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REVIEW

SPECIAL ISSUE: CELL BIOLOGY OF HOST–PATHOGEN INTERACTIONS

Viral use and subversion of membrane organization and trafficking

Miguel Hernandez-Gonzalez[1], Gabrielle Larocque[1] and Michael Way[1,2,*]

ABSTRACT

Membrane trafficking is an essential cellular process conserved across all eukaryotes, which regulates the uptake or release of macromolecules from cells, the composition of cellular membranes and organelle biogenesis. It influences numerous aspects of cellular organisation, dynamics and homeostasis, including nutrition, signalling and cell architecture. Not surprisingly, malfunction of membrane trafficking is linked to many serious genetic, metabolic and neurological disorders. It is also often hijacked during viral infection, enabling viruses to accomplish many of the main stages of their replication cycle, including entry into and egress from cells. The appropriation of membrane trafficking by viruses has been studied since the birth of cell biology and has helped elucidate how this integral cellular process functions. In this Review, we discuss some of the different strategies viruses use to manipulate and take over the membrane compartments of their hosts to promote their replication, assembly and egress.

KEY WORDS: Infection, Membrane trafficking, Virus

Introduction

Viruses are obligate intracellular parasites that are completely dependent on their hosts for their continued survival, replication and spread. Consequently, they have evolved exquisite strategies to manipulate different cellular systems not only to replicate but also to get in and out of cells, while at the same time avoiding or suppressing the intrinsic defence mechanisms of the host. Every virus has their own unique cellular challenges, which vary depending on whether they have an RNA or DNA genome, are enveloped or not, and whether they replicate in the nucleus or cytoplasm. Although the precise mechanisms vary between different virus families, there are many common themes, including how viruses hijack and repurpose membrane organisation and trafficking, the topic of this Review.

Membrane trafficking is a dynamic and versatile process consisting of the interconnected secretory and endocytic pathways (Fig. 1). In the secretory pathway, proteins destined to be sent out of the cell or delivered to the plasma membrane or intermediate membrane compartments are first translocated into the endoplasmic reticulum (ER) (Gemmer and Förster, 2020), where new lipids are also synthesised (Balla et al., 2020; Yang et al., 2018). ER cargoes are subsequently transported to the Golgi complex, which acts as a central transport hub, sorting them into distinct transport carriers in the trans-Golgi network (TGN), to ensure they are correctly delivered to their final destinations (Glick and Luini, 2011; Guo et al., 2014; Miller and Schekman, 2013; Pantazopoulou and Glick, 2019). In the endocytic pathway, extracellular or plasma-membrane-bound cargoes are internalised into endosomes and sorted to be recycled back to the plasma membrane, sent to other destinations, such as the Golgi, or degraded in lysosomes (Grant and Donaldson, 2009; Kirchhausen et al., 2014). The identity of all these different membrane compartments is determined by their lipid composition and by Rab and Arf GTPases, which recruit specific effector proteins (Donaldson et al., 2016; Langemeyer et al., 2018; Mizuno-Yamasaki et al., 2012; Thomas and Fromme, 2020).

Studies on the involvement and impact of viral infection on the regulation, dynamics and function of membrane trafficking exist for almost every viral family, although some of them have been studied more than others (Ketter and Randall, 2019; Robinson et al., 2018). Understanding how viruses take advantage of membrane trafficking offers the promise of obtaining fundamental mechanistic insights into the regulation and function of this essential cellular process. It can also provide a critical understanding into the underlying cause of disease and help to identify potential targets for therapeutic interventions. In this Review, we discuss examples from different viral families to illustrate how viruses subvert and reorganise membrane trafficking to suit their own needs.

Transport and access to replication sites

Enveloped viruses gain access to the cell by direct fusion of their envelope with the plasma membrane, which gives immediate access to the cytoplasm, or by piggybacking on cellular internalisation pathways (Helenius, 2018; Mercer et al., 2020; Yamauchi and Helenius, 2013). In the second strategy, the viral capsid or genome is subsequently released into the cytosol through fusion of the viral envelope with the limiting endosomal membrane, in a process driven by viral fusion proteins (Kielian, 2014). In the case of non-enveloped viruses, after endocytosis, cytoplasmic access is achieved by the disruption of the endosomal membrane (Daussy and Wodrich, 2020; Moyer and Nemerow, 2011).

Once in the cytoplasm, the capsid or genome often needs to be transported to a particular cellular location to initiate replication (Fig. 2). In general, RNA viruses replicate in the cytoplasm, frequently in specialised structures assembled on the surface of membrane-bound compartments (Miller and Krijnse-Locker, 2008; Neufeldt et al., 2018). In contrast, DNA viruses typically replicate in the nucleus, which provides easy access to the replication and transcription machinery of the host, the possibility of genome integration and some degree of protection against cellular defence mechanisms (El-Jesr et al., 2020; Rathinam and Fitzgerald, 2011). As an exception, giant DNA viruses, including poxviruses, encode their own replication machinery and generally replicate in the cytoplasm (Schramm and Locker, 2005), although there is increasing evidence that they still require host factors from the nucleus (Postigo et al., 2017; Senkevich et al., 2017).

For viruses that undergo nuclear replication, the immediate task after entry is actually getting to the nucleus, which can be a considerable distance away from the site of infection, especially in neurons (Miranda-Saksena et al., 2018). Fortunately, the cell

[1]Cellular Signalling and Cytoskeletal Function Laboratory, The Francis Crick Institute, 1 Midland Road, London NW1 1AT, UK. [2]Department of Infectious Disease, Imperial College, London W2 1PG, UK.

*Author for correspondence (michael.way@crick.ac.uk)

M.W., 0000-0001-7207-2722

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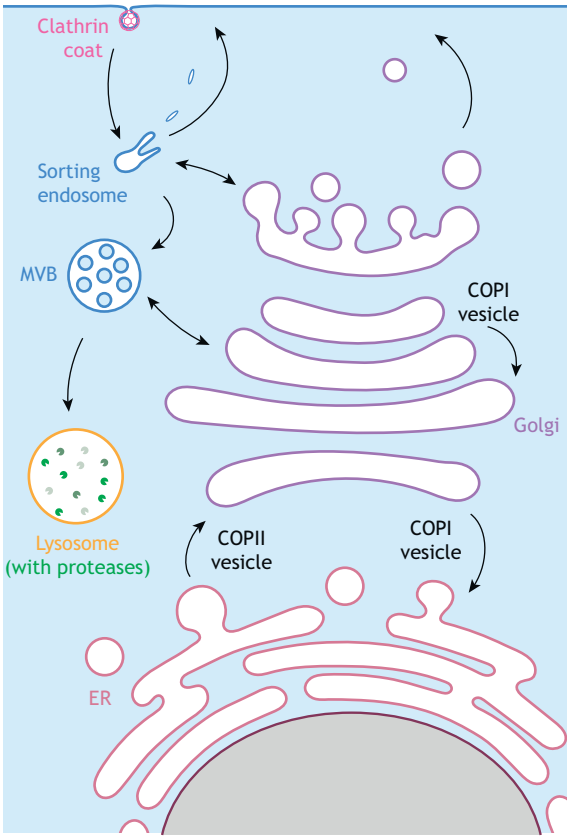

