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Enterovirus Replication Organelles; function, formation and the role of viral proteins

Abstract

Enteroviruses extensively manipulate cellular functions during infection. One of the most prominent changes is in membrane morphology. Enteroviral infection causes the formation of Replication Organelles (ROs) that increase viral replication. ROs are rearranged membranes with different properties to any cellular membranes and serve as platforms for assembly of replication complexes and new virions. Many cellular pathways are modulated by viral proteins to create an environment suited for viral replication. These include manipulation of intracellular trafficking and lipid metabolism. Lipid droplets also play a role in effective infection by shuttling lipids towards ROs. Late during infection autophagy plays a role in non-lytic release by wrapping ROs in more membranes and allowing for the egress of vesicles filled with virions. Finally, recent advances in cryo-Electron Tomography (cryo-ET) are discussed that can allow the study of viral replication *in situ* in previously unachievable detail.

Layman's summary

Enteroviruses are able to affect the way cells work upon infection. One way they do this is by changing the shape of intracellular membranes, which helps the virus to reproduce on structures called Replication Organelles (ROs). These structures are shaped differently than the ones found in non-infected cells and they act as places where the virus can replicate efficiently. Enteroviruses also change the way cells work by modifying many cellular processes. In particular, lipid synthesis is increased and lipids are transported to ROs. Lipid droplets, which are organelles where lipids are stored, are important during infection by supplying lipids to the ROs. Late in the infection process, the virus uses a process called autophagy to release new copies of itself without damaging the cell. This involves wrapping the ROs in additional membranes to create vesicles that can be released from the cell. Recently, a new technique called cryo-Electron Tomography has been developed which allows scientists to study how viruses reproduce inside cells at a very high level of detail which can be used to gain new insights in the way enteroviruses replicate during infection.

Introduction

In the introduction the enterovirus classifications and diseases they cause will be shortly discussed as well as the enteroviral genome organization.

Enterovirus classification and disease

Picornaviridae is a large family of positive-stranded non-enveloped RNA viruses. Picornaviruses can infect a wide range of animals including humans and cause many different diseases ranging from milder to more severe. Within the picornavirus family there are 68 genera of viruses of which *Enteroviruses* are the best known. Other well-known genera within the family include *Aphtoviruses* (e.g. foot-and-mouth disease virus), *Cardioviruses* (e.g. encephalomyocarditis virus), and *Hepatoviruses* (e.g. hepatitis A virus). The genus *Enterovirus* contains 10 species (Enterovirus species A-K) with over 100 different serotypes and 3 species of Rhinoviruses, together more than 150 different serotypes, mostly responsible for common colds. Enteroviruses are important pathogens for both human and animals and primarily infect the gastrointestinal tract. Enteroviruses are the major cause of hand-foot-mouth disease. They are also known to disseminate and infect the central nervous system which can lead to more severe conditions such as poliomyelitis, meningitis, encephalitis and paralytic disease. Currently, there are no approved enterovirus vaccines except for poliovirus and enterovirus A71 and no antiviral drugs to treat enterovirus infection.

Enterovirus genome organization

Enteroviruses have a 7.5 kb RNA genome which contains one open reading frame that encodes for a single polyprotein that can be divided into P1, P2 and P3 regions. The P1 region consists of the structural proteins VP1 through VP4 and the other two regions are made up of 2A-2C and 3A-3D, respectively. When the viral RNA is translated during infection as a polyprotein, it is proteolytically processed by viral proteases $2A^{pro}$ and $3C^{pro}$ to release individual viral proteins and some stable precursors. VP1-4 are structural proteins that assemble into icosahedral capsids and are important in packaging of viral RNA. Because the enterovirus genome is so small compared to other viruses, many enteroviral proteins have multiple functions in replication and all are essential for viral replication. $2A^{pro}$ is a viral protease that releases itself from the polyprotein. 2B is a multimeric membrane protein that forms pores in cellular membranes. 2C is an ATPase with helicase activity that can associate with membranes. 3A is a trans-membrane protein that that has functions in RNA replication and membrane remodeling. 3B is a viral peptide that serves as the primer for initiation of genome replication. $3C^{pro}$ is the main viral protease involved in proteolytic processing of the viral polyprotein. $3D^{pol}$ is the viral RNA-dependent RNA polymerase responsible for genome replication.

Enterovirus life cycle

Entry: attachment and uncoating

Entry into cells is the start of the life cycle of viruses, that as obligatory intracellular parasites only replicate within host cells. Enteroviruses, like many other viruses, require receptor binding on cellular surfaces to enter cells via receptor-mediated endocytosis. Enteroviruses have similar icosahedral capsid structures but bind to different cellular receptors. This partly explains the wide range of diseases they can cause. Enteroviral receptors can broadly be classified into two groups: attachment receptors and uncoating receptors. Attachment receptors are important to facilitate binding to cells and virus internalization, whereas uncoating receptors induce conformational changes of the capsid upon binding to release the viral genome. Most uncoating receptors bind in the region of the capsid that is

called the canyon. The canyon is a region of the capsid located around the fivefold symmetry axis and binding of it by a receptor leads to conformational changes in the capsid structure (Baggen et al., 2018).

Almost all enteroviruses require binding to a specific proteinaceous host cell receptor. The first identified viral receptor was for poliovirus: the uncoating receptor CD155 (Mendelsohn et al., 1989). After that, many receptors were discovered. Besides protein receptors used by all enteroviruses, sialic acid has been identified as an additional uncoating receptor for EV-D68 (Baggen et al., 2018). For some enteroviruses, pH is additionally required for uncoating.

The endocytic routes taken upon internalization can be very diverse depending on cell type and receptor for different enteroviruses. However, after internalization the mechanism of delivery of the viral genome into the cytosol is conserved among different enteroviruses. Genome release requires the crossing of the endosomal membrane. Enteroviruses are naked viruses that don't have a membrane, unlike enveloped viruses. Enveloped viruses can fuse the endosomal membrane with their envelope to escape into the cytosol whereas this process is very different for enteroviruses. Upon internalization, gradual destabilization of the enterovirus capsid leads to genome release at a regulated location and time. Binding to uncoating receptors aids in this process by locking the virion in a naturally occurring expanded state. This leads to the insertion of an amphipathic helix from capsid protein VP1 into the endosomal membrane forming a pore through which the viral genome can escape into the cytosol (Fricks & Hogle, 1990).

Translation

Directly after release of the viral genome into the cytosol, translation by ribosomes commences. Enteroviruses are positive-stranded RNA viruses so their genome can be directly translated to yield viral proteins. For all enteroviruses, translation is initiated on an internal ribosome entry site (IRES). The IRES precedes the viral polyprotein in the genome and is a part of the 5' untranslated region. The IRES is composed of unique secondary and tertiary structures formed by the viral RNA genome. The IRES allows for cap-independent translation by ribosomes requiring a subset of the host cell translation machinery. Enterovirus translation requires several IRES trans-acting factors (ITAFs) (K. M. Lee et al., 2017). Multiple ITAFs have been identified that aid in translation by interacting with viral RNA. These include PCBP2, PTB and hnRNP (Kafasla et al., 2009), (Sweeney et al., 2014). Many ITAFS are under non-infected conditions mainly localized in the nucleus but during infection relocated to the cytosol. Several ITAFs are targets of enteroviral proteases and upon cleavage have altered functions to aid in viral replication. The truncated versions gain or lose functions upon cleavage. Before proteolysis, PCBP2 and PTB stimulate IRES-dependent translation. Upon proteolysis they contribute to switching from translation to replication of the viral RNA because the truncated versions can not stimulate translation anymore. However, they can still bind the viral RNA (Back et al., 2002). A switch from translation to replication is required for enteroviruses because the positive-stranded viral RNA genome serves as template for both processes. The translation and replication machinery go over the viral RNA in opposite directions as translation occurs in the 5' to 3' direction and replication in the 3' to 5' direction over the template. This means when both are executed simultaneously, it would lead to a clash.

