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Virus factories, double membrane vesicles and viroplasm generated in animal cells

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Many viruses reorganise cellular membrane compartments and the cytoskeleton to generate subcellular microenvironments called virus factories or 'viroplasm'. These create a platform to concentrate replicase proteins, virus genomes and host proteins required for replication and also protect against antiviral defences. There is growing interest in understanding how viruses induce such large changes in cellular organisation, and recent studies are beginning to reveal the relationship between virus factories and viroplasm and the cellular structures that house them. In this review, we discuss how three supergroups of (+)RNA viruses generate replication sites from membrane-bound organelles and highlight research on perinuclear factories induced by the nucleocytoplasmic large DNA viruses.

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Introduction

Many viruses replicate within subcellular microenvironments or 'mini-organelles' called virus factories or 'viroplasm'. Formation of these structures involves rearrangement of host cell membranes and cytoskeleton and induces a 'cytopathic effect' indicative of virus infection. It is generally believed that factories and viroplasm create a platform to concentrate replicase proteins, virus genomes and host proteins required for replication, and at the same time physically separate replication sites from a myriad of cellular antiviral defences. The subversion of membrane trafficking pathways during the formation of replication sites may add further benefit by slowing the transport of immunomodulatory proteins to the surface of infected cells to protect against immune responses, while disruption of the cytoskeleton may enhance release of viruses from cells. There is growing interest in understanding how viruses induce such large changes in cellular organisation, and recent advances in electron microscopy coupled with tomography and live cell imaging are beginning to reveal the relationship between virus factories and viroplasm and the cellular structures that house them. In this review, we discuss how three supergroups of (+)strand RNA viruses generate replication sites from membrane-bound organelles and highlight research on perinuclear factories induced by the nucleocytoplasmic large DNA viruses (NCLDV).

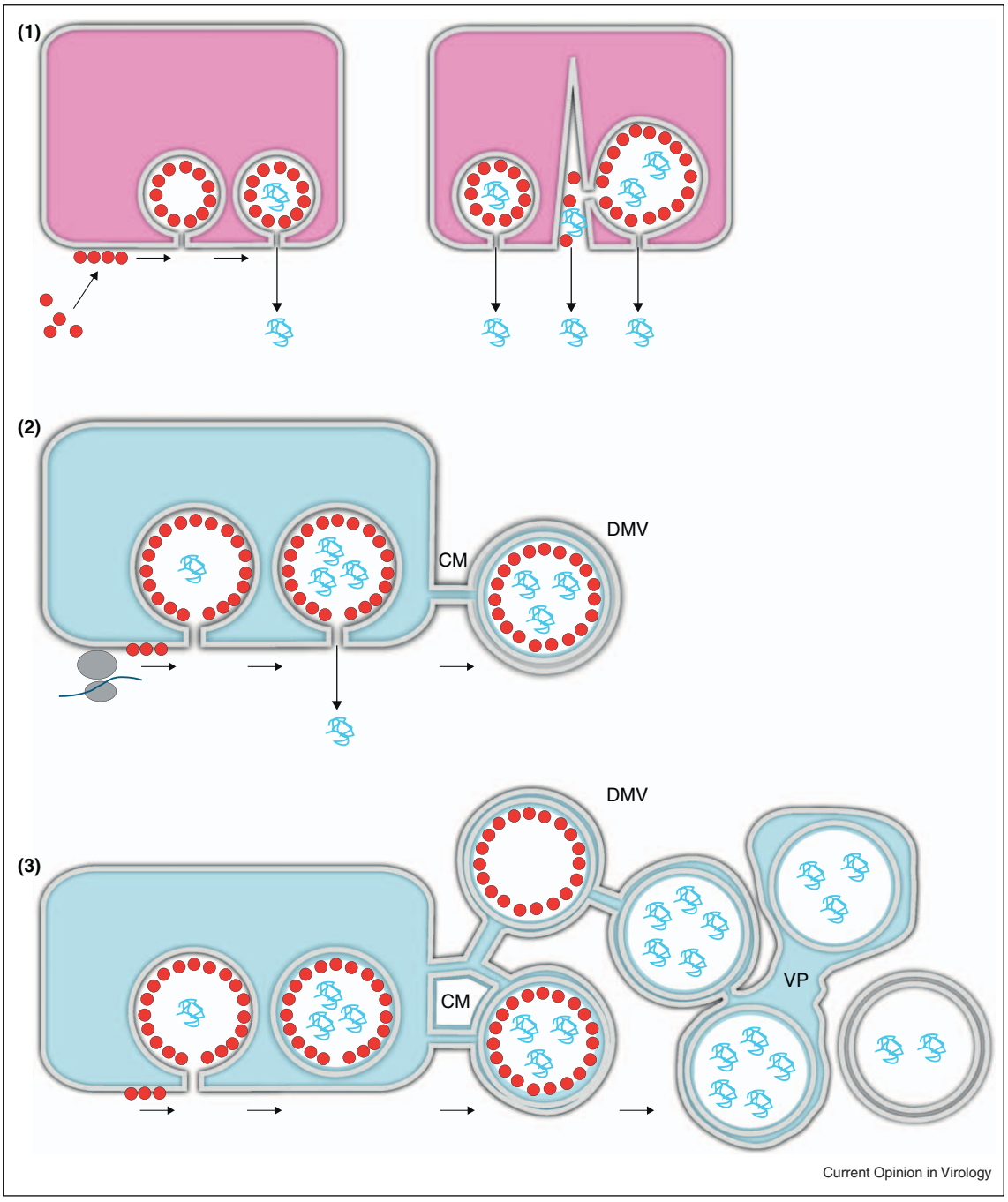
(+)RNA viruses form factories from membranes derived from the secretory pathway