Fig. 1. Main intracellular trafficking pathways. Specialised regions of the endoplasmic reticulum (ER) serve as a departure station for newly synthesised cargo that travels to the Golgi in COPII vesicles. At the Golgi, cargo proteins mature and leave from the trans-Golgi network towards the plasma membrane or endosomes. COPI vesicles help Golgi organisation by trafficking within the Golgi and from the Golgi to the ER. The protein composition at the plasma membrane is also regulated by the endocytic pathway. Clathrin-coated vesicles bring internalised cargoes to sorting endosomes where these cargoes are either sorted in recycling endosomes en route to the plasma membrane or in late endosomes/multivesicular bodies (MVBs), going towards protease-containing lysosomes for degradation. Secretory and endocytic pathways are highly interconnected, which is represented by a bidirectional arrow.

provides a convenient transport system, and viruses encode proteins that recruit the minus-end-directed microtubule motor dynein to be actively transported to a perinuclear location (Dodding and Way, 2011; Naghavi and Walsh, 2017; Wang et al., 2018). The next challenge is the physical barrier imposed by the nuclear envelope. Viruses bypass this in different ways (Fay and Pante, 2015). Some retroviruses, such as murine leukemia virus, gain access during mitosis when the nuclear envelope is absent (Goff, 2007). The drawback of this strategy is that, during interphase, they are unable to integrate into the host's genome to establish long-term viral replication (Goff, 2007; Katz et al., 2005). Penetration through one of the many nuclear pores circumvents this limitation. However, the nuclear pore complex (NPC) has an upper size limit of ∼39 nm (Lin and Hoelz, 2019; Panté and Kann, 2002), and only some viruses, such as hepatitis B virus, are small enough to penetrate intact (Jiang and Hildt, 2020). In contrast, larger viruses such as Herpesvirus simplex 1 (HSV1) and adenoviruses disassemble their capsid in the cytoplasm or in association with the NPC, allowing the viral genome to pass through (Fay and Pante, 2015; Li et al., 2019).

Replication and assembly on membrane compartments

Cytoplasmic viral replication often takes place at specialised replication organelles (ROs), which are assembled on the surface of a membrane-bound compartment (Miller and Krijnse-Locker, 2008; Neufeldt et al., 2018). Viral infection alters these membranes to generate invaginations or cavities in which the replication machinery is concentrated and organised to efficiently perform viral replication in a controlled location. They also serve as a framework to coordinate replication and assembly. The cellular source and location of membranes for RO formation are diverse, varying between different virus families (Fig. 2). For example, *Togaviridae* form ROs at the plasma membrane and endosomes, although endosomal ROs may not be fully functional in the Togavirus genus alphavirus (Frolova et al., 2010). The structure of ROs also varies depending on the viral family. *Flaviviridae*, such as Dengue virus (DENV) and Zika virus (ZKV), form slightly opened invaginations at the ER, with a 11-nm pore connecting the lumen to the cytosol, providing access to the cytosolic components which are essential for replication (Miorin et al., 2013; Neufeldt et al., 2018; Welsch et al., 2009). In contrast, hepatitis C virus (HCV) forms cup-shaped ROs called double-membrane vesicles (DMVs) that protrude from ER membranes (Cortese et al., 2017; Neufeldt et al., 2018; Romero-Brey et al., 2012). Although the lumina of some of these DMVs are connected to the cytosol, most of them are closed (Romero-Brey et al., 2012). It is possible that DMVs close after replication and therefore represent a later stage, such as assembly or envelopment. *Coronaviridae* also induce characteristic DMVs that likely contain newly synthesised RNA genomes (Cortese et al., 2020; Gosert et al., 2002; Klein et al., 2020; Snijder et al., 2020). These are also connected to the cytosol through a pore, suggesting the existence of pore-stabilising viral proteins, such as the nsp3 protein in mouse hepatitis coronavirus (Wolff et al., 2020). Finally, lipid droplets are hijacked at the ER by flaviviruses and SARS-CoV-2 for efficient replication, suggesting a general role of these lipid storage organelles in early stages of virus replication (Cloherty et al., 2020; Dias et al., 2020; Paul and Bartenschlager, 2015).

ROs assemble with the assistance of host proteins and a limited number of non-structural viral proteins, but how they are initiated and organised remains a mystery. It is also not understood how membrane curvature is induced to form ROs, but the answer may lie in specialised viral proteins. In the case of DENV, the integral viral membrane proteins NS4A and NS4B drive RO formation (Paul and Bartenschlager, 2015). These two proteins contain amphipathic helices which, when inserted in a single leaflet of the ER membrane, would induce membrane curvature to facilitate the formation of ROs. Membrane curvature may also be increased by the oligomerisation of these proteins (Stern et al., 2013; Zou et al., 2014). The ER-tubulation factor reticulon 3.1A (Voeltz et al., 2006) and atlastins (dynamin-related GTPases) have been linked to membrane remodelling in *Flaviviridae* (Aktepe et al., 2017; Monel et al., 2019; Neufeldt et al., 2019), highlighting the role of cellular membrane-bending proteins in RO formation.

In many cases, RO formation substantially modifies the organisation and function of cellular membranes, resulting in the establishment of a new *status quo* that favours virus replication and assembly. A good example of this is seen in infections caused by *Picornaviridae* members, such as poliovirus and other enteroviruses, that heavily affect Golgi organisation (Belov, 2016; Belov and Sztul, 2014; Jackson, 2014). In normal conditions, Golgi maintenance depends on a continuous supply of membranes from the ER, mainly in the form of COPII vesicles, whose formation is driven by the Sar1 GTPase (herein referring generically to SAR1A

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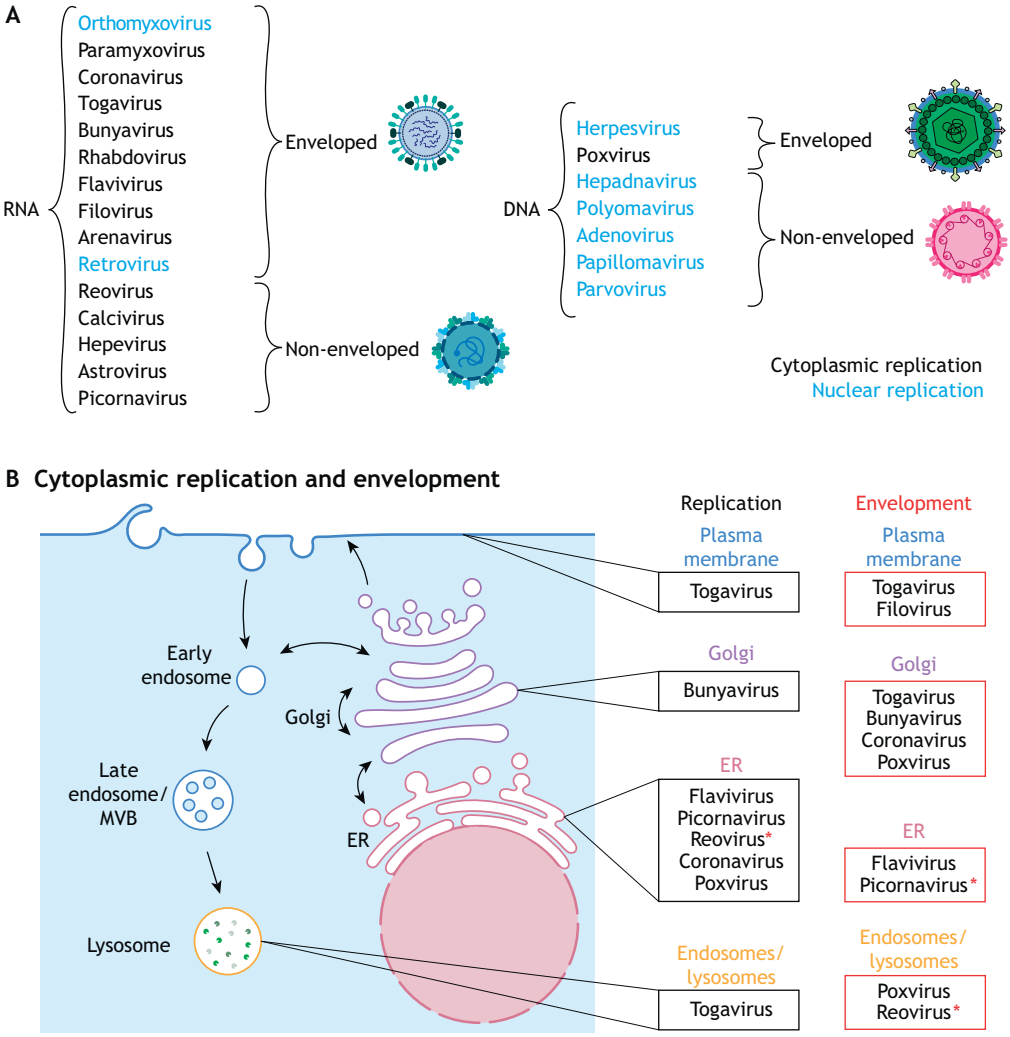