Replication

Enteroviral genome replication occurs via synthesis of a negative RNA strand with the positive strand as template. The negative strand is then used as a template for the generation of new positive-stranded viral genomes. The process of genome replication has double-stranded RNA (dsRNA) intermediates. Genome replication is initiated on higher-order RNA structures at 5' and 3' ends of the

viral genome and requires the RNA-dependent RNA polymerase $3D^{pol}$ as well as a primer. This primer is the viral peptide 3B, which becomes covalently attached to the 5'-end of the viral genome by two uridines during genome replication (Baggen et al., 2018). All non-structural viral proteins are required for successful replication and thus play an important role. $3CD^{pro}$ and 3AB bind to structural elements of the viral RNA involved in priming, and 2C has helicase activity dependent on its ATPase activity as well as RNA chaperoning activity independent of its ATPase activity (Z. Chen et al., 2022). Other viral proteins act less directly on replication such as 2B that permeabilizes cellular membranes such as ER and Golgi. During infection, membrane rearrangements are induced by viral proteins. These correspond to sites of replication and are termed Replication Organelles (ROs). The viral proteins that are responsible for these rearrangements will be discussed in further detail later. Recently, replication was shown to occur in distinct cycles instead of in a more random pattern. During early infection, after a few initial rounds of translation, a switch from translation to replication occurs. This is triggered by the presence of sufficient viral proteins present. After the viral RNA has switched from translation to replication, approximately 15 to 20 new positive-sense RNA strands are produced per viral genome (Boersma et al., 2020).

Virion assembly and release

To complete the viral life cycle, the production of new infectious virions is required. This process requires the assembly of new capsids and the loading of viral RNA into them. The binding of heat shock protein 90 (Hsp90) to capsid precursor protein P1 is essential for the folding and maturation of capsid proteins. This catalyzes proteolytic processing of P1 into VP0, VP1 and VP3 (Geller et al., 2007). The capsid proteins assemble into pentameric particles (Ansardi et al., 1992) and after assembly virions go through maturation. They mature by viral RNA-mediated cleavage of VP0 into VP2 and VP4. This yields mature and infectious new virions.

Enteroviruses were thought to spread exclusively via host cell lysis which releases newly produced virions into the environment. More recently, it was revealed that there is a role in transmission for virus-containing vesicles that are released in a non-lytic manner (Y. H. Chen et al., 2015). Viral infection has been shown to trigger autophagy which leads to the engulfment of virions and release of vesicles containing multiple virions. The vesicles can bind neighboring cells and be internalized but ultimately require presence of the viral receptor for effective infection (Y. H. Chen et al., 2015).

Enteroviruses extensively manipulate cellular functions

Enteroviruses rapidly and extensively manipulate many cellular processes upon infection to create an environment suitable for viral replication. Examples of viral manipulation include host translation shutdown and dysregulation of nucleocytoplasmic trafficking which both lead to suppression of innate immune responses and increased viral replication.

Host translational shutdown

Cellular mRNAs are translated by ribosomes in the cytosol to yield proteins. This requires assembly of the ribosomal pre-initiation complex on the cap of the mRNA molecule. Several host initiation factors are required for the assembly and subsequent translation. During infection one of the most rapidly cleaved targets of enteroviral proteases is translation initiation factor eIF-4G. This efficiently shuts down initiation of translation of capped host mRNA molecules (Kempf & Barton, 2008) but not translation of viral RNA which is cap-independent because of its IRES. Ribosomes remain available for translation of viral RNA in this manner.

Dysregulation of nucleocytoplasmic trafficking

Another mechanism of viral manipulation is disruption of nucleocytoplasmic transport. Because of their small genome, enteroviruses rely on host cells to provide many of the proteins required for replication of their genome. Many of these proteins bind RNA and are mostly located in the nucleus. Upon infection relocation of these factors to the cytosol can be observed (Gustin & Sarnow, 2001). This is due to proteolytic degradation of nucleoporins. $2A^{pro}$, and less prominently $3C^{pro}$, rapidly cleaves nup98 followed later in infection by nup62 and nup153 (Gustin & Sarnow, 2001). Interestingly, nup98 has also been demonstrated to be a transcription factor for anti-viral genes in *drosophila* (Panda et al., 2014). Cleavage of nucleoporins leads to a loss of integrity of the central channel of the nuclear pore complex, which is responsible for coordinating nucleocytoplasmic trafficking. This central channel is filled with unstructured nucleoporins extending into the channel and forming a mesh-like barrier for large biomolecules. The rapid cleavage suggests a highly specific and efficient mechanism since 2A protein levels are low early in infection. Besides the role of relocating host cell factors for viral replication from the nucleus to the cytoplasm, the disruption of nucleocytoplasmic transport helps in repressing innate immune responses. MDA5 recognizes viral RNAs (Kato et al., 2006) and initiates an anti-viral signaling cascade that activates a set of type I interferons transcription factors (these include IFN regulatory factor 3 (IRF-3), nuclear factor (NF)-κ-B and transcription factor AP-1). The transcription factors trigger increased transcription of anti-viral factors (Younessi et al., 2012). The dysregulation of nucleocytoplasmic transport is an important mechanism for successful viral infection because it antagonizes host innate immune responses by interfering with transcription of anti-viral genes (Chinsangaram et al., 2001).

The host cell translation and dyregulation of nucleocytoplasmic transport are only two examples of the many cellular functions enteroviruses manipulate during infection. Other examples include the disruption of ionic gradients between different cellular compartments by viral protein 2B (Van Kuppeveld et al., 2005), degradation of the cytoskeleton (Barnabei et al., 2015) and disruption of vesicular transport within the cell. Collectively, these effects lead to the cytopathic effects underlying cell lysis and viral transmission.

Replication organelles

Enteroviruses induce large-scale membrane rearrangements to create an environment suitable for virus replication. These membranous structures are associated with dsRNA which is an intermediate of viral replication and are termed Replication Organelles (ROs). Enterovirus RO morphology has been studied using 3D Electron Tomography (ET) and surprisingly membrane rearrangements of Poliovirus and Coxsackie B3 virus were similar to one another (Belov et al., 2012), (Limpens et al., 2011). In this chapter, what is currently known about RO function, formation and the viral and host proteins involved will be discussed.

Replication Organelle function

All positive-stranded RNA viruses remodel host cell membranes into viral factories. ROs have been implicated as sites of viral replication because their emergence coincides with the exponential phase of viral RNA synthesis (Limpens et al., 2011). Additionally double-stranded RNA, which is an intermediate of viral replication, has been demonstrated to be associated with RO membranes (Belov et al., 2012). ROs are thought to contribute to viral replication in multiple ways. They may increase the concentration of proteins required for viral RNA replication, serve as a platform where replication

complexes can assemble and play a role in coordinating different parts of the viral replication to be carried out efficiently (Ravindran et al., 2016). Several viral proteins are membrane-associated and this could increase their local concentration and thereby boost viral replication. There are conflicting reports on the importance of each of the proposed functions of ROs. However, perturbing the membrane rearrangements underlying RO formation have been shown to inhibit viral replication (J. R. P. M. Strating et al., 2015), (Belov et al., 2007).

The constituents of RO membranes determine biophysical properties of these membranes. These are important for viral replication and are modulated by viral proteins to create a distinct environment that favors enteroviral replication. RO membranes are enriched in several membrane constituents such as phosphatidylinositol-4-phosphate (PI4P). This will be discussed in more detail later. PI4P enrichment is required for the correct proteolytic processing at the 3AB junction and liberating 3B for its function as primer in viral genome replication. When 3AB is present as a stable precursor, the 3A-B cleavage site is membrane-associated and effectively hidden from proteolytic processing by $3C^{pro}$. The phosphate groups present in the PI4P-enriched RO membranes have a different affinity for the hydrophobic linker between 3A and 3B. This changes the 3AB conformation and makes the 3A-B cleavage site more accessible for $3C^{pro}$ increasing cleavage rates (Melia et al., 2018).