The RNA-dependent RNA polymerases (RdRp) of the (+)strand RNA viruses are targeted to the cytoplasmic face of membrane-bound organelles and subsequent assembly of the replicase complex induces membrane curvature and the formation of densely packed membrane vesicles (reviewed in [1,2]) (Figure 1). The alphaviruses generate membrane invaginations called spherules [3], while the flaviviruses and coronaviruses generate networks of double membrane vesicles (DMVs) connected to a complex of convoluted membranes (CM) derived from the endoplasmic reticulum (ER). The picornaviruses also generate DMVs and a heterogeneous series of membrane vesicles and membrane rosettes [4,5]. The formation of spherules, and possibly DMVs, parallels mechanisms of capsid assembly where ordered assembly of replicase proteins induces membrane curvature and invaginations of uniform diameter [2]. The more complex membrane rearrangements involving ER networks and CM may involve additional recruitment of host proteins that modulate SNAP (Soluble NSF [N-ethylmaleimide sensitive factor] Attachment Protein) Receptor (SNARE) proteins and the Rab and Arf GTPases that control the secretory pathway (reviewed in [6]).

Spherules produced by alphaviruses and nodaviruses

RdRp supergroup 3 viruses such as the animal alphaviruses, Semliki Forest virus (SFV) and Sindbis virus produce 50 nm diameter invaginations called spherules that are aligned along the inside face of the limiting membrane of endosomes and lysosomes [7]. Flock house virus (FHV) is a member of the *Nodaviridae* family (RdRp supergroup 1), which generates spherules in the outer membrane of mitochondria [8]. Each spherule contains approximately 100 copies of the replicase protein packed along the inner membrane surface [9••]. Tomographic

Figure 1



Models for formation of sperules and double membrane vesicles during replication of (+)strand RNA viruses. Panel 1: Spherule produced by alphaviruses. Replicase proteins (red spheres) are recruited to the cytoplasmic face of membrane-bound organelles. Assembly of replicase proteins induces membrane curvature and invagination forming a spherule. The spherule remains connected to the limiting membrane of the organelle and a pore allows new genomes to enter the cytosol (adapted from [2]). Panel 2: Virus-induced vesicles and double membrane vesicles generated by DENV flavivirus. Replicase proteins (red spheres) are recruited to the cytoplasmic face of membrane-bound organelles. Assembly of replicase proteins induces membrane curvature and invagination into the ER forming a large spherule. The invagination remains connected to the limiting membrane of the organelle and a pore allows new genomes to enter the cytosol. Close apposition of ER membranes leads to the formation of DMVs connected to the ER by convoluted membranes (CM) (adapted from [10••]). This may close the pore leading to the cytosol. Panel 3: Virus-induced vesicles and double membrane vesicles generated by SARS-CoV coronavirus. Replicase proteins (red spheres) are recruited to the cytoplasmic face of membrane-bound organelles. Assembly of replicase proteins induces membrane curvature and invagination into the ER forming a large spherule. It has been difficult to find evidence for a pore connecting invaginations to cytosol. Close apposition of ER membranes leads to the formation of DMVs connected to the ER by CM. These may exclude replicase proteins and become sites for storage of viral RNA. In some cases the close apposition of ER membranes is lost and single membraned vesicles arranged in vesicle packets (VP) appear within membrane networks connected to the ER (adapted from [11••]).

