at room temperature. Nuclei were counterstained with DAPI. Coverslips were mounted with FluorSave (Calbiochem). Images were acquired with a Nikon Ti Eclipse microscope equipped with an Andor DU-897 EMCCD-camera.

Image analysis. To quantify colocalization of OSBP and filipin with 3AB, images containing Z-stacks taken at ~100nm intervals throughout the depth of the cells were first deconvoluted using NIS software (20 iterations) and further processed using ImageJ as follows. Individual infected cells were outlined and a mask was created, and the signal outside the mask was cropped to exclude it from the calculations. The Pearson's coefficient of colocalization was determined for a number of at least 15 cells per condition using the Coloc 2 plugin with default settings.

siRNA treatment. HeLa R19 cells were reverse-transfected with 2 pmoles of siRNA per well of a 96-well plate (2000 cells/well) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's indications. Scrambled siRNA (AllStars Neg. Control, Qiagen) was used as a control. siRNA against hPI4KA (cat. no. S102777390) and hPI4KB (target sequence: 5'-UGUUGGGGCUUCCCUGCCCTT-3') were from Qiagen. siRNA against hOSBP (two siRNAs mixed at 1:1 ratio, target sequences: 5'-CGCUAAUGGAAGAAGUUUA[dT][dT]-3' and 5'-CCUUUGAGCUGGACCGAUU[dT][dT]-3') was from Sigma. siRNA against PI4K2A and PI4K2B were from Ambion [21]. 48 h p.t., cells were either infected with virus or analyzed in a cell viability assay.

Cell viability assay. Cell viability was determined in parallel with virus infection. The medium was replaced with CellTiter 96 AQueous One Solution Reagent (Promega) and optical densities were measured at 490 nm following a 2 h incubation at 370C, 5% CO2. The obtained raw values were converted to percentage of samples transfected with scrambled siRNAs, after correction for background absorbance.

Statistical analyses. Where indicated, unpaired one-tailed Student's t-test, one-way ANOVA or two-tailed Mann–Whitney test were applied as statistical analyses using the GraphPad Prism software.

Bioinformatics analyses. Multiple sequence alignment (MSA) of 3A proteins of cardio- and senecaviruses were generated using the Viralis platform [56] and assisted by HMMER 3.1 [57], Muscle 3.8.31 [58], and ClustalW 2.012 [59] programs in default modes. A subset of this MSA including one virus per species, in total four sequences, was then prepared using GeneDoc 2.7 [60] to highlight conservation. This MSA and/or its separate sequences were used as input to predict secondary protein structure by PsiPred [30], transmembrane domains by TMHMM2 [32], and coiled-coils by Paircoil2 [31].

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Tyrphostin AG1478 inhibits encephalomyocarditis virus and hepatitis C virus by targeting phosphatidylinositol 4-kinase IIIα

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ABSTRACT

Encephalomyocarditis virus (EMCV), like hepatitis C virus (HCV), requires phosphatidylinositol 4-kinase IIIα (PI4KA) for genome replication. Here, we demonstrate that tyrphostin AG1478, a known EGFR inhibitor, also inhibits PI4KA activity, both *in vitro* and in cells. AG1478 impaired replication of EMCV and HCV, but not that of an EMCV mutant previously shown to escape PI4KA inhibition. This work uncovers novel cellular and antiviral properties of AG1478, a compound previously only regarded as cancer chemotherapy agent.

Tyrphostin AG1478 (N-(3-Chlorophenyl)-6,7-dimethoxy-4-quinazolinamine) is best known as a potent and specific inhibitor of epidermal growth factor receptor (EGFR) signaling. AG1478 inhibits EGFR by blocking its protein tyrosine kinase activity and by promoting the formation of inactive EGFR dimers [1,2]. A previous study demonstrated that AG1478 induces Golgi dispersal and proposed GBF1 (Golgi-specific brefeldin A resistance factor 1) as the target [3]. GBF1 is an essential host factor for genome replication of picornaviruses from the genus enterovirus, such as poliovirus (PV) and coxsackievirus B3 (CVB3) [4–6]. GBF1 inhibitors such as brefeldin A (BFA) or golgicide A (GCA) completely block enterovirus replication [7]. However, we demonstrated that AG1478 does not have any effect on enterovirus replication, thus questioning the validity of AG1478 as bona-fide GBF1 inhibitor [7]. Notably, AG1478 contains a 4-anilinoquinazoline core, similar as AL-9 (Fig. 1A), an established inhibitor of phosphatidylinositol 4-kinase type III isoform α (PI4KA) [8]. PI4KA is one of the four mammalian PI4K isoforms that generate phosphatidylinositol 4-phosphate (PI4P) from PI [9–11]. PI4KA activity was shown to be indispensable for the replication of the hepatitis C virus (HCV) of the *Flaviviridae* family [12–18], and more recently we demonstrated it to be also essential for encephalomyocarditis virus (EMCV), a picornavirus of the genus *Cardiovirus* [19]. These considerations prompted us to investigate whether AG1478 might be a PI4KA inhibitor.

We examined whether AG1478 inhibits EMCV replication in a single-cycle assay. To this end, HeLa cells were infected with either EMCV or CVB3 for 30 min, after which virus-containing medium was replaced with compound-containing medium. 8 hours later, cells were lysed by freeze-thawing to determine

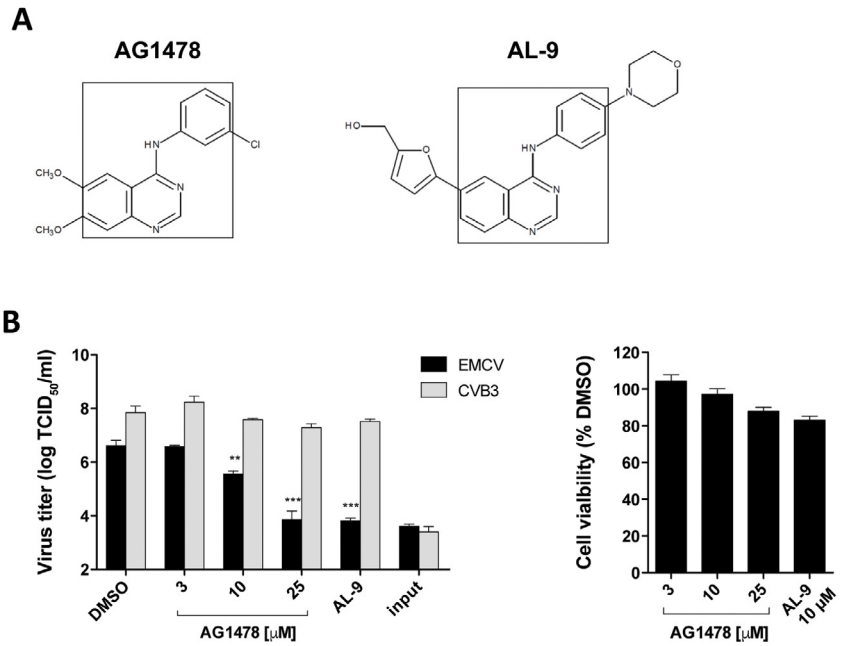

Figure 1. Tyrphostin AG1478 inhibits EMCV replication. A) Shown are the chemical structures of AG1478 and the PI4KA inhibitor AL-9. Outlined is the common 4-anilino-quinazoline core. B) Sensitivities of EMCV and CVB3 to AG1478 treatment. HeLa cells were infected with for 30 min with EMCV or CVB3 at MOI 1, after which virus-containing medium was replaced with fresh (compound-containing) medium. Cells were freeze-thawed to release intracellular virus particles and the total virus titers at 0.5 (input) and 8h p.i. were determined by endpoint dilution. Cytotoxicity of the treatment was measured separately in a cell viability assay. Shown are mean values ± SEM. Means were statistically compared using unpaired t test. **, P<0.01; ***, P<0.001.

the total virus titers by end-point dilution. As we have previously shown [7], AG1478 did not perturb CVB3 replication (Fig. 1B). However, EMCV was inhibited by AG1478 in a dose-dependent manner, with a complete inhibition at 25 μM. The inhibition observed with AG1478 was comparable to that obtained with 10 μM AL-9 (Fig. 1B). In parallel, a cell viability assay was performed to verify that the antiviral activity of AG1478 was not due to cytotoxic effects (Fig 1B).

Having established that AG1478 impairs EMCV replication and shares structural similarities with the known PI4KA inhibitor AL-9, we next wondered whether AG1478 targets PI4KA. To address this question, we first investigated if AG1478 can directly inhibit the lipid kinase activity of PI4KA in an *in vitro* assay. Using a previously described protocol [20], we measured the *in vitro* activities of immunoprecipitated PI4KA or commercially available purified PI4KA, in the absence or presence of increasing concentrations of AG1478 or the established PI4KA inhibitor "Compound A" [21]. Both compounds reduced PI4KA activity, although AG1478 was less potent than Compound A (Fig 2A).

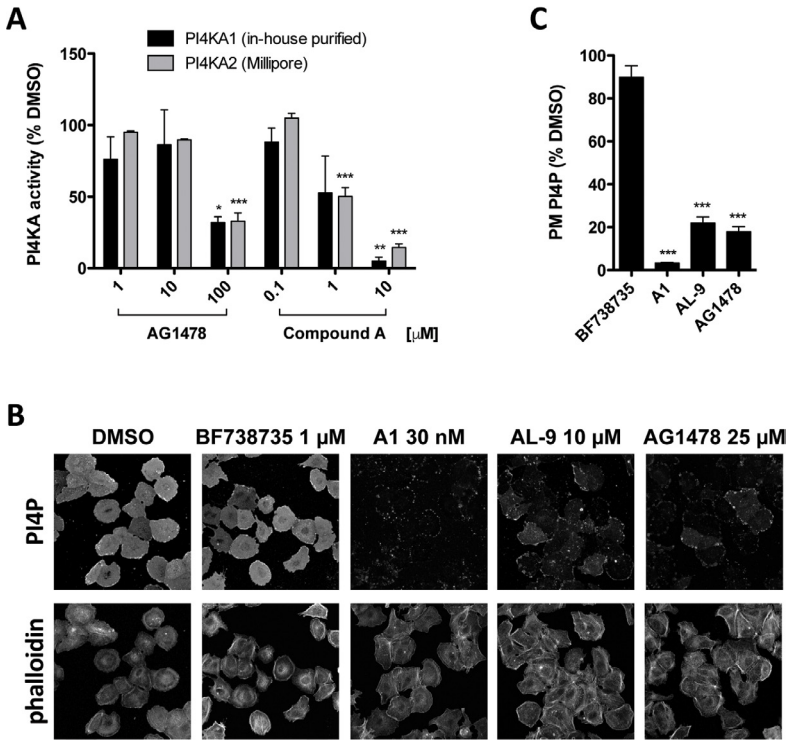

Figure 2. AG1478 targets PI4KA activity. A) PI4KA lipid kinase *in vitro* assay. HA-tagged PI4KA purified by immunoprecipitation as described previously (20), or purified commercially available PI4KA (Merck Millipore, Darmstadt, Germany), were preincubated for one hour with the indicated concentrations of AG1478 or Compound A before starting the lipid kinase reaction as previously described (20). PI4KA activity was measured as incorporation of [32P] and is expressed as percent of the DMSO control (100%). Shown are mean values ± SEM. B) AG1478 inhibits PI4KA-dependent PI4P production in intact cells. Huh7-Lunet/T7 cells were treated for 3 hours with the indicated concentrations of AG1478 or PI4KA inhibitors AL-9 and A1, or the PI4KB inhibitor BF738735, included here as negative control. Following compound treatment, cells were fixed with paraformaldehyde and further subjected to immunofluorescence analysis to visualize plasma membrane PI4P (22). Alexa Fluor 488-phalloidin was used as counterstain. C) The intensities of PI4P signals in B) were quantified using ImageJ, as previously described (19). Shown are mean values ± SEM of at least 40 cells per condition, expressed as percent of the DMSO control (100%). (A, C) Means were statistically compared using unpaired t test. ***, P<0.001.