ROs have also been thought to contribute to successful viral transmission by shielding viral RNA from host innate immune responses (Ravindran et al., 2016). For poliovirus, it was demonstrated that ROs are essential for successful replication and transmission. That is because they shield viral RNA replication from innate immune responses (Viktorova et al., 2018). This was shown by infecting cells grown on choline-depleted medium inhibiting the bulk activation of phospholipid synthesis. The activation of phospholipid synthesis is a major driver of RO formation and choline is required for the synthesis of the main phospholipid making up cellular and RO membranes. Only a small difference in viral replication was observed at 4 hours post infection after one round of replication before host cell lysis causes transmission to neighboring cells. In contrast, for a 24 hour infection spanning multiple viral life cycles, a significant decrease in viral replication was observed. This could be due to increased innate immune responses by infected cells protecting uninfected neighboring cells from infection by excreting interferons. The innate immune response could be increased because the viral replication machinery is less well protected from cellular anti-viral proteins in the absence of ROs. Another study similarly demonstrated that innate immune responses did not increase in severity or were accelerated during one cycle of replication for a mutant Coxsackie B3 virus (CVB3) uncapable of forming ROs (Melia et al., 2017). Both of these studies showed no significant difference in viral replication when RO formation is perturbed. However, both studies only assessed viral replication during one cycle of replication. It is conceivable that ROs are involved in suppressing innate immune responses and preventing the production and release of interferon molecules that enable the infection of neighboring cells. Another possible role of ROs in viral replication is aiding in the release of virions during late infection, which will be discussed in more detail later. Both the suppression of innate immune responses and the release of virions would only become apparent in transmission of viral infection to neighboring cells during an infection on a population level.

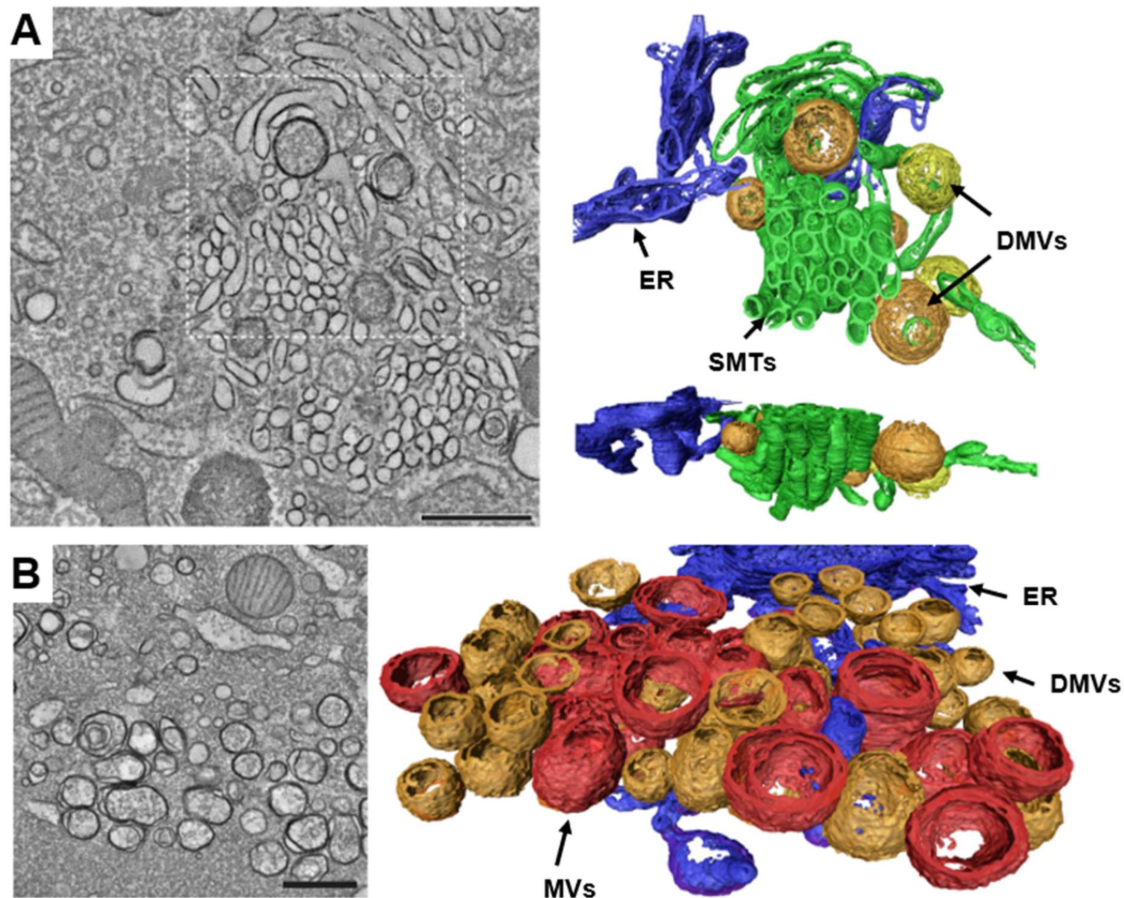

Figure 1. Replication Organelles from Coxsackie B3 infected cells. Left side is a tomographic slice, right side is the segmented ROs of A) early ROs and B) late ROs. Scale bar: 500 nm. Adapted from (Limpens et al., 2011)

Morphology of Replication Organelles

If enterovirus ROs play a role in shielding of viral RNA, their morphology should separate cellular factors from replication machinery. In early studies enterovirus ROs have been described as clusters of vesicles (K et al., 1992). Only much more recently has the ultrastructure of ROs been elucidated for poliovirus and CVB3 using Electron Tomography (ET) (Belov et al., 2012), (Limpens et al., 2011). This demonstrated that enterovirus ROs form a cellular landscape of membranes. The RO morphology changes over the course of infection. Early in infection, RO membranes are mostly present as single-membrane tubules (SMTs) (*Fig. 1A*) and during infection there is progression to double-membrane vesicles (DMVs). During late stages of infection (*Fig. 1B*) multilamellar vesicles (MVs) can also be observed. It was thought to be conceivable that the crowded environment of RO membranes could shield the viral RNA from the innate immune response. RO membranes are often found in close proximity of Endoplasmatic Reticulum tubules (ER) and often make membrane contacts sites (MCSs) with them. SMTs are wrapped by membranes during the progression of infection to form DMVs and MVs (Limpens et al., 2011). Upon observing the ultrastructure of ROs, there was debate on the role of the three types of RO membranes in viral replication. Viral RNA synthesis is high during the moment of infection when there are only SMTs present (Belov et al., 2012). This indicates that DMVs are not a prerequisite of replication and suggests that they might play a more important role during later stages of the viral life cycle. For instance, they could be involved in viral transmission which will be discussed in more detail later.

ROs are formed by rearranging host cell membranes and inducing curvature. There are two types of membrane curvature and the one used by all picornaviruses is known as positive curvature (J. R. P. Strating et al., 2013). This is in contrast to negative curvature which is used by flaviviruses to form ROs. Positive curvature can be imagined as a budding vesicle with membranes extending outwards whereas negative curvature can be imagined as an invagination of a membrane creating a vesicle-like space. Membrane curvature to form ROs is induced by viral proteins in combination with host factors.

