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Highlights

1. The replication of coronaviruses, as in other positive-strand RNA viruses, is closely tied to the formation of membrane-bound replicative organelles (DMOs)
2. The proteins responsible for rearranging cellular membranes to form the organelles are conserved not just among the *Coronaviridae* family members, but across the order *Nidovirales*.
3. Here, we collect and interpret the recent experimental evidence about the role and importance of membrane-bound organelles in coronavirus replication.

New insights on the role of paired membrane structures in coronavirus replication

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Abstract

The replication of coronaviruses, as in other positive-strand RNA viruses, is closely tied to the formation of membrane-bound replicative organelles inside infected cells. The proteins responsible for rearranging cellular membranes to form the organelles are conserved not just among the *Coronaviridae* family members, but across the order *Nidovirales*. Taken together, these observations suggest that the coronavirus replicative organelle plays an important role in viral replication, perhaps facilitating the production or protection of viral RNA. However, the exact nature of this role, and the specific contexts under which it is important have not been fully elucidated. Here, we collect and interpret the recent experimental evidence about the role and importance of membrane-bound organelles in coronavirus replication.

Paired membranes associated with viral RNA

All positive-stranded RNA viruses (+RNA) that infect eukaryotes are believed to form membrane-bound replicative organelles, though this remains to be formally tested for several families of viruses (1). One of the most widespread membrane modifications caused by +RNA viruses results in the formation of paired membranes, i.e. two closely apposed lipid bilayers. A growing body of evidence, presented in Table 1 indicates that the paired membrane structures are induced by the expression of viral proteins – most typically by parts of the viral replicase. Table 1 lists the virus lineages for which there is evidence that some form of virus-induced paired-membrane structure is associated with viral replication. The wide distribution of membrane pairing in +RNA viruses suggests that this is an effective strategy for successfully producing new viruses, and that membrane pairing may somehow increase the competitive fitness of these viruses.

While we can speculate that +RNA viruses may gain a fitness advantage by replicating on the membranes of dedicated viral organelles, this has been difficult to test experimentally. However, there are several lines of experimental and genetic evidence that suggest that RNA synthesis is tied to the formation of replicative organelles. Viral RNA accumulates in the

coronavirus organelles, suggesting that the organelles may be a site of RNA synthesis (2-5). Furthermore, viral organelles are not formed when RNA synthesis is stopped (6, 7). While it is clear that RNA synthesis is linked with the organelles, it has proved difficult to directly test whether or to what extent the process of organelle formation is necessary for the process of RNA synthesis, because of the practical difficulty in separating the two processes in an experimental setting.

Structure of the organelles

Electron tomography studies have revealed that the replicative organelles of different nidoviruses are drawn from a repertoire of paired-membrane structures, including (paired) convoluted membranes, pouch-like double-membrane spherules, long paired membranes and double-membrane vesicles (2, 5, 8), though studies of the more recently discovered mesoniviruses and roniviruses remain poorly characterized (9, 10). A catalog of the virus-induced membrane structures that have been observed for each coronavirus is shown at right in Figure 1.

The common element in nidovirus-like membrane rearrangement is that the membranes are paired, usually maintaining a consistent-sized gap between the two membranes (reviewed here (11)). Since protein-induced membrane pairing appears to be a consistent feature associated with nidovirus replication, and in the absence of data carefully dissecting the relationship between the shape and function of these different paired membrane structures, it makes sense to refer to the resulting structures collectively as double-membrane organelles (DMO).

Despite a relative wealth of structural data, it has proved difficult to test hypotheses about the role of DMOs in viral replication and fitness directly because DMO formation is linked so closely to replication and expression of replicase proteins. Here, we will discuss the implications of two recent studies that address questions about the role of DMOs in nidovirus replication (12), and characterize the effects of a new DMO-blocking drug against a variety of coronaviruses (13).

Viral proteins involved in organelle formation

Further evidence of the probable importance of nidovirus replicative organelles for viral RNA replication comes in the form of genetic conservation. Nidoviruses, and most particularly coronaviruses, are highly genetically variable and contain several genus-specific or even species-specific genes (14). However, there are two clusters of genes that are conserved in all known nidoviruses (11, 14). The first is a highly conserved cluster of genes homologous to the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) nsp3-6 (Figure 1). Expression of the membrane-anchored proteins nsp3, nsp4 and nsp6 is sufficient to induce the formation of SARS-like paired-membrane replicative organelles (15). The second conserved gene cluster encodes the viral RNA polymerase and superfamily 1 helicase (16). The conservation of membrane-pairing genes in the context of an otherwise hypervariable group of viruses is a strong argument in favour of the importance of at least the membrane-pairing genes for RNA synthesis

The proteins that form SARS-CoV replicative organelles have several features in common with distant homologs found throughout the *Nidovirales*. We will refer to the transmembrane proteins homologous to SARS-CoV nsp3, nsp4 and nsp6 a as TM1, TM2, and TM3, respectively. The relative genomic positions and functions attributed to TM1-3 in nidoviruses are shown in Figure 1.

Of the three proteins involved in SARS-CoV replicative organelle formation, the least conserved is TM1, which has a multidomain architecture (17). Many nidovirus and all coronavirus TM1 proteins contain one or more ubiquitin-like domains which may help to anchor the viral RNA to the membranes where replication takes place (18). Potentially RNA-binding macrodomains (19-25), papain-like proteinases (26-28), other RNA binding domains (29) and a well conserved but poorly understood region known only as the Y domain (17) are also commonly but not ubiquitously found in nidovirus TM1 proteins. All putative TM1 proteins are predicted to contain one or more transmembrane domains, as shown in Figure 1. The C-terminal region of TM1, from the first

transmembrane region to the end of the Y domain induces membrane proliferation, which in some ways resembles an autophagy response (30).

TM2 and TM3 are recognizable because they contain four or more predicted transmembrane regions, and are encoded immediately before and after the viral main protease (M^{pro}). Bioinformatics generally predicts an even number of transmembrane spans in these proteins, which would be necessary to localize M^{pro} on the same side of the membrane as all of its predicted upstream and downstream cleavage sites. However there are additional hydrophobic regions that are strongly predicted to span the membrane, but which do not for several viruses, including most coronaviruses (31-33).

TM2 contains two potential conserved domains located between the first and second transmembrane domains in coronavirus, and after the final transmembrane domain in most nidoviruses. Mutations in the first non-hydrophobic domain of TM2, which is the largest part of the coronavirus replicase to localize on the luminal face of the membrane, have been demonstrated to disrupt RNA replication and may cause defects in membrane pairing (34). Deletion of the latter conserved domain of TM2, which has been structurally solved (35, 36), was surprisingly well tolerated (35, 37). TM2 localizes to membranes, but does not induce any recognizable change to intracellular membranes in the absence of other viral proteins (30). However, co-expression of TM2 with full-length TM1 results in extensive pairing of perinuclear membranes in both coronavirus (30) and arterivirus (38, 39). Additionally, it has recently been shown that co-expression of a fragment of MHV TM1 including the transmembrane region and the C-terminus with TM2 induced ER membrane zippering and curvature similar to the phenotype observed after SARS-CoV TM1 and TM2 co-expression (40). In that report TM1 and TM2 were demonstrated to interact via protein loops on the luminal face of the membrane.

