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Author(s): Borges, Iara; Rodrigues, Rodrigo Araújo Lima; Dornas, Fábio Pio; De Freitas Almeida, Gabriel; Aquino, Isabella; Bonjardim, Cláudio Antônio; Kroon, Erna Geessien; Scola, Bernard La; Abrahão, Jônatas Santos

Title: Trapping the enemy : Vermamoeba vermiformis circumvents Faustovirus mariensis dissemination by enclosing viral progeny inside cysts

Year: 2019

Version: Accepted version (Final draft)

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Please cite the original version:

Borges, I., Rodrigues, R. A. L., Dornas, F. P., De Freitas Almeida, G., Aquino, I., Bonjardim, C. A., Kroon, E. G., Scola, B. L., & Abrahão, J. S. (2019). Trapping the enemy : Vermamoeba vermiformis circumvents Faustovirus mariensis dissemination by enclosing viral progeny inside cysts. *Journal of Virology*, 93(14), Article e00312-19. <https://doi.org/10.1128/JVI.00312-19>

1 **Title: Trapping the enemy: *Vermamoeba vermiformis* circumvents Faustovirus mariensis**
2 **dissemination by enclosing viral progeny inside cysts**

3 **Running title:** *Vermamoeba vermiformis* traps faustovirus inside cysts

4 **Authors:** Iara Borges¹, Rodrigo Araújo Lima Rodrigues¹, Fábio Pio Dornas², Gabriel Almeida³,
5 Isabella Aquino¹, Cláudio Antônio Bonjardim¹, Erna Geessien Kroon¹, Bernard La Scola⁴,
6 Jônatas Santos Abrahão^{1*}

7 **Affiliations**

8 1 Laboratório de Vírus, Instituto de Ciências Biológicas, Departamento de Microbiologia,
9 Universidade Federal de Minas Gerais, Belo Horizonte, MG, 31270-901, Brazil.

10 2 Universidade Federal dos Vales do Jequitinhonha e Mucuri, Diamantina, Brazil

11 3 Department of Biological and Environmental Science, University of Jyväskylä, FI-40014
12 Jyväskylä, Finland.

13 4 URMITE, Aix Marseille Université, UM63, CNRS 7278, IRD 198, INSERM 1095, IHU -
14 Méditerranée Infection, 19-21 boulevard Jean Moulin, 13005 Marseille, France

15 * Corresponding author: jonatas.abrahao@gmail.com

16 **Keywords:** faustovirus; vermoameba; cysts; antiviral; virus control

17 **Abstract**

18 Viruses depend on cells to replicate and can cause considerable damage to their hosts.
19 However, hosts have developed a plethora of antiviral mechanisms to counter-attack or prevent
20 viral replication and to maintain homeostasis. Advantageous features are constantly being
21 selected, affecting host-virus interactions, and constituting a harsh race for supremacy in nature.
22 Here we describe a new antiviral mechanism unveiled by the interaction between a giant virus
23 and its amoebal host. Faustovirus mariensis infects *Vermamoeba vermiformis*, a free-living

24 amoeba, and induces cell lysis to disseminate into the environment. Once infected, the cells
25 release a soluble factor that triggers the encystment of neighbor cells, preventing their infection.
26 Remarkably, infected cells stimulated by the factor encyst and trap the viruses and viral
27 factories inside cyst walls, which are no longer viable and cannot excyst. This unprecedented
28 mechanism illustrates that a plethora of antiviral strategies remains to be discovered in nature.

29

30 **Importance**

31 Understanding how viruses of microbes interact with its hosts is not only important from a basic
32 scientific point of view, but also for a better comprehension of the evolution of life. Studies
33 involving large and giant viruses have revealed original and outstanding mechanisms
34 concerning virus-host relationships. Here we report a mechanism developed by *Vermamoeba*
35 *vermiformis*, a free-living amoeba, to reduce Faustovirus mariensis dissemination. Once
36 infected, *V. vermiformis* cells release a factor that induces the encystment of neighbor cells,
37 preventing infection of further cells and/or trapping the viruses and viral factories inside the cyst
38 walls. This phenomenon reinforces the need for more studies regarding large/giant viruses and
39 their hosts.

40

41 **Introduction**

42 Virion release and dissemination are *sine qua non* conditions for the maintenance of
43 most of viral species in nature. Evolutionary constraints have shaped a variety of mechanisms
44 promoting viral particle release and dissemination from infected cells; these range from mature
45 virions budding through the cell membrane to virus-induced cell lysis. As a response, hosts have
46 developed mechanisms to block or reduce viral particle propagation, replication, or both through
47 host-populations in a constant struggle against viruses. The Red Queen theory illustrates such a
48 race for supremacy, in which advantageous features are selected, and these changes, at least

temporarily and spatially, the balance of the interaction to one of the sides. Examples of hosts limiting viruses can be found in many groups of organisms, from bacterial anti-bacteriophage defenses (1), to plants silencing viral genes (2), and to the importance of pattern recognition receptors (PRRs), capable of triggering immune responses for multicellular host species (3). As remarkable example, the interferon (IFN) system acts as a major player against viral propagation in vertebrates (4–6). Host cells recognize viral molecules through PRRs, resulting in signaling pathways that lead to the production of IFN molecules. These are secreted and act in paracrine and autocrine ways by activating a second round of signaling, this time responsible for establishing an antiviral state, which leads to cell death in the case of infection. Although some cells are infected and lysed, IFN signaling reduces virus propagation and total viral load (6).