Nidoviruses are a family of viruses that include coronaviruses. These viruses also form ROs by inducing positive curvature in host cell membranes. Nidovirus ROs are mainly DMVs and dsRNA had long been observed within them. This posed a problem on where viral replication takes place. Recent studies using cryo-Electron Tomography (cryo-ET) elucidated the existence of pores spanning the DMV membranes (Wolff et al., 2020). These membrane-spanning pores are made up of viral proteins situated on the neck of ROs that actively participate in viral replication by creating a means of transport between cytosol and RO interior. The pores directly interact with replication machinery to support viral replication (Nishikiori et al., 2022). The coronavirus pores were only identified by cryo-ET recently and were never observed in earlier ET studies with fixated samples. The increased resolution from using cryo-ET compared to classical ET on fixated samples allowed for the discovery of the pores. In contrast, the lack of pores for enteroviruses could suggest replication actually occurs on the cytoplasmic side of the RO membranes and not within the RO structures (Belov & Van Kuppeveld, 2012). However, very few studies on enterovirus infection *in situ* have been performed with cryo-ET. More *in situ* cryo-ET work on enteroviruses could provide new insightful discoveries for this genus of viruses as well.

Formation of Replication Organelles

The proteins responsible for RO formation consist of a combination of viral and host accessory proteins. The combination of viral proteins 2BC and 3A have been reported to induce changes in ER membranes leading to RO formation (Suhy et al., 2000). 2B is a membrane protein that has viroporin activity. It acts by inducing positive curvature by inserting its amphipathic α-helix in membranes (Van Kuppeveld et al., 2005). The inserted helices form pores in cellular membranes and permeabilize Golgi and ER membranes. This leads to a disruption in ionic gradients between these organelles and the cytosol (Van Kuppeveld et al., 2005). 2C is involved in modulating membranes via an amphipathic α-helix that can associate with membranes. 2C can also bind host factor reticulon 3 which is involved in the shaping of ER into tubules by inducing membrane curvature possibly allowing 2C to indirectly influence membrane shape. Both of these viral proteins can induce membrane curvature, either directly or indirectly. The 2C protein from the aphtovirus hand-foot-mouth virus has been demonstrated to induce autophagy by directly binding to autophagy factor beclin1 (Gladue et al., 2012). This direct interaction is essential for virus survival and could aid in the formation of DMVs and MVs observed in later stages of viral infection. The structure of MVs form in a manner that resembles the formation of autophagosomes. For enteroviruses, no direct interaction has been observed but there is colocalization of autophagy factor LC3 with 2C during infection (Y. R. Lee et al., 2014). This suggests a similar mechanism of autophagy stimulation could be used by enteroviruses during infection. Enteroviral 3A has been implicated in the formation of ROs by interacting with several host factors such as GBF1, ACBD3 and c10orf76. What is known about the interactions of 3A with each factor will be discussed below.

Cellular membranes are made up of five main phospholipid classes. These are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (SM) (Spector & Yorek, 1985). Another component of membranes is sterols of which cholesterol is the main type in mammalian cells. Enterovirus ROs are considerably enriched in PC, PI4P

and cholesterol compared to cellular organelle membranes (Ilnytska et al., 2013). Enteroviral 3A can modulate the activity of phosphatidylinositol 4-kinase type IIIb (PI4KB). PI4KB is one of the four mammalian kinases that phosphorylates PI to yield PI4P and is mainly located at the Golgi. PI4KB is a key enzyme in regulating membrane transport through local enrichment of PI4P on membranes. All enteroviruses rely on PI4KB activity for viral replication (Van Der Schaar et al., 2013). PI4KB activity is regulated by binding of different effector proteins and this is exploited by different viruses during infection. Enterovirus 3A does not directly interact with PI4KB but recruits PI4KB to RO membranes via interactions with host cell factors (*Fig. 2*) (Dorobantu et al., 2015).

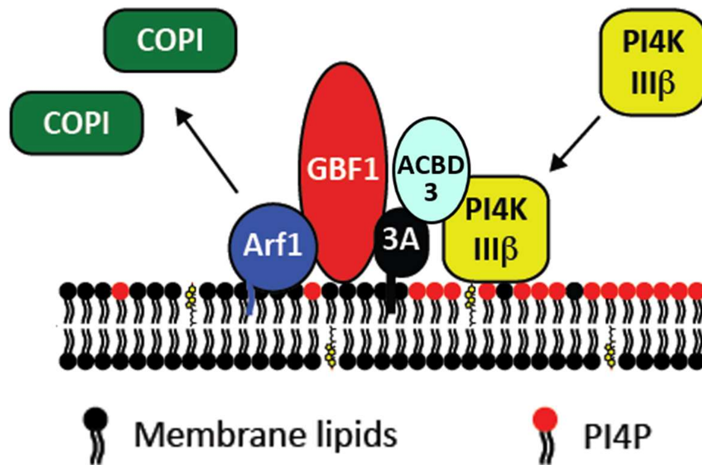

Figure 2. 3A interacts with multiple host factors to induce membrane rearrangements (Hsu et al., 2010)

ACBD3

Golgi-resident protein acyl-CoA-binding domain-containing protein 3 (ACBD3) has been demonstrated to be essential in PI4KB recruitment to ROs. ACBD3 recruits PI4KB to Golgi-membranes in non-infected cells. ACBD3 only requires three of its domains for enterovirus replication. It requires a Golgi-binding, 3A-binding and PI4KB-binding domain for successful infection (Lyoo et al., 2019). The other domains are dispensable for viral replication. Interestingly, a knockdown of ACBD3 is not sufficient to repress viral replication (Téoulé et al., 2013) and only a knockout of the gene is. This indicats that low levels of ABCD3 compared to endogenous levels are sufficient for succesful viral infection.

GBF1

Golgi brefeldin A resistant guanine nucleotide exchange factor 1 (GBF1) is an essential host factor for enterovirus replication (Wessels et al., 2006) and it interacts with 3A through its N-terminus. It is a Golgi-localized protein that plays a role in the secretory pathway by exchanging GDP for GTP in the GTPase ADP-ribosylation factor 1 (Arf1) which activates it. GBF1 regulates Arf1 activity by binding of Arf1 through its catalytic sec7 domain (Kaczmarek et al., 2017). Arf1 is a member of a family of Arf GTPases that are crucial in regulating eukaryotic cell organization by organizing intracellular transport. In non-infected cells, active Arf1 recruits effector proteins such as COPI factors to the intermediate compartment between ER and Golgi and induces transport by coating of membranes by COPI factors. This does not occur during enterovirus infection as COPI proteins are gradually depleted during infection (Hsu et al., 2010). It was initially thought that 3A interacts with GBF1 directly leading to PI4KB recruitment, but it was later found to recruit PI4KB independently of GBF1/Arf1 (Dorobantu et al., 2015), (Dorobantu et al., 2014). The role of GBF1 during infection remains largely unclear. GBF1 is a large multi-domain protein with many different functions. However, only a few GBF1 domains are required for enteroviral replication such as the catalytically active sec7 domain and the N-terminus

that binds 3A (Viktorova et al., 2019). This suggests that the role of GBF1 in enteroviral infection is different than its role in uninfected cells where it utilizes different domains it possesses as well.

C10orf76

The protein c10orf76 has also been found to contribute to PI4P levels in Golgi. It interacts with PI4KB and is essential for the replication of several enteroviruses (McPhail et al., 2020). It is not essential for all enteroviruses for reasons that are poorly understood. C10orf76 binding to PI4KB is what drives its relocalization to ROs but it remains to be established why there are differences between different enteroviruses in the importance of c10orf76 during viral infection.