The maze-like paired-membrane structures that resulted from coexpression of SARS-CoV TM1 and TM2 have not ever been reported in coronavirus-infected cells, suggesting that this should be interpreted as a conditional, or

perhaps partial phenotype, that is not observed when the full viral replicase polyprotein is expressed. This suggests that membrane pairing is caused by heterotypic interactions between TM1 and TM2 on opposing membranes, but that the final architecture of the paired membranes is dependent on additional viral proteins.

TM3 largely consists of transmembrane regions, without the hallmarks of amino acid conservation or predicted structural conservation that would be expected for an enzyme. Overexpression of TM3 alone disturbs intracellular membrane trafficking (41, 42), resulting in an accumulation of single-membrane vesicles around the microtubule organization complex (30). However, quantitative electron microscopy revealed that expression of TM2 with TM3 prevents the membrane disruption seen with TM3 expression alone (30). When SARS-CoV TM1, TM2 and TM3 are coexpressed, membrane-containing bodies which resembled authentic SARS-CoV replicative organelles were formed. However, in each of the cell sections where DMV-like membranes were observed, the membrane proliferation phenotype of TM1, the paired membrane phenotype of TM1+TM2 and the single membrane vesicle accumulation from TM3 were each visible, suggesting that these proteins do not always colocalize efficiently when expressed from plasmids in different parts of the cell instead of being expressed in the natural form as a polyprotein (BWN, personal communication). This suggests that while TM3 is not necessary for membrane pairing, TM3 may be necessary to induce the formation of the double-membrane vesicles (DMVs) that are characteristic of coronavirus replicative organelles.

Interactions among DMV-making proteins

The formation of large intracellular structures such as the maze-like TM1+TM2 bodies and DMV-like TM1+TM2+TM3 bodies suggests that nsp3, nsp4 and nsp6 may interact both homotypically and heterotypically. SARS-CoV nsp3-nsp3 interactions have been detected in cells by yeast two-hybridization (43) and GST pulldown (44), and in purified protein by perfluorooctanoic acid polyacrylamide gel electrophoresis (17). While SARS-CoV nsp4-nsp4 interactions were not found in yeast-two hybrid or mammalian

two-hybrid screens (43, 45) studies with another coronavirus did detect nsp4-nsp4 interactions by Venus reporter fluorescence (46). To date, homotypic interactions have not been demonstrated for nsp6 despite several attempts (43-45).

Heterotypic interactions between coronavirus TM1-3 proteins have been demonstrated biochemically: a TM1-TM2 interaction was detected by mammalian two-hybridization (43) and weakly detected by Venus reporter fluorescence (46). A TM2-3 interaction has been demonstrated by Venus reporter fluorescence (46), though it did not appear in other hybridization studies. A one-way interaction between the amino-terminal 192 amino acid domain of TM1 and TM3 detected by yeast two-hybridization (44) has also been reported. However, the apparent independence of TM1 and TM3 phenotypes after coexpression, coupled with the abrupt change in both phenotypes in the presence of TM2 suggests that interactions between these proteins may be largely mediated by TM2 (30).

Virus-host interactions

Molecular interactions between host and viral factors are observed in virtually every step of the viral life cycle. Viruses rely on and manipulate established cellular pathways to accommodate their needs during replication and to counteract host innate immune signalling. Replication of coronaviruses is no exception; while some host factors have been described in the context of viral RNA replication and transcription (47), few studies have looked closely at the complex interplay of host pathways in the establishment of virus-induced membrane-bound replication complexes.

To date, the precise origin of DMO membranes remains elusive. DMO membranes were initially suggested to derive from the early secretory pathway, although the absence of conventional ER, ERGIC and Golgi protein markers on viral replicative membranes argues against this hypothesis (48, 49). Since DMVs are reminiscent of the double-membranes of autophagosomes, several lines of controversial evidence hypothesized a diversion of Atg (autophagy-related) proteins and autophagosome function

during coronavirus replication, as it is the case for other +RNA viruses (50-54). The involvement of autophagy was recently investigated in the context of the avian CoV Infectious Bronchitis Virus (IBV) infections (41). The authors conclude that the presence of exogenous, individually expressed IBV nsp6, which localizes to the ER, induces the formation of autophagosomes in contrast to other IBV replicase proteins. Additionally, although autophagosomes induced by IBV nsp6 or IBV infection appeared smaller than conventional autophagosomes observed after starvation of cells, they were similar in size to DMVs (42). However, the data reported here do not appear to support the assumption that there is a functional link between IBV nsp6 and autophagosomes, and a role of the autophagy in the formation of IBV replicative structures can hereby not be demonstrated. Moreover, neither induction nor inhibition of autophagy seems to affect IBV replication (55).

New evidence concerning the source of membranes for CoV-induced DMOs was proposed, in which Mouse Hepatitis Virus (MHV) probably co-opts a cellular degradation pathway of ER-associated degradation (ERAD) regulators, known as the ERAD tuning pathway (56). The ERAD pathway is responsible for the turnover of folding-defective polypeptides in the ER and is modulated by stress-inducible positive regulators of ERAD-mediated protein disposal such as EDEM1 (ER degradation-enhancing alpha mannosidase-like 1) and OS-9 (osteosarcoma amplified 9). The latter assist in transporting misfolded proteins into the cytosol for subsequent degradation by the proteasomal system. Under physiological conditions, however, low concentrations of EDEM1 and OS-9 are maintained in the ER lumen in order to avoid premature degradation of proteins that are undergoing folding programs (57). In this case, EDEM1 and OS-9 are selectively confined by interacting with the transmembrane-anchored cargo receptor SEL1L (suppressor of lin-12-like protein 1) and later released from the ER lumen in small short-lived vesicles, called EDEMosomes, which rapidly fuse with the endolysosomal compartments (58). This steady-state disposal of EDEM1 and OS-9 is known as ERAD tuning pathway. While not relying on the coat protein complex II (COPII) or Atg7, it critically depends on the non-lipidated form of LC3 (LC3-I), which is recruited to EDEMosomes. However, the specific

autophagosomal marker GFP-LC3 does not associate with EDEMosomes, which are therefore distinct structures (59).

The coronavirus MHV is hypothesized to divert the ERAD tuning machinery for the generation of DMOs. Similarly to EDEMosomes, colocalization of EDEM1, OS-9, SEL1L, LC3-I and double-stranded (ds) RNA is observed upon MHV infection. Moreover, replication of MHV, which does not require an intact autophagy pathway, is impaired upon knockdown of LC3 or SEL1L (58). DMVs furthermore lack conventional ER markers and do not associate with GFP-LC3 (56). Altogether, the evidence from this study strongly suggests that MHV exploits the ERAD-tuning machinery to establish its replicative structures.