Our *in vitro* data suggested that AG1478 is a direct PI4KA inhibitor with lower potency than other PI4KA inhibitors. We next examined whether AG1478 inhibits PI4KA activity also in living cells, and how this compares to inhibition by known PI4KA inhibitors. PI4KA is responsible for the production of the plasma membrane (PM) pool of PI4P; thus, monitoring the PM PI4P levels provides a direct and reliable measure of the PI4KA activity in cells. Using a previously established quantitative immunofluorescence staining protocol [19,22], we measured the intensity of PM PI4P signals in Huh7 cells. Unlike HeLa cells, Huh7 cells are relatively flat and exhibit a continuous sheet-like PI4P pattern covering the entire cell surface, which enables imaging of the entire PM-associated signal for accurate quantification. We compared the effects of AG1478 on PM PI4P with those of AL-9 and "A1" (Fig. 2B, upper panels), the latter a recently described very potent PI4KA inhibitor [11] (of note, A1 should not be confused with Compound A, as they represent distinct PI4KA inhibitors). Quantification of the PI4P intensity signals (Fig. 2C) revealed that AG1478 treatment led to a significant reduction of PI4P levels (up to 83%) compared to the DMSO control, very similar to AL-9 (79% inhibition), while the impact of A1 treatment was even greater (97%). In contrast, treatment with BF738735, an inhibitor of the PI4K type IIIβ isoform [23], responsible for generating PI4P at Golgi membranes [24,25], did not have a significant effect on PM PI4P levels. Alexa Fluor 488-coupled phalloidin (Invitrogen) was used as counterstain, to facilitate delineation of the cell margins where the PI4P signal was almost completely lost upon treatment (Fig. 2B, lower panels). These results demonstrated that AG1478 indeed impairs PI4KA activity in intact cells.

Recently, we discovered that single point mutations in the viral protein 3A render EMCV replication resistant to PI4KA inhibitors [26]. To validate that AG1478 inhibits EMCV by targeting PI4KA, we tested whether the previously identified mutation in EMCV 3A (A32V) conferring resistance to PI4KA inhibitors AL-9 and A1 would also provide cross-resistance to AG1478. Furthermore, we evaluated if AG1478 blocks EMCV at the step of viral genome replication by using wild-type (wt) or the 3A-A32V mutant EMCV expressing *Renilla* luciferase upstream of the capsid coding region (RLuc-EMCV) [26]. Determining the *Renilla* luciferase activity in this assay provides a direct measurement of viral RNA replication. Cells were infected with wt or mutant RLuc-EMCV for 30 min, followed by treatment with AG1478 or the established EMCV replication inhibitor dipyridamole (Dip) [27]. 7 hours later, cells were lysed to allow quantification of the intracellular amount of luciferase. Dip treatment completely inhibited the genome replication of both wt and mutant virus. In contrast, AG1478 only blocked the wt virus, but not the mutant, which replicated to almost full extent in the presence of AG1478 (Fig. 3). These data strongly indicated that AG1478 inhibits EMCV genome replication by targeting PI4KA.

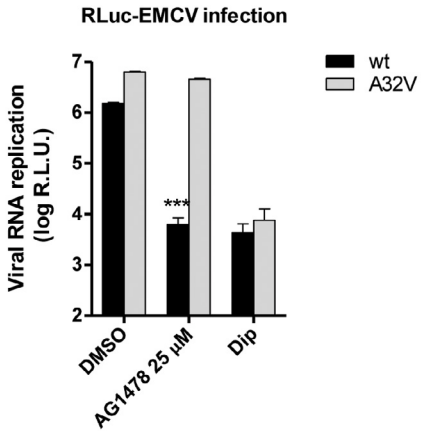

Figure 3. The PI4KA-escape mutant EMCV-3A-A32V is cross-resistant to AG1478. HeLa cells were infected with virus at MOI 1 for 30 min. After removal of the inoculum, fresh (compound-containing) medium was added to the cells. After 8 hours, cells were lysed to determine the intracellular *Renilla* luciferase activity as a measure of viral RNA replication. Shown are mean values ± SEM. Means were statistically compared using unpaired t test. ***, P<0.001.

Finally, we tested whether AG1478 also exerts antiviral activity against HCV. To this end, we compared the effects of AG1478 with those of Compound A, in Huh7 cells stably expressing genotype 1b (gt1b, Con1-ET) or 2a (gt2a, JFH-1) subgenomic HCV replicons [28–30], by measuring viral RNA replication after three days of treatment. Similar as Compound A, AG1478 treatment had greater effects on gt1b than on gt2a (Fig. 4A). The observed antiviral effects of AG1478 on HCV were not due to cytotoxicity, as demonstrated by a cell viability assay (Fig. 4B). Unfortunately, we were unable to test concentrations of AG1478 higher than 10 uM in this assay, because these were cytotoxic (data not shown), impairing further assessment of the antiviral effects on gt2a. Notably, AG1478 was less potent than Compound A in inhibiting HCV, which correlates with the potencies of the two compounds in inhibiting PI4KA activity observed in the *in vitro* kinase assay (Fig. 2A). These results suggested that AG1478 likely inhibits HCV by targeting PI4KA.

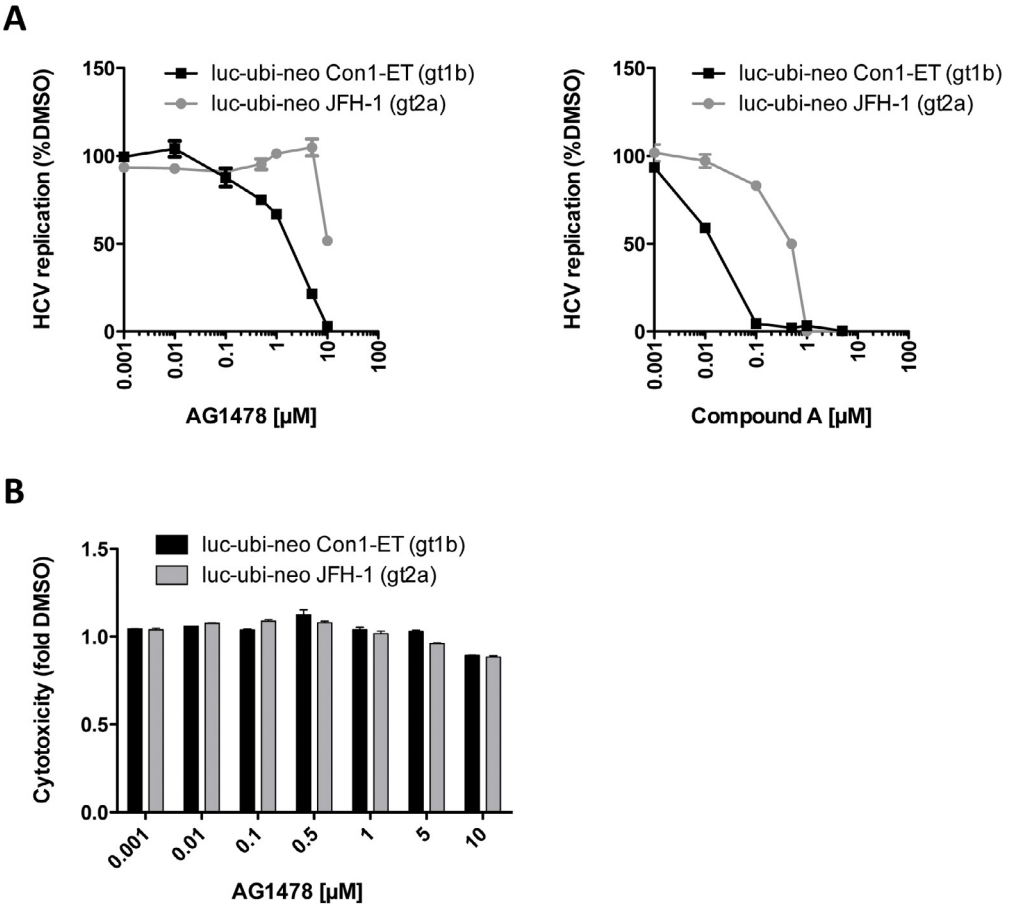

Figure 4. AG1478 inhibits HCV replication in a genotype-dependent manner. Stable Huh7 cell lines harboring HCV reporter replicons of either genotype Con1-ET (gt1b) or JFH-1 (gt2a) were incubated with the indicated amounts of compound for 72h, after which firefly luciferase activity was determined as a measure of virus RNA replication (A). Cytotoxic effects of the AG1478 treatment were evaluated separately in a cell viability assay (B). (A,B) Shown are mean values ± SEM.