PI4P enrichment leads to cholesterol accumulation

Enteroviral proteins affect several host factors leading to the formation of replication organelles enriched in PI4P in their membranes. PI4P-enriched membranes occur under in non-infected cells mainly in the Golgi where they attract certain host factors. They also recruit some viral proteins such as $3CD^{pol}$ that can also associate specifically with PI4P (Banerjee et al., 2018). As discussed before, 3A induces the synthesis of PI4P on RO membranes by recruiting several host factors implicated in PI4P production to those membranes. The accumulation of PI4P leads to the recruitment of host factor oxysterol-binding protein (OSBP). OSBP is attracted to PI4P-rich membranes through binding of PI4P with a pleckstrin homology domain (De Matteis et al., 2013). OSBP recruitment leads to the formation of membrane contact sites (MCSs) between ROs and ER. At MCSs lipids are shuttled between membranes. OSBP exchanges PI4P from RO membranes for cholesterol from the ER leading to an accumulation of cholesterol in RO membranes (J. R. P. M. Strating et al., 2015). In uninfected cells, MCSs connect the ER to the PI4P-enriched Golgi network were it can serve as a master regulator of lipid homeostasis by shuttling cholesterol to Golgi membranes (Mesmin et al., 2013). After being shuttled to the ER PI4P is hydrolyzed by OSBP and later trafficked towards RO membranes by ER-resident proteins completing the cycle of PI4P shuttling.

The effect of 3CD on PI4KB activity

3CD has also been implicated in RO formation by increasing PI4P synthesis after 2BC and 3A were implicated in PI4KB activation. 3CD was found to achieve this by acting on the GBF1/Arf1 pathway at different steps. The 3C domain of 3CD acts upstream of Arf1 activation and the 3D domain of 3CD acts downstream of Arf1 activation in increasing PI4P levels. 3CD affects more than PI4P levels as synthesis of phosphatidylinositol-4,5- bisphosphate (PIP2) and PC is also increased by 3CD (Banerjee et al., 2018). It achieves this independently of its $3C^{pro}$ protease activity and is suggested to serve as a master regulator of cellular membrane biogenesis by acting on cellular pathways. Besides the GBF1/Arf1 pathway 3CD has also been implicated in activating different Arfs using the Arf-activating Guanine nucleotide Exchange Factors (GEFs) BIG1 and BIG2 (Belov et al., 2007). 3CD increases Arf recruitment to RO membranes and increases Arf activity which is implicated in the formation of the ROs. The modulation of host factors by enteroviral proteins leading to the formation of ROs remains a very complex subject where much is still unclear.

Replication starts at ER and later dissolves Golgi as well

During infection there are massive changes in the membrane landscape of the cell. Not only are ROs formed but also are cellular organelles altered. For example, the Golgi apparatus becomes fragmented over the course of infection. This interferes with the secretory pathway of the host cell. This serves as an additional mechanism of interference with host cell functioning that inhibits several anti-viral host defenses such as MHC presentation and cytokine, chemokine and interferon secretion (Mousnier et al., 2014). Later during infection, the Golgi apparatus completely collapses and becomes undetectable.

This suggest a role for Golgi membranes as a possible source of membrane building blocks for ROs (van der Schaar et al., 2016). Early in infection, replication could possibly take place on the intact Golgi membranes since the disassembly of the Golgi apparatus coincides with the formation of early ROs (Limpens et al., 2011). For Coxsackie B3 virus, it was shown that ROs started emerging on the ER and only later in infection started including the trans-Golgi network by whole cell electron microscopy on fixated cells (*Fig. 3*) (Melia et al., 2019). This elucidated the origins of replication during enterovirus infection by examining the membranous ultrastructure of a whole cell. However, this method decreased the amount of information that could be obtained to the high contrast membranous structures of ROs.

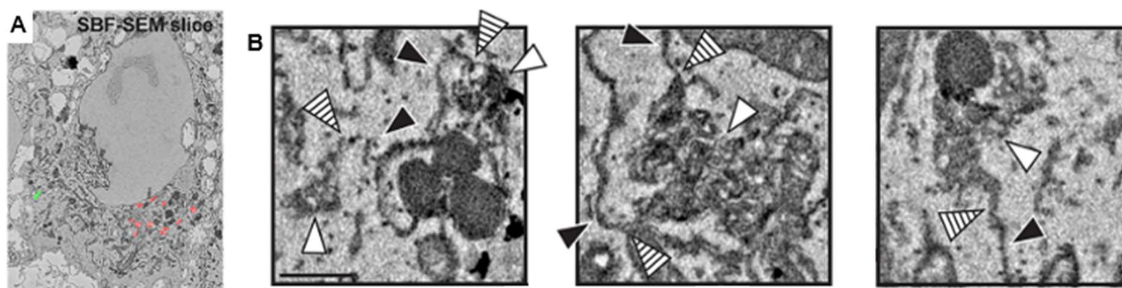

Figure 3. Whole cell EM of cell infected with CVB3 fixated at 6 hours post-infection. A) Representative slice of the whole cell and B) Magnifications at locations of 3A foci. White arrows indicate RO membranes, striped arrows membrane continuities and black arrows ER membranes. Scale bar: 500 nm. Adapted from (Melia et al., 2019)

Lipid droplets and lipid homeostasis

Several cellular organelles play an important role in RO formation or viral replication. In this chapter the role of lipid droplets will be discussed as well as the modulation of lipid homeostasis by viral proteins during infection.

Lipid droplets during infection

Lipid droplets are cellular organelles that store neutral lipids, mainly triacyl-glycerides (TAGs) and cholesterol-esters. They are dynamic organelles that are the main supply of long chain fatty acids for the increased membrane synthesis that underlies the formation of ROs during enteroviral infection (Viktorova et al., 2018). It has been suggested that lipid droplets are depleted during enterovirus infection to sustain viral replication and provide the phospholipids for RO membranes (Melia et al., 2019). The lipids stored within lipid droplets are mostly neutral and are wrapped in a phospholipid monolayer which forms the outer layer of the organelle. The outer phospholipid layer associates with different cellular proteins to balance between hydrolysis and synthesis of the lipids stored within. Recruitment of the lipases HSL and ATGL to lipid droplets during infection is a clear sign of the strong upregulation of lipolysis induced during enteroviral infection. It was demonstrated new MCSs are formed between RO membranes and lipid droplets (Laufman et al., 2019). MCSs serve as locations of lipid exchange between organelles and those formed during infection are different than in non-infected cells. It was demonstrated that 2C and 2BC are responsible for the formation of MCSs during infection and that the ATPase activity of 2C is required for MCS function and RO formation. The self-association of 2C proteins was also shown to be necessary suggesting that the binding of two 2C molecules on opposite membranes anchors the membrane contact site together. However, it remains unclear how viral proteins can specifically bind to the lipid droplet psospholipid outer monolayer. This is likely dependent on the 2C amphipathic helix that can associate with membraens. It has been reported that lipid droplets themselves can more efficiently bind amphipathic helices because of a

larger amount of packing defects in their outer layer compared to other cellular membranes (Prévost et al., 2018).

Glycerol and fatty acids import is increased during infection

Phospholipid biosynthesis is very much upregulated during enterovirus infection. The pathway that leads to the synthesis of neutral TAGs as well as phospholipids is stimulated by viral proteins during infection. The first molecule in this lipid pathway is glycerol. The glycerol import was shown to be increased during infection (Mosser et al., 1972). This suggests a higher cellular consumption of fatty acids which would be added onto the glycerol backbone during the subsequent metabolic reactions. This was supported for multiple picornaviruses where during infection import of long chain fatty acids is activated and they are being channeled towards PC synthesis (Nchoutmboube et al., 2013). In non-infected cells, imported fatty acids would be transported into lipid droplets where they are used to synthesize neutral lipids and kept for storage. This indicates that RO membranes are likely made up of newly synthesized phospholipids. The increase in fatty acid import was found to be linked to an increase in acyl-CoA synthetase activity. This increase occurs very rapidly upon infection and was not found to be sensitive to transcriptional activity. This suggests that the mechanism is reliant on modulation of proteins already present in the cell (Belov, 2014). 2A was identified as the viral protein required for the increased import of fatty acids. Strikingly, it was found to be independent of its protease activity. However, it was not sufficient by itself, requiring the full P2-P3 polyprotein for a strong activation. Interestingly, infected cells also have a different preference for certain fatty acids compared to uninfected cells for internalization. They internalize fatty acids with shorter chains. Phospholipids with shorter fatty acid chains have a higher fluidity in membranes (Nchoutmboube et al., 2013). This suggests a mechanism via which viruses can influence biophysical properties of RO membranes such as fluidity through manipulating host functions such as lipid homeostasis and fatty acid import. Moreover, RO morphology itself could be an effect of properties of the phospholipids making up RO membranes. Enterovirus ROs, consisting of a network of membranous tubules, closely resemble so-called myelin figures (*Fig. 4*). These figures form spontaneously at the interface of concentrated phospholipids and water (Huang et al., 2005). They resemble the membranous network of ROs formed during infection. It's tempting to speculate that this process is similar to the formation of ROs upon increased synthesis of phospholipids. That would suggest that the morphology of ROs is reliant on the increased phospholipid synthesis directly. This would hint at a universal mechanism of RO formation that explains the nearly identical phenotype for different enteroviruses even though there are large differences in factors suggested to be implicated in RO formation.