In order to learn whether this mechanism might be common to other nidoviruses, other viruses that use a similar replication strategy to MHV were examined. One of these, the arterivirus Equine Arteritis Virus (EAV) has been shown to require the same subset of ERAD tuning factors as MHV to ensure replication (60). Recently, investigations of the even more distantly-related Japanese Encephalitis Virus (JEV), which belongs to the *Flaviviridae* family, revealed that it may usurp the same components of the ERAD-tuning pathway as well (61). Consistent with this hypothesis, both viruses were shown to replicate independently of a functional autophagy pathway. The non-lipidated LC3 marker protein, which is essential for the replication of EAV and JEV, associated with their replication complexes together with EDEM1 whereas GFP-LC3 did not label these structures. These observations parallel the ones seen for MHV but raise further questions whether this feature is even more widespread amongst +RNA viruses.

Despite the resemblance of MHV, EAV and JEV in the requirement of host factors for efficient replication, diversion of the ERAD tuning pathway cannot be considered as a generic way of inducing replicative membranes by these viral families. Probable variations within families have to be kept in mind as exemplified by the comparison of DMOs from two different coronavirus genus members. Indeed, IBV's recently described spherules derived from paired ER

membranes significantly differ from the DMO structures observed upon alpha- and beta-coronaviruses infections (8, 62) and their generation might require a different set of factors. Furthermore, the morphology of DMOs induced by flaviviruses such as Hepatitis C Virus, Dengue virus or West Nile Virus is highly heterogeneous and the identification of a common, conserved membrane diversion strategy seems unlikely (63). However, it is possible that the diversion of one pathway could lead to the generation of the different arrangements of membrane that we collectively refer to as the DMO.

Importantly, it has been shown that, in contrast to what is observed during EAV infection, endogenous LC3 does not colocalize with membrane puncta induced by expression of EAV nsp2 and nsp3, and the membrane modifications induced by the latter are not affected by LC3 knockdown (60). Similarly, LC3 and EDEM1 were not recruited to rearranged membranes induced by co-expression of MHV TM1 and TM2 (40). While this still has to be proven in the context of CoV TM1, TM2 and TM3 expression, it raises the questions whether LC3 participates to the biological function of DMVs rather than its generation. A novel hypothesis has been recently suggested for Poliovirus, according to which the virus might not only co-opt a host pathway, but also divert the functional network of individual proteins (64). Host factors could therefore have a proviral function during infection, distinct from the function for which they have been initially described. Accordingly, this is reminiscent with novel functions attributed to LC3 during cellular homeostasis, cytoprotection against invading pathogens or during Chlamydia trachomatis' intracellular life cycle (65).

Natural variation in DMV structure

The DMOs of the model coronavirus MHV take the form of perinuclear DMVs which appear either singly, or grouped around and interconnected with a region of paired, convoluted membrane (CM;). A recent study examined DMV formation by *wild-type* MHV-inf-1 (*wt*) and five *temperature-sensitive* (*ts*) MHV mutants, each of which differed from *wt* by a single amino acid substitution. The panel of *ts* viruses chosen contained mutations in an interdomain linker of nsp3 (TM1), M^{pro}, the viral RNA polymerase, cap N-methyltransferase and

cap O-methyltransferase, respectively (6, 12, 66). With the exception of the polymerase mutant, which was attenuated tenfold, these viruses produced the same amount of infectious progeny as *wt* (12).

All of the mutants produced significantly smaller DMVs than *wt* virus, varying from almost *wt* size to 17% smaller (Table 2). In two of the mutants that produced normal amounts of infectious progeny, not only were the DMVs smaller, there were only about half as many DMVs per visibly infected cell compared to *wt* (Table 2). Examination of the size and number of intracellular virus particles from the same samples did not reveal corresponding changes, suggesting that the observed DMV phenotypes were not an artifact of sample preparation. The number of CMs remained in a constant ratio to the number of DMVs present, suggesting that the mutations affected production of the entire DMO.

Induced variation in DMVs

The DMOs of human coronavirus 229E (HCoV-229E) include DMVs similar to those observed after MHV infection (13). In testing a new antiviral called K22, it was observed that infectivity, viral RNA, and DMV formation were all blocked by treatment with 4 μM K22. A time of addition study revealed that K22 did not block viral entry, and had the greatest antiviral effects after virus entry during the first few hours of infection, leading to the interpretation that K22 inhibits a cellular or viral component involved in a post-entry, early stage of viral replication.

After serial passage of the virus in the presence of K22, resistant mutants were selected. Surprisingly, two independently isolated resistance mutations mapped to opposite ends of transmembrane helices in TM3 (nsp6) at positions H121L and M159V. The resistant viruses released similar amounts of new progeny compared to *wt*, but produced only about half as many DMVs per infected cell. In addition, the DMVs induced by resistance mutants appeared structurally impaired. Similarly to MHV nsp4 mutants) K22 escape mutants induced DMV with partially collapsed inner membranes, even when K22 was not present. Moreover, the specific infectivity of those newly

364 released virions was about ten-fold lower for TM3 mutants than for wt. This
365 suggested that the mutations in nsp6 conferred resistance to K22 at a cost of
366 impairing an early intracellular step in the establishment of infection.
367
368 **Fitness consequences**
369 From these experiments it was clear that HCoV-229E viruses with K22
370 resistance mutations in TM3 incurred a steep fitness cost, in the form of
371 decreased specific infectivity. There were also indications of a similar
372 decrease in efficiency in the MHV nsp3 mutant Br*ts*31, which produced
373 significantly more intracellular RNA than *wt*, but without a corresponding
374 increase in infectious progeny.
375
376 To find out if the MHV mutants also incurred a fitness cost associated with
377 producing smaller and fewer DMVs, competitive fitness assays were carried
378 out. To do this, equal infectivities of two viruses were added to the same flask
379 at a temperature where both viruses could grow normally. After 24h in direct
380 competition, the amount of each virus was quantified either by sequencing to
381 look for the *ts* mutation, or by phenotypically screening for *ts* and non-*ts* virus.
382 None of the MHV mutants tested was significantly less fit than *wt* in
383 continuous or primary fibroblasts, and two mutants were significantly fitter
384 than *wt* under the assay conditions. One of the viruses with increased fitness
385 compared to wild-type was the N-methyltransferase mutant Br*ts*105, which
386 produced only half as many DMVs as *wt*. These results demonstrated that at
387 least under these experimental conditions, producing larger or more
388 numerous DMVs did not confer a corresponding fitness advantage.
389
390 **Implications for coronavirus replication**
391 When interpreting these findings, it is important to consider that none of the
392 HCoV-229E or MHV mutants tested to date has been able to replicate entirely
393 without DMOs. And while some of these tests were carried out in primary
394 cells, work in animal models was not possible because of the lack of a small
395 animal model for HCoV-229E, and because the mutations restricted the
396 growth of MHV mutants at physiological temperatures. These two studies do
397 not disprove the fundamental connectedness between coronavirus RNA

replication and DMO formation, but together, they reveal an unexpected plasticity in the size and number of DMVs that are needed to carry out wild-type amounts of RNA synthesis.