Fig. 2. Virus classification. (A) Main RNA and DNA viral families causing human diseases. Enveloped and non-enveloped classification is shown. Enveloped viruses are those in which at least one type of infectious form of the virion is enveloped by a lipid membrane, which can be acquired from a variety of cellular origins. Blue and black indicates nuclear and cytoplasmic replication, respectively. (B) Sites of replication and envelopment or budding for the viral families that replicate in the cytoplasm. In *Reoviridae*, association of viral factories with ER membranes has been reported. Red asterisks indicate that these families are classified as non-enveloped, although a non-lytic egress has been described (see text for details).

and SAR1B) (Peotter et al., 2019; Ward et al., 2001). In contrast, Golgi-localised Arf GTPases, which are activated by different GTPase exchange factors, including GBF1, recruit the COPI complex to control Golgi-to-ER and intra-Golgi trafficking (Fig. 1), as well as other effectors such as the lipid-modifying enzyme phosphatidylinositol-4-kinase IIIβ (PI4KIIIβ) (Godi et al., 1999). Impairing Sar1 or Arf1 functions disrupts the Golgi, which is partly reabsorbed by the ER (Ward et al., 2001). Despite work from many groups, it is still not clear how the Golgi organisation is maintained (Glick and Luini, 2011; Pantazopoulou and Glick, 2019) and understanding how viruses manipulate the Golgi may provide additional molecular insights.

During *Picornaviridae* infection, ER-to-Golgi trafficking is shut down and the COPII and Golgi-organising machinery, including Arf1, GBF1 and PI4KIIIβ, is recruited to ROs (Doedens et al., 1997; Doedens and Kirkegaard, 1995; Rust et al., 2001). This machinery, together with Sar1, is then hijacked and used during viral replication: Sar1 is required as its loss inhibits foot-and-mouth disease virus infection, whereas a dominant-negative Sar1 impairs poliovirus RNA replication (Hsu et al., 2010; Midgley et al., 2013). However, the precise function of Sar1 in the replication cycle of *Picornaviridae* and other RNA viruses is still not fully clear. In contrast, we have a much better understanding of the role of Arf1, GBF1 and PI4KIIIβ (Hsu et al., 2010; Lanke et al., 2009). It is proposed that, early in infection, the conserved enteroviral protein 3A localises to the Golgi, where it enhances the recruitment of PI4KIIIβ by Arf1 and GBF1 at the expense of COPI. The increase in PI4KIIIβ activity leads to higher phosphatidylinositol 4-phosphate (PI4P) levels in Golgi and ER-Golgi intermediate compartment (ERGIC) membranes, and the displacement of COPI disrupts the normal organisation of the Golgi (Hsu et al., 2010; Melia et al., 2019; Wessels et al., 2006). This rise in PI4P triggers the recruitment of viral RNA polymerase $3D^{pol}$, contributing to the formation of functional ROs, which associate with ER membranes, although they contain Golgi components (Hsu et al., 2010; Moghimi et al., 2020). A high-PI4P microenvironment is also important for efficient replication of the *Flaviviridae* member HCV (Harak et al., 2014; Hsu et al., 2010; Reiss et al., 2011). In fact, the formation of RO membranes rich in PI4P and cholesterol with mixed ER-Golgi identity seems common in RNA viruses that replicate in association with the ER (Belov, 2016). This PI4P and cholesterol enrichment is typical of Golgi membranes in non-infected cells and may serve for recruitment of viral and cellular proteins required to generate ROs. Finally, hijacking the Golgi machinery for RO formation may be also a common theme for RNA viruses, as GBF1 is also crucial for the replication of HCV, *Coronaviridae* and *Phlebovirus* (Goueslain et al., 2010; Lebsir et al., 2019; Martinez and Arias, 2020; Uckeley et al., 2019; Verheije et al., 2008). Although manipulation of the Golgi is common, there are likely degrees of Golgi disruption as enveloped

RNA viruses, such as HCV or SARS-Cov-2, still need to traverse the Golgi to exit the cell (Coller et al., 2012; Ghosh et al., 2020). In fact, Arf1 is required for replication of mouse hepatitis coronavirus, even though it is not as drastically displaced to ROs as is the case in cells infected with *Picornavirus* (Belov et al., 2005; Verheije et al., 2008).

The investigation of ROs is an exciting and rapidly growing field, with new membrane-associated ROs continuously being described for a number of viruses, including orthoreoviruses, which were initially thought to replicate in membrane-less viral inclusions (Fernández de Castro et al., 2014). In all cases, further extensive molecular, morphological and functional characterisation is still required because we lack a complete understanding of the composition and organisation of these essential replication structures.

Egress – getting the best out of the cell

To spread the infection, new viral progeny need to get out of the cell. Virus release can occur after cell lysis, but this is a dangerous option, since it is likely to trigger an increased immune response. Alternatively, viruses can leave the cell by budding at the plasma membrane or after getting enveloped by a cellular membrane compartment (Fig. 3). Historically, the term budding has been preferred for small viruses, whereas envelopment or wrapping is more often used for bigger viruses, such as HSV1 or poxviruses. In this Review, we use the term budding when viruses acquire membrane at the plasma membrane during their release. In contrast, we utilise envelopment when the membrane is obtained from an intracellular compartment. During envelopment, which can occur at a number of organelles, including the ER, the Golgi or even secretory autophagosomes, viruses acquire a number of membranes, the outermost of which will eventually fuse with the plasma membrane, releasing an enveloped virion (Fig. 2). Independently of which membrane is used for envelopment, the process presents similar challenges. First, it frequently requires the presence of viral proteins in the acceptor membrane to mediate the initial interaction with the non-enveloped virion. Second, the membrane must be deformed to accommodate the virus. Finally, the virus has to close the membrane, which may involve viral manipulation of the endosomal sorting complex required for transport (ESCRT) machinery of the host (Box 1) (Barouch-Bentov et al., 2016; Corless et al., 2010; Tabata et al., 2016). Once the enveloped virus is in the lumen of an organelle, it can behave like a cellular cargo that is sorted and secreted by exocytosis either via the canonical pathway (ER–Golgi–exocytic vesicles) or through less conventional pathways, involving secretory lysosomes or even autophagosomes.