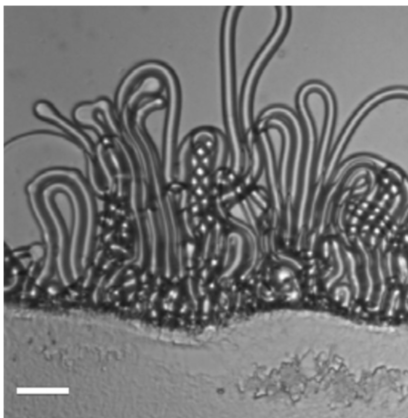

Figure 4. Myelin figures form at the contact area between concentrated phospholipids (below) and water (above). Scale bar: 100 µm. (Huang et al., 2005)

PC synthesis is upregulated by 2A

Phosphatidylcholine is the phospholipid making up the bulk of cellular membranes. As previously discussed, PC synthesis is highly upregulated during infection. To accommodate increased phospholipid synthesis, more fatty acids are imported by infected cells. However, these are not immediately shuttled towards RO membranes for use in PC synthesis. First, the fatty acids are metabolically incorporated into TAG molecules and deposited into lipid droplets. The neutral lipids stored in lipid droplets are used lipolyzed and used for PC synthesis (Laufman et al., 2019). This gradually depletes lipid droplets over the course of infection. It has been demonstrated that the bottleneck in PC synthesis is the presence of enough CDP-choline. This molecule provides a choline group to a diacyl-glycerol molecule to yield a phospholipid. CDP-choline is produced by the enzyme CCTα (Viktorova et al., 2018). In uninfected cells, CCTα is mostly present in the nucleus. However, viral protease $2A^{pro}$ disrupts nucleocytoplasmic trafficking during infection. This drives relocation of CCTα to the cytosol where it is activated by binding to membranes. The amount of CCTα translocated to the cytosol is sufficient to increase PC synthesis. Interestingly, binding of CCTα to RO membranes could itself contribute to RO morphology. CCTα has been demonstrated to rearrange membranes by inducing curvature via an amphipathic helix it possesses (Taneva et al., 2012).

Cholesterol metabolism is modulated by viral proteins

Cholesterol has long been found to be essential for efficient viral replication in infected cells (Strating & van Kuppeveld, 2017). Cholesterol is crucial for regulating the fluidity of membranes and for the organization of lipid rafts. Lipid rafts are assemblies that separate on membranes and serve as platforms for proteins to assemble on. They have important functions in processes such as membrane signaling and trafficking. The assembly of certain proteins specifically on lipid rafts is based on different biophysical properties that membranes possess by a different composition of lipids and proteins locally (Lingwood & Simons, 2010). It was long known that many viruses rely on lipid rafts for entry into cells (Lorizate & Kräusslich, 2011). For enteroviruses, it has also been shown that the rate of viral replication correlates with the amount of free cholesterol in cells indicating its importance for other parts of the viral life cycle besides entry (Ilnytska et al., 2013). Non-infected cells rely largely on lipid metabolism to synthesize lipids they require for membrane biogenesis. However, for picornaviruses it was shown that they do not rely on cholesterol synthesis to sustain viral replication. Instead, they actively use clathrin-mediated endocytosis to traffic cholesterol from the plasma membrane and cholesterol imported extracellularly to ROs during infection (Belov, 2014).

Cholesterol is enriched in RO membranes by two mechanisms. It is exchanged at MCSs by OSBP from ER to ROs. This is driven by PI4KB-mediated PI4P-enrichment in RO membranes. A second mechanism that accumulates cholesterol at ROs was found to also depend on PI4KB. It relies on binding of viral protein 3A to recycling endosomes. In non-infected cells, recycling endosomes traffic a portion of endocytosed cholesterol back to the plasma membrane which is also enriched in cholesterol. However, 3A can reroute the recycling endosomes to ROs. 3A accomplishes this by binding to Rab11 which is present on recycling endosomes. Rab11 and PI4KB interact in non-infected cells but expression of 3A increases this interaction and recruitment of both PI4KB and rab11 to the same membranes (Ilnytska et al., 2013). However, this mechanism does not rely on PI4KB activity since it probably does not require increased PI4P levels. Instead, it relies on a physical interaction with PI4KB.

Early in infection, the large amount of cholesterol trafficked from the plasma membrane towards RO membranes could lead to a decrease in cholesterol biosynthesis. A large part of the cholesterol travels by recycling endosome to ROs but the remainder will be trafficked through the ER. Cholesterol entering the ER leads to negative feedback on its synthesis (Ilnytska et al., 2013). Because the cholesterol-rich

Golgi membranes are dissolved by 3A during infection, they also increase the amount of cholesterol trafficking through the ER. This decreases cholesterol synthesis as well. Cholesterol is of importance for maintaining rigidity of membranes which could be especially important for PI4P-enriched membranes such as RO membranes. PI4P membranes are known to be very fluent (Zhendre et al., 2011). Besides being more fluent through PI4P-enrichment, RO membranes are more fluent through shorter fatty acid chains as explained before. This overall increase in membrane fluidity could require large amounts of cholesterol to maintain rigidity (Nchoutmboube et al., 2013). Maintaining sufficient rigidity in RO membranes could be important for the efficient assembly of viral protein complexes. $3CD^{pro}$, $3C^{pro}$ and $3D^{pol}$ have all been shown to specifically locate to PI4P-rich membranes by binding PI4P. They are also a part of the viral replication machinery and could require more rigid membranes for a $3CD^{pro}$ conformation that attenuates further proteolytic processing of $3CD^{pro}$ into $3C^{pro}$ and $3D^{pol}$ (Ilnytska et al., 2013).

The role of autophagy in viral release

Enteroviruses are traditionally thought to be lytic viruses that egress from cells by rupturing the plasma membrane and killing the host cell in the process. Recently, there has been more attention for the role of a non-lytic release in transmission.