In this context, comprehensive studies involving large and giant viruses have revealed original and outstanding mechanisms concerning virus-host relationships. A complex interaction involving three players has been described for the free-living protist *Cafeteria roenbergensis*, in which a proviophage (mavirus), integrated in the genome of the protist, imparts a partial protection to *C. roenbergensis* populations in case of an eventual infection by a lytic giant virus called Cafeteria roenbergensis virus (CroV) (7). It has been also demonstrated that haploid cells (but not diploid cells) of *Emiliana huxleyi*, a free-living marine protist, are refractory to *Emiliana huxleyi* virus 86 infection in an evasion mechanism known as Cheshire Cat (8). Analogously, our team described the same phenomenon for free-living amoebas belonging to the *Acanthamoeba* genus, in which the cysts, but not the trophozoitic forms, are resistant to mimivirus infection (9, 10). We have demonstrated that once infected by mimivirus, *Acanthamoeba* trophozoites are no longer able to encyst, because mimivirus blocks the expression of a serine proteinase gene, a canonical element involved in the encystment process(10). Although it has been described that *Acanthamoeba* trophozoites are able to encyst in the presence of some intracellular bacteria (11), this phenomenon has never been described during amoebal virus infections.

76 Here we report a mechanism developed by *Vermamoeba vermiformis*, a free-living
77 amoeba, to reduce Faustovirus mariensis dissemination and consequently protect neighbor cells.
78 Once infected, *V. vermiformis* cells release a non-proteic soluble factor that induces the
79 encystment of neighbor cells, preventing infection of further cells, since Faustovirus is only able
80 to infect trophozoites. Interestingly, if cells already infected are exposed to the soluble
81 encystment factor, they encyst and trap the viruses and viral factories inside the cyst walls.
82 Unlike what has been described for amoebal cysts containing intracellular bacteria, cysts
83 enclosing Faustovirus particles, factories, or both are no longer viable and cannot excyst, thus
84 trapping viral progeny irreversibly inside the thick cyst walls and promoting an effective
85 reduction of viral load on, and dissemination to, the amoebal population.

86 Results

87 1. Faustovirus mariensis: isolation and genomic analysis

88 Attempting to isolate new amoebal viruses, we performed collections of surface water samples
89 at Pampulha Lagoon in Belo Horizonte, Brazil. One of the samples, collected in front of
90 Pampulha Art Museum (Figure 1A), induced a cytopathic effect (CPE) in *V. vermiformis*, a
91 free-living amoeba occurring worldwide, and already described as one of the hosts of some
92 giant or large viruses, including Faustovirus, Tupanvirus, Kaumobavirus, and Orpheovirus.
93 Cells presenting CPE were submitted to transmission and scanning electron-microscopy (TEM
94 and SEM), revealing viral particles similar to Faustoviruses, with approximately 190 nm,
95 icosahedral symmetry and a capsid containing an electrodense central region (genome) (Figures
96 1B–1D). We named this new isolate Faustovirus mariensis. During virus propagation and
97 purification, we observed that F. mariensis induces the formation of plaque forming units, a
98 CPE never described for any other amoebal giant virus, according to our knowledge (Figures
99 1E–1F). During the time-course of infection, plaque units expand and coalesce in *V.*
100 *vermiformis* monolayers (Figure 1G).

101 The genome of *F. mariensis* is a circular double-stranded DNA molecule of 466,080-bp
102 length (See Fig. S1 at [https://5c95043044c49.site123.me/my-blog/supp-material-trapping-the-](https://5c95043044c49.site123.me/my-blog/supp-material-trapping-the-enemy-vermamoeba-vermiformis-circumvents-faustovirus-mariensis-dissemination-by-enclosing-viral-progeny-inside-cysts)
103 [enemy-vermamoeba-vermiformis-circumvents-faustovirus-mariensis-dissemination-by-](https://5c95043044c49.site123.me/my-blog/supp-material-trapping-the-enemy-vermamoeba-vermiformis-circumvents-faustovirus-mariensis-dissemination-by-enclosing-viral-progeny-inside-cysts)
104 [enclosing-viral-progeny-inside-cysts](https://5c95043044c49.site123.me/my-blog/supp-material-trapping-the-enemy-vermamoeba-vermiformis-circumvents-faustovirus-mariensis-dissemination-by-enclosing-viral-progeny-inside-cysts)). The GC content is 36%, and it was predicted to encode
105 483 genes (210 located at negative strand; 273 at positive strand), with a coding density of 90%.
106 The predicted proteins had a mean length (\pm standard deviation) of 279 ± 258 amino acids
107 (ranging from 53 to 2980 amino acids). A total of 374 proteins (77.4%) had no known function
108 (Figure 2A). The major functional gene categories were represented by DNA replication,
109 recombination and repair, as well as transcription and RNA processing, with 22 and 25 genes,
110 respectively (Figure 2A), with additional genes for DNA polymerase, D5 primase helicase,
111 topoisomerase II, different subunits of DNA-directed RNA polymerase, mRNA capping
112 enzymes, transcription factors, among others. No tRNA was predicted and no ORFans were
113 detected. Differently from other giant viruses, only one translation factor is encoded by *F.*
114 *mariensis*, the translation initiation factor SUI1, which was the only translation-related gene also
115 observed in Faustovirus E12, the first member of this new group of viruses to be described (12).
116 Other genes previously described for faustoviruses were also encoded in *F. mariensis*. These
117 include two adjacent polyproteins of 220 kDa and 60 kDa, a ribosomal protein acetyltransferase,
118 and some genes belonging to large paralogous families, such as membrane occupation and
119 recognition nexus (MORN) repeat-containing proteins, and ankyrin repeat-containing proteins,
120 although only two genes were found to have this repeat domain (Figure 2A). Synteny analysis
121 revealed a highly conserved genome organization among faustoviruses. However, the *F.*
122 *mariensis* genome exhibited a small region (~11.5 kb) that was rearranged in its genome in
123 regards to the other described faustoviruses, i.e., the region was located after a conserved block
124 of >30 kb in *F. mariensis* and before that block in other viruses (See Fig. S2 at
125 [https://5c95043044c49.site123.me/my-blog/supp-material-trapping-the-enemy-vermamoeba-](https://5c95043044c49.site123.me/my-blog/supp-material-trapping-the-enemy-vermamoeba-vermiformis-circumvents-faustovirus-mariensis-dissemination-by-enclosing-viral-progeny-inside-cysts)
126 [vermiformis-circumvents-faustovirus-mariensis-dissemination-by-enclosing-viral-progeny-](https://5c95043044c49.site123.me/my-blog/supp-material-trapping-the-enemy-vermamoeba-vermiformis-circumvents-faustovirus-mariensis-dissemination-by-enclosing-viral-progeny-inside-cysts)
127 [inside-cysts](https://5c95043044c49.site123.me/my-blog/supp-material-trapping-the-enemy-vermamoeba-vermiformis-circumvents-faustovirus-mariensis-dissemination-by-enclosing-viral-progeny-inside-cysts)). This region comprised 18 genes, most of them with unknown functions. The
128 analysis of DNA polymerase, a marker used for phylogenetic analysis of nucleocytoplasmic