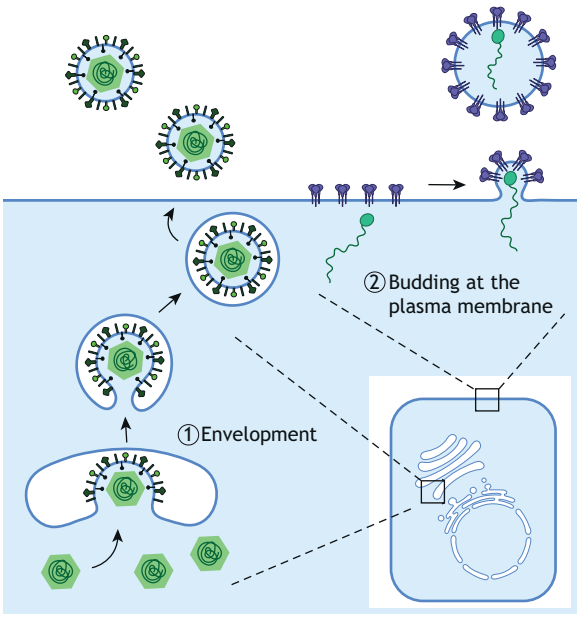

Fig. 3. Envelopment and budding during viral egress. Viral particles assembled in the nucleus or the cytoplasm are enveloped by a cellular membrane to leave the cell in the absence of cell lysis. There are two different ways to acquire this membrane. (1) Virions can be enveloped using an intracellular compartment, such as the Golgi, and travel through the membrane trafficking system to be released after fusion of its outer membrane with the cell surface. (2) In contrast, budding at the plasma membrane directly releases extracellular viral particles. Both strategies involve similar processes that require membrane bending toward the luminal or extracellular space and scission of the neck to generate membrane-bound virions.

Envelopment at the early secretory pathway

The early secretory pathway comprises the ER, the ERGIC and the cis-Golgi (Gomez-Navarro and Miller, 2016). RNA viruses, such as *Flaviviridae* and *Coronaviridae*, which replicate in ROs associated with the ER or the ER/ERGIC, respectively, are enveloped at the early secretory pathway (Klein et al., 2020; Knoops et al., 2008; Krijnse-Locker et al., 1994; Stertz et al., 2007), suggesting there is a strong coupling between the sites of replication and envelopment. In fact, in the *Flavivirus* genus, the pore of ROs is adjacent to enveloping particles at the ER. In this genus, which includes Dengue and Zika viruses, the nucleocapsids are enveloped with ER membranes decorated with the viral proteins prM and E, forming enveloped particles in the lumen of the ER. Expression of prM and E in non-infected cells leads to the formation of subviral particles, suggesting that prM and E drive envelopment at the ER (Ferlenghi et al., 2001). From their site of envelopment, flaviviruses and coronaviruses use the host trafficking machinery to get to the Golgi before leaving the cell. Ultrastructural analysis demonstrated that viral particles from β-coronaviruses (a group that includes SARS-CoV-2) accumulate at the Golgi and in nearby vesicles, leading to the suggestion that they travel through the Golgi and then leave the cell by vesicular transport (Stertz et al., 2007; Ulasli et al., 2010). However, more recent work has found that β-coronaviruses travel through the Golgi and the TGN to reach the endosomal system and leave the cell from secretory lysosomes (Ghosh et al., 2020), which is similar to the non-lytic egress pathway used by the non-enveloped genus Orthoreovirus (Fernández de Castro et al., 2020; see below). In contrast to β-coronaviruses, HCV, an enveloped flavivirus, does not seem to travel through lysosomes. Instead, after trafficking from the ER to the Golgi in COPII vesicles, HCV uses the membrane trafficking machinery to leave the cell using the secretory pathway, similar to that used by cellular cargoes (Coller et al., 2012; Syed et al., 2017). In fact, egress of HCV is tightly linked to the secretion of very-low-density lipoprotein (VLDL) (Bartenschlager et al., 2011; Gastaminza et al., 2008; Huang et al., 2007). Furthermore, before envelopment at the ER, HCV assembly, which takes place in association with lipid droplets, has parallels with VLDL production, as both require triglycerides and cholesterol (Aizaki et al., 2008; Maillard et al., 2011; Shelness and Sellers, 2001). Both are also rich in apolipoproteins, such as apoE, which is essential for HCV assembly (Bartenschlager et al., 2011). Finally, the restricted production of VLDL in the liver could contribute to HCV hepatotropism (Huang et al., 2007), suggesting that membrane trafficking requirements may influence organ tropisms of viral infections.

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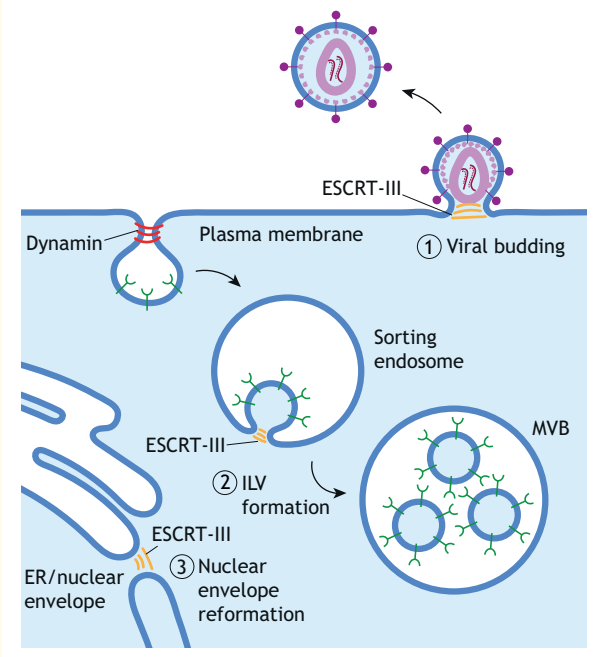
Box 1. The ESCRT pathway