Summarizing, we here identify PI4KA as a novel cellular target of tyrphostin AG1478, a compound previously only recognized as an EGFR inhibitor and Golgi-dispersing agent. We reveal that AG1478 exerts antiviral properties against EMCV and HCV, and demonstrate that its mode-of-action involves inhibition of PI4KA activity. Our *in vitro* data suggested that AG1478 is a direct inhibitor of PI4KA, however, we cannot exclude the possibility that AG1478 targets PI4KA activity indirectly, or a combination of both. The antiviral properties of AG1478 are most likely not linked to its effects on EGFR signaling, since AG1478 was shown to inhibit EGFR in the low nanomolar range [31], whereas inhibition of virus replication (and PI4KA activity) requires micromolar concentrations. Although EGFR inhibition unlikely accounts for the antiviral activity of AG1478, it would be interesting to investigate in the future whether AL-9 (and other structurally-related inhibitors) may also exhibit anti-EGFR properties. In conclusion, our study uncovers important cellular effects and antiviral properties of tyrphostin AG1478, a compound proposed earlier as promising treatment in cancer chemotherapy.

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Summary and General Discussion

parts of this chapter have been published in:

Fat(al) attraction: picornaviruses usurp lipid transfer at membrane contact sites to create replication organelles

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SUMMARY

Like all viruses, picornaviruses critically depend on the host cell resources for their propagation. To ensure a favorable environment for the replication of their +RNA genomes, picornaviruses, similar as other +RNA viruses, transform the intracellular membrane landscape into viral replication organelles (ROs). These virus-induced ROs are novel membranous structures displaying a unique architecture, protein and lipid composition. Requirements for picornavirus RO formation and functioning are currently poorly characterized. This thesis project aimed to identify novel host factors essential for picornavirus genome replication and to dissect the molecular mechanism of host factor recruitment to enterovirus ROs. A better understanding of the virus-host interactions and mechanisms underlying the biogenesis and functionality of the ROs may set fertile grounds for the development of future antiviral therapies.

This thesis investigates the viral and cellular requirements for RO formation and genome replication by distinct picornaviruses. **Chapters 2** and **3** deal with enteroviruses, using coxsackievirus B3 (CVB3) and rhinovirus (RV) as a model, while **Chapters 4-6** focus on the distantly-related cardioviruses, employing encephalomyocarditis virus (EMCV) as a model.

In **Chapter 2**, we investigated the mechanism by which CVB3 recruits the Golgi-localized enzyme PI4KB to its ROs. PI4KB was proposed to be recruited to enterovirus ROs by the viral protein 3A via GBF1/Arf1 or via ACBD3, which were shown to be important for the recruitment of the kinase to Golgi membranes in non-infected cells. We performed a systematic analysis of PI4KB recruitment to membranes in both *in vitro* systems and in intact cells. Genetic depletion of GBF1 or Arf1 by siRNA treatment or pharmacological inhibition of GBF1-mediated Arf1 activation by BFA did not interfere with PI4KB recruitment to membranes by 3A. Furthermore, we demonstrate that 3A mutants that cannot interact with GBF1 can still recruit the kinase to membranes in an *in vitro* system, and that some 3A mutants that retain their interaction with GBF1 are impaired in PI4KB recruitment, collectively indicating that PI4KB recruitment occurs independently of GBF1/Arf1. Although multiple enteroviral 3A proteins interacted with ACBD3 in a mammalian two-hybrid system, ACBD3 depletion by siRNA treatment did not hamper PI4KB recruitment to CVB3 ROs or virus replication, which was rather enhanced upon ACBD3 knockdown. PI4KB was still recruited in ACBD3-depleted cells by a 3A mutant that is impaired in GBF1 binding, suggesting that ACBD3 and GBF1/Arf1 are simultaneously dispensable for PI4KB recruitment. Using purified recombinant proteins in co-IP assays, we could detect direct interactions between ACBD3 and 3A and between ACBD3 and PI4KB, but not between 3A and PI4KB. Thus, the mechanism of PI4KB recruitment to ROs by 3A, which may occur via an unknown cellular co-factor or via a transient direct interaction, remains obscure.

Chapter 3 studies the importance of PI4KB in RV replication and the mechanism by which it is recruited to RV ROs. A first evidence that GBF1 is not involved in PI4KB recruitment by RV was the finding that RV 3A proteins establish little, if any interaction with GBF1, and yet induce PI4KB accumulation at membranes when expressed *in vitro* or in intact cells. However, GBF1 was present at RV ROs and was required for virus replication, since treatment with the GBF1 inhibitors golgicide A (GCA) and BFA inhibited RV replication, an effect that could be rescued by ectopic expression of the BFA-resistant GBF1 mutants M832L and A795E. Importantly, these data suggested that the mechanism that positions GBF1 at RV ROs to function in virus replication is likely 3A-independent. Although RV 3A proteins interacted with ACBD3, PI4KB recruitment was found to be ABCD3-independent, since knockdown of ACBD3 did not affect the kinase recruitment or virus replication. These data are consistent with the results from

Chapter 2, strongly arguing that PI4KB recruitment to enterovirus ROs is a process dependent on 3A, but not on GBF1/Arf1 or ACBD3.

Chapter 4 describes for the first time how cardioviruses develop their ROs, using EMCV as a prototype virus. We demonstrate that cardioviruses also manipulate the PI4P metabolism for their own benefit, not by utilizing PI4KB like enteroviruses do, but instead by hijacking the ER-localized PI4KA. By chemical and genetic inhibition we show that PI4KA activity is required for EMCV genome replication. In EMCV-infected cells, PI4KA, but not PI4KB redistributed to ROs, greatly overlapping with the viral protein 3A(B). By radioactive co-IP, we identified 3A and its precursor 3AB as the only viral non-structural proteins interacting with PI4KA, a finding also confirmed by co-IP coupled with WB analysis. Overexpression of 3A or 3AB was sufficient to induce PI4KA recruitment to discrete cytoplasmic punctae where the viral proteins also localized, suggesting that in infected cells, PI4KA is recruited to EMCV ROs by 3A. Detailed IF analysis of the PI4P profile revealed profound changes in both the distribution and amounts of this lipid in infected cells, which contained elevated PI4P levels. Using PI4KA inhibitors, we demonstrated that PI4P is important for the recruitment of OSBP to the ROs. OSBP is an essential cardiovirus host factor, since OSBP-targeted siRNA treatment reduced EMCV genome replication. Using PI4KA and OSBP inhibitors, we demonstrated that OSBP functions at membrane contact sites (MCSs) to shuttle cholesterol to the ROs in exchange for PI4P. Blocking the PI4P/cholesterol shuttling function of OSBP proved detrimental to both EMCV genome replication and the overall integrity of the ROs. Last, but not least, our findings revealed a striking convergence of EMCV and HCV with respect to host factor usage, since HCV was also shown to hijack the PI4KA-OSBP pathway in order to develop PI4P-and cholesterol-enriched viral replication structures. While this study provided the first detailed mechanistic insight into the development of cardiovirus ROs, the role of PI4P and cholesterol in virus replication require further investigation.

In **Chapter 5** we demonstrate that EMCV can escape PI4KA inhibition by acquiring single point mutations in the viral protein 3A. Using a genetic approach by propagating wt EMCV in a cell line with stable PI4KA knockdown, we selected two independent virus cultures that harbored point mutations in 3A, namely A32V and A34V. When introduced back in the wt genome, these mutations recapitulated the resistant phenotype against both PI4KA inhibitors and PI4KA depletion. Remarkably, the mutant viruses did not display altered replication kinetics compared to wt virus, nor did they resort to using another PI4K isoform. Consistent with these results, we observed that PI4KA inhibition throughout infection resulted in the generation of altered ROs phenotype with a clustered distribution, as previously shown in **Chapter 4** for the wt virus upon acute PI4KA inhibition. Notably, these altered ROs were devoid of PI4P, OSBP and cholesterol, but could still support virus RNA replication. Although this suggested that the mutants no longer depended on OSBP, we instead observed very little, if any cross-resistance to OSBP chemical inhibition or depletion by siRNA treatment. These data argued that the mutants still require a yet unknown function of OSBP, perhaps not related to PI4P/cholesterol shuttling, which remains to be determined. This study not only provides a strong genetic evidence for the involvement of EMCV 3A in modulating PI4P metabolism, but also highlights the complex functions of host factors, such as OSBP, in virus replication.

Chapter 6 reports the identification of tyrphostin AG1478 as a novel PI4KA inhibitor. AG1478 is well known for its anti-cancer properties due to its potent inhibition of EGFR tyrosine kinase activity. Based on the structural resemblance of AG1478 with AL-9, a known PI4KA inhibitor, we set out to investigate whether AG1478 also has anti-PI4KA activity. AG1478 inhibited PI4KA activity both in intact cells and *in*

vitro, suggesting that it acts by binding to the kinase directly. Consequently, AG1478 exhibited antiviral properties against EMCV, but not CVB3. Furthermore, the PI4KA-escape mutant EMCV-3A-A32V was cross-resistant to AG1478, substantiating that the antiviral mode-of-action is based on PI4KA inhibition. AG1478 can prove a useful tool to explore the role of PI4KA both in the context of infection as well as under normal physiological conditions.

GENERAL DISCUSSION

Replication organelle formation is a highly complex process and a critical step in the life cycle of a picornavirus, since it ensures the "stage" where viral and host players concentrate to orchestrate viral RNA replication. Enteroviruses and cardioviruses, which represent distantly related picornaviruses, have evolved to engage distinct cellular PI4-kinases in the formation of their RO. How and why they achieve this is discussed below. The first part (I) of this discussion will focus on the mechanism of PI4KB recruitment to enterovirus ROs, whereas the second part (II) will address the role of PI4KA and PI4P in cardiovirus replication.

Part I. Mechanism of PI4KB recruitment to enterovirus ROs

The Golgi-localized, lipid-modifying enzyme PI4KB is an essential host factor for enterovirus replication [1]. During enterovirus infection, PI4KB and its product, PI4P, accumulate at viral ROs. PI4KB recruitment to the ROs is mediated by the small enterovirus protein 3A, but the underlying mechanism is unclear.

Role of GBF1/Arf1

Several lines of evidence implied an involvement of GBF1/Arf1 in PI4KB recruitment by enteroviruses. First, 3A of PV and CVB3 can directly interact with GBF1. Second, PI4KB is one of Arf1's effectors in non-infected cells. Finally, GBF1, Arf1 and PI4KB colocalized with viral proteins at the ROs, and PI4KB co-immunoprecipitated with 3A from lysates of infected cells. A recruitment mechanism was envisaged, whereby 3A directly interacts with GBF1 to modulate Arf1 effector recruitment in such a way that PI4KB is specifically recruited at ROs at the cost of another Arf1 effector, COPI. In **Chapter 2** we demonstrate that 3A-GBF1 interaction and PI4KB recruitment are not functionally linked in enterovirus infection. CVB3 3A alanine mutants that did not bind GBF1 still recruited PI4KB, and vice-versa, 3A mutants no longer recruiting PI4KB could still accumulate GBF1/Arf1 on membranes. Furthermore, chemical inhibitors or siRNA treatment targeted at GBF1/Arf1 did not interfere with PI4KB recruitment to 3A-positive membranes in an *in vitro* assay or in intact cells, suggesting that CVB3 3A does not recruit PI4KB via GBF1/Arf1.

