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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6

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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

2

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

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 a 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

4

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^{pro}). Bioinformatics generally predicts an even number

132 of transmembrane spans in these proteins, which would be necessary to

133 localize M^{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

6

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

8

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^{pro}, the viral RNA polymerase, cap N-methyltransferase and

10

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 µM 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

11

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

12

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

436 **Acknowledgements**

437

440

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

Order	Family	Host[a]	Origin[b]	Type[c]	Proteins[d]	References
Nidovirales	*Arteriviridae*	A	ER	V,Z	nsp2, 3	(38, 39, 68-70)
	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)

456 [a]Animals (A) or Plants (P)
457 [b]Membranes from the endoplasmic reticulum (ER), chloroplast (Cp), mitochondria (Mt),
458 lysosome (Ly) or peroxisome (Px)
459 [c]Paired membranes in the form of double-membrane vesicles (V), zippered ER (Z), open-
460 necked spherules (S), or convoluted membranes (C)
461 [d]Proteins implicated in membrane rearrangements
462 [e]Not reported (*nr*)
463
464

465
466 **Table 2.** Differences in size and prevalence of MHV DMVs and intracellular virions (IV).

Virus	Conditions	*ts*	Cells	Prevalence				Size (nm)			
				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		--	*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	*17Cl-1 33ºC 10 hpi*	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

467 [a]Calculated by two-tailed Fisher's exact test
468 [b]Calculated by two-tailed Mann-Whitney test
469 [c]Not significantly different from the appropriate wild-type control
470 [d]Attenuated growth at 33°C compared to wild-type
471

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).

493

494

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