reconstructions show that spherule membranes are continuous with the outer mitochondrial membrane and that a membrane neck connecting the lumen of the spherule to the cytosol surrounds a channel wide enough to allow passage of (+)RNA into the cytosol [9••]. Rubella virus is the only member of the Rubivirus genus of the *Togaviridae*, which are distantly related to the alphaviruses. Assembly of the rubella RdRp on the cytoplasmic face of endosomes and lysosomes generates spherules containing replicase proteins and double-stranded RNA. In some cases, the spherules grow into large vacuoles and rigid membrane rods and sheets possibly coated with RdRp. Freeze fracture studies and tomographic reconstitution have identified inter-connections between the vacuoles [7] and protein bridges connecting vacuoles to ER.

Double membrane vesicles, convoluted membranes and membrane webs

Flaviviruses

The flavivirus genus are part of RdRp supergroup 2. Yellow fever virus, West Nile virus and dengue virus (DENV) generate 80–100 nm diameter invaginations into the ER. Tomographic reconstructions of membranes induced by DENV show a continuous network of ER membrane connected to spherical vesicles and CM [10••]. Virus-induced vesicles contain replicase proteins and dsRNA and are found within the lumen of the ER. Most have double membranes suggesting that they are formed from invaginations into ER cisternae [10••]. Each spherical vesicle is connected to the ER membrane by a neck with a pore opening to the cytosol that could allow transit of viral RNA. In some images, the pores lie adjacent to sites of virus assembly making it possible that viral RNA passes directly from the spherical vesicles to budding viruses [10••].

Arteriviruses and coronaviruses

Arterivirus and coronaviruses also generate densely packed membrane vesicles. Three-dimensional reconstructions of vesicles induced by severe acute respiratory syndrome coronavirus (SARS-CoV) show DMVs between 150 and 300 nm in size bounded by two tightly apposed membranes connected to ER [11••]. SARS-CoV also induces CM containing small tubular and reticular membranes connected to the ER. Later during infection cells contain 'vesicular packets' where single membraned vesicles are surrounded by a common outer ER membrane [11••]. The interior of the DMVs contains dsRNA but surprisingly, unlike DMVs generated by flaviviruses, the replicase proteins are absent from the DMVs but locate to the CM that lie between DMVs. This suggests that virus replication occurs in the CM rather than DMVs. Neck-like structures extending from DMVs to the outer ER membrane are visible, but evidence for a pore connected to the cytosol is lacking. Enveloped SARS-CoV can be detected in the vesicular packets. This suggests that replication may take place on CM and that genomes are transferred to vesicular packets for envelopment and budding, while excess viral RNA may be stored in DMVs.

Picornaviruses

Picornaviruses generate densely packed DMVs between 200 and 400 nm in diameter, a series of single membraned vesicles resulting from fragmentation of the Golgi, and autophagosomes possibly generated as a bystander response to infection [11••,12–16]. The nature and relative numbers of vesicles vary greatly depending on the picornavirus family and it is not clear which population of vesicles house the replication complex. The DMVs generated during picornavirus replication lack an obvious opening to the cytosol making it possible that, as suggested for coronaviruses, they are a by-product of replicase assembly and are used as a storage site for viral RNA.

Mechanisms of membrane rearrangement

Lipid biosynthesis plays an important role in both alphavirus replication and nodavirus replication as cerulenin treatment inhibits alphavirus and nodavirus replication [17,18] and both FHV and SFV appear to upregulate phosphatidylcholine synthesis [18]. Similarly, hepatitis C virus (HCV) replication requires fatty acid synthesis and geranylgeranylation [19] and cerulenin also inhibits poliovirus (PV) viral RNA synthesis [20] and coxsackievirus B3 replication [21]. This suggests that lipid synthesis is required for efficient replication of many different (+)strand RNA viruses.