large-DNA viruses (NCLDV) confirmed that *F. mariensis* was clustered with other Faustovirus isolates, and was more related to the clade containing Faustovirus E12 (Figure 2B).

2. Faustovirus mariensis replication cycle

The analyses of the *F. mariensis* replication cycle in *V. vermiformis* were performed based on asynchronal infection (multiplicity of infection (MOI) of 0.1), starting from fresh trophozoites, and purified viral particles. We analyzed whether *V. vermiformis* cysts would be permissive to *F. mariensis* infection, but neither cytopathic effects nor increases in viral loads were observed; therefore, Faustovirus, as has been described for mimivirus, needs to infect amoebal trophozoites to start its replication cycle. In the cytoplasm of trophozoites, *F. mariensis* induced the formation of large electron-lucent viral factories (about 3.5 μm length) frequently observed close to the cell nucleus (Figures 3A–3C). Viral infection induced the recruitment of mitochondria to the periphery of viral factory, suggesting virus-induced energy optimization during viral morphogenesis (Figures 3A and 3C). Curiously, intranuclear particles were also observed but further studies are necessary to determine their role in the *F. mariensis* replication cycle (See Fig. S3 at <https://5c95043044c49.site123.me/my-blog/supp-material-trapping-the-enemy-vermamoeba-vermiformis-circumvents-faustovirus-mariensis-dissemination-by-enclosing-viral-progeny-inside-cysts>).

As observed for other viruses of free-living amoebae (13–16), *F. mariensis* morphogenesis began with crescents, open structures of approximately 50 nm, which grew as the electron-dense material of the viral factory fulfilled them (Figure 4A). Particles of almost 200 nm without genomic content were observed in the late phases of morphogenesis, when the genome was incorporated and centralized within several newly formed viral particles (Figure 4A). Faustovirus *mariensis* progeny were organized in a honeycomb fashion inside viral factories, as previously described for other Faustovirus isolates (12) (Figure 4B-E). Small honeycombs expanded as new mature viruses formed and coalesced to others in the cytoplasm (Figure 4B). By the end of the replication cycle, the cytoplasm was fully taken by new *F. mariensis* particles (See Fig. S4 at <https://5c95043044c49.site123.me/my-blog/supp-material->

trapping-the-enemy-vermamoeba-vermiformis-circumvents-faustovirus-mariensis-dissemination-by-enclosing-viral-progeny-inside-cysts). Contrary to what has been described for Marseillevirus and Pandoravirus, exocytosis was not observed (16). Cellular lysis is likely the most important form of liberation of newly formed *F. mariensis* particles. Isolated particles or aggregates of *F. mariensis* associated with cellular structures were observed extracellularly, and aggregates became as large as a trophozoite of *V. vermiformis* (See Fig. S4 at <https://5c95043044c49.site123.me/my-blog/supp-material-trapping-the-enemy-vermamoeba-vermiformis-circumvents-faustovirus-mariensis-dissemination-by-enclosing-viral-progeny-inside-cysts>).

3. *V. vermiformis* cells may encyst and trap *F. mariensis* particles and factories inside cyst wall

Interestingly, during routine production and titration of *F. mariensis*, we observed a substantial formation and accumulation of *V. vermiformis* cysts, especially when cells were infected at high MOIs (1 and 10). This curious effect drew our attention, and we decided to verify whether other viruses would be able to trigger encystment. However, *V. vermiformis* infection with Tupanvirus or Orpheovirus did not induce the formation and accumulation of cysts in the culture flasks, regardless the MOI (data not shown). As such phenomena seemed to be a singular characteristic of *F. mariensis*, we decided to further characterize it.