For these reasons, along with the observation that RNA replication is detectable before the first appearance of organelles (67), we favour an interpretation in which the organelles are a late manifestation of accumulated viral proteins resulting from abundant RNA expression. In this interpretation, DMOs could still play an obligate role in viral replication under specific conditions or in specific cell types, but the primary role for DMOs would be to increase the efficiency of either RNA production, delivery of newly synthesized RNA to sites where it could be translated or packaged, and/or shielding abundantly synthesized viral RNA from host cell innate immune sensing pathways. These studies also suggest that at least half of the DMVs present in infected cells may be in excess of what is strictly needed to sustain normal levels of RNA synthesis, given that both MHV and HCoV-229E mutants replicated normally despite producing only half the normal complement of DMVs.

Before these studies, very little was known about the potential for natural and induced variation in intracellular membrane rearrangement. The viruses described in these studies all produced normal amounts of progeny virus particles, and were all selected for analysis for reasons unrelated to DMO formation. These represent only a handful of the available nidovirus replicase mutants that have been published. From this work we can hypothesize that other MHV ts mutants, or K22-resistant HCoV-229E mutants with replicase defects would probably make either smaller or fewer DMVs, and a larger collection of such mutants will like be highly informative to further our understanding on the pivotal role(s) of DMOs in the coronavirus life cycle. Hopefully the unique insight provided by these results, together with the relative ease of analysis will make quantitative electron microscopy a routine part of the characterization of new virus mutants. In addition, the accumulated knowledge on the nature of coronavirus DMOs and the possibility to experimentally interfere with DMO formation by using small compound

inhibitors, such as K22, will allow us to dissect similarities and differences between viral DMOs and related cellular organelles.

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Table 1. Evidence paired membrane structures in +RNA virus infection.

Order	Family	Host[a]	Origin[b]	Type[c]	Proteins[d]	References
Nidovirales	*Arteriviridae*	A	ER	V,Z	nsp2, 3	(38, 39, 44-46)
	Coronaviridae	A	ER	V,Z,S,C	nsp3+4+6	(2, 8, 30)
	Mesoniviridae	A	ER	V?	*nr*[e]	(71)
Picornavirales	*Picornaviridae*	A	ER	V	2BC, 3A	(72-75)
	Secoviridae	P	ER	V?	*nr*	(76)
Tymovirales	*Betaflexiviridae*	P	ER	V	*nr*	(77, 78)
	Tymoviridae	P	Cp, Mt	V	*nr*	(79)
Unclassified	*Astroviridae*	A	ER	V	nsp1a	(80, 81)
	Bromoviridae	P	ER	Z,S	1a+2a^{pol}	(82-84)
	Closteroviridae	P	*nr*	V	*nr*	(85)
	Flaviviridae	A	ER	V,S,C	NS4A+4B	(86-91)
	Nodaviridae	A	Mito	S	pA+RNA	(92, 93)
	Togaviridae	A	Ly, ER	V,S?	P123	(94-96)
	Tombusviridae	P	Px	S	*nr*	(97)

[a]Animals (A) or Plants (P)
[b]Membranes from the endoplasmic reticulum (ER), chloroplast (Cp), mitochondria (Mt), lysosome (Ly) or peroxisome (Px)
[c]Paired membranes in the form of double-membrane vesicles (V), zippered ER (Z), open-necked spherules (S), or convoluted membranes (C)
[d]Proteins implicated in membrane rearrangements
[e]Not reported (*nr*)

Table 2. Differences in size and prevalence of MHV DMVs and intracellular virions (IV).

				Prevalence				*Size (nm)*			
Virus	**Conditions**	***ts***	**Cells**	**DMV**	**P value[a]**	**IV**	**P value**	**DMV**	**P value[b]**	**IV**	**P value**
Wild-type	*DBT 33ºC 5.5 hpi*	--	*n=323*	6%	--	7%	--	228 ± 45	--	69 ± 8	--
Br*ts*31		nsp3	*n=753*	2%	8×10^{-4}	7%	ns[c]	195 ± 38	2×10^{-6}	69 ± 9	ns
Wild-type	*17Cl-1 33ºC 10 hpi*	--	*n=161*	40%	--	29%	--	228±36	--	68±10	--
Br*ts*31		nsp3	*n=238*	24%	4×10^{-4}	25%	ns	208±34	5×10^{-19}	68±10	ns
Alb*ts*16		nsp5	*n=120*	37%	ns	19%	ns	189±33	8×10^{-66}	70±8	ns
Wü*ts*18		nsp16	*n=140*	36%	ns	20%	ns	211±35	2×10^{-15}	67±12	ns
Br*ts*105		nsp14	*n=230*	22%	1×10^{-4}	32%	ns	220±36	2×10^{-4}	69±10	ns
Alb*ts*22[d]		nsp12	*n=320*	13%	1×10^{-5}	9%	1×10^{-5}	204±43	2×10^{-13}	68±11	ns

[a]Calculated by two-tailed Fisher's exact test
[b]Calculated by two-tailed Mann-Whitney test
[c]Not significantly different from the appropriate wild-type control
[d]Attenuated growth at 33°C compared to wild-type

Figure Legend

Figure 1. Conservation and functional organization of the carboxyl-terminal region of nidovirus polyprotein 1a. Domains that are homologous at the amino acid level are shown at left in solid colors. More distantly related potential homologs identified by genome position and comparison of predicted secondary structures are marked with stripes. Positions of transmembrane regions (black bars) and hydrophobic non-transmembrane regions (striped bars) were predicted by TMHMM 2.0 (98) and amended to reflect known topologies (31-33) wherever possible. Clusters of conserved cysteine and histidine residues that may bind metal ions are marked with white ovals. A jagged line denotes the uncertain position of the amino terminus. Regions that induce membrane pairing, proliferation or vesiculation in betacoronavirus SARS-CoV and arterivirus EAV are shown above and below the domain annotation, respectively, and all annotations come from the references listed for Table 1. Double-membrane organelles observed (x) or uncertainly observed (?) in infected cells are marked at right. Virus names are abbreviated as follows: white bream virus (WBV), fathead minnow nidovirus (FHMNV), equine arteritis virus (EAV), lactate dehydrogenase elevating virus (LDV), porcine reproductive and respiratory syndrome virus (PRRSV), simian hemorrhagic fever virus (SHFV) and wobbly possum nidovirus (WPNV).