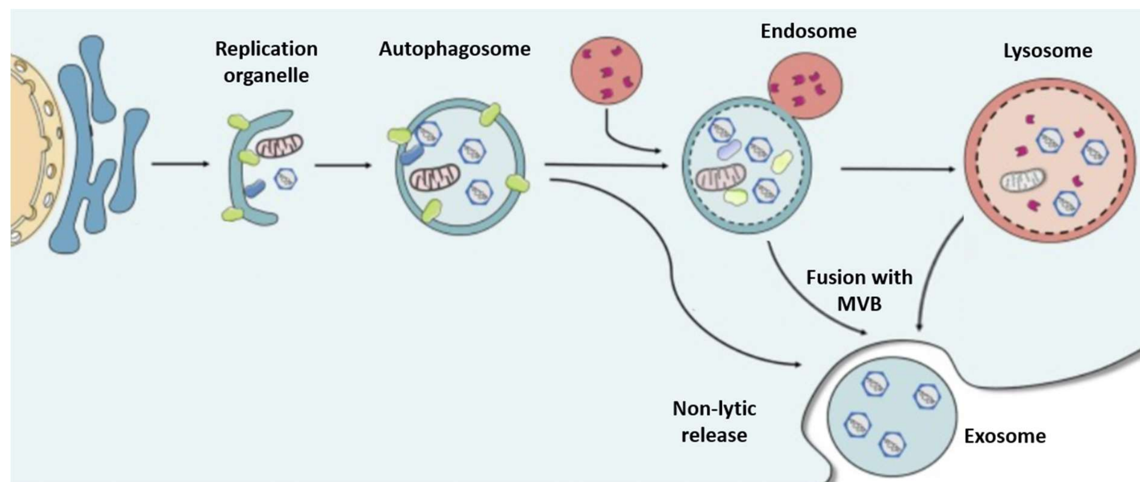

Figure 5. Schematic overview of non-lytic virion release by the autophagic pathway. Adapted from (Sun et al., 2019)

Replication Organelles during late infection are implicated with autophagy

It has long been reported that RO morphology changes during progression of enterovirus infection. During the peak of viral RNA synthesis, RO membranes are rearranged by viral proteins in ensemble with host factors to generate factories for viral reproduction. Early during infection, ROs are mostly single membrane tubules (SMTs) and later in infection they progress towards a higher proportion of double membrane vesicles (DMVs) and multilamellar vesicles (MVs) (Hsu et al., 2010). This process resembles autophagic wrapping of cargo by membranes and has been suggested to be reliant on the cellular autophagy pathway (Richards & Jackson, 2012). Evidence came from the observation that poliovirus genome replication was decreased by non-specifically inhibiting autophagy through blocking of acidification of cellular components. Acidification of phagosomes or endosomes leads to their fusion with lysosomes and eventually degradation of their content but many viruses have managed to evolve mechanisms exploiting the autophagic pathway. They can use it to their advantage in transmission and/or replication. Viruses can inhibit fusion of autophagosomes with lysosomes to

prevent degradation of viral proteins. DMVs and MVs, which are created from DMVs by wrapping of more membranes, were demonstrated to contain autophagy marker proteins such as BECLIN1 and LC3 (de Armas-Rillo et al., 2016) thus earning them the name of autophagosome-like vesicles. It was originally thought that autophagosomal structures could also serve as sites of genome replication. However, recent evidence suggests they do not play a critical role in genome replication but rather contribute to virion assembly (Zimina et al., 2021). The inhibition of fusion of autophagosomal-like vesicles with endosomes and lysosomes facilitates their fusion with Multivesicular Bodies (MVBs). This in turn leads to the release of exosomes containing infectious virions (*Fig. 5*) (Zhang et al., 2022).

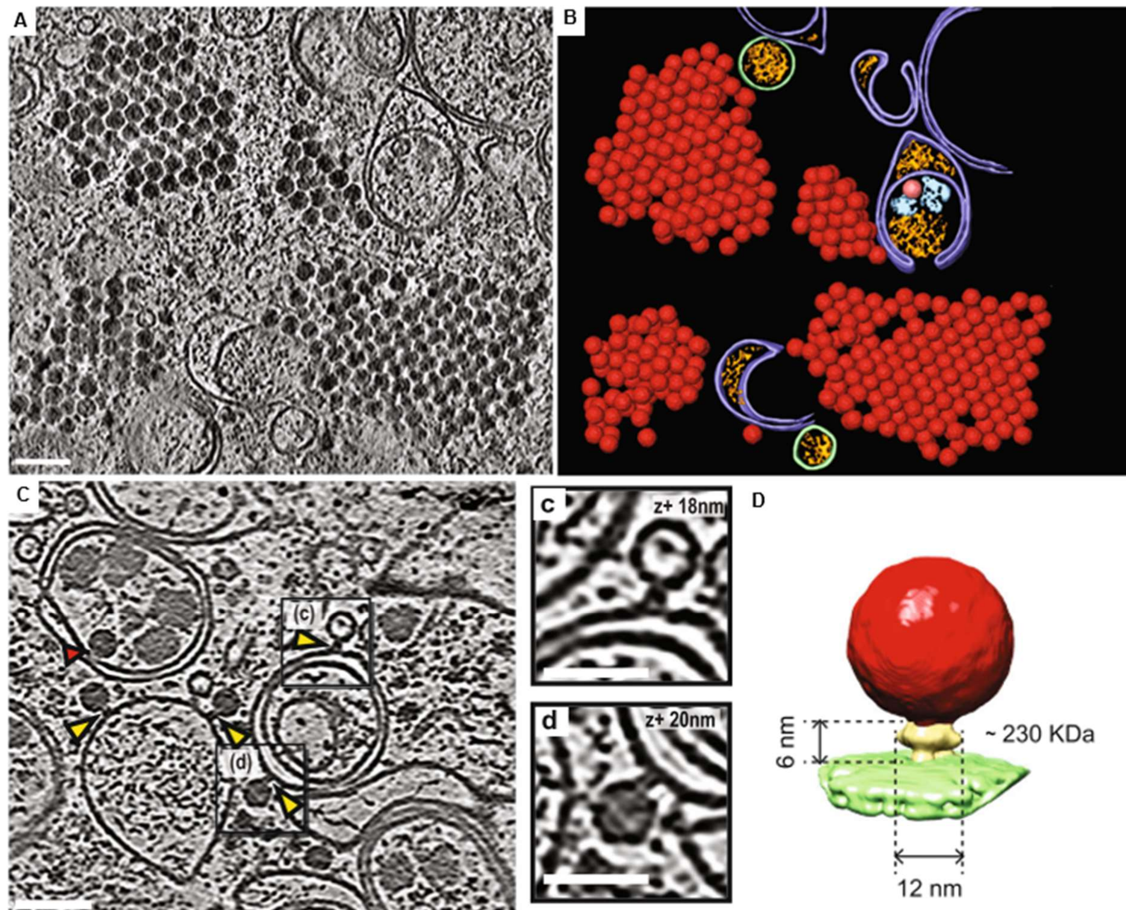

Figure 6. Poliovirus replication observed by cryo-ET. A) tomographic slice of infected cell where ULK1 is inhibited, B) segmentation of membranes and virions. C) Tomographic slice of an infected cell. Red arrow indicates loaded virion unattached to membrane and yellow arrows indicate virions tethered to RO membranes. D) Subtomogram average of viriosn tethered to RO membranes with a molecular weight estimate. Scale bars: 100 nm. Adapted from (Dahmane et al., 2022)

It has been known for a long time that during late stages of infection, virions can pack very into very tight arrays within cells (Nishikiori et al., 2022). In a recent study, cryo-ET was used to study how autophagy aids in the packaging and assembly of poliovirus virions for non-lytic transmission. They demonstrated that there were especially many closely packed arrays of virions when the classical autophagy pathway was inhibited by knocking out autophagy initiator ULK1 (*Fig. 6a and b*). The cells in which ULK1 was inhibited, released more virions late during infection compared to infected cells with an intact autophagy pathway. This suggests that the canonical autophagy pathway decreases virion release and that enteroviruses shut it down in order to take over downstream autophagy factors to increase viral production and release (Dahmane et al., 2022). Furthermore, they observed that

virions loaded with RNA were selectively packaged in autophagosome-like vesicles over empty capsids (*Fig. 6c*). How autophagosomal membranes can selectively package virions over empty capsids is unknown. Due to very small differences in structure and properties between loaded and unloaded capsids, it's likely that correct packaging might already be correlated with packaging by autophagic membranes. Interestingly, capsid proteins have been recently demonstrated to interact with autophagy proteins directly. VP0 was shown to interact with autophagy factor LC3A (Zimina et al., 2021). This direct interaction could increase the amount of virions as cargo in autophagosomal vesicles. It was suggested that this interaction also aids in balancing virion assembly and genome replication by aiding in not packaging viral RNAs into virions that are actively contributing in genome replication and translation. It is conceivable that being in close proximity to membranes is helpful for correct packaging of virions. This hints at membrane-associated factors aiding in efficient loading of viral RNA into capsids. Membrane-associated factors connecting capsids to RO membranes have been observed by cryo-ET in the same study. However, the resolution obtained from subtomogram averaging of the factors was too low to identify the proteins (*Fig. 6d*) (Dahmane et al., 2022). It is tempting to speculate that viral protein 2C plays a role in this process. 2C is membrane-associated and has RNA-binding activity as a helicase. It even has been shown to interact with capsid protein VP3 (*Fig. 7*). Binding of RNA, membranes and capsid makes it an interesting candidate as a factor aiding in capsid loading.