The endosomal sorting complex required for transport (ESCRT) pathway participates in essential processes, such as the formation of intraluminal vesicles in multivesicular bodies (MVBs), abscission during cell division and reformation of the nuclear envelope (Carlton and Martin-Serrano, 2009; McCullough et al., 2015; Remec Pavlin and Hurley, 2020). The function of the ESCRT pathway is to catalyse the scission of a cytoplasm-filled vesicle or to close a cytoplasm-filled hole at a specific membrane-bound compartment. The scission catalysed by ESCRT machinery is topologically opposed to that of dynamin. Both components act from within the cytoplasm; however, dynamin encircles and constricts a membrane neck whereas ESCRT acts from the inside of a topologically opposed and cytoplasm-filled neck or stalk. Mechanistically, the site of action of ESCRT is determined by the recruitment of adaptor proteins to specific membranes, which in turn recruit early-acting ESCRT factors via conserved motifs known as late domains. These early factors include Bro1 domain proteins, such as Alix, and the ESCRT-I and -II complexes. Afterwards, the ESCRT-III complex is recruited. ESCRT-III forms filaments that, together with VPS4 ATPases, drive membrane remodelling and scission. ESCRT is involved in the budding of HIV at the plasma membrane and in the envelopment of a number of viral families at different cellular organelles, such as the nuclear envelope, the ER and the Golgi (Votteler and Sundquist, 2013). In HIV-infected cells, the viral polyprotein Gag acts as an adaptor at the plasma membrane to activate the ESCRT pathway to facilitate virus budding (McCullough et al., 2018; Votteler and Sundquist, 2013). Gag contains YPXL and P(T/S)AP late domains that bind the early-acting factor Alix and TSG101, respectively. The PPXY motif in other retroviral Gag proteins (e.g. murine leukemia virus) also helps initiate the ESCRT pathway by interacting with the E3 ubiquitin-protein ligase NEDD4. Ubiquitin could also act as a late domain that recruits early-acting factors that contain ubiquitin-binding domains. At the end of the process, ESCRT-III and VPS4 ATPases are recruited, and catalyse the scission of virions budding at the plasma membrane. Proteins containing these late domains have been found in many enveloped viruses, and we are just starting to understand the importance of the ESCRT pathway in virus maturation and egress (Chen and Lamb, 2008; Votteler and Sundquist, 2013).



As with cellular cargoes, once inside the Golgi lumen, viruses frequently undergo additional maturation steps, which can result in morphological changes appreciable by electron microscopy, such as in the case of *Coronaviridae*, *Togaviridae* and *Bunyaviridae* (Risco et al., 2003; Salanueva et al., 1999, 2003). This maturation is probably the consequence of physical rearrangements of the viral structure due to posttranslational modifications, including the action of pro-convertases such as furin, an essential step for activating the fusion machinery of many enveloped viruses (Braun and Sauter, 2019; Thomas, 2002).

Envelopment at the late secretory pathway

Envelopment at late compartments takes place in a number of viral families. Here, we focus on *Poxviridae* and *Herpesviridae* to illustrate how this process occurs. Vaccinia virus (*Poxviridae*) undergoes a complex replication and assembly process in cytoplasmic ROs – known as viral factories – located close to the nucleus at the microtubule-organising centre of the cell (Leite and Way, 2015; Ploubidou et al., 2000). Replication initially results in the assembly of intracellular mature virions (IMVs), consisting of a core of genomic DNA and viral proteins, surrounded by a single membrane (Chichón et al., 2009; Chlanda et al., 2009). This single membrane bilayer is not acquired by envelopment, but is derived from the ER membrane by a mechanism that is still not fully understood (Chlanda et al., 2009; Krijnse Locker et al., 2013; Moss, 2015; Weisberg et al., 2017). A limited number of studies suggest that IMVs, which are infectious, are capable of leaving infected cells by directly budding at the plasma membrane (Meiser et al., 2003; Tsutsui, 1983). However, it is more generally accepted that the majority of IMVs are released when infected cells lyse. In addition, some IMVs are capable of leaving the cell by an alternative route that involves their envelopment with a membrane cisterna derived from the TGN or an endosomal compartment (Schmelz et al., 1994; Smith et al., 2002; Tooze et al., 1993). The identity of these membranes is modified by the presence of integral and peripheral viral membrane proteins, some of which, such as B5 and the lipid-modifying enzyme F13 are essential for IMV envelopment (Blasco and Moss, 1991; Engelstad and Smith, 1993; Smith et al., 2002; Wolffe et al., 1993). The molecular basis of IMV envelopment is still largely obscure, although recent observations point to the possible involvement of ESCRT-III components and the ATPase vacuolar protein sorting-associated protein 4B (VPS4B) (Huttunen et al., 2020 preprint). The ESCRT-associated proteins Alix (also known as PDCD6IP) and tumor susceptibility gene 101 protein (TSG101) may also have a role, as their depletion leads to a reduction in virus release (Honeychurch et al., 2007). The hijacking of the ESCRT machinery by vaccinia is maybe not surprising given it is frequently used by multiple viral families during their envelopment or budding (Box 1). However, some viruses, such as influenza, seem to carry out these processes in an ESCRT-independent manner (Chen et al., 2008; Rossman et al., 2010). Interestingly, F13 contains a conserved YXXL motif that may be recognised by Alix to facilitate the recruitment of ESCRT components (Honeychurch et al., 2007). It is possible that ESCRT mediates the final closing step in IMV envelopment by the TGN. However, it has been proposed that ESCRT brings about envelopment of IMVs by multivesicular bodies (MVBs), which subsequently fuse with the plasma membrane to release viral particles (Huttunen et al., 2020 preprint). Interestingly, the use of MVBs for envelopment has also been recently reported for the herpesvirus human cytomegalovirus (HCMV) (Flomm et al., 2021 preprint). It is of course possible that ESCRT participates in budding of IMVs at the plasma membrane, as it does for HIV (McCullough et al., 2018; Meiser et al., 2003; Tsutsui, 1983; Votteler and Sundquist, 2013).

Envelopment of IMVs by TGN-derived cisternae results in the addition of two membranes and the formation of intracellular enveloped virions (IEVs) (Smith et al., 2002). Once formed, the integral IEV membrane protein A36 recruits kinesin-1 to drive microtubule-dependent transport of virions to the cell periphery (Dodding et al., 2011; Rietdorf et al., 2001; Ward and Moss, 2001). It is thought that the outermost membrane of the IEV fuses with the plasma membrane, but the molecular basis of this process remains unknown. During this fusion step, A36 relocates to the plasma membrane beneath the cell-associated extracellular virion (CEV) attached to the outside of the cell (van Eijl et al., 2000). These CEVs locally activate Src and Abl family kinases, resulting in the phosphorylation of A36 and the induction of a signalling network that activates Arp2/3-driven actin polymerisation (Donnelly et al., 2013; Frischknecht et al., 1999; Moreau et al., 2000; Newsome et al., 2004; Reeves et al., 2005), which enhances the cell-to-cell spread of the virus (Cudmore et al., 1995; Doceul et al., 2010; Horsington et al., 2013; Ward and Moss, 2001). Interestingly, immediately after fusion with the plasma membrane, both septins and clathrin are recruited beneath CEVs prior to induction of actin polymerisation (Pfanzelter et al., 2018; Snetkov et al., 2016). Septins, a family of cytoskeletal proteins, act as a restriction factor to suppress release of CEVs from plasma membrane invaginations (Pfanzelter et al., 2018; Spiliotis and McMurray, 2020). In contrast, clathrin promotes A36 clustering to enhance actin assembly (Humphries et al., 2012; Snetkov et al., 2016). Although beneficial to promoting vaccinia spread, the main function of clathrin is most likely to promote endocytosis of A36 and associated IEV proteins from the plasma membrane back to the TGN, to facilitate additional viral assembly once CEVs are released from the cell surface (Husain and Moss, 2003).