References

1. ***Neuman BW, Angelini MM, Buchmeier MJ.*** *2014. Does form meet function in the coronavirus replicative organelle? Trends Microbiol.*
2. ***Knoops K, Kikkert M, Worm SH, Zevenhoven-Dobbe JC, van der Meer Y, Koster AJ, Mommaas AM, Snijder EJ.*** *2008. SARS-coronavirus replication is supported by a reticulovesicular network of modified endoplasmic reticulum. PLoS biology* ***6:****e226.*
3. ***Gosert R, Kanjanahaluethai A, Egger D, Bienz K, Baker SC.*** *2002. RNA replication of mouse hepatitis virus takes place at double-membrane vesicles. Journal of virology* ***76:****3697-3708.*
4. ***Hagemeijer MC, Vonk AM, Monastyrska I, Rottier PJ, de Haan CA.*** *2012. Visualizing coronavirus RNA synthesis in time by using click chemistry. Journal of virology* ***86:****5808-5816.*
5. ***Knoops K, Barcena M, Limpens RW, Koster AJ, Mommaas AM, Snijder EJ.*** *2012. Ultrastructural characterization of arterivirus replication structures: reshaping the endoplasmic reticulum to accommodate viral RNA synthesis. Journal of virology* ***86:****2474-2487.*
6. ***Stokes HL, Baliji S, Hui CG, Sawicki SG, Baker SC, Siddell SG.*** *2010. A new cistron in the murine hepatitis virus replicase gene. Journal of virology* ***84:****10148-10158.*
7. ***Verheije MH, Raaben M, Mari M, Te Lintelo EG, Reggiori F, van Kuppeveld FJ, Rottier PJ, de Haan CA.*** *2008. Mouse hepatitis coronavirus RNA replication depends on GBF1-mediated ARF1 activation. PLoS pathogens* ***4:****e1000088.*
8. ***Maier HJ, Hawes PC, Cottam EM, Mantell J, Verkade P, Monaghan P, Wileman T, Britton P.*** *2013. Infectious bronchitis virus generates spherules from zippered endoplasmic reticulum membranes. mBio* ***4:****e00801-00813.*
9. ***Zirkel F, Kurth A, Quan PL, Briese T, Ellerbrok H, Pauli G, Leendertz FH, Lipkin WI, Ziebuhr J, Drosten C, Junglen S.*** *2011. An insect nidovirus emerging from a primary tropical rainforest. MBio* ***2:****e00077-00011.*
10. ***Spann KM, Vickers JE, Lester RJG.*** *1995. Lymphoid organ virus of Penaeus monodon from Australia. Diseases of aquatic organisms* ***23:****127-134.*

11. ***Angelini MM, Neuman BW, Buchmeier MJ.*** *2014. Untangling membrane rearrangement in the nidovirales. DNA and cell biology* ***33:****122-127.*

12. ***Al-Mulla HM, Turrell L, Smith NM, Payne L, Baliji S, Zust R, Thiel V, Baker SC, Siddell SG, Neuman BW.*** *2014. Competitive fitness in coronaviruses is not correlated with size or number of double-membrane vesicles under reduced-temperature growth conditions. MBio* ***5:****e01107-01113.*

13. ***Lundin A, Dijkman R, Bergstrom T, Kann N, Adamiak B, Hannoun C, Kindler E, Jonsdottir HR, Muth D, Kint J, Forlenza M, Muller MA, Drosten C, Thiel V, Trybala E.*** *2014. Targeting membrane-bound viral RNA synthesis reveals potent inhibition of diverse coronaviruses including the middle East respiratory syndrome virus. PLoS pathogens* ***10:****e1004166.*

14. ***Lauber C, Goeman JJ, Parquet Mdel C, Nga PT, Snijder EJ, Morita K, Gorbalenya AE.*** *2013. The footprint of genome architecture in the largest genome expansion in RNA viruses. PLoS pathogens* ***9:****e1003500.*

15. ***Angelini MM, Akhlaghpour M, Neuman BW, Buchmeier MJ.*** *2013. Severe acute respiratory syndrome coronavirus nonstructural proteins 3, 4, and 6 induce double-membrane vesicles. MBio* ***4****.*

16. ***Deng Z, Lehmann KC, Li X, Feng C, Wang G, Zhang Q, Qi X, Yu L, Zhang X, Feng W, Wu W, Gong P, Tao Y, Posthuma CC, Snijder EJ, Gorbalenya AE, Chen Z.*** *2014. Structural basis for the regulatory function of a complex zinc-binding domain in a replicative arterivirus helicase resembling a nonsense-mediated mRNA decay helicase. Nucleic acids research* ***42:****3464-3477.*

17. ***Neuman BW, Joseph JS, Saikatendu KS, Serrano P, Chatterjee A, Johnson MA, Liao L, Klaus JP, Yates JR, 3rd, Wuthrich K, Stevens RC, Buchmeier MJ, Kuhn P.*** *2008. Proteomics analysis unravels the functional repertoire of coronavirus nonstructural protein 3. Journal of virology* ***82:****5279-5294.*

18. ***Hurst KR, Koetzner CA, Masters PS.*** *2013. Characterization of a critical interaction between the coronavirus nucleocapsid protein and nonstructural protein 3 of the viral replicase-transcriptase complex. Journal of virology* ***87:****9159-9172.*

19. ***Serrano P, Johnson MA, Almeida MS, Horst R, Herrmann T, Joseph JS, Neuman BW, Subramanian V, Saikatendu KS, Buchmeier MJ, Stevens RC, Kuhn P, Wuthrich K.*** *2007. Nuclear magnetic resonance structure of the N-terminal domain of nonstructural protein 3 from the severe acute respiratory syndrome coronavirus. Journal of virology* ***81:****12049-12060.*

20. ***Xu Y, Cong L, Chen C, Wei L, Zhao Q, Xu X, Ma Y, Bartlam M, Rao Z.*** *2009. Crystal structures of two coronavirus ADP-ribose-1''-monophosphatases and their complexes with ADP-Ribose: a systematic structural analysis of the viral ADRP domain. Journal of virology* ***83:****1083-1092.*

21. ***Wojdyla JA, Manolaridis I, Snijder EJ, Gorbalenya AE, Coutard B, Piotrowski Y, Hilgenfeld R, Tucker PA.*** *2009. Structure of the X (ADRP) domain of nsp3 from feline coronavirus. Acta crystallographica. Section D, Biological crystallography* ***65:****1292-1300.*

22. ***Saikatendu KS, Joseph JS, Subramanian V, Clayton T, Griffith M, Moy K, Velasquez J, Neuman BW, Buchmeier MJ, Stevens RC, Kuhn P.*** *2005. Structural basis of severe acute respiratory syndrome coronavirus ADP-ribose-1''-phosphate dephosphorylation by a conserved domain of nsP3. Structure* ***13:****1665-1675.*