In **Chapter 3**, PI4KB is shown to be an essential host factor enriched at the ROs of human rhinovirus 2 and 14, whose 3A proteins establish very little (RV14) or no interaction (RV2) with GBF1. GBF1 was also present at the replication sites, despite of a lack of interaction with 3A, suggesting that its recruitment may be mediated by another viral protein. RV 3A alone was sufficient to induce PI4KB recruitment to membranes, demonstrating that RVs, like PV and CVB3, employ their viral protein 3A to bring PI4KB to their RO in a GBF1/Arf1-independent fashion.

If GBF1 is not important for PI4KB recruitment, then what role does it play in enterovirus infection? Activated Arf proteins significantly accumulate at replication membranes upon poliovirus infection [2,3], however, multiple observations point towards an Arf–independent function of GBF1 in enterovirus replication. Although one study reported that depletion of Arf1 expression by siRNA treatment inhibited CVB3 RNA replication by 70% [1], in another study, Arf1 knockdown had no effect on CVB3 progeny virion production [4]. Overexpression of wt or BFA-resistant GBF1, but not that of different Arf proteins or constitutively active Arf, can restore enterovirus replication in the presence of BFA [4,5]. BFA acts on GBF1 by locking it in an abortive complex with Arf-GDP on membranes [6,7]. Given the strong evidence

that enteroviruses do not require an active Arf to establish infection, one possible explanation for the mechanism of BFA inhibition of enterovirus replication is that GBF1 is trapped on membranes in a state that impairs its engagement in virus replication [8]. Another proof that BFA-mediated inhibition of virus replication is likely not linked to the GBF1 enzymatic activity was the finding that overexpression of a GBF1 deletion mutant lacking the catalytic Sec7 domain was still able to rescue (to a certain extent) PV replication from BFA inhibition [8]. Removal of the first 37 amino acids of GBF1 was detrimental to poliovirus replication, suggesting that the far N-terminus of GBF1 is absolutely required in infection [8]. It appears that the same deletion also abolishes the association of GBF1 with membranes [9], thus implicating a tight crosstalk between the membrane-binding properties of GBF1 and its role in virus replication. Fine-scale mapping of the N-terminal amino acid residues of GBF1 required for virus replication and/or membrane association could be revealing more about its function in infection.

Enteroviruses are believed to initiate replication at Golgi membranes [1,10,11]. It is unlikely that GBF1 plays a structural role in virus replication by maintaining Golgi integrity, since the small-molecule compound AG1478 induces Golgi disassembly and yet does not impair enterovirus replication ([12] and **Chapter 2**). Whatever role GBF1 performs in enterovirus replication, it is rather related to the functioning than the formation of ROs. Poliovirus proteins expressed in the presence of BFA induced membrane rearrangements equivalent in morphology to those formed in control-treated cells, but these structures induced in the presence of BFA failed to support viral RNA replication [5]. Furthermore, these findings also suggest that an intact Golgi may not be a prerequisite for the establishment of enterovirus infection in target cells.

The role of GBF1 in enterovirus replication is not understood. A possible clue might stem from a recent study demonstrating an implication of lipid droplet (LD) metabolism in RV replication [13]. The homology downstream of Sec7 domain 1 (HDS1) of GBF1 has lipid-binding properties and was shown to be sufficient and required for its stable targeting to LDs [14]. Furthermore, both GBF1 and the Arf/COPI machinery function directly on LDs, where they regulate LD homeostasis by locally targeting key enzymes involved in lipolysis [15,16]. One could imagine that RVs exploit the GBF1/Arf1/COPI machinery to promote LD degradation, a process that seems to support virus replication by providing essential lipids (e.g. cholesterol) serving as building blocks for RO membrane biogenesis [13]. Whether this hypothesis is true and if it also applies to PV or CVB3 infection remains to be determined. Nevertheless, one should leave open the possibility that GBF1 may be involved in multiple processes that promote virus replication, of which regulation of LD homeostasis may be one.

Surely, elucidating the function of GBF1 in enterovirus replication will require substantial future efforts. Perhaps a strategy for future studies should include mapping of the GBF1 interactome in infected cells and subsequent evaluation of the importance of these GBF1-binding factors in virus replication. Some of the known GBF1 interactors, such as p115[17] and Rab1b [18], have already been demonstrated to be dispensable for enterovirus replication [4,8]. Another interaction partner of GBF1 is the COPI subunit γ-COP. Interestingly, γ-COP binds directly to the N-terminus of GBF1 [19], which is involved in 3A interaction and virus replication. γ-COP is part of the same COPI subcomplex as the β-COP subunit [20], which was observed to be depleted from the ROs, concomitant with PI4KB enrichment [1]. Thus, one probable explanation for COPI removal from RO membranes is that 3A displaces COPI upon binding to the N-terminus of GBF1, instead of 3A modulating Arf1 selective effector recruitment as initially proposed.

GBF1 is not only important for the replication of enteroviruses. The picornavirus AiV of the genus *Kobuvirus* was also shown to require GBF1 [21]. Interestingly, kobuviruses are not sensitive to BFA treatment [22], which again argues for a non-enzymatic role of GBF1 in picornavirus replication. Furthermore, GBF1 was also identified as an important host factor for the replication of hepatitis C virus and coronaviruses [23–27]. A significant challenge that should be tackled by future research is to determine whether GBF1 plays a synonymous role in the replication of these distinct +RNA viruses.

Role of ACBD3

Kobuviruses recruit PI4KB to their ROs via the Golgi-localized protein ACBD3, a novel interactor of PI4KB [22]. Together with the finding that multiple enterovirus 3A proteins (including PV, CV, and RV 3A) co-purify with ACBD3 and PI4KB [21], this suggested that enteroviruses might also recruit PI4KB using ACBD3 as a cofactor. However, the experimental data we provide in **Chapters 2** and **3** fail to support this model. ACBD3 depletion by siRNA treatment did not impede PI4KB recruitment to membranes in CVB3-infected cells (**Chapter 3**), nor in cells in which CVB3 or RV 3A proteins were overexpressed (**Chapter 3**). In line with these results, we observed that ACBD3 knockdown did not inhibit CVB3 or RV replication.

Thus, we considered the possibility that 3A recruits PI4KB via a direct interaction. However, using purified recombinant proteins, we could not detect a direct interaction between 3A and PI4KB, but we did detect direct interactions between CVB3 3A and ACBD3 and between ACBD3 and PI4KB. The direct CVB3 3A-ACBD3 interaction and the localization of ACBD3 at CVB3 ROs (**Chapter 2**) strongly suggests that ACBD3 should be directly involved in virus replication, which is contradicted by the rest of our results showing that ACBD3 is in fact not required. What could explain this puzzling phenotype? First, one could consider the possibility that ACBD3 is essential for virus replication and that the amount of ACBD3 protein contained in the host cell exceeds the minimal level required to ensure an efficient viral replication by far. Having this in mind, we cannot exclude that the residual level of ACBD3 present in the cell after the siRNA treatment we applied in the knockdown experiments from **Chapters 2** and **3** is still sufficient to recruit PI4KB and to support virus replication. Therefore, a more reliable loss-of-function approach would be to assess the extent of virus replication in cells where ACBD3 has been depleted by gene knockout.

Second, there may be functional redundancy between ACBD3 and the other 6 "acyl-CoA-binding protein (ACBP) domain-containing" (ACBD) proteins found in mammalian cells (reviewed in [28]). These proteins, dubbed ACBD1 to ACBD7, share a sequence- and structure-wise conserved ACBP domain. The region of ACBD3 important for 3A interaction mapped to the Golgi-dynamics (GOLD) domain (**Chapter 2** and [29]). Although the GOLD domain is unique to ACBD3 among the ACBD members, this does not imply that other regions of ACBD3, such as the acyl-CoA-binding domain (ACBD), are also of relevance in the context of infection, in which case our results could be explained by an eventual redundancy among ACBD proteins.

Third, ACBD3 may be regarded as a restriction factor for enterovirus infection. In contrast with the results of Greninger et al. [21], we consistently observed that ACBD3 knockdown actually augments enterovirus replication instead of inhibiting it, albeit modestly (**Chapter 2**). Our data are corroborated by another study demonstrating similar effects of ACBD3 depletion on PV replication [30]. PI4KB and a novel ACBD3 interactor, TBC1D22, were shown to bind in a competitive and mutually exclusive manner to the same domain of ACBD3 [29]. Interestingly, kobuviral 3A proteins seem to prevent TBC1D22A binding to ACBD3, while enterovirus 3A proteins do not. Thus, a model can be imagined in which enteroviral 3A

proteins somehow promote the binding of TBC1D22 to ACBD3 in order to release PI4KB from a PI4KB-ACBD3 complex that hampers the involvement of the kinase in replication. This hypothesis seems to be supported by data from the HCV field. ACBD3 was shown to negatively regulate the replication of HCV genotypes that are dependent on PI4KB [31]. Furthermore, the same study revealed that NS5A competed with PI4KB for binding to ACBD3, suggesting that NS5A might disrupt a preexisting ACBD3-PI4KB complex to form the NS5A-ACBD3 complex, and by this relocate PI4KB to HCV replication sites in order to facilitate virus replication.

Notably, it has been proposed that ACBD3 participates in lipid homeostasis in at least two different ways. ACBD3 was suggested to influence the cellular lipogenic pathway by inhibiting the maturation and activity of the sterol regulatory element binding protein 1 (SREBP1), a transcription factor that, among others, controls the expression of the enzyme fatty acid synthase (FASN) [32]. Inhibition of SREBP1 by ACBD3 through its ACBD domain resulted in a marked reduction of FASN protein levels and consequently *de novo* palmitate biosynthesis. Since *de novo* fatty acid synthesis was suggested to be required for membrane development in picornavirus-infected cells [33–35] and FASN was found to be upregulated in CVB3-infected cells [36], the observed elevation of enterovirus replication (**Chapter 2** and [30]) upon ACBD3 knockdown may be linked to ACBD3's modulation of the fatty acid metabolism. On the other hand, ACBD3 has also been attributed a role in the transport of ceramide at ER-Golgi membrane contact sites (MCSs) [37]. Based on experimental data, a working model was proposed in which ACBD3, via the GOLD domain, interacts with the protein phosphatase PPM1L and recruits it to ER-Golgi MCS to promote dephosphorylation of the ceramide transporter (CERT). This triggers CERT activation and subsequent transfer of ceramide from its site of synthesis (ER) to the Golgi [37]. Currently, it is not known whether ceramide is an important lipid in enterovirus infection. Enteroviruses could therefore modulate ACBD3 to either promote ceramide import at ROs, or to diverge it away from the ROs. Clearly, ACBD3 constitutes a controversial aspect in the field. Future research should be aimed at reconciling the present conflicting data on the role of ACBD3 in enterovirus replication and at clarifying the functional importance of the enteroviral 3A-ACBD3 interaction.