In contrast to vaccinia, HSV1 egress has the additional hurdle of traversing the nuclear envelope, since viral replication and initial assembly occur in the nucleus (Bigalke and Heldwein, 2016; Lv et al., 2019). To achieve this task, the virus disassembles the nuclear lamina before enveloping itself in nuclear membrane, only to shed this envelope to gain access to the cytoplasm (Fig. 4). Members of the *Herpesviridae* family accomplish this feat using the nuclear egress complex (NEC), consisting of two viral proteins (pUL31 and pUL34 in HSV1). The NEC, which is anchored to the inner nuclear membrane (INM), promotes both viral envelopment and disruption of the nuclear lamina (Bigalke and Heldwein, 2016). During mitosis, phosphorylation of lamins results in the disassembly of the nuclear lamina (Margalit et al., 2005). A similar process occurs during *Herpesviridae* infection, as the NEC recruits protein kinase C, which is thought to phosphorylate lamin B, whereas the viral kinase pUS3 phosphorylates lamin A/C (Mou et al., 2007; Park and Baines, 2006). Lamin-associated proteins, such as emerin, also get phosphorylated, contributing to nuclear lamina disassembly (Leach et al., 2007; Leach and Roller, 2010). After local lamina dissolution, the virus is enveloped by the INM in a process that topologically resembles the formation of intraluminal vesicles during the formation on MVBs (Fig. 4). The NEC controls viral envelopment by the INM, since it is sufficient to induce membrane budding *in vitro* and forms perinuclear vesicles when expressed in the absence of other viral proteins (Bigalke et al., 2014; Draganova et al., 2020; Klupp et al., 2007; Lorenz et al., 2015). The ESCRT complex, which is also involved in the formation of intraluminal vesicles in MVBs, is recruited by the NEC to facilitate INM envelopment closure, although molecular details are still lacking (Arii et al., 2018; Lee et al., 2012). Finally, to reach the cytosol, enveloped capsids in the nuclear envelope lumen fuse with the outer nuclear membrane in a process reminiscent of cell entry (Fig. 4). During the initial establishment of HSV1 infection, the viral proteins gB, gD and the heterodimer gH–gL are required for membrane fusion during entry (Connolly et al., 2020). These proteins are also present in the nuclear envelope and are incorporated into viral particles when they get enveloped by the INM (Ahmad and Wilson, 2020; Cai et al., 1987; Forrester et al., 1992). Loss of gB and gH results in the accumulation of enveloped viral particles in the perinuclear space, whereas single deletions impair viral entry, but not nuclear egress (Farnsworth et al., 2007b). This suggests that both fusion mechanisms are subtly different even though they both release capsids into the cytoplasm. The main difference is that, during the initial establishment of infection, incoming non-enveloped capsids have a set of viral proteins between the capsid and the envelope that form the tegument, whereas those undergoing egress only acquire tegument in the cytosol before being enveloped at the TGN. As with vaccinia, the TGN cisternae that envelop egressing HSV-1 capsids require the presence of viral membrane proteins, including gE–gI and gD (Farnsworth et al., 2003, 2007a; McMillan and Johnson, 2001) as well as the ESCRT complex (Barnes and Wilson, 2019). Recent biochemical and structural analysis of the herpesvirus tegument complex pUL7–pUL51, which is involved in envelopment (Albecka et al., 2017; Roller and Fetters, 2015), reveals that the pUL51 structure resembles that of the ESCRT-III component CHMP4B and also forms similar filaments (Butt et al., 2020). This homology suggests that pUL51 may promote membrane scission in a similar way to ESCRT, thus providing a redundant pathway to guarantee envelopment (Butt et al., 2020). Once enveloped, the virus follows the route used by cellular cargoes to leave the cell.

When exiting polarised cells, it is important to leave at the right plasma membrane domain as this determines the accessibility to surrounding tissues, which will influence virus invasiveness and pathogenicity (Cong and Ren, 2014; Tamhankar and Patterson, 2019; Tucker and Compans, 1993). In polarised cells, the cargo-sorting machinery ensures proteins destined for export are directed to follow either basolateral or apical routes (Deborde et al., 2008; Weisz and Rodriguez-Boulan, 2009). *Herpesviridae* and other families have evolved to use the same cargo-sorting machinery to ensure they spread from the right plasma membrane domain. In the case of HSV1, exit occurs at cell junctions, a part of the basolateral domain (Johnson et al., 2001; Sugimoto et al., 2008). This exit strategy is controlled by the gE–gI viral complex at the TGN; when gE function is impaired, viral release occurs at the apical plasma membrane and spread is reduced (Johnson et al., 2001). Interestingly, gE contains a YXXΦ motif (where Φ is a bulky hydrophobic amino acid) in its cytoplasmic tail, which is required for its TGN localisation (Alconada et al., 1999). When recognised by the plasma membrane adaptor AP2, this endocytic motif YXXΦ would recycle gE back to the TGN. However, this scenario cannot explain how gE directs HSV1 to the basolateral domain. Hypothetically, the YXXΦ motif could be recognised at the TGN by other adaptor protein (AP) complexes and/or accessory factors to direct viral egress to lateral membranes. In fact, basolateral sorting of cargoes involves the recognition of the YXXΦ motif by AP complexes (Bonifacino, 2014). It is also noteworthy that, during HSV1 infection, many TGN markers relocate to cell junctions, whereas other trafficking components disperse cytoplasmically (McMillan and Johnson, 2001; Wisner and Johnson, 2004). This redistribution is not a by-product of envelopment or egress, as it also occurs in the absence of viral envelopment (Wisner and Johnson, 2004), suggesting the TGN function and organisation is altered to favour the virus in infected cells.

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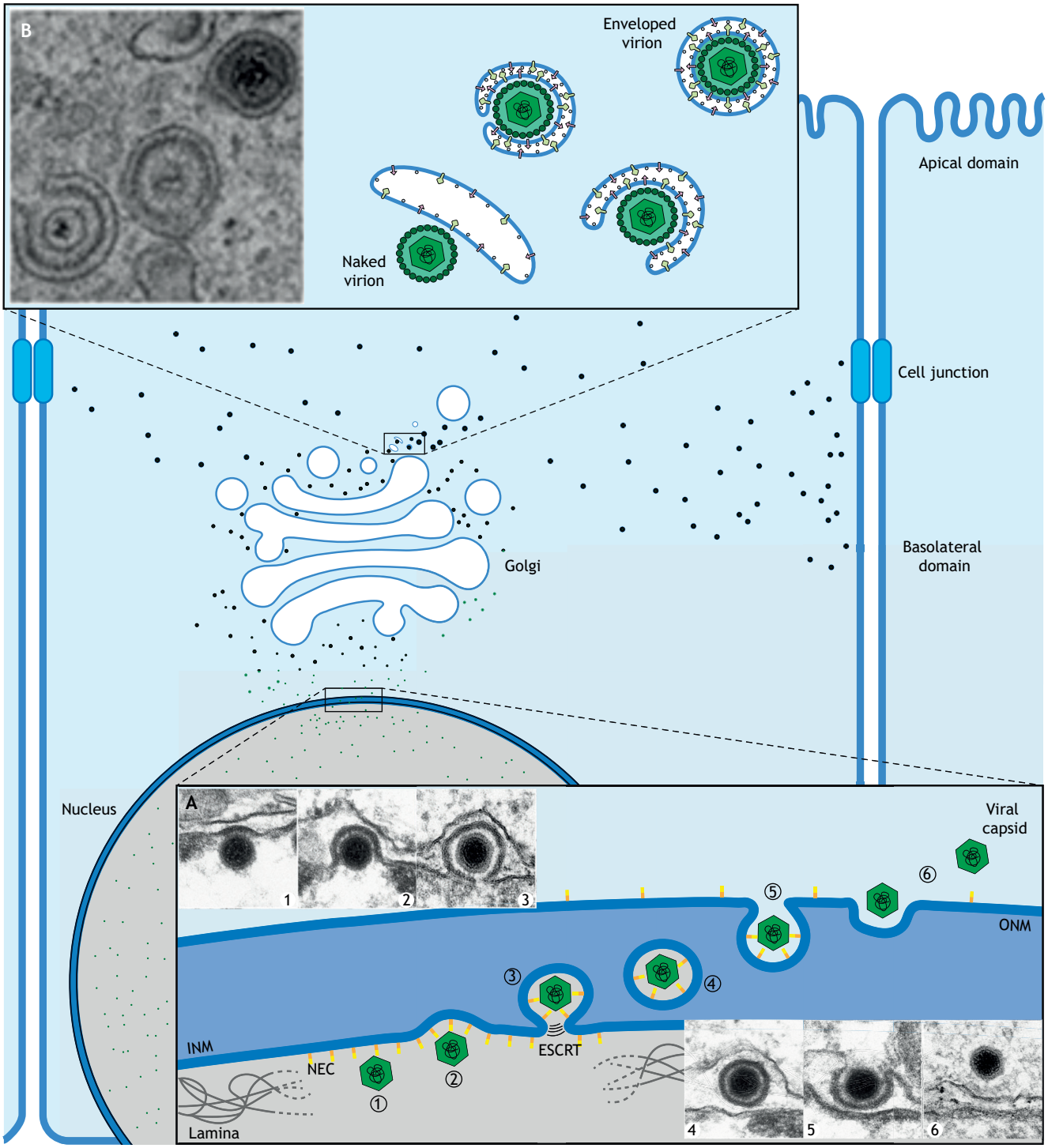