23. ***Tan J, Vonrhein C, Smart OS, Bricogne G, Bollati M, Kusov Y, Hansen G, Mesters JR, Schmidt CL, Hilgenfeld R.*** *2009. The SARS-unique domain (SUD) of SARS coronavirus contains two macrodomains that bind G-quadruplexes. PLoS pathogens* ***5:****e1000428.*

24. ***Chatterjee A, Johnson MA, Serrano P, Pedrini B, Joseph JS, Neuman BW, Saikatendu K, Buchmeier MJ, Kuhn P, Wuthrich K.*** *2009. Nuclear magnetic resonance structure shows that the severe acute respiratory syndrome coronavirus-unique domain contains a macrodomain fold. Journal of virology* ***83:****1823-1836.*

25. ***Johnson MA, Chatterjee A, Neuman BW, Wuthrich K.*** *2010. SARS coronavirus unique domain: three-domain molecular architecture in solution and RNA binding. Journal of molecular biology* ***400:****724-742.*

26. ***Ratia K, Saikatendu KS, Santarsiero BD, Barretto N, Baker SC, Stevens RC, Mesecar AD.*** *2006. Severe acute respiratory syndrome coronavirus papain-like protease: structure of a viral deubiquitinating enzyme.*

Proceedings of the National Academy of Sciences of the United States of America ***103:****5717-5722.*

27. ***Wojdyla JA, Manolaridis I, van Kasteren PB, Kikkert M, Snijder EJ, Gorbalenya AE, Tucker PA.*** *2010. Papain-like protease 1 from transmissible gastroenteritis virus: crystal structure and enzymatic activity toward viral and cellular substrates. Journal of virology* ***84:****10063-10073.*

28. ***van Kasteren PB, Bailey-Elkin BA, James TW, Ninaber DK, Beugeling C, Khajehpour M, Snijder EJ, Mark BL, Kikkert M.*** *2013. Deubiquitinase function of arterivirus papain-like protease 2 suppresses the innate immune response in infected host cells. Proceedings of the National Academy of Sciences of the United States of America* ***110:****E838-847.*

29. ***Serrano P, Johnson MA, Chatterjee A, Neuman BW, Joseph JS, Buchmeier MJ, Kuhn P, Wuthrich K.*** *2009. Nuclear magnetic resonance structure of the nucleic acid-binding domain of severe acute respiratory syndrome coronavirus nonstructural protein 3. Journal of virology* ***83:****12998-13008.*

30. ***Angelini MM, Akhlaghpour M, Neuman BW, Buchmeier MJ.*** *2013. Severe acute respiratory syndrome coronavirus nonstructural proteins 3, 4, and 6 induce double-membrane vesicles. MBio* ***4:****e00524-00513.*

31. ***Kanjanahaluethai A, Chen Z, Jukneliene D, Baker SC.*** *2007. Membrane topology of murine coronavirus replicase nonstructural protein 3. Virology* ***361:****391-401.*

32. ***Oostra M, Hagemeijer MC, van Gent M, Bekker CP, te Lintelo EG, Rottier PJ, de Haan CA.*** *2008. Topology and membrane anchoring of the coronavirus replication complex: not all hydrophobic domains of nsp3 and nsp6 are membrane spanning. Journal of virology* ***82:****12392-12405.*

33. ***Oostra M, te Lintelo EG, Deijs M, Verheije MH, Rottier PJ, de Haan CA.*** *2007. Localization and membrane topology of coronavirus nonstructural protein 4: involvement of the early secretory pathway in replication. Journal of virology* ***81:****12323-12336.*

34. ***Gadlage MJ, Sparks JS, Beachboard DC, Cox RG, Doyle JD, Stobart CC, Denison MR.*** *2010. Murine hepatitis virus nonstructural protein 4 regulates*

virus-induced membrane modifications and replication complex function. Journal of virology ***84:****280-290.*

35. ***Manolaridis I, Wojdyla JA, Panjikar S, Snijder EJ, Gorbalenya AE, Berglind H, Nordlund P, Coutard B, Tucker PA.*** *2009. Structure of the C-terminal domain of nsp4 from feline coronavirus. Acta crystallographica. Section D, Biological crystallography* ***65:****839-846.*

36. ***Xu X, Lou Z, Ma Y, Chen X, Yang Z, Tong X, Zhao Q, Xu Y, Deng H, Bartlam M, Rao Z.*** *2009. Crystal structure of the C-terminal cytoplasmic domain of non-structural protein 4 from mouse hepatitis virus A59. PloS one* ***4:****e6217.*

37. ***Sparks JS, Lu X, Denison MR.*** *2007. Genetic analysis of Murine hepatitis virus nsp4 in virus replication. Journal of virology* ***81:****12554-12563.*

38. ***Snijder EJ, van Tol H, Roos N, Pedersen KW.*** *2001. Non-structural proteins 2 and 3 interact to modify host cell membranes during the formation of the arterivirus replication complex. The Journal of general virology* ***82:****985-994.*

39. ***Posthuma CC, Pedersen KW, Lu Z, Joosten RG, Roos N, Zevenhoven-Dobbe JC, Snijder EJ.*** *2008. Formation of the arterivirus replication/transcription complex: a key role for nonstructural protein 3 in the remodeling of intracellular membranes. Journal of virology* ***82:****4480-4491.*

40. ***Hagemeijer MC, Monastyrska I, Griffith J, van der Sluijs P, Voortman J, van Bergen en Henegouwen PM, Vonk AM, Rottier PJ, Reggiori F, de Haan CA.*** *2014. Membrane rearrangements mediated by coronavirus nonstructural proteins 3 and 4. Virology* ***458-459:****125-135.*

41. ***Cottam EM, Maier HJ, Manifava M, Vaux LC, Chandra-Schoenfelder P, Gerner W, Britton P, Ktistakis NT, Wileman T.*** *2011. Coronavirus nsp6 proteins generate autophagosomes from the endoplasmic reticulum via an omegasome intermediate. Autophagy* ***7:****1335-1347.*

42. ***Cottam EM, Whelband MC, Wileman T.*** *2014. Coronavirus NSP6 restricts autophagosome expansion. Autophagy* ***10:****1426-1441.*

43. ***Pan J, Peng X, Gao Y, Li Z, Lu X, Chen Y, Ishaq M, Liu D, Dediego ML, Enjuanes L, Guo D.*** *2008. Genome-wide analysis of protein-protein*

interactions and involvement of viral proteins in SARS-CoV replication. PloS one ***3:****e3299.*

44. ***Imbert I, Snijder EJ, Dimitrova M, Guillemot JC, Lecine P, Canard B.*** *2008. The SARS-Coronavirus PLnc domain of nsp3 as a replication/transcription scaffolding protein. Virus research* ***133:****136-148.*

45. ***von Brunn A, Teepe C, Simpson JC, Pepperkok R, Friedel CC, Zimmer R, Roberts R, Baric R, Haas J.*** *2007. Analysis of intraviral protein-protein interactions of the SARS coronavirus ORFeome. PloS one* ***2:****e459.*