Viral proteins that generate DMVs are beginning to be identified. When expressed separately the picornavirus 2BC, 2C and 3A proteins generate ER vesicles and tubules but these differ from the vesicles produced during infection. For PV, coexpression of 2BC and 3A can generate DMVs similar to those seen in infected cells [16]. The arterivirus nsp3 and the coronavirus equivalent nsp4 proteins are multispanning ER membrane proteins. Coexpression of equine arterivirus nsp2 and 3 [22] can generate DMVs from the ER, and protein interactions involving cysteine residues in the first loop domain are required for generating correct curvature [23]. DMV formation may also require host proteins that regulate the formation of vesicles within the ER and Golgi. Inhibition of Sar1 and Arf1 GTPases inhibits RNA replication, and inhibition of the Arf GTP exchange factor, GBF1, by brefeldin A reduces the number of DMVs in infected cells [24•]. The downstream effector of Arf1 required by coronaviruses remains unknown but may be the phosphatidylinositol-4-kinase-IIIβ (PI4KIIIβ) shown to play a role in picornavirus and HCV replication (see below).

For enteroviruses such as PV and coxsackieviruses, the nonstructural protein 3A may play a crucial role in the recruitment of the RdRp, $3D^{pol}$, to the cytoplasmic face of membrane-bound organelles by increasing the recruitment of PI4KIIIβ [25••]. The mechanism hinges on the

ability of the enterovirus 3A protein to modulate the activity of the Arf1 GTPase and its guanine nucleotide exchange factor GBF1. Arf1 and GBF1 play a central role in regulating membrane traffic between the ER and Golgi. Activation of Arf1 by GBF1 catalyses recruitment of COP1 proteins to the Golgi to facilitate formation of the COP1-coated vesicles that carry proteins from the Golgi to the ER. Binding of enterovirus 3A protein to GBF1 activates Arf1 and increases recruitment of an alternative Arf1 effector protein, PI4KIIIβ, to membranes [24•]. Since $3D^{pol}$ binds to phosphatidylinositol-4-phosphate (PI4-P), the localised production of PI4-P increases recruitment of the $3D^{pol}$ at the expense of COP1. This results in disassembly of the Golgi providing membrane vesicles enriched in PI4-P for replication. This role for 3A may not hold for other picornaviruses where replication is not dependent on an active GBF1, and for picornaviruses that express 3A proteins unable to disrupt ER to Golgi transport [26–29].

HCV also promotes recruitment of PI4K to replication sites [25••,30••]. HCV generates a membranous web with many similarities to the network of spherical vesicles and CM produced by DENV. PI4-P lipids are colocalised with HCV replicase protein NS5A, and replication is reduced following PI4KIIIβ knockdown or overexpression of the Sac1 phosphatase that removes phosphate from PI4-P. The hepatitis NS5A protein also binds PI4KIIIα and stimulates enzyme activity and knockdown of PI4KIIIα prevents formation of the membranous web associated with virus replication [30••,31•]. The NS5A protein is anchored to the cytoplasmic face of the membranous web and recruits NS5B and a series of cellular proteins that regulate membrane vesicle formation. These include vesicle-associated membrane protein-associated proteins [32] which bind SNAREs involved in ER to Golgi transport, and a Rab1-GAP protein, TBC1D20. NS4B can recruit Rab5 and Rab5 effectors EEA1, rabaptin 5 and Rab4 [31•] suggesting the web may fuse with endosomes [33].