Alternative PI4KB recruitment mechanisms

C10ORF76. C10orf76 is a protein with an unknown function that has been identified as an interaction partner of PI4KB by two separate affinity-purification mass spectrometry (AP-MS) studies [29,38]. A recent study discovered C10orf76 as a novel coxsackievirus host factor [39]. In non-infected cells, C10orf76 associated with PI4KB and mediated PI4KB-dependent PI4P synthesis at Golgi membranes. However, C10orf76 was only required for the genome replication of coxsackievirus A10, but not coxsackievirus B1, suggesting a species-specific dependence on this host factor. Future studies should investigate to which extent different enteroviruses require C10orf76, and whether C10orf76 acts as cofactor in PI4KB recruitment also in infected cells.

NCS-1. Other known PI4KB interactors that deserve attention are the neuronal calcium sensor 1 (NCS-1, or frequenin in yeast) and 14-3-3 proteins. In theory, these binding partners of PI4KB could play a role in its recruitment to membranes. NCS-1 is an N-terminally myristoylated protein that binds Ca2+ and plays an important role in exocytosis [40]. NCS-1 myristoylation was shown to influence its conformation and Ca2+ binding, which in turn regulates its physiological functions [41]. The role of NCS-1 in exocytosis is intimately linked with PI4KB. NCS-1 directly interacts with PI4KB and enhances the kinase activity both *in vitro* and in cells [42–46]. NCS-1 myristoylation was demonstrated to be important for the direct interaction with PI4KB and upregulation of its activity [43]. Interestingly, NCS-1-PI4KB complexes could

be purified from both membrane and cytosolic fractions [45]. While the role of NCS-1 in stimulating PI4KB activity is clear, as yet, it is not understood whether and how NCS-1 contributes to PI4KB recruitment to membranes, because NCS-1 primarily localizes in the cytosol [46]. Nevertheless, PI4KB and NCS-1 also colocalized at Golgi membranes [43], suggesting that the physical interaction between NCS-1 and PI4KB could also account for the translocation of the kinase to target membranes.

14-3-3. 14-3-3 proteins are multifunctional scaffolding proteins that recognize and bind phosphorylated cellular proteins [47]. 14-3-3 has been also identified to bind PI4KB, following phosphorylation of the kinase by PKD at residue Ser294, which increases PI4KB activity [48,49]. The site-specific binding of 14-3-3 to phosphorylated PI4KB influences the kinase activity by stabilizing its active conformation. However, a role of 14-3-3 proteins in the recruitment of PI4KB to membranes has not been explored yet.

In conclusion, a direct interaction between the enteroviral protein 3A and PI4KB could not be shown (**Chapter 2**) and – although only a limited number of PI4KB interaction partners have been investigated for their involvement in kinase recruitment to RO (**Chapters 2** and **3**) – a pathway for indirect PI4KB recruitment has also not been identified yet. Of note, a role in PI4KB recruitment to the ROs has also been attributed to enterovirus 2BC, which was shown to interact with the kinase in a mammalian two-hybrid system [50]. However, more work is needed to elucidate whether this interaction is relevant in the context of infection. Thus, the detailed molecular events underlying the mechanism of PI4KB recruitment to enterovirus ROs remain to be clarified.

Part II. Role of PI4KA/PI4P in cardiovirus replication

EMCV replicates at PI4P-rich ROs

Picornaviruses from the genus *Cardiovirus* were long believed to employ replication strategies substantially distinct from those of enteroviruses, as members of this genus did not show sensitivity to treatment with inhibitors of GBF1 or PI4KB [12,51–53]. While this implied that cardioviruses do not rely on GBF1/PI4KB-controlled pathways, in **Chapter 4** we report that in fact cardiovirus replication also depends on PI4P lipids. Similar as shown previously for HCV [54], EMCV induces the formation of PI4P-enriched ROs by recruiting the ER-localized enzyme PI4KA. Recruitment of PI4KA to EMCV ROs is mediated by the viral protein 3A (**Chapter 4**). It is remarkable that members belonging to three distinct picornavirus genera, namely *Enterovirus*, *Cardiovirus* and *Kobuvirus*, all employ their 3A protein to recruit distinct PI4K isoforms to their RO. The 3A proteins of enteroviruses, cardioviruses and kobuviruses are similar in size, but their sequences are very poorly conserved between genera. Likewise, PI4KA and PI4KB are not only different in size and sequence, but they also localize at distinct subcellular compartments [55]. Considering this, it is reasonable to assume that exploitation of PI4P metabolism by these distantly related picornaviruses for RO development might have provided an evolutionary advantage.

PI4KA co-purified with EMCV 3A and its precursor 3AB, but not with other EMCV non-structural proteins (**Chapter 4**). Furthermore, ectopic expression of 3A or 3AB led to PI4KA redistribution to discrete cytoplasmic punctae that also stained positive for the viral proteins, suggesting that 3A is sufficient to recruit PI4KA to membranes. To our knowledge, this is the first study that firmly assigns a direct function of cardiovirus 3A in virus replication, since PI4KA activity is shown to be essential for EMCV genome replication (**Chapter 4**). It remains to be established whether EMCV 3A recruits PI4KA via a

direct interaction or with the help of a cellular cofactor.

HCV protein NS5A directly binds PI4KA and stimulates its activity *in vitro* and in intact cells, which results in the accumulation of intracellular PI4P at HCV replication membranes [54]. Moreover, one study showed that the enrichment of PI4P at the HCV membranous web correlated with a concomitant depletion of PI4P in the plasma membrane [56]. Since the PM pool of PI4P is PI4KA-derived [57–59], it has been suggested that the observed PM PI4P depletion may be a consequence of PI4KA being sequestered at the MW and prevented from synthesizing PI4P at the PM [56]. In contrast with these data, we did not observe any alterations of the PM PI4P pool upon infection with EMCV, whereas the intracellular PI4P presented a markedly changed distribution and elevated levels (**Chapter 4**). This phenotype may have several explanations. One possibility is that (a fraction of) PI4KA still interacts with its cellular partners that position it transiently at PM to catalyze local PI4P synthesis. Another scenario is that PI4P lipids synthesized elsewhere in the cell by the other PI4K isoforms are transported to the PM. Finally, the unchanged PM PI4P levels may have reflected a slow turnover of the lipid at this compartment. However, we observed that acute treatment of the cells with PI4KA inhibitors results in a dramatic decrease of PM PI4P amounts (**Chapter 6** and unpublished data), suggesting instead a highly dynamic turnover of PI4P at this compartment. Since PI4P lipids are critical for organelle identity and functioning [60], and EMCV has been demonstrated to upregulate fatty acid import – a PM-dependent process [61], it is conceivable that EMCV ensures a fully functional PM by maintaining its PI4P pool unaltered, which in turn allows the import of fatty acids believed to be facilitate membrane biogenesis. The elevated levels of PI4P in EMCV-infected cells most likely reflect an enhanced PI4KA activity, which may be promoted by EMCV 3A or another viral protein.

OSBP is a PI4KA effector in cardiovirus replication

The large amounts of PI4P lipids in EMCV-infected cells play a central role in redistributing OSBP to the viral ROs (**Chapters 4** and **5**). OSBP has recently emerged as an essential player in non-vesicular intracellular lipid transport and homeostasis [62]. In non-infected cells, OSBP participates in the formation of membrane contact sites (MCSs) between the ER and trans-Golgi membranes [63]. OSBP tethers these membranes by binding to the integral ER membrane proteins VAP-A and VAP-B via its FFAT motif, while it docks to the trans-Golgi membranes by binding to PI4P and Arf1 via its PH domain. Via its OSBP-related domain (ORD), a domain conserved across the OSBP family, OSBP transfers PI4P to the ER, where it is hydrolyzed by Sac1 to provide the energy required for the transport of cholesterol against the concentration gradient, that is, from the ER to the Golgi. Cholesterol binding and transport also occurs via the ORD. The PI4P/cholesterol lipid transfer function of OSBP at MCSs is similarly exploited by EMCV (**Chapter 4**), HCV [64], and enteroviruses [13,65]. It is highly likely that the PI4P-enriched membranes of AiV replication sites [66] also serve to recruit OSBP and subsequently cholesterol, but this remains to be established. By siRNA treatment and chemical inhibitors, we demonstrate that the function of OSBP is critical for EMCV genome replication (**Chapters 4** and **5**). VAP proteins and Sac1 have been shown to be important for enterovirus [1,13] and HCV replication [1,23,67,68], thus they are likely also involved in EMCV replication.

Interestingly, upon 3A overexpression, endogenous OSBP loses its typical Golgi localization and instead concentrates at 3A-positive punctae distributed throughout the cytoplasm (**Chapter 4**), suggesting that 3A alone is sufficient to induce OSBP recruitment to membranes. The 3A-mediated OSBP recruitment to ROs is a PI4P-dependent process (**Chapter 4**), however, additional, PI4P-independent mechanisms may also be involved, such as an interaction between 3A (or another viral protein) and OSBP. This may also

be suggested by our observation that acute inhibition of PI4KA activity in EMCV-infected cells leads to a decrease, but not a complete loss of OSBP localization from the ROs (**Chapter 4**).

It is remarkable that OSBP can accommodate two lipids as structurally distinct as PI4P and cholesterol in its ORD, which raises the possibility that the ORD of OSBP may also exhibit affinity for other lipid species than PI4P/cholesterol, some of which could potentially be important for virus replication and delivered to the ROs in an OSBP-dependent manner. OSBP is one of the 12 members of the OSBP-related protein (ORP) family, which are thought to play important roles in lipid homeostasis at diverse MCSs [69,70], however, the precise functions of the various ORP proteins remain poorly characterized. Recently, it has been suggested that besides OSBP, other ORPs may also be involved in enterovirus replication [13,65], but strong evidence to support these findings is still lacking. It remains to be established whether EMCV replication requires the function of other ORP proteins.