Hence, *V. vermiformis* cells were infected with *F. mariensis* at a MOI of 10 and prepared for transmission electron microscopy (TEM) (24 hpi). Remarkably, TEM images revealed *F. mariensis* particles and viral factories not only inside a few *V. vermiformis* trophozoites, but also within the cytoplasm of many cysts and cells under encystment (Figure 5). This phenomenon, previously described for some bacteria (*Salmonella enterica*, *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Escherichia coli*) (11), had never been described for viruses to our knowledge. The observation of more than one hundred cysts revealed the presence of *F. mariensis* under distinct phases of the replication cycle, including the early viral factory formation, late morphogenesis, honeycomb formation/coalescence and, at last,

cytoplasm fulfilled by mature viral particles (Figure 6 A-F). In case of cells fulfilled by viral particles (late cycle), some cellular structures seemed to be degraded, including mitochondria and plasma membrane (Figure 6F). Despite the observation of variations in wall thickness among infected cysts, the cyst walls appeared intact and with no damage (Figure 6).

We then quantified the number of cysts of *V. vermiformis* formed after the inoculation of *F. mariensis* at different MOIs. A total of 3×10^6 trophozoites were added to T25 flasks with 5 ml of Peptone Yeast Extract Glucose (PYG) medium (this medium favors the propagation of amoebas) and inoculated at MOIs of 0.01, 0.1, 1, or 10. After adsorption, cells were washed and then incubated for 48 h with PYG medium. Uninfected cells were used as controls, and after 48 h of incubation, we measured the propagation of those cells, which reached approximately 7.8×10^6 cells; among those cells, we observed a natural encystment rate of approximately 21%, which is related to the reduction of nutrients in the PYG medium during the time of incubation (Figure 7A). However, *V. vermiformis* trophozoites infected by *F. mariensis* presented a higher rate of cysts 48 hpi, and this number was positively related to the MOI. Flasks inoculated at MOIs of 0.01 or 0.1 resulted in 63.6% and 75% of cysts, respectively; while flasks inoculated at higher MOIs, 1 or 10, both presented 100% cysts at 48 hpi (Figure 7A). Interestingly, infections with high MOIs (1 and 10) presented an encystment ratio (number of cysts obtained divided by the number of trophozoites imputed) of approximately 1:1, close to the initial input of trophozoites. These results indicated that trophozoites infected at high MOIs (1 or 10) with *F. mariensis* were almost totally converted to cysts (Figure 7A). We also analyzed whether *F. mariensis* inactivated particles (same MOI as above described) would be able to trigger *V. vermiformis* encystment, but we could not observe differences in the number of cysts between inoculated and non-inoculated cells, indicating that the triggering of encystment is dependent on virus entry and replication.

The next step was to measure *F. mariensis* replication in *V. vermiformis* inoculated at different MOIs. Therefore, *V. vermiformis* cells were infected with *F. mariensis* at MOIs of 0.01, 0.1, 1, and 10, and incubated for one week. At this stage, trophozoites were no longer observed, regardless the MOI, since they either were lysed (releasing viruses to supernatant) or

converted to cysts. This longer time of incubation was designed to allow the lysis or encystment of all trophozoites in the system; therefore, at the end of the experiments, we would find viruses only in the supernatants (originated from lysis of infected trophozoites) or inside cysts. The supernatants and cysts were separated by centrifugation and viral genome load was quantified by qPCR. A substantial replication of the virus was correlated with low MOIs, with larger amounts of DNA detected in the supernatant at MOIs of 0.01 and 0.1 (Figure 7B). Higher MOIs (1 and 10) however, presented relatively low amounts of viral DNA in the supernatant but a substantial amount inside cysts, indicating that the virus was able to initiate the infection but not to release its progeny, after having its genome (and particles) imprisoned within the newly formed cysts (Figure 7B).

4. Faustovirus infected cysts fail excystment

Studies demonstrated that several intracellular bacteria are able to survive and take advantage of amoebal encystment (11, 17). It has been suggested this feature would guarantee bacteria to be protected during adverse environmental conditions while inside the cyst. Once good environmental conditions are available, these bacteria could return to multiply and maintain their life cycle upon excystment. At a first glance, *F. mariensis* could possibly use this same stratagem to eventually return to replicate in new trophozoites.

To test this hypothesis, trophozoites of *V. vermiformis* were infected with different MOIs of *F. mariensis* diluted ten-fold from 0.01 to 10. Cysts produced after the infection were collected, washed to remove external viral particles, and then their potential of excystment was evaluated. Two different methods to trigger excystment were used: exposure of cysts to PYG media with 5% of fetal bovine serum (FBS) in T25 cell flasks, and plating of cysts on petri dishes with Bacto® Agar covered with a monolayer of heat-inactivated *Escherichia coli*.

Surprisingly, cysts derived from the higher MOIs (1 and 10) did not excyst upon either method employed (Figure 8A). Those cysts were then analyzed by TEM, revealing viral particles inside almost all of the cysts. Curiously, some of those cysts, in spite of appearing to have a typical thick wall, had a reduced diameter (about 3.5 μm) and had lost their rounded

238 shape (Figures 8C–8E). For cysts derived from infections with MOIs of 0.01 and 0.1,
239 excystment was observed for nearly 24% of cells. The TEM analysis revealed that only cysts
240 not presenting viral particles, factories, or both in their cytoplasm were able to excyst (Figure
241 8C). This data was confirmed by subculturing individual trophozoites obtained after excystment
242 (from cultures inoculated at MOIs of 0.01 and 0.1), which did not show any cytopathic effect or
243 detection of viral particles. As described for cells infected at MOIs of 1 and 10, we also
244 observed infected cysts with reduced diameters and irregular shapes in flasks inoculated at
245 MOIs of 0.01 and 0.1 (Figures 8C–8E).