Role of autophagy

Double-membraned vesicles are usually rare in cells, but DMVs are induced during autophagy. This makes it possible that autophagosomes may provide a source for DMVs associated with virus replication. A role for autophagosomes in supporting replication of the coronavirus mouse hepatitis virus (MHV) was first provided by studies where mouse embryonic stem cells lacking crucial autophagy protein Atg5 showed a 1000-fold reduction in MHV replication and reduced numbers of DMVs [34]. Less clear-cut results come from studies of primary fibroblasts or macrophages where loss of Atg5 has little impact on virus replication [35]. Vesicles labelled with autophagy marker LC3 are, however, produced during coronavirus infection suggesting that the virus activates autophagy. Autophagy may be activated by the nsp6 proteins of cornaviruses, or in the case of the arteriviruses, the equivalent nsp5–7 protein. The nsp6 (nsp5–7) proteins locate to the ER where they generate small vesicles enriched in phosphatidylinositol-3-phosphate and early autophagy marker Atg5 [36••]. These vesicles closely resemble cellular organelles called omegasomes that are formed from the ER during the initial stages of autophagy [37], and mature into autophagosomes labelled with autophagy marker Atg8/LC3. Autophagy is activated during cornavirus infection but this does not mean that all the DMVs generated in the cytoplasm are autophagosomes. Most of the DMVs are smaller than autophagosomes and may be formed from invaginations into the ER (Figure 1). Vesicles formed from the ER during coronavirus infection can also recruit a non-lipidated autophagy marker LC3 (LC3I) by a pathway, that is, paradoxically, independent of autophagy, and linked to the export of ER chaperones from the ER to endosomes [38••]. Autophagosomes may not therefore play a direct role in the formation of virus-induced DMVs, but may represent a defence against infection.

Picornaviruses activate autophagy in cell culture models [39] and in some cases inhibition of autophagy reduces replication while activation increases virus yields. The autophagosome marker LC3 colocalises with PV replicase proteins suggesting replication on autophagosomes. Translocation of LC3 to vesicles can be induced by expression of PV 2BC alone, but formation of DMVs resembling autophagosomes requires coexpression of 2BC with 3A [16,39]. As with the coronaviruses, the picornavirus DMVs are approximately one third the diameter of cellular autophagosomes making it difficult to determine if assembly of the replicase complex results in the formation of DMVs directly, or if the DMVs represent modified autophagosomes.

Aggresomes and pericentriolar factories formed by nucleocytoplasmic large DNA viruses

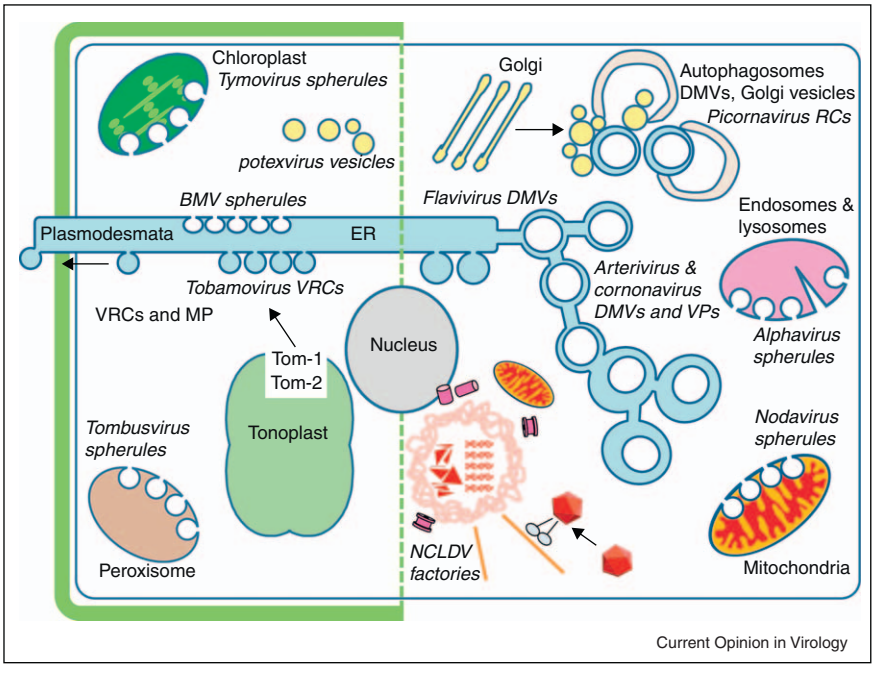
Virus assembly and replication can also occur in virus factories close to the microtubule organising centre (MTOC) [40]. These inclusions lack cellular membranes and resemble inclusions called aggresomes that form at the MTOC in response to protein aggregation. Aggresome inclusions such as Lewy and Mallory bodies are a pathological hallmark of protein misfolding diseases, and protect cells from the damage associated with protein aggregation. Aggresomes and factories share many features in common including recruitment of mitochondria, cellular chaperones and confinement within cages of rearranged vimentin filaments (reviewed in [40,42,43]). Many viruses are delivered to the MTOC after entering cells, and in common with protein aggregates, this involves recognition by the microtubule motor protein, dynein. It is possible that viruses may appear foreign or misfolded to cells and stimulate an aggresome response. For the NCLDV this may be beneficial and provide a site