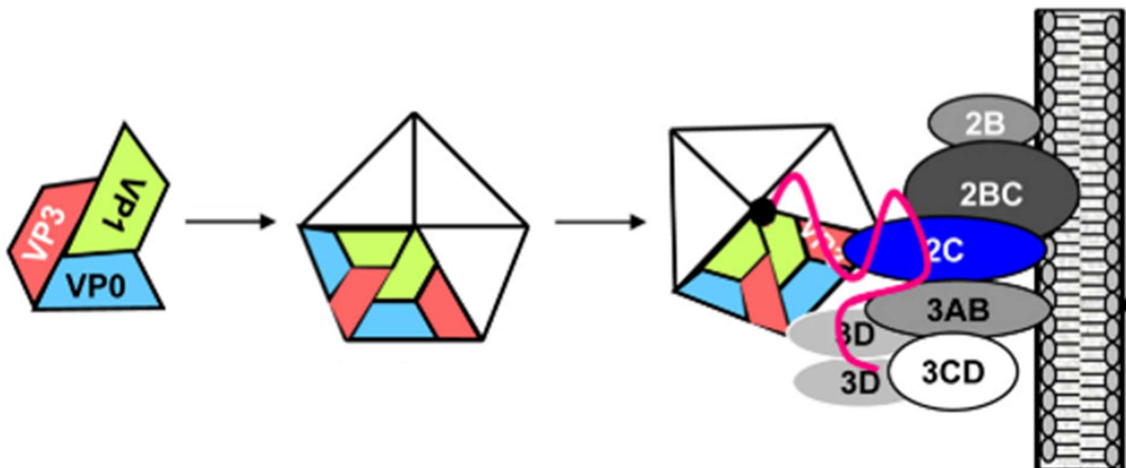

Figure 7. Schematic overview of the viral proteins involved in capsid assembly and loading of the viral genome. Adapted from (Jiang et al., 2014)

Recently, there has been more attention on additional roles structural proteins could play in the viral life cycle. For example, it was shown that expression of VP1 induces ER stress which triggers autophagy in neuronal cells which could play a role in viral egress (Wen et al., 2019). This is another example of the different roles viral proteins play to increase infection efficiency.

Viral proteins implicated in triggering autophagy

Viral proteins 2B, 2BC, 3A and 3AB have been demonstrated to possess the ability of triggering autophagy and colocalizing with LC3 on autophagosomal membranous structures. This increased induction of autophagy leading to an increase in enterovirus replication (Li et al., 2020). However, autophagosomal-like structures induced during infection are quite different from those in uninfected cells. They were demonstrated to be rather immobile due to tethering to microtubules via the viral 3A protein (Taylor et al., 2009). This presumably prevents migration and maturation of these autophagosomal vesicles preventing breakdown of their cargo and likely halts their release until the cytoskeleton breaks down during late stages of infection.

The release of autophagosomal vesicles requires fusion with MVBs. Recently, it was demonstrated that enteroviral 3A is sufficient to induce exosome secretion. It stimulates MVB fusion with the plasma membrane by interaction with Rab27a (Wu et al., 2023). This fusion generates a structure called an amphisome. Fusion of the outer membrane of the amphisome with the plasma membrane releases virions that are still encapsulated in the membrane of a vesicle. For their transmission, enteroviruses are still dependent on their respective host cell receptors expressed on neighboring cells. The likely advantage they gain from this mode of transmission is the protection from neutralizing antibodies present in the extracellular environment. These could neutralize virions during naked egress to uninfected cells (Feng et al., 2013). Actin filaments have also been implicated in viral transmission. They form membrane protrusions that can contact adjacent cells and aid in transmission to neighboring cells (Paloheimo et al., 2011). It is unknown how this transmission occurs exactly but it is possible that autophagosomal vesicles can use the cytoskeleton for transport to the cell periphery and egress at sites like these. Whether this process is lytic or non-lytic, remains unknown.

Cryo-Electron Tomography to study Enterovirus replication

Until recently, enterovirus ROs have only been studied by classical Electron Tomography (ET) with fixated samples. This lead to many insights into RO formation and the viral life cycle in general. However, fixating samples creates artefacts. Besides inducing artefacts, the resolution that can be obtained with classical ET is quite low compared to cryo-ET. This makes it very difficult to study more than membrane rearrangements in samples. Recent advances in hardware and software have enabled the *in situ* study of cells under aqueous conditions. This reduces the amount of artefacts observed because the cellular environment is still under native conditions. The achievable resolution has also greatly improved allowing for the identification and study of proteins in their cellular context.

During the last decade the field of cryo-electron microscopy has gone through what is called the resolution revolution. Improvements in software and hardware have been immense during this time, allowing for much higher resolution information to be obtained from biological samples. For the branch of single particle analysis, this entailed solving the structures of many proteins that were previously stuck at a too low resolution. Within cryo-ET, improvements in hardware such as direct electron detectors allowed for a much lower dose on samples. This reduces radiation-induced sample damage, achieving a much more favorable signal-to-noise ratio. This greatly improved the resolution that could be achieved from tomographic data acquisition. In combination with advancements in sample preparation, such as the development of cryo Focused Ion Beam milling (cryoFIB-milling), this opened many doors towards the structural study of proteins in their native cellular environment. More recently, advances in software regarding subtomogram averaging have made it possible to achieve sub-nanometer resolution on protein complexes within cells (Tegunov et al., 2021). There have also been many advances within cryo-Correlative Light and Electron Microscopy (cryo-CLEM) allowing for correlation of fluorescence microscopy data and cryo-ET data. This allows for evermore precise identification of proteins or regions of interest from the crowded cellular environment, making it possible to investigate rare events. Some events occur less frequently or are transient in nature making their study by cryo-ET very difficult. These techniques have allowed for a new wave of research previously hampered by technological advances and allowed for many new questions to be investigated and answered. Only recent work using cryo-ET, on coronavirus infection identified membrane-spanning pores on ROs finally elucidating the site of viral replication within the previously observed DMVs (Wolff et al., 2020). This example indicates that there remains much work to be done

on viral replication and that the improved resolution that can be obtained allow for the study of processes that remained unclear previously.

An interesting avenue of research would be to study the replication of enteroviruses early in infection. Previous studies have mainly focused on ROs late during infection because the changes induced by the virus are so large that it was possible to study with current techniques at the time. It was previously impossible to identify sites of viral replication due to limitations in localization and identification of early ROs when the membrane rearrangements are still relatively small. It will be interesting to investigate the ROs early in their formation and compare their morphology to later during infection. It's both conceivable that the morphology will be different or that the morphology is already comparable to ROs at peak RNA synthesis but at a smaller scale. Studying formation of ROs using cryo-CLEM should offer new insights. Another thing one could look into, would be to try and identify new host factors associated with ROs and replication complexes. Using subtomogram averaging would allow for the direct observation of proteins associated with ROs and replication complexes. Newly identified host factors could then be used as targets to design anti-viral strategies and therapeutics.

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