246 When the viability of cysts was assayed with 0.4% trypan blue, it was revealed that
247 100% of cysts from cells inoculated at MOIs of 1 and 10 were no longer viable, while 46% and
248 57% of cysts from flasks inoculated at MOIs of 0.01 and 0.1 were not viable, respectively
249 (Figure 8B). Although not viable, infected cysts were as resistant as uninfected cysts against
250 hydrochloric acid and heating treatments followed by 3 cycles of sonication. They kept their
251 walls visually intact, and preserved intracellular structures, including viruses and factories (See
252 Fig. S5 at [https://5c95043044c49.site123.me/my-blog/supp-material-trapping-the-enemy-](https://5c95043044c49.site123.me/my-blog/supp-material-trapping-the-enemy-vermamoeba-vermiformis-circumvents-faustovirus-mariensis-dissemination-by-enclosing-viral-progeny-inside-cysts)
253 [vermamoeba-vermiformis-circumvents-faustovirus-mariensis-dissemination-by-enclosing-viral-](https://5c95043044c49.site123.me/my-blog/supp-material-trapping-the-enemy-vermamoeba-vermiformis-circumvents-faustovirus-mariensis-dissemination-by-enclosing-viral-progeny-inside-cysts)
254 [progeny-inside-cysts](https://5c95043044c49.site123.me/my-blog/supp-material-trapping-the-enemy-vermamoeba-vermiformis-circumvents-faustovirus-mariensis-dissemination-by-enclosing-viral-progeny-inside-cysts)). Taken together, these results indicate that *V. vermiformis* encystment was
255 triggered by *F. mariensis* infection, in a MOI-dependent way. Once encysted, infected cells
256 were no longer able to excyst, thus irreversibly imprisoning the viral progeny within their thick
257 walls.

258

259 **5. Faustovirus mariensis is unable to circumvent *V. vermiformis* encystment**

260 Our team has previously shown that mimivirus was capable of inhibiting the process of
261 encystment in *Acanthamoeba castellanii* by down-regulating the expression of a cellular serine
262 protease gene, which is an essential enzyme responsible for triggering the encystment pathway
263 in these cells (10). Once infected by mimivirus, a given trophozoite of *A. castellanii* inevitably
264 suffers from lysis due to viral replication, since the virus blocks encystment. In contrast, when

265 *A. castellanii* trophozoites were incubated with an encystment stimulation factor (e.g. NEFF
266 solution) previous to Mimivirus inoculation, serine proteinase gene expression was stimulated,
267 which triggered encystment and circumvented Mimivirus replication. Therefore, avoiding or
268 blocking the triggering of encystment is critical for amoebal virus replication, since they are
269 able to replicate only in non-encysting trophozoites. Herein, we investigated whether *F.*
270 *mariensis* could be able to block *V. vermiformis* encystment.

271 Briefly, *V. vermiformis* trophozoites were treated with NEFF solution, and 2 h later this
272 solution was removed; the purified virus was inoculated at a MOI of 10, then flasks were added
273 with PYG medium and incubated for 24 h. Virus genome replication was assayed by qPCR, and
274 cells were analyzed by TEM and optical microscopy. Cell viability was analyzed using trypan
275 blue. After 24 h, virus genome replication was not observed, and all trophozoites were
276 converted into viable uninfected cysts (Figures 9A–9D and see Fig. S6 at
277 [https://5c95043044c49.site123.me/my-blog/supp-material-trapping-the-enemy-vermamoeba-](https://5c95043044c49.site123.me/my-blog/supp-material-trapping-the-enemy-vermamoeba-vermiformis-circumvents-faustovirus-mariensis-dissemination-by-enclosing-viral-progeny-inside-cysts)
278 [vermiformis-circumvents-faustovirus-mariensis-dissemination-by-enclosing-viral-progeny-](https://5c95043044c49.site123.me/my-blog/supp-material-trapping-the-enemy-vermamoeba-vermiformis-circumvents-faustovirus-mariensis-dissemination-by-enclosing-viral-progeny-inside-cysts)
279 [inside-cysts](https://5c95043044c49.site123.me/my-blog/supp-material-trapping-the-enemy-vermamoeba-vermiformis-circumvents-faustovirus-mariensis-dissemination-by-enclosing-viral-progeny-inside-cysts)). Alternatively, we inoculated *V. vermiformis* trophozoites with *F. mariensis* at a
280 MOI of 10; then, after 2 h, we treated the cells with NEFF solution, and then cells were
281 incubated for 24 h. In this case, we detected viral genome replication inside cysts (about 6 logs,
282 arbitrary units); and most of cysts (non-viable) had viral particles or factories in the cytoplasm
283 (Figures 9A–9D and Fig. S6 at weblink informed above). As an experimental control, we
284 submitted Tupanvirus, a Mimivirus relative, to the same experiments (MOI of 10). It was not
285 possible to detect Tupanvirus genome replication in *V. vermiformis* pre-treated with NEFF;
286 there were neither viral particles nor genomes inside cysts (Figures 9A–9D and Fig. S6 at
287 weblink informed above). On the other hand, when *V. vermiformis* trophozoites were infected
288 with Tupanvirus and then treated with NEFF, the virus genome was able to replicate (about four
289 logs, arbitrary units), cells were lysed, and no cyst formation was observed (Figures 9A–9D and
290 Fig. S6 at weblink informed above).