Origin of EMCV ROs

Accruing evidence suggests that RO formation is a complex process based on both *de novo* lipid synthesis and the remodeling of pre-existing intracellular membranes [71,72]. In an effort to assign a possible origin to the EMCV ROs, we have screened a number of cellular organelle markers for colocalization with viral proteins 3A or 2C, as shown in **Chapter 4**. The signal for the ER-resident protein calreticulin overlapped with EMCV 3A to a certain degree, whereas none of the other tested organelle markers – including cis- and trans-Golgi, ER-Golgi intermediate compartment (ERGIC) and ER exit site (ERES) – showed any apparent colocalization with viral proteins, suggesting that EMCV ROs are derived from the ER rather than from the Golgi. Nevertheless, EMCV-infected cells displayed an obvious dispersal of Golgi markers throughout the cytoplasm, with greater effects on the TGN than the cis-Golgi compartment, suggesting that the Golgi integrity is affected upon infection. This poses an intriguing question: why would EMCV trigger dispersal of the Golgi complex if it does not utilize it as a membrane source, like enteroviruses do? A plausible explanation may be that EMCV-induced Golgi dispersal is part of a mechanism that contributes to OSBP recruitment to the ROs. TGN/Golgi dispersal is likely to disrupt the MCSs where OSBP normally localizes and operates, consequently rendering OSBP "available" for recruitment to and functioning at ER-ROs. Although less likely, it cannot be excluded that EMCV is highly selective in recruiting some Golgi factors at the ROs, a process that may very well interfere with the Golgi architecture.

Putative roles of PI4P/cholesterol in cardiovirus replication

Structural role. PI4KA and OSBP are essential host factors for EMCV replication by virtue of their functions at the ROs in lipid synthesis (PI4KA) and transport (OSBP). What can explain the critical importance of PI4P and cholesterol for virus genome replication? Our experimental data presented in **Chapters 4** and **5** suggest that PI4P/cholesterol are important for the global organization of EMCV ROs, since treatment with either PI4KA or OSBP inhibitors resulted in an altered RO appearance, represented by a more clustered distribution. Since PI4KA and OSBP inhibitors inhibit virus genome replication, taken together, these results suggest that the clustered ROs may no longer be capable of supporting viral RNA synthesis. A similar clustering effect has also been reported in the case of the HCV MW upon PI4KA or OSBP inhibition [54,64,73], arguing for a central role of PI4P/cholesterol in maintaining the integrity of virus replication structures. The structural role of PI4P/cholesterol could be a direct one, such as influencing the membrane curvature (PI4P and cholesterol) or fluidity (cholesterol).

However, by recruiting host proteins other than OSBP to the ROs, PI4P may also play an indirect role in replication. Numerous cellular proteins have been shown to bind PI4P, including the PH-domain containing proteins CERT and FAPP2, which intimately couple PI4P binding with lipid transfer between membranes [74]. Additionally, PI4P lipids can bind cellular proteins without a canonical PH-domain, like GOLPH3 [75] or BAR-domain-containing proteins called arfaptins [76], which have intrinsic membrane-shaping properties. Thus, by directly impacting the membrane lipid composition or shape of the ROs, these proteins may play a role in virus replication. While the importance of these PI4P-interacting proteins in picornavirus replication is not known yet, some have previously been assigned a role in HCV life cycle, i.e. FAPP2 was shown to play a PI4P-dependent role in RNA replication [77], whereas CERT and GOLPH3 seem to be involved in particle assembly [78,79].

Polyprotein processing. Accruing evidence suggests that cholesterol is needed to ensure the correct processing of the enterovirus polyprotein. Cyclodextrin-mediated cholesterol depletion or cholesterol disruption by filipin was shown to influence polyprotein processing by promoting the cleavage of precursor protein 3CDpro into individual proteins $3C^{pro}$ and $3D^{pol}$ [80]. Enhanced 3CDpro processing was also observed upon treatment with the kinase inhibitor GW5074 [81], which was shown to inhibit PI4KB activity [82,83]. Whether PI4P lipids and/or cholesterol are required for proper polyprotein processing in EMCV-infected cells remains to be investigated.

Role in phosphorylation. Interestingly, PI4KA was also demonstrated to be involved in regulating the phosphorylation status of HCV NS5A, but the underlying mechanism remains unknown [84]. It was recently shown that PI4P can bind to proteins and promote their phosphorylation [85], but the PI4KA-mediated NS5A phosphorylation was suggested to be independent of (high levels of) PI4P lipids [84]. Regardless of the mechanism, modulation of NS5A phosphorylation represents yet another mechanism by which PI4KA contributes to HCV MW morphogenesis and viral RNA replication [84]. As yet, no evidence exists that EMCV non-structural proteins are phosphorylated, but this does not exclude the possibility that PI4KA/PI4P also promote EMCV infection by modulating the phosphorylation of cellular proteins required for efficient replication.

Virus egress. Apart from its vital importance for virus genome replication, PI4P could also support other steps in the picornavirus life cycle, such as virus egress. It has been recently shown that newly formed PV virions are packaged in autophagosome-like DMV vesicles enriched in phosphatidylserine (PS) [86]. These vesicles are closely juxtaposed to ROs, which also contain PS. By which mechanism these DMVs are formed and how they and the ROs acquire PS is not understood. One possibility is that the DMVs emerge from the tubular ROs, as explained in **Chapter 1**. A clue for how PS may become enriched at the ROs is provided by a recent study showing that ORP5 and ORP8 mediate PS/PI4P counterflow at ER-PM MCSs to deliver PS to the PM [87]. Possibly, in infected cells, this mechanism is hijacked so that ORP5 and ORP8 operate at ER-RO MCSs to shuttle PS to the ROs, which then progress to DMVs. Fusion of these virus-containing DMVs with the PM releases a SMV that presents PS, enabling en-bloc transmission of multiple virions into neighboring cells, a process dependent on both the virus-specific receptor and PS lipids, possibly via the PS receptor, although the latter was not verified yet [86]. Whether PI4P and ORP5/8 are involved in the egress of cardioviruses or enteroviruses remains to be determined.

PI4KA-independent EMCV mutants

Development of viral resistance against inhibitors targeting essential host factors is believed to be a less likely phenomenon than resistance acquired in response to therapies based on direct-acting

antivirals (DAAs). In **Chapter 5**, we describe the isolation and characterization of two EMCV mutants that exhibit resistance to PI4KA inhibitors or to PI4KA depletion by siRNA treatment. This work is the first achieving the isolation of a +RNA virus that can overcome the need for the essential host factor PI4KA, since the isolation of a PI4KA-independent hepatitis C virus mutant has not been reported yet. EMCV mutants have been selected by passaging the wt virus in a cell line with a stable knockdown of PI4KA expression. This alternative genetic approach, although technically challenging, represents a more "clean" and reliable resistance culturing strategy than the standard, compound-based one, which is more susceptible to the putative off-target effects of inhibitors.

Resistance mutations. Two independent single-point mutations acquired in the viral protein 3A, namely A32V and A34V, were identified to be responsible for the observed resistance against PI4KA depletion or inhibition (**Chapter 5**). Mapping of these mutations to the 3A protein provides a direct genetic link for the involvement of 3A in PI4KA-mediated PI4P metabolism in infection. Remarkably, the mutations had no effect on virus replication kinetics and apparently did not alter EMCV host requirements in the absence of inhibitors, since both EMCV-3A-A32V and EMCV-3A-A34V recruited PI4KA and OSBP and replicated at PI4P and cholesterol-enriched ROs (**Chapter 5**). The 3A mutations rendered EMCV independent of high levels of PI4P and cholesterol, which no longer accumulated at viral ROs upon PI4KA inhibition. Strikingly, although OSBP was apparently also not enriched at ROs in cells with inhibited PI4KA activity, the mutants were still sensitive to OSBP depletion or inhibition (**Chapter 5**). This puzzling phenotype is in stark contrast with the phenotype of the previously described CVB3 3A mutants (i.e. H57Y or V45A), which are cross-resistant to PI4KB and OSBP inhibitors [65,88]. Considering that the functions of PI4KA and OSBP in EMCV replication are coupled in the PI4KA/PI4P-OSBP/cholesterol pathway and that OSBP and cholesterol are not enriched at ROs upon PI4KA inhibition, it is intriguing that the EMCV mutants still require OSBP. Although improbable, these data may be suggestive of a non-canonical, yet undiscovered function of OSBP in virus replication, as also discussed earlier in this chapter. The mechanism behind the resistance of the enterovirus mutants to PI4KB (and/or OSBP inhibitors) is not deciphered yet, but, as in the case of EMCV, it may be based on bypassing the need for high levels of PI4P, since these mutants no longer induce PI4P in the presence of PI4KB inhibitors [82].

Resistance mechanism. Unfortunately, the data we have obtained thus far does not allow us to provide a satisfactory explanation of the resistance mechanism employed by the EMCV 3A single-point mutants. Our bioinformatics analysis (**Chapter 5**) revealing that both mutations are located in a region of 3A putative determinant of intermolecular interactions suggests that the resistance mechanism may involve modulation of 3A interactions with host or viral factors. Recent evidence from the enterovirus field argues that the A70T mutation in PV 3A alleviates virus replication from PI4KB inhibitors by restoring polyprotein processing [81,89], substantiating the role of PI4P/cholesterol in this step of the virus life cycle. One study showed that the mutation A70T increases the basal levels of 3A by promoting processing of the precursor 3AB [89]. Treatment with PI4KB inhibitors led to accumulation of 3AB in wt PV-infected cells, whereas in cells infected with the mutant PV, 3A was accumulated instead [89]. Moreover, in two other resistant PV 3A mutants (3A-T41M and H86Y), a positive correlation was found between the extent of the resistance phenotype and 3A protein levels, suggesting that indeed the elevated 3A levels may contribute to rescuing virus replication [89]. It was thus proposed that the "target" of these mutations is not 3A, but in fact the 3AB precursor. Indeed, results of a preliminary analysis of 3AB cleavage events in cells infected with the mutant CVB3-3A-H57Y are consistent with the PV data (Heyrhyoung Lyoo, Hilde van der Schaar, Frank van Kuppeveld, unpublished data). Thus, enterovirus polyprotein processing seems to be a membrane-dependent process, which is altered in

the absence of a proper lipid microenvironment and rescued by mutations in 3A providing resistance to PI4K/OSBP inhibitors. Unfortunately, the lack of available tools for a comprehensive detection of viral proteins has hampered a similar analysis of the resistance mechanism employed by EMCV mutants. Hopefully, future research will shed light on this important aspect of cardiovirus replication.