46. ***Hagemeijer MC, Ulasli M, Vonk AM, Reggiori F, Rottier PJ, de Haan CA.*** *2011. Mobility and interactions of coronavirus nonstructural protein 4. Journal of virology* ***85:****4572-4577.*

47. ***Zhong Y, Tan YW, Liu DX.*** *2012. Recent progress in studies of arterivirus- and coronavirus-host interactions. Viruses* ***4:****980-1010.*

48. ***Verheije M, Raaben M, Mari M, Te Lintelo E, Reggiori F, van Kuppeveld F, Rottier P, de Haan C.*** *2008. Mouse hepatitis coronavirus RNA replication depends on GBF1-mediated ARF1 activation. PLoS pathogens* ***4****.*

49. ***Knoops K, Swett-Tapia C, van den Worm S, Te Velthuis A, Koster A, Mommaas A, Snijder E, Kikkert M.*** *2010. Integrity of the early secretory pathway promotes, but is not required for, severe acute respiratory syndrome coronavirus RNA synthesis and virus-induced remodeling of endoplasmic reticulum membranes. Journal of virology* ***84:****833-846.*

50. ***Prentice E, Jerome W, Yoshimori T, Mizushima N, Denison M.*** *2004. Coronavirus replication complex formation utilizes components of cellular autophagy. The Journal of biological chemistry* ***279:****10136-10141.*

51. ***Snijder E, van der Meer Y, Zevenhoven-Dobbe J, Onderwater J, van der Meulen J, Koerten H, Mommaas A.*** *2006. Ultrastructure and origin of membrane vesicles associated with the severe acute respiratory syndrome coronavirus replication complex. Journal of virology* ***80:****5927-5940.*

52. ***Zhao Z, Thackray L, Miller B, Lynn T, Becker M, Ward E, Mizushima N, Denison M, Virgin H.*** *2007. Coronavirus replication does not require the autophagy gene ATG5. Autophagy* ***3:****581-585.*

53. ***Maier HJ, Britton P.*** *2012. Involvement of autophagy in coronavirus replication. Viruses* ***4:****3440-3451.*

692 54. ***Richards AL, Jackson WT.*** *2013. How positive-strand RNA viruses benefit*
693 *from autophagosome maturation. J Virol* ***87:****9966-9972.*
694 55. ***Maier HJ, Cottam EM, Stevenson-Leggett P, Wilkinson JA, Harte CJ,***
695 ***Wileman T, Britton P.*** *2013. Visualizing the autophagy pathway in avian*
696 *cells and its application to studying infectious bronchitis virus. Autophagy*
697 ***9:****496-509.*
698 56. ***Reggiori F, Monastyrska I, Verheije MH, Cali T, Ulasli M, Bianchi S,***
699 ***Bernasconi R, de Haan CA, Molinari M.*** *2010. Coronaviruses Hijack the*
700 *LC3-I-positive EDEMosomes, ER-derived vesicles exporting short-lived ERAD*
701 *regulators, for replication. Cell host & microbe* ***7:****500-508.*
702 57. ***Calì T, Galli C, Olivari S, Molinari M.*** *2008. Segregation and rapid*
703 *turnover of EDEM1 by an autophagy-like mechanism modulates standard*
704 *ERAD and folding activities. Biochemical and biophysical research*
705 *communications* ***371:****405-410.*
706 58. ***Bernasconi R, Galli C, Noack J, Bianchi S, de Haan CA, Reggiori F,***
707 ***Molinari M.*** *2012. Role of the SEL1L:LC3-I complex as an ERAD tuning*
708 *receptor in the mammalian ER. Mol Cell* ***46:****809-819.*
709 59. ***Noack J, Bernasconi R, Molinari M.*** *2014. How viruses hijack the ERAD*
710 *tuning machinery. J Virol.*
711 60. ***Monastyrska I, Ulasli M, Rottier PJ, Guan JL, Reggiori F, de Haan CA.***
712 *2013. An autophagy-independent role for LC3 in equine arteritis virus*
713 *replication. Autophagy* ***9:****164-174.*
714 61. ***Sharma M, Bhattacharyya S, Nain M, Kaur M, Sood V, Gupta V, Khasa R,***
715 ***Abdin M, Vrati S, Kalia M.*** *2014. Japanese Encephalitis Virus Replication is*
716 *Negatively Regulated by Autophagy and Occurs on LC3-I- and EDEM1-*
717 *containing Membranes. Autophagy* ***10:****54-53.*
718 62. ***Neuman BW.*** *2013. How the double spherules of infectious bronchitis virus*
719 *impact our understanding of RNA virus replicative organelles. mBio*
720 ***4:****e00987-00913.*
721 63. ***Romero-Brey I, Bartenschlager R.*** *2014. Membranous Replication*
722 *Factories Induced by Plus-Strand RNA Viruses. Viruses* ***6:****2826-2857.*
723 64. ***Belov GA, Sztul E.*** *2014. Rewiring of Cellular Membrane Homeostasis by*
724 *Picornaviruses. J Virol* ***88:****9478-9489.*

65. ***Bestebroer J, V'Kovski P, Mauthe M, Reggiori F.*** *2013. Hidden behind autophagy: the unconventional roles of ATG proteins. Traffic* ***14:****1029-1041.*

66. ***Sawicki SG, Sawicki DL, Younker D, Meyer Y, Thiel V, Stokes H, Siddell SG.*** *2005. Functional and genetic analysis of coronavirus replicase-transcriptase proteins. PLoS pathogens* ***1:****e39.*

67. ***Ulasli M, Verheije MH, de Haan CA, Reggiori F.*** *2010. Qualitative and quantitative ultrastructural analysis of the membrane rearrangements induced by coronavirus. Cellular microbiology* ***12:****844-861.*

68. ***Wood O, Tauraso N, Liebhaber H.*** *1970. Electron microscopic study of tissue cultures infected with simian haemorrhagic fever virus. The Journal of general virology* ***7:****129-136.*

69. ***Pedersen KW, van der Meer Y, Roos N, Snijder EJ.*** *1999. Open reading frame 1a-encoded subunits of the arterivirus replicase induce endoplasmic reticulum-derived double-membrane vesicles which carry the viral replication complex. Journal of virology* ***73:****2016-2026.*

70. ***Pol JM, Wagenaar F, Reus JE.*** *1997. Comparative morphogenesis of three PRRS virus strains. Veterinary microbiology* ***55:****203-208.*

71. ***Thuy NT, Huy TQ, Nga PT, Morita K, Dunia I, Benedetti L.*** *2013. A new nidovirus (NamDinh virus NDiV): Its ultrastructural characterization in the C6/36 mosquito cell line. Virology* ***444:****337-342.*