291 As previously mentioned, the encystment process is triggered after the expression of
292 cellular serine proteinases. Such proteases' catalytic site is composed by a triad, consisting of

three amino acids: His 57, Ser 195 and Asp 102. The importance of such enzymes on *Acanthamoeba* encystment has been demonstrated by measuring the levels of serine-proteinase transcripts and protein during encystment process (9, 10). Inhibition of serine-proteinase genes transcription negatively affect encystment. We measured (by qPCR) the expression levels of two serine proteinase mRNA isotypes present in *V. vermiformis* upon infection (MOI of 10) with *F. mariensis*, Tupanvirus, or NEFF treatment. Five hours post-infection, *F. mariensis* induced significant expression of both analyzed serine proteinase isotypes ($p < 0.001$), in levels comparable to those detected in cells treated with the encystment stimulation solution NEFF (Figure 9E). In contrast, Tupanvirus circumvented the expression of *V. vermiformis* serine proteinases, keeping the levels similar to uninfected or untreated trophozoites (Figure 9E). Altogether, these results suggest that *F. mariensis* was incapable of down-regulating factors that trigger the encystment of *V. vermiformis* as has been described for Mimivirus. Tupanvirus however, demonstrated a behavior in *V. vermiformis* similar to that of Mimivirus in *A. castellanii*.

6. Infected trophozoites release soluble factors which trigger the encystment

Previous studies have demonstrated that the secretion of soluble factors can modulate the process of encystment in *A. castellanii* (18). Although the nature of such factors remains to be better characterized, it has been suggested they could act as major players in a communication system among amoebas, stimulating cell encystment as a response to harsh conditions. To investigate whether *V. vermiformis* cells infected by *F. mariensis* secrete factors involved with encystment, the supernatant of a culture infected at a MOI of 10 was collected 4 dpi. The supernatant was filtered with a 0.1- μ m pore size filter to remove all viral particles and subsequently diluted 2-fold to inoculate new cultures of *V. vermiformis* trophozoites.

Remarkably, we observed a strong induction of encystment in *V. vermiformis* cells in a dose-dependent fashion by the virus-free supernatant from previous *F. mariensis* infections. Undiluted (pure) supernatant induced the encystment of approximately 67% of cells (Figure 10A). Supernatants from *V. vermiformis* cells infected by *F. mariensis* at MOIs of 0.01, 0.1, and

1 were also collected, filtered, and inoculated (undiluted) to fresh *V. vermiformis* cells. Supernatants with MOIs of 0.01, 0.1, and 1 caused encystment of nearly 35%, 43%, and 65% of cells, respectively (Figure 10B). To determine the nature of this encystment factor, the supernatant was treated with different concentrations of proteinase K or bromelain (to remove proteins) and inoculated into fresh cultures of *V. vermiformis* trophozoites. The enzymatic treatment did not affect the encystment of *V. vermiformis*, suggesting that such encystment factor(s) were not proteins.

We measured the concentration of different inorganic factors in the supernatants of infected cells (MOI of 10, 10 hpi) with *F. mariensis*, Tupanvirus, and uninfected cells (control). Supernatants of all studied groups had similar concentrations of potassium and calcium (Figures 10C and 10D). However, we observed a significant increase in magnesium ion (Mg^{2+}) concentrations in cells infected with *F. mariensis* (Figure 10E). This result is consistent with previous studies that have shown Mg^{2+} as a factor able to trigger encystment in *A. castellanii* (9, 19). We then tested the potential of Mg^{2+} to induce encystment in *V. vermiformis*. First, 3 nmol of Mg^{2+} was put in a culture of 4×10^4 amoeba trophozoites, and the concentration of Mg^{2+} was measured, as well as the rate of cyst formation at different times. The Mg^{2+} input not only stimulated the encystment of 79% of the cells after 9 hours, but it also promoted a gradual increase of Mg^{2+} concentration during the experimental period (Figure 10F). These results suggest that once stimulated by Mg^{2+} , *V. vermiformis* trophozoites trigger an encystment program, and secrete more Mg^{2+} as an encystment stimulus for neighbor trophozoites.

Considering these results, we evaluated the effect of a bivalent cation inhibitor, ethylenediaminetetraacetic acid (EDTA), on cyst formation during *F. mariensis* infection, at a MOI of 10. EDTA is well known to act as an Mg^{2+} chelating agent. EDTA caused a significant reduction in cyst formation rates (Figure 10G). Our results demonstrated that EDTA promoted a significant increase in the viral genome load in the supernatants of cultures, since the viral progeny succeeded in being released from infected trophozoites, because they were not being trapped inside cysts (Figure 10H). We also observed that EDTA affected the capacity of supernatant collected from cells infected with *F. mariensis* to induce encystment in fresh

348 trophozoites in a dose-dependent way (Figure 10I), reinforcing the idea that Mg^{2+} may be one of
349 the soluble factors released by *V. vermiformis* to stimulate encystment in neighbor cells.