EMCV mutants replicate on altered ROs. When investigating the replication of EMCV mutants in the presence of PI4KA inhibitors (**Chapter 5**), we made the surprising observation that their ROs displayed the altered, clustered phenotype observed previously with the wt virus upon acute PI4KA inhibition (**Chapter 4**). This observation further supports that lack of high levels of PI4P and cholesterol can lead to the disruption of the global organization of the EMCV ROs. It is remarkable that the mutants have adapted to efficiently replicate their genome on these apparently disrupted ROs. An in-depth electron microscopy analysis of both wt and EMCV mutant-infected cells in the presence and absence of PI4KA is required to better understand the ultrastructural changes associated with this altered RO phenotype. Electron microscopy analysis of the clustered HCV MW phenotype revealed significant morphological changes in HCV MW ultrastructure, characterized by clusters of accumulated homogeneous DMVs with reduced diameter [54,64]. Preliminary electron microscopy data of EMCV wt-infected cells suggests the accumulation of cytoplasmic SMVs and DMVs that establish direct contacts with ER tubules (Charlotte Melia, Montserrat Bárcena, Bram Koster, Hilde van der Schaar, Frank van Kuppeveld, unpublished data). Together with our data presented in **Chapters 4** and **5**, this result is indicative of the presence of ER-ROs MCSs. Thus, detailed EM investigation of the mutant EMCV ROs in the presence of PI4KA inhibitors may not only reveal morphological alterations, but also novel features such as perhaps novel MCSs between ROs and other subcellular compartments. Interestingly, PI4KB inhibitors do not seem to induce similar clustering effects in CVB3-3A-H57Y infected cells, instead, upon PI4KB inhibition, this mutant seems to replicate at an intact Golgi (Hilde van der Schaar, Frank van Kuppeveld, Charlotte Melia, Montserrat Bárcena, Bram Koster, unpublished results). This aspect further delineates the interesting differences between the enterovirus and cardiovirus mutants, which may stem from their differential host cell requirements and/or viral replication mechanisms.

A novel PI4KA inhibitor

In **Chapter 6**, we describe the antiviral properties of tyrphostin AG1478 against EMCV. AG1478 is a well-established inhibitor of epidermal growth factor receptor (EGFR) tyrosine kinase activity and thereby widely explored in the field of anti-cancer therapy [90–92]. Since AG1478 inhibited EMCV only in the micromolar range (**Chapter 6**), we excluded that its antiviral effects could be linked to EGFR, which is already inhibited by AG1478 at nanomolar concentrations [91]. AG1478 is also known to have dramatic effects on the Golgi integrity [93]. Treatment of cells with micromolar amounts of AG1478 was shown to induce a rapid dispersal of the Golgi, similar to the Golgi dispersal induced by BFA [93]. However, BFA does not affect EMCV replication [12,51,52], indicating that Golgi disruption *per se* does not account for the antiviral effects of AG1478. Since many kinase inhibitors exhibit promiscuous activity, we reasoned that AG1478 might be targeting a host factor essential for EMCV replication. The structural resemblance of AG1478 with the established PI4KA inhibitor AL-9 [56] (they both have a 4-anilinoquinazoline core) prompted us to consider the possibility that AG1478 inhibits EMCV replication by targeting PI4KA. Indeed, AG1478 inhibited PI4KA activity both *in vitro* and in intact cells. Furthermore, the PI4KA-escape mutant EMCV-3A-A32V was cross-resistant to AG1478 treatment, further supporting the idea that the mode-of-action of AG1478 is through PI4KA inhibition. To the best of our knowledge, AG1478 is the only PI4KA inhibitor commercially available, making it a convenient tool to study the function of PI4KA in both cellular and virus-induced processes.

PI4KA and PI4KB as antiviral targets

The discovery of PI4Ks as essential host factors for enteroviruses and HCV has triggered the interest of laboratories from both industry and academia in developing specific PI4KA/PI4KB inhibitors with the hope of their valorization on the market as antiviral drugs. Historically, antiviral drug discovery has been focusing primarily on developing inhibitors against viral targets, such as viral proteases or viral polymerases. Consequently, most of the antiviral drugs licensed thus far target viral proteins. The host-targeted antiviral approach has been much less explored. The only host-targeted antiviral inhibitor approved to date is Maraviroc, an antagonist of the HIV receptor CCR5 [94]. The growing interest in host-targeted antiviral strategies promoted the successful development of another drug, namely the cyclosporine analogue Alisporivir (DEB025), a non-immunosuppressive inhibitor of the host factor cyclophilin, currently in the final stage of clinical development for the treatment of Hepatitis C [95–97]. Direct targeting of host factors instead of viral proteins may present the advantage of imposing a higher barrier to the development of resistance, as well as the potential for broad-spectrum activity, given that multiple viruses rely on the same host factor. For instance, cellular cyclophilins were shown to be important for many different viruses including SARS coronavirus [98,99], HBV [100], and HIV (reviewed in [97]), and thus development of cyclophylin inhibitors may constitute a key antiviral strategy for the treatment of diverse viral infections. Unfortunately, accumulating evidence substantiates that viruses can also become resistant against host-targeted therapy. For instance, HIV-1 could gain resistance to Maraviroc by adapting to interact with the antagonist-bound form of CCR5 [101,102]. Furthermore, results of previous studies [82,103–106] and of this thesis (**Chapter 5**) highlight the ability of picornaviruses to rapidly acquire mutations that render them resistant to inhibitors of essential host factors, such as PI4Ks and OSBP.

A potential disadvantage of targeting host factors is the occurrence of adverse side effects, as many cellular proteins are important for cellular function. Indeed, toxicity-related issues have precluded the development of PI4K inhibitors as treatment for viral infections, casting serious doubts on the feasibility of targeting these host factors as antiviral therapies. Novartis reported the identification of highly selective PI4KB inhibitors that did not exhibit toxicity when tested across hundreds of cell lines and primary cells, but instead exerted antiproliferative effects on lymphocytes *in vitro* [107]. Another study by Boehringer Ingelheim reported strong adverse effects of PI4KB targeting *in vivo*, since mortality was induced in mice by PI4KB inhibitors with otherwise promising pharmacokinetic profiles and very good selectivity *in vitro* [108]. However, an analogue of the PI4KB inhibitor BF738735 that we also employed in our studies from this thesis (**Chapters 4, 5** and **6**) was well tolerated in mice and protective in a CVB4-induced pancreatitis model [53], raising the possibility that PI4KB targeting is not deleterious *per se*. Genetic or pharmacological inhibition of PI4KA also induced lethality in mice as revealed by two separate studies [58,109], which indicated that targeting this host factor is indeed deleterious to the host and may preclude the long-term treatment required for the cure of HCV infections. However, this does not necessarily rule out targeting PI4Ks as a suitable treatment against picornaviruses. First, adverse effects may be prevented by the use of a short treatment duration, since picornaviruses establish acute infections requiring only short-term therapy. Second, an efficient therapy might not necessarily require complete inhibition of the PI4K activity, but rather the administration of a non-toxic, suboptimal dose that does not completely inhibit viral replication, but is sufficient to reduce it to a level that allows the immune system to combat the infection. Alternatively, applying a suboptimal PI4K inhibitor dose could be part of a combination therapy (with another viral or host-targeted inhibitor), which would greatly reduce the risks of resistance development. Thus, while the development of PI4K inhibitors as antiviral therapies is associated with risks and challenges, it may still prove a worthwhile effort.

Concluding remarks and future prospects

The past decade has witnessed major advances in the identification of novel picornavirus host factors and understanding the nature of their essential role in virus genome replication. Given that +RNA viruses from distantly related families employ apparently common strategies to develop membranous viral replication structures, the knowledge generated by studying picornaviruses may be extrapolated to benefit other +RNA research fields as well. The present thesis not only contributes to this knowledge by deciphering the mechanisms underlying picornavirus replication and RO formation, but also contributes to a deeper understanding of the mechanisms that govern complex cellular processes. In spite of these major advances in the field, much still remains to be elucidated regarding cellular requirements for virus replication.

The dependence of distinct picornaviruses on common cellular pathways opens the possibility for the development of host-targeted, broad-spectrum antivirals. With the emergence of enteroviruses EV-A71 and EV-D68, which pose serious threats to public health, and no available antiviral therapies to combat them, there is an urgent need to identify potential new targets for novel, hopefully pan-anti-enteroviral drugs.

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Nederlandse Samenvatting

Picornavirussen hebben, net zoals alle andere virussen, gastheercellen nodig om zichzelf te vermenigvuldigen. Zij behoren tot de groep van virussen die een positief-strengs RNA molecuul als genoom bij zich dragen. Deze virusgroep transformeert de intracellulaire membranen van de gastheercellen in zogeheten "replicatie organellen" (RO's) om ervoor te zorgen dat ze een optimale omgeving hebben voor genoom replicatie. De virus-geïnduceerde RO's zijn geheel nieuwe membraanstructuren met een unieke architectuur en eiwit- en lipidesamenstelling. De benodigdheden voor de vorming van picornavirus RO's zijn tot nu toe nauwelijks bekend. Het onderzoek beschreven in dit proefschrift heeft als doel om nieuwe gastheerfactoren te identificeren die noodzakelijk zijn voor de picornavirus genoom replicatie, als ook om de achterliggende moleculaire mechanismen te ontrafelen die belangrijk zijn voor het recruteren van bekende gastheerfactoren naar enterovirus RO's. Een beter begrip van de interacties tussen picornavirussen en hun gastheer, alsmede van de mechanismen die ten grondslag liggen aan de vorming en het functioneren van RO's, kan een bijdrage leveren aan de ontwikkeling van een toekomstige antivirale therapie.

In dit proefschrift worden de virale en cellulaire vereisten voor genoom replicatie en vorming van RO's van verschillende picornavirussen onderzocht. **Hoofdstuk 2** en **3** gaan over enterovirussen, waarin coxsackievirus B3 (CVB3) en rhinovirus (RV) als prototype virussen worden gebruikt, terwijl **Hoofdstuk 4, 5,** en **6** is gericht op de verwante cardiovirussen, waarin encephalomyocarditis virus (EMCV) als een representatief virus wordt gebruikt.