Fig. 4. Nuclear and cellular egress of HSV-1. (A) Viral capsids assembled in the nucleus interact with the inner nuclear membrane (INM) via the viral nuclear egress complex (NEC). During primary envelopment, the NEC locally disassembles the nuclear lamina (1) and helps the INM envelop the capsid (2), which is sealed with the help of the ESCRT complex (3–4). Next, enveloped capsids fuse with the outer nuclear membrane (ONM) (5) to release the virions into the cytoplasm in a process called de-envelopment (6). (B) In the cytoplasm, capsids acquire tegument before getting enveloped by trans-Golgi or endosomal membranes enriched in viral proteins and sealed by the ESCRT machinery during this secondary envelopment. Enveloped virions are transported to the basolateral domain of the plasma membrane, including cell junctions, for release. Electron micrographs in A are reproduced from Mettenleiter et al. (2013) [©2012 Blackwell Publishing Ltd] and those in B are from Maringer et al. (2012), and are reproduced with permission from the American Society for Microbiology. Note that the width of the EM figure in B measures 660 nm.

HCMV, another member of the *Herpesviridae* family, also induces extensive remodelling of cellular membranes (Das and Pellett, 2007, 2011; Das et al., 2007; Villinger et al., 2015). HCMV envelopment takes place in a complex perinuclear assembly compartment, consisting of a set of cisternae and vesicles that have membrane identities associated with the cis-Golgi, TGN, endosomes and ER (Alwine, 2012; Buchkovich et al., 2009; Das and Pellett, 2011; Das et al., 2007). Exactly how HCMV

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manipulates the cell to form this complex assembly compartment is still a mystery, while membrane organisation is understudied in other *Herpesviridae* infections. Future studies into the molecular mechanisms that govern these complex membrane reorganisations will uncover whether they constitute a general effect of *Herpesviridae* infection or whether each member has its own unique strategy.

Egress of non-enveloped viruses in the absence of cell lysis

Non-enveloped viruses typically spread after cell lysis; however, there are examples of non-lytic egress. In the case of *Picornaviridae*, such as poliovirus and Coxsackievirus, this pathway involves manipulation of autophagosomes (Mutsafi and Altan-Bonnet, 2018) (Fig. 5). Autophagosomes normally capture organelles, cellular components and intracellular pathogens to direct their recycling or destruction in lysosomes (Yu et al., 2018). However, poliovirus particles found in autophagosomes labelled with lipidated microtubule-associated protein light chain 3 (LC3, also known as MAP1LC3) family proteins (lipdated form is denoted LC3-II) do not follow this degradative pathway (Bird et al., 2014; Chen et al., 2015; Robinson et al., 2014). Rather, autophagosomes that surround and capture virions at the ER become secretory, fusing