350 Discussion

351 Viral sensing as well as the molecular communication within multicellular organisms or
352 among unicellular individuals are important factors for survival in the never-ending
353 evolutionary struggle between viruses and hosts. The ability to sense that a virus is present and
354 respond to an infection is crucial for the host population survival. Bacterial enzymes
355 discriminate between endogenous and foreign nucleic acids by using epigenetic cues; *Sulfolobus*
356 *islandicus* (Archaea) is able to enter dormancy in response to phage presence, and quorum
357 sensing has been shown to be important for anti-phage defense strategies in *Vibrio anguillarum*
358 (20–22). PRRs are conserved molecules found in multicellular organisms such as plants, insects
359 and vertebrates (23). The activation of these receptors by pathogens results in signaling
360 pathways that lead to innate immune mechanisms aimed at controlling the infections. As the
361 complexity of organisms increases, the immune responses become more robust. When a
362 pathogen is sensed by PRRs in a vertebrate cell, soluble molecules (IFNs) are secreted and
363 establish an antiviral state in any other cell that receives the IFN, besides acting on immune
364 cells to mount an adaptive immune response (4). Although much is known about viral sensing
365 and antiviral responses on multicellular organisms, the bases of pathogen recognition and
366 response to infection in unicellular eukaryotes is still obscure. Understanding how viruses of
367 microbes interact with its hosts is not only important from a basic scientific point of view, but
368 also for a better comprehension of the evolution of life as well as discovering novel mechanisms
369 and molecules that could be used for applied science. In recent examples, communication
370 between viruses has been shown and described to be mediated by small peptides, while the
371 investigation of bacterial DNA-based acquired immunity led to the recent CRISPR revolution in
372 science and biomedicine (24–26).

Here we show that, when *V. vermiformis* faces infection by Faustovirus, it is able to trap the viruses inside cysts. The virus-containing cysts are non-viable, so the trapping process protects the uninfected amoebal population and could be considered a novel type of antiviral strategy. Our data also show that the encystment response to *F. mariensis* was mediated by at least one unknown encystment factor released by infected cells in conjunction with Mg^{2+} . An overview of the process is shown in Figure 10J. The putative encystment factor was not a protein, since a proteinase K/bromelain treatment did not abolish the cyst inducing ability of conditioned media. Its secretion was dependent on Faustovirus entry and replication, as conditioned media from *V. vermiformis* exposed to inactivated virions did not enhance cyst formation. Since it is unlikely that the encystment factor was derived from the virus, due to its negative impact on viral success, it is tempting to assume that viruses are being recognized by the infected amoebal cell, which in turn produces the encystment factor as a response. When this factor is secreted, nearby cells are warned of viral presence and either reversibly encyst before the infection happens or trap the viruses inside damaged cysts, thus protecting the rest of the population. This could be considered analogous to the IFN response, in which virus sensing leads to molecular communication between cells and a subsequent sacrifice of those infected in order to minimize damage to the organism population. In the case of giant viruses such as Tupanvirus and Mimivirus, escape mechanisms against the production of the encystment factor would have appeared, analogous to the myriad of evasion mechanisms possessed by viruses of vertebrates against the IFN system. Recently, Yoshikawa and co-workers demonstrated that a new remarkable giant virus called Medusavirus is able to induce encystment of *Acanthamoeba* cells as well (27). However, the mechanisms involved in such phenomenon remain to be investigated. New studies on amoebas (including *V. vermiformis*) cyst wall composition, synthesis and dynamics would be welcome to a better understanding of such processes (28). Ecological approaches would be very interesting as well, concerning how encystment factors (as Mg^{+2}) would act on lakes, rivers and oceans, and its impact on the communication of amoebas' communities, on narrow and broad scales.

400 The complexity of the amoebal host cell, along with the large genomes of giant viruses,
401 makes a reductionist approach to study *V. vermiformis* and *F. mariensis* interactions
402 challenging. The non-protein nature of the encystment factor(s) makes isolating it from the
403 complex metabolomics of an amoebal cell difficult, while the large genome and elaborate
404 particle of the virus makes pinpointing specific targets for recognition or other interactions
405 challenging. However, our data can be considered a starting point for better comprehending
406 ancient and unique virus-host interactions. The antiviral strategy described here is a new
407 example of the constant race for supremacy between parasites and hosts. This unprecedented
408 mechanism illustrates that many different antiviral strategies are yet to be unveiled in the
409 biosphere.

410 **Methods**

411 **Virus isolation**

412 In August 2015, 15 water samples were collected from Pampulha Lagoon, Belo Horizonte,
413 Brazil. The collection was performed with sterile tubes, and the samples were stored at 4 °C
414 until the inoculation process. The samples were then subjected to filtrations through a paper
415 filter and then through a 5 µm filter to remove large particles of sediment. For viral isolation, we
416 used *Acanthamoeba polyphaga* (ATCC 30461), *Acanthamoeba castellanii* (ATCC 30234), and
417 *Vermamoeba vermiformis* (ATCC CDC19). Amoebae were grown in 75 cm² Nunc™ Cell
418 Culture Treated Flasks (Thermo Fisher Scientific, USA) with 30 mL of Peptone Yeast Extract
419 Glucose (PYG) medium (29) supplemented with 0.14 mg/mL penicillin (Sigma-Aldrich, USA),
420 50 mg/mL gentamycin (Thermo Fisher Scientific, USA), and 2.5 mg/mL amphotericin (Bristol-
421 Myers Squibb, New York, USA) at 32 °C. For virus isolation, amoebae were re-suspended in 10
422 mL of PYG supplemented with an antibiotic mix containing 0.004 mg/mL ciprofloxacin
423 (Cellofarm, Brazil), 0.004 mg/mL vancomycin (Sigma-Aldrich, U.S.A), and 0.020 mg/mL
424 doxycycline (Sigma-Aldrich, U.S.A). Then, the suspension was diluted 1:10 in PBS, and
425 inoculated in 96-well plates containing 4×10^4 cells per well. The plates were incubated for 7