72. ***Hsu NY, Ilnytska O, Belov G, Santiana M, Chen YH, Takvorian PM, Pau C, van der Schaar H, Kaushik-Basu N, Balla T, Cameron CE, Ehrenfeld E, van Kuppeveld FJ, Altan-Bonnet N.*** *2010. Viral reorganization of the secretory pathway generates distinct organelles for RNA replication. Cell* ***141:****799-811.*

73. ***Richards AL, Soares-Martins JA, Riddell GT, Jackson WT.*** *2014. Generation of unique poliovirus RNA replication organelles. MBio* ***5:****e00833-00813.*

74. ***Teterina NL, Bienz K, Egger D, Gorbalenya AE, Ehrenfeld E.*** *1997. Induction of intracellular membrane rearrangements by HAV proteins 2C and 2BC. Virology* ***237:****66-77.*

75. ***Suhy DA, Giddings TH, Jr., Kirkegaard K.*** *2000. Remodeling the endoplasmic reticulum by poliovirus infection and by individual viral*

proteins: an autophagy-like origin for virus-induced vesicles. Journal of virology ***74:****8953-8965.*

76. ***Roberts IM, Harrison BD.*** *1970. Inclusion bodies and tubular structures in Chenopodium amaranticolor plants infected with strawberry latent ringspot virus. The Journal of general virology* ***7:****47-54.*

77. ***Edwardson JR, Christie RG.*** *1978. Use of virus-induced inclusions in classification and diagnosis. Ann Rev Phytopathol* ***16:****31-55.*

78. ***Rudzinska-Langwald A.*** *1990. Cytological changes in phloem parenchyma cells of Solanum rostratum (Dunal.) related to the replication of potato virus M (PVM). Acta Societatis Botanicorum Poloniae* ***59:****45-53.*

79. ***Lesemann DE.*** *1977. Virus group-specific and virus-specific cytological alterations induced by members of the tymovirus group. J Phytopathol* ***90:****315-336.*

80. ***Guix S, Caballero S, Bosch A, Pinto RM.*** *2004. C-terminal nsP1a protein of human astrovirus colocalizes with the endoplasmic reticulum and viral RNA. Journal of virology* ***78:****13627-13636.*

81. ***Mendez E, Aguirre-Crespo G, Zavala G, Arias CF.*** *2007. Association of the astrovirus structural protein VP90 with membranes plays a role in virus morphogenesis. Journal of virology* ***81:****10649-10658.*

82. ***Moreira AG, Kitajima EW, Rezende JAM.*** *2010. Identification and partial characterization of a Carica papaya-infecting isolate of Alfalfa mosaic virus in Brazil. J Gen Plant Pathol* ***76:****172-175.*

83. ***Schwartz M, Chen J, Janda M, Sullivan M, den Boon J, Ahlquist P.*** *2002. A positive-strand RNA virus replication complex parallels form and function of retrovirus capsids. Molecular cell* ***9:****505-514.*

84. ***Schwartz M, Chen J, Lee WM, Janda M, Ahlquist P.*** *2004. Alternate, virus-induced membrane rearrangements support positive-strand RNA virus genome replication. Proceedings of the National Academy of Sciences of the United States of America* ***101:****11263-11268.*

85. ***Medina V, Tian T, Wierzchos J, Falk BW.*** *1998. Specific inclusion bodies are associated with replication of lettuce infectious yellows virus RNAs in Nicotiana benthamiana protoplasts. The Journal of general virology* ***79 (Pt 10):****2325-2329.*

86. ***Gillespie LK, Hoenen A, Morgan G, Mackenzie JM.*** *2010. The endoplasmic reticulum provides the membrane platform for biogenesis of the flavivirus replication complex. Journal of virology* ***84:****10438-10447.*

87. ***Welsch S, Miller S, Romero-Brey I, Merz A, Bleck CK, Walther P, Fuller SD, Antony C, Krijnse-Locker J, Bartenschlager R.*** *2009. Composition and three-dimensional architecture of the dengue virus replication and assembly sites. Cell host & microbe* ***5:****365-375.*

88. ***Romero-Brey I, Merz A, Chiramel A, Lee JY, Chlanda P, Haselman U, Santarella-Mellwig R, Habermann A, Hoppe S, Kallis S, Walther P, Antony C, Krijnse-Locker J, Bartenschlager R.*** *2012. Three-dimensional architecture and biogenesis of membrane structures associated with hepatitis C virus replication. PLoS pathogens* ***8:****e1003056.*

89. ***Miller S, Kastner S, Krijnse-Locker J, Buhler S, Bartenschlager R.*** *2007. The non-structural protein 4A of dengue virus is an integral membrane protein inducing membrane alterations in a 2K-regulated manner. J Biol Chem* ***282:****8873-8882.*

90. ***Roosendaal J, Westaway EG, Khromykh A, Mackenzie JM.*** *2006. Regulated cleavages at the West Nile virus NS4A-2K-NS4B junctions play a major role in rearranging cytoplasmic membranes and Golgi trafficking of the NS4A protein. Journal of virology* ***80:****4623-4632.*

91. ***Egger D, Wolk B, Gosert R, Bianchi L, Blum HE, Moradpour D, Bienz K.*** *2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. Journal of virology* ***76:****5974-5984.*

92. ***Kopek BG, Settles EW, Friesen PD, Ahlquist P.*** *2010. Nodavirus-induced membrane rearrangement in replication complex assembly requires replicase protein a, RNA templates, and polymerase activity. Journal of virology* ***84:****12492-12503.*

93. ***Kopek BG, Perkins G, Miller DJ, Ellisman MH, Ahlquist P.*** *2007. Three-dimensional analysis of a viral RNA replication complex reveals a virus-induced mini-organelle. PLoS biology* ***5:****e220.*

822 *94.* ***Magliano D, Marshall JA, Bowden DS, Vardaxis N, Meanger J, Lee JY.***
823 *1998. Rubella virus replication complexes are virus-modified lysosomes.*
824 *Virology* ***240:****57-63.*
825 *95.* ***Fontana J, Lopez-Iglesias C, Tzeng WP, Frey TK, Fernandez JJ, Risco C.***
826 *2010. Three-dimensional structure of Rubella virus factories. Virology*
827 ***405:****579-591.*
828 *96.* ***Salonen A, Vasiljeva L, Merits A, Magden J, Jokitalo E, Kaariainen L.***
829 *2003. Properly folded nonstructural polyprotein directs the semliki forest*
830 *virus replication complex to the endosomal compartment. Journal of*
831 *virology* ***77:****1691-1702.*
832 *97.* ***Barajas D, Jiang Y, Nagy PD.*** *2009. A unique role for the host ESCRT*
833 *proteins in replication of Tomato bushy stunt virus. PLoS pathogens*
834 ***5:****e1000705.*
835 *98.* ***Krogh A, Larsson B, von Heijne G, Sonnhammer EL.*** *2001. Predicting*
836 *transmembrane protein topology with a hidden Markov model: application*
837 *to complete genomes. Journal of molecular biology* ***305:****567-580.*
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839
840

Figure 1

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