for replication, for other viruses it may lead to confinement at the MTOC and degradation. Delivery of incoming African swine fever virus (ASFV) to the MTOC is important for the initiation of replication [41] and replication of both the ASFV and the iridovirus frog virus 3 appears to require rearrangement of vimentin [44,45]. Vimentin may provide a scaffold to prevent diffusion of viral components into the cytoplasm. DNA and RNA are spatially separated within vaccinia virus (VACV) and ASFV factories [46•,47] and individual factories appear to be distinct entities within the cell. Although virus factories recruit many host-cell proteins to facilitate their replication, factories also represent effector sites for antiviral activity as VACV and ASFV replication sites are targeted by stress granule components and Mx proteins respectively [48,49].

Conclusions

A number of clear similarities between the membrane rearrangements generated by the positive strand RNA viruses are beginning to emerge [1,2] and these are shared between animal and plant viruses. Spherules and DMVs differ morphologically but may be formed by similar mechanisms involving ordered assembly of replicase proteins on membrane-bound organelles leading to membrane invagination (Figure 1). DMVs may be modified spherules where close apposition of ER-derived membranes follows initial invagination into the ER lumen. For cornaviruses, and possibly picornaviruses, this may result in loss of the pore connecting the spherule to the cytosol and conversion of the spherule into a site for storage of viral RNA to suppress innate immune responses to double-stranded RNA. The site of spherule formation differs between viruses (Figure 2) and is determined by membrane targeting sequences in the nonstructural proteins that recruit the RdRp. This has been demonstrated by the work of Miller *et al.* who could retarget FHV replication complexes from the mitochondria to the ER in yeast [50]. Recent work has shown that recruitment of RdRp may also involve modification of membrane lipids by Arf1-dependent recruitment of PI4KIIIβ to membranes to generate PI4-P. This mechanism is shared between the enteroviruses of the picornavirus supergroup and the flavivirus HCV [25••,31•]. The membrane vesicles generated by picornaviruses are most heterogeneous and varied between subgroups and may be derived from membrane compartments that fragment during infection, rather than be formed for virus replication. It is uncertain which

Figure 2



Sites of replication generated by plant and animal viruses. The diagram is in two halves. The left represents a plant cell and is surrounded by a cell wall (green). Viral replication complexes (VRC) are generated by plant viruses at the ER, chloroplast and peroxisomes. Tobamovirus VRCs are initiated at the tonoplast. VRCs of many plant viruses can be transported within the cell along the ER and between cells using the plasmodesmata. This movement is directed by plant movement proteins (MP); see accompanying article by Jeanmarie Verchot for details. The right represents an animal cell. Alphavirus and nodaviruses form spherules at the endosomal–lysosomal system and mitochondria respectively. Coronavirus, arterivirus and flaviviruses form double membrane vesicles (DMVs) from the ER and coronaviruses and arteriviruses also form vesicle packets (VPs). The membranes used to generate sites of picornavirus replication are unclear but may involve the Golgi, ER and/or autophagosomes. Nucleo-cytoplasmic large DNA viruses (NCLDV) factories are formed after microtubule-mediated delivery of incoming viruses to the microtubule organising centre next to the nucleus. Thereafter they recruit host chaperones, mitochondria and intermediate filaments.

membranes house the virus replicase and replication may occur on the cytoplasmic face of the ER rather than in vesicles [5]. Parallels between plant and animal picornaviruses are therefore difficult to define. The large DNA viruses of animals such as poxviruses and other members of the NCLDV generate perinuclear inclusions called virus factories that assemble at the MTOC and are maintained by dynein microtubule motor proteins. Replication complexes generated in plants often move through cells onboard microtubule motor proteins (Figure 2), but perinuclear inclusions are not found for plant viruses. As pointed out in the accompanying review by Jeanmarie Verchot, plants lack a MTOC to concentrate motor cargoes, and replication sites are therefore dispersed throughout the cell.

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