

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

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

### 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.

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

3

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19

20

21 Keywords: RNA virus replication, membrane rearrangement, replicative  
22 organelle, virus factory

23

24

25 **Abstract**

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

37

38 **Paired membranes associated with viral RNA**

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

52

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

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

65

### 66 **Structure of the organelles**

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

75

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

84

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

92

### 93 **Viral proteins involved in organelle formation**

94 Further evidence of the probable importance of nidovirus replicative  
95 organelles for viral RNA replication comes in the form of genetic conservation.  
96 Nidoviruses, and most particularly coronaviruses, are highly genetically  
97 variable and contain several genus-specific or even species-specific genes  
98 (14). However, there are two clusters of genes that are conserved in all  
99 known nidoviruses (11, 14). The first is a highly conserved cluster of genes  
100 homologous to the Severe Acute Respiratory Syndrome Coronavirus (SARS-  
101 CoV) nsp3-6 (Figure 1). Expression of the membrane-anchored proteins  
102 nsp3, nsp4 and nsp6 is sufficient to induce the formation of SARS-like paired-  
103 membrane replicative organelles (15). The second conserved gene cluster  
104 encodes the viral RNA polymerase and superfamily 1 helicase (16). The  
105 conservation of membrane-pairing genes in the context of an otherwise  
106 hypervariable group of viruses is a strong argument in favour of the  
107 importance of at least the membrane-pairing genes for RNA synthesis  
108

109 The proteins that form SARS-CoV replicative organelles have several features  
110 in common with distant homologs found throughout the *Nidovirales*. We will  
111 refer to the transmembrane proteins homologous to SARS-CoV nsp3, nsp4  
112 and nsp6 as TM1, TM2, and TM3, respectively. The relative genomic  
113 positions and functions attributed to TM1-3 in nidoviruses are shown in Figure  
114 1.

115

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

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

128

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

137

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

156

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



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

165

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

185

### 186 **Interactions among DMV-making proteins**

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

194 two-hybrid screens (43, 45) studies with another coronavirus did detect nsp4-  
195 nsp4 interactions by Venus reporter fluorescence (46). To date, homotypic  
196 interactions have not been demonstrated for nsp6 despite several attempts  
197 (43-45).

198

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

210

### 211 **Virus-host interactions**

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

220

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

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

241

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

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

264

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

274

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

289

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

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

304

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

321

### 322 **Natural variation in DMV structure**

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

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

333

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

344

#### 345 **Induced variation in DMVs**

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

354

355 After serial passage of the virus in the presence of K22, resistant mutants  
356 were selected. Surprisingly, two independently isolated resistance mutations  
357 mapped to opposite ends of transmembrane helices in TM3 (nsp6) at  
358 positions H121L and M159V. The resistant viruses released similar amounts  
359 of new progeny compared to *wt*, but produced only about half as many DMVs  
360 per infected cell. In addition, the DMVs induced by resistance mutants  
361 appeared structurally impaired. Similarly to MHV nsp4 mutants) K22 escape  
362 mutants induced DMV with partially collapsed inner membranes, even when  
363 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 *Brts31*, 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 *Brts105*, 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

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

401

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

416

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



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

434

435

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437

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439 project 149784; VT and PV).

440

441 **Table 1.** Evidence paired membrane structures in +RNA virus infection.  
 442

Order	Family	Host <sup>a</sup>	Origin <sup>b</sup>	Type <sup>c</sup>	Proteins <sup>d</sup>	References
<i>Nidovirales</i>	<i>Arteriviridae</i>	A	ER	V,Z	nsp2, 3	(38, 39, 443)
	<i>Coronaviridae</i>	A	ER	V,Z,S,C	nsp3+4+6	(2, 8, 30, 445)
	<i>Mesoniviridae</i>	A	ER	V?	<i>nr</i> <sup>e</sup>	(71) 446
<i>Picornavirales</i>	<i>Picornaviridae</i>	A	ER	V	2BC, 3A	(72-75) 447
	<i>Secoviridae</i>	P	ER	V?	<i>nr</i>	(76) 448
<i>Tymovirales</i>	<i>Betaflexiviridae</i>	P	ER	V	<i>nr</i>	(77, 78) 449
	<i>Tymoviridae</i>	P	Cp, Mt	V	<i>nr</i>	(79) 450
Unclassified	<i>Astroviridae</i>	A	ER	V	nsp1a	(80, 81) 451
	<i>Bromoviridae</i>	P	ER	Z,S	1a+2a <sup>pol</sup>	(82-84) 452
	<i>Closteroviridae</i>	P	<i>nr</i>	V	<i>nr</i>	(85) 453
	<i>Flaviviridae</i>	A	ER	V,S,C	NS4A+4B	(86-91) 454
	<i>Nodaviridae</i>	A	Mito	S	pA+RNA	(92, 93) 455
	<i>Togaviridae</i>	A	Ly, ER	V,S?	P123	(94-96) 454
	<i>Tombusviridae</i>	P	Px	S	<i>nr</i>	(97) 455

456 <sup>a</sup>Animals (A) or Plants (P)

457 <sup>b</sup>Membranes from the endoplasmic reticulum (ER), chloroplast (Cp), mitochondria (Mt),  
 458 lysosome (Ly) or peroxisome (Px)

459 <sup>c</sup>Paired membranes in the form of double-membrane vesicles (V), zippered ER (Z), open-  
 460 necked spherules (S), or convoluted membranes (C)

461 <sup>d</sup>Proteins implicated in membrane rearrangements

462 <sup>e</sup>Not reported (*nr*)

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

Virus	Condition s	ts	Cells	Prevalence				Size (nm)			
				DMV	P value <sup>a</sup>	IV	P value	DMV	P value <sup>b</sup>	IV	P value
Wild-type	DBT 33°C	--	n=323	6%	--	7%	--	228 ± 45	--	69 ± 8	--
Brts31	5.5 hpi	nsp3	n=753	2%	8×10 <sup>-4</sup>	7%	ns <sup>c</sup>	195 ± 38	2×10 <sup>-6</sup>	69 ± 9	ns
Wild-type		--	n=161	40%	--	29%	--	228±36	--	68±10	--
Brts31		nsp3	n=238	24%	4×10 <sup>-4</sup>	25%	ns	208±34	5×10 <sup>-19</sup>	68±10	ns
Albts16	17Cl-1	nsp5	n=120	37%	ns	19%	ns	189±33	8×10 <sup>-66</sup>	70±8	ns
Wüts18	33°C	nsp16	n=140	36%	ns	20%	ns	211±35	2×10 <sup>-15</sup>	67±12	ns
Brts105	10 hpi	nsp14	n=230	22%	1×10 <sup>-4</sup>	32%	ns	220±36	2×10 <sup>-4</sup>	69±10	ns
Albts22 <sup>d</sup>		nsp12	n=320	13%	1×10 <sup>-5</sup>	9%	1×10 <sup>-5</sup>	204±43	2×10 <sup>-13</sup>	68±11	ns

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<sup>a</sup>Calculated by two-tailed Fisher's exact test

<sup>b</sup>Calculated by two-tailed Mann-Whitney test

<sup>c</sup>Not significantly different from the appropriate wild-type control

<sup>d</sup>Attenuated growth at 33°C compared to wild-type

472 **Figure Legend**

473

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

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**Figure 1**  
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