In **Hoofdstuk 2** wordt het mechanisme bestudeerd waarmee CVB3 het Golgi-gelokaliseerde enzym PI4KB recruteert naar zijn RO's. Het was eerder gesuggereerd dat PI4KB door het 3A eiwit naar enterovirus RO's werd gerecruteerd ofwel via GBF1/Arf1 ofwel via ACBD3, omdat deze factoren het kinase naar de membranen van het Golgi apparaat recruteren in niet-geïnfecteerde cellen. De recrutering van PI4KB naar membranen is hier systematisch geanalyseerd, zowel *in vitro* als ook in intacte cellen. Genetische depletie van GBF1 of Arf1 met siRNA's of farmacologische remming van GBF1-gemedieerde Arf1 activatie door brefeldin A (BFA) had geen invloed op PI4KB recrutering naar membranen door 3A. Daarnaast is aangetoond dat 3A-mutanten, die geen interactie kunnen aangaan met GBF1, nog steeds PI4KB kunnen recruteren *in vitro*. Andere 3A-mutanten, die nog wel een binding met GBF1 konden aangaan, konden het kinase juist niet recruteren. Deze resultaten tezamen toonden aan dat de PI4KB recrutering door 3A onafhankelijk is van GBF1/Arf1. Ondanks het feit dat de 3A eiwitten van verschillende enterovirussen een interactie aangingen met ACBD3 in een "mammalian two-hybdrid assay", verhinderde de depletie van ACBD3 door siRNA's niet dat PI4KB naar CVB3 RO's werd gerecruteerd. Tevens werd CVB3 replicatie niet door ACBD3 depletie geremd, maar juist enigszins gestimuleerd. PI4KB werd nog steeds gerecruteerd in ACBD3-gedepleteerde cellen door een 3A-mutant die geen GBF1 meer kon binden, wat impliceert dat ACBD3 en GBF1/Arf1 allebei niet nodig zijn voor PI4KB recrutering. In co-immunoprecipitatie experimenten met gezuiverde recombinante eiwitten werden er directe interacties gevonden tussen ACBD3 en 3A, evenals tussen ACBD3 en PI4KB, maar niet tussen 3A en PI4KB. Het mechanisme van PI4KB recrutering naar RO's door 3A, dat ofwel via een nog onbekende cellulaire co-factor ofwel via een transiënte directe interactie plaatsvindt, blijft tot dusver onopgehelderd.

In **Hoofdstuk 3** wordt de rol van PI4KB in RV replicatie onderzocht en het mechanisme waarmee het kinase naar RO's wordt gerecruteerd. De eerste aanwijzing dat GBF1 niet betrokken is bij PI4KB recrutering naar RO's was de bevinding dat de 3A eiwitten van RV nauwelijks tot geen interactie hebben met GBF1, maar toch zorgen voor accumulatie van PI4KB naar membranen, zowel wanneer het *in vitro* werd geëxpresseerd als ook in intacte cellen. GBF1 was echter wel aanwezig op RV RO's en was ook

essentieel voor virus replicatie, aangezien remmers van GBF1, te weten golgicide A en BFA, een reductie in RV replicatie teweeg brachten. Expressie van BFA-resistente GBF1 mutanten M832L en A795E hief het remmende effect van BFA op replicatie op. Deze resultaten suggereerden dat het mechanisme dat ervoor zorgt dat GBF1 is gepositioneerd op RV RO's om zijn functie uit te oefenen in replicatie waarschijnlijk onafhankelijk is van 3A. Ongeacht de bevinding dat RV 3A een interactie aanging met ACBD3, was PI4KB recrutering onafhankelijk van ACBD3, omdat depletie van ACBD3 geen effect hierop had en ook niet op replicatie. Deze resultaten zijn in overeenstemming met de resultaten beschreven in **Hoofdstuk 2**, wat tezamen een sterk bewijs vormt dat het recruteren van PI4KB naar enterovirus RO's een proces is dat gemedieerd wordt door 3A, maar dat onafhankelijk is van GBF1/Arf1 en ACBD3.

In **Hoofdstuk 4** wordt voor het eerst beschreven hoe cardiovirussen hun RO's ontwikkelen. Cardiovirussen manipuleren ook het PI4P metabolisme voor replicatie, echter niet door PI4KB te kapen zoals enterovirussen, maar juist het ER-gelokaliseerde PI4KA. Door middel van chemische of genetische remming wordt aangetoond dat de activiteit van PI4KA essentieel is voor EMCV genoom replicatie. In EMCV-geïnfecteerde cellen wordt PI4KA, maar niet PI4KB, herverdeeld naar RO's waar het een grote overlap vertoont met het 3A(B) eiwit. Met radio-actieve co-immunoprecipitatie werd aangetoond dat 3A en zijn precursor 3AB de enige niet-structurele eiwitten zijn die interacteren met PI4KA, wat werd bevestigd met co-immunoprecipitatie in combinatie met Western Blot analyse. Overexpressie van 3A of 3AB bleek voldoende te zijn om PI4KA te recruteren naar specifieke "punctae" in het cytoplasma waar de virale eiwitten ook lokaliseerden, wat impliceert dat 3A tijdens infectie ook verantwoordelijk is voor het recruteren van het kinase naar RO's. Gedetailleerde immunofluorescentie analyse van het PI4P patroon liet zien dat er grote veranderingen waren in de distributie van PI4P in geïnfecteerde cellen ten opzichte van niet- geïnfecteerde cellen en dat er een duidelijke stijging was in de hoeveelheid PI4P. Gebruikmakend van PI4KA remmers werd vervolgens aangetoond dat PI4P belangrijk was voor het aantrekken van OSBP naar RO's. OSBP bleek een essentiële factor te zijn voor cardiovirussen, aangezien OSBP depletie de genoom replicatie van EMCV sterk reduceerde. OSBP bleek belangrijk te zijn voor het uitwisselen van PI4P voor cholesterol op zogeheten "membrane contact sites" tussen de RO's en het ER. Het blokkeren van deze lipide-uitwisseling door OSBP remde genoom replicatie en verstoorde de globale structuur van de RO's. Tenslotte laten deze resultaten een buitengewone convergentie van EMCV met HCV zien betreffende hun gebruik van gastheerfactoren, aangezien HCV ook PI4KA en OSBP gebruikt om replicatie structuren met een hoge concentratie van PI4P en cholesterol te creëren. Deze studie heeft het eerste mechanistische inzicht gegeven in de ontwikkeling van cardiovirus RO's, maar de rol van PI4P en cholesterol in virus replicatie vereist verder onderzoek.

In **Hoofdstuk 5** wordt aangetoond dat EMCV kan ontsnappen aan remming van PI4KA door het verkrijgen van één puntmutatie in het 3A eiwit. Door het passeren van wild-type EMCV op een cellijn met een stabiele depletie van PI4KA werden er twee virus culturen verkregen, waarvan een de mutatie 3A-A32V had en de ander 3A-A34V. Wanneer deze mutaties werden teruggeplaatst in het wild-type genoom, werd het resulterende virus resistent tegen remming of depletie van PI4KA. Opmerkelijk genoeg hadden deze mutante virussen dezelfde groeikinetiek als het wild-type virus. Ook werden ze niet afhankelijk van een ander isoform van PI4K. In overeenstemming met deze resultaten bleek dat de mutante virussen tijdens langdurige PI4KA inhibitie geclusterde RO's vormden, net zoals geobserveerd werd voor het wild-type virus tijdens acute PI4KA remming in **Hoofdstuk 4**. In deze RO's was geen PI4P, OSBP en cholesterol aanwezig, maar desalnietemin ondersteunden ze wel genoom replicatie. Ondanks dat deze observatie suggereerde dat de mutante virussen niet langer afhankelijk waren OSBP, werd

opmerkelijk genoeg gevonden dat ze niet kruisresistent waren tegen remming of depletie van OSBP. Met deze laatste resultaten kan worden beargumenteerd dat de mutante virussen juist wel afhankelijk zijn van OSBP, zij het misschien een nog onbekende functie die wellicht niet gerelateerd is aan het uitwisselen van PI4P voor cholesterol. Deze studie geeft niet alleen een sterk genetisch bewijs voor de rol van EMCV 3A in het moduleren van PI4P metabolisme, maar illustreert tevens de complexe functies van gastheerfactoren zoals OSBP in virus replicatie.

Hoofdstuk 6 beschrijft de identificatie van tyrphostin AG1478 als een nieuwe PI4KA remmer. AG1478 staat bekend om zijn werking tegen kanker dankzij zijn sterke remming van de activiteit van EGFR tyrosine kinase. Aangezien er een aanzienlijke overeenkomst was qua molecuulstructuur tussen AG1478 en AL-9, een bekende PI4KA remmer, werd onderzocht of AG1478 ook PI4KA kon remmen. AG1478 remde inderdaad de kinase activiteit van PI4KA, zowel in intacte cells als *in vitro*, wat aangeeft dat AG1478 mogelijk direct aan het kinase zou kunnen binden. Als een gevolg van deze activiteit remde AG1478 de replicatie van EMCV, maar niet van CVB3. Bovendien was EMCV 3A-A32V, de mutant die onafhankelijk is van PI4KA, kruisresistent tegen AG1478, wat verder bevestigt dat het antivirale werkingsmechanisme is gebaseerd op PI4KA remming. AG1478 zou goed van pas kunnen komen in het verdere onderzoek naar de rol van PI4KA, zowel in geïnfecteerde cellen als onder fysiologische condities.

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Curriculum vitae

Curriculum vitae

Cristina Dorobantu was born on 16 April in Bucharest, Romania, by the name Radulescu. She obtained her high school degree (Bacalaureat) in 2004 at the National College "Mihai Viteazul" in Bucharest, after which she started her study of Biochemistry at the University of Bucharest. After obtaining her Bachelor's degree in Biochemistry in 2008, she enrolled in the Master's programme Biochemistry and Molecular Biology at the University of Bucharest, which she graduated in 2010. For her Bachelor's and Master's thesis projects, Cristina performed 9-month internships at the Institute of Biochemistry of the Romanian Academy. There, under the supervision of Dr. Norica Nichita, she studied the entry and morphogenesis steps in the hepatitis B virus life cycle. Following her Bachelor's internship, she joined the institute as research assistant, until the autumn of 2011.

In 2011, Cristina moved to the Netherlands to join the group of Dr. Frank van Kuppeveld at Radboud University Nijmegen as a PhD student and Marie Curie fellow of the European Initial Training Network EUVIRNA, working on the role of host lipid kinases in picornavirus replication. One year later, she moved with Frank's lab to Utrecht and continued her PhD research at the Virology Division of the Faculty of Veterinary Medicine, Utrecht University. During her PhD, Cristina performed 2 industrial training stages, at Janssen Infectious Diseases (Beerse, Belgium) and RiboxX (Dresden, Germany), and one academia training stage in the lab of Prof. Ralf Barteschlager in Heidelberg, Germany.

In July 2016, Cristina has joined the team of Dr. Raphael Gaudin as post-doc at INSERM U1110 in Strasbourg, to investigate the entry steps of hepatitis B virus and hepatitis C virus by live cell microscopy.

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