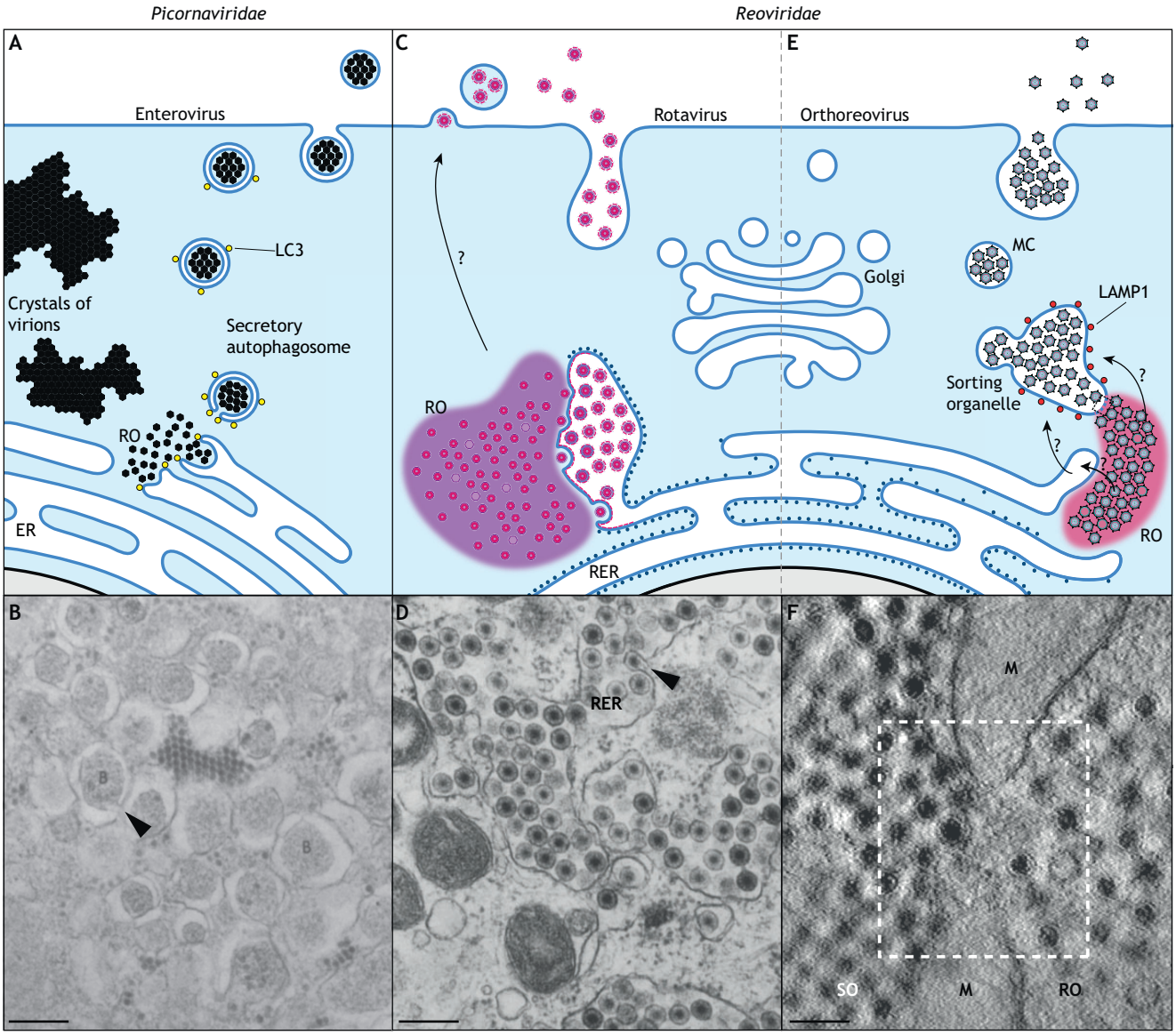

Fig. 5. Non-lytic egress of non-enveloped viruses. (A,B) Enteroviruses replicate in cytoplasmic replication organelles (ROs) associated with the ER. A proportion of mature virions are packaged into LC3-positive secretory autophagosomes [arrowhead in B; reprinted from Dales et al. (1965) with permission from Elsevier], whereas others accumulate intracellularly. (C,D) Rotavirus also replicates and assembles in ROs associated with ER membranes, which are used for envelopment (see arrowhead in D; republished with permission from Rockefeller University Press from Poruchynsky et al. (1991); permission conveyed through Copyright Clearance Center, Inc.]. This membrane is subsequently removed in the ER lumen before virions are released, possibly by a non-conventional secretory pathway (Jourdan et al., 1997). (E) Orthoreovirus replicates in ROs associated with the ER and other membranes before accumulating inside a sorting organelle (SO) with lysosomal identity (LAMP1 positive) by an unknown mechanism. Membrane carriers (MC) then bud from the SO to transport virions to the plasma membrane. (F) The electron micrograph shows the close proximity between the RO, the SO and mitochondrial membranes (M) in orthoreovirus-infected cells. Republished with permission from Rockefeller University Press from Fernandez de Castro et al. (2020); permission conveyed through Copyright Clearance Center, Inc.]. RER, rough ER. Scale bars: 200 nm.

with the plasma membrane to release a vesicle containing viral particles (Chen et al., 2015; Ponpuak et al., 2015; Robinson et al., 2014). This mechanism explains how the bulk of poliovirus release occurs before cell lysis (Jackson et al., 2005). Importantly, hepatitis A virus, a distant member of the *Picornaviridae* family, has also been shown to leave cells with a membranous envelope (Feng et al., 2013). These data appear to blur the distinction between enveloped and non-enveloped viruses.

Other non-enveloped viruses have evolved alternative strategies of egress. These include orthoreoviruses, which replicate and assemble in ROs known as viral inclusions (Fernández de Castro et al., 2014; Garcés Suárez et al., 2019) (Fig. 5). These replication sites are associated with ER and mitochondrial membranes, as well as lipid droplets (Fernández de Castro et al., 2014; Tenorio et al., 2018). More recently, non-lytic egress of mammalian orthoreovirus has been found to occur via membranous carriers, which fuse with the plasma membrane to release non enveloped viral particles (Fernández de Castro et al., 2020). These membrane carriers seem to bud from larger membrane-bound organelles with a lysosomal identity (LAMP1 positive) that are associated with viral inclusions (Fernández de Castro et al., 2020) (Fig. 5). In contrast to what is seen with *Picornaviridae*, these structures lack autophagic identity. It is not immediately obvious how the lysosomal identity of this compartment is maintained in the absence of virion degradation. Moreover, exactly how assembled orthoreovirus virions translocate from the cytoplasm into the lumen of these larger sorting organelles without acquiring an envelope remains to be established. The answer may be found in the analysis of the close relative rotavirus, which replicates and assembles in RNA–protein condensates known as cytoplasmic inclusions (Garcés Suárez et al., 2019; Geiger et al., 2020 preprint). Rotaviruses induce and hijack the early stages of autophagy and the anterograde COPII machinery to transport viral proteins from the ER to the sites of viral assembly (Crawford et al., 2019). This blocks ER-to-Golgi traffic, as seen with many other RNA viruses (Doedens et al., 1997; Xu et al., 2000). Rotavirus virions access the lumen of the ER by envelopment. However, the envelope is thought to be subsequently removed by an unknown mechanism in the lumen of the ER (Tian et al., 1996) (Fig. 5). It is possible that orthoreoviruses enter sorting organelles (or a previous compartment, like the ER) by envelopment and, similarly to rotaviruses, shed this envelope once they are luminal. The situation actually appears more complex as extracellular vesicles containing infectious rotavirus have been recently described (Santiana et al., 2018). This observation suggests that rotavirus release occurs via multiple mechanisms, and may be cell type dependent.

Conclusions and future perspectives

Historically, early insights into the organisation and function of membrane trafficking were linked to the analysis of viral infections or the trafficking of viral proteins (Butt et al., 2020; Fries and Rothman, 1980; Helenius, 2020; Rothman and Fine, 1980). Today, we have a far greater molecular understanding of how membrane trafficking regulates the movement of cellular cargoes and lipids and the maintenance of organelles and cellular architecture (Emr et al., 2009; Guo et al., 2014; Pantazopoulou and Glick, 2019). However, it is clear from the few examples we have discussed that, in the majority of cases, we still lack a full molecular understanding of how even relatively simple viruses manipulate cellular membranes for their own ends. It is also evident that viruses can still provide additional molecular insights into the regulation and function of membrane trafficking.

Ideally, we should all be examining viral infections at a subcellular level in a living organism. However, this presents many technical problems including the ability to image the right tissue with sufficient resolution and speed. Going forward, the use of organoids offers the possibility of examining the cellular impact of viral infection in a more complex 3D situation that is closer to the real physiological situation than a cell monolayer in a culture dish (Cugola et al., 2016; Ettayebi et al., 2016; Garcez et al., 2016; Ramani et al., 2018). Another frustration in working with viruses is their size, which limits our ability to fully resolve what is happening in the light microscope. Because of this, studies have traditionally relied heavily on morphological analysis using electron microscopy. However, these static snapshots do not provide the full picture as they lack true dynamic information. The use of super-resolution microscopy combined with image analysis is now revolutionising multiple aspects of cellular virology by allowing us to see dynamic events in ever-increasing detail (Gray et al., 2019; Scherer et al., 2020; Sekine et al., 2017). In addition to light microscopy, improvements in electron microscopy techniques, such as focused ion beam scanning electron microscopy (FIB-SEM), are allowing us to image whole cells or even tissues, whereas cryo-electron tomography is providing even higher resolution snapshots and unprecedented molecular insights into viral structures and membrane rearrangements in their native state in the cell (Calder and Rosenthal, 2016; Ibiricu et al., 2011; Quemin et al., 2020). These technological advances in combination with functional assays, such as CRISPR screens, are undoubtedly driving a renaissance for membrane trafficking and virus research.

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Competing interests

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