426 days at 32 °C, and observations of the cytopathic effects were done daily using an inverted
427 optical microscope (Quimis, Brazil). The well contents were then collected, frozen, and thawed
428 three times to help release the viruses from intact amoeba cells. The samples were re-inoculated
429 for two new sub-cultures on fresh amoebae, as described above (blind passages). The contents
430 of wells with cytopathic effects were collected and inoculated into new 25 cm² Nunc™ Cell
431 Culture Treated Flasks with Filter Caps (Thermo Fisher Scientific, USA) cultures containing 1
432 million cells. The cytopathic effects were confirmed, and these cultures were centrifuged at
433 1200 x g for lysate clearance and were further analyzed for giant viruses. Negative controls with
434 no sample-inoculated amoeba were used in all microplates. From this collection of samples, we
435 isolated two amoeba viruses: Niemeyer virus (Mimivirus), which was able to infect
436 *Acanthamoeba* cells, and *F. mariensis*, which was able to replicate in *V. vermiformis*. These new
437 isolates were registered at the Brazilian Biological Resources Bank, SISBIO number A237F1B.

438 **Virus production, purification and cytopathic effect analysis**

439 For Faustovirus production and purification, twenty T175 flasks (Thermo Fisher Scientific,
440 USA) containing 20 million *V. vermiformis* in PYG medium were inoculated with *F. mariensis*
441 at a MOI of 0.01 and incubated for 5 days, at 28°C. The lysate was centrifuged at 1200 x g.
442 Then, the supernatant was collected, inoculated onto a 24% sucrose (Merck, Germany) cushion,
443 and centrifuged at 8000 x g for 2 hours. The pellet was re-suspended in PBS and stored in an -
444 80°C freezer. Three aliquots of the virus stock were titrated to the 50% end-point and calculated
445 by the Reed-Muench method (30). Half of the viral production (600 µl) was used for biological
446 assays and the other half (600 µl) was used for genome sequencing. For plaque forming unit
447 (PFU) analysis, four million *V. vermiformis* cells were added to 6-well plates, forming cell
448 monolayers. Then, the monolayers were inoculated with *F. mariensis* diluted ten-fold, and
449 observed for 48 h with an optical microscope with association a digital camera (Quimis,
450 Moticam 2300, Brazil). To test whether *F. mariensis* would be able to infect *V. vermiformis*
451 cysts, T25 flasks containing 4 million cysts were inoculated with *F. mariensis* at MOIs of 0.01,
452 0.1, 1, and 10 in PBS and observed for 48h. In addition, 1 ml of these cultures were collected at

453 0, 4, 8, 12, and 24 hpi, and submitted to PCR targeting Faustovirus DNA polymerase to check
454 virus replication. As previously mentioned, we had no evidence that *F. mariensis* was able to
455 initiate its cycle by infecting *V. vermiformis* cysts.

456 Tupanvirus soda lake and Orpheovirus IHUMI LCC2 were propagated and purified as described
457 for *F. mariensis*, except a 46% sucrose cushion was used during ultracentrifugation. The pellets
458 were re-suspended in PBS and stored in an -80°C freezer. Three aliquots of each virus stock
459 were titrated to the end-point, which was calculated by the Reed-Muench method (30).

460 **Electron microscopy**

461 For transmission electron microscopy (TEM) assays, *V. vermiformis* cells were
462 infected/encysted and then were prepared for microscopy. Briefly, the medium was discarded
463 and the monolayer gently washed twice with 0.1 M phosphate buffer. Glutaraldehyde (2.5%,
464 v/v) was added to the system, followed by incubation for 1 h at room temperature for fixation.
465 The cells were then collected, centrifuged at 1200 x g for 10 min, the medium discarded, and the
466 cells were stored at 4 °C in phosphate buffer until electron microscopy analyses.

467 For the scanning electron microscopy (SEM) assays, the cells or virus particles were
468 prepared onto round glass blades covered by poly-L-lysine and fixed with 2.5% glutaraldehyde
469 in 0.1 M cacodylate buffer for 1 h at room temperature. Samples were then washed three times
470 with 0.1 M cacodylate buffer and post-fixed with 1.0% osmium tetroxide for 1 h at room
471 temperature. After a second fixation, the samples were washed three times with 0.1 M
472 cacodylate buffer and immersed in 0.1% tannic acid for 20 min. Samples were then washed in
473 cacodylate buffer and dehydrated by serial passages in ethanol solutions with concentrations
474 ranging from 35–100%. They were dried at the critical CO₂ point, transferred onto stubs, and
475 metalized with a 5 nm gold layer. The analyses were completed with scanning electronic
476 microscopy (FEG Quanta 200 FEI) at the Center of Microscopy of UFMG, Brazil.

477 **Genome sequencing and analyses**

478 The *F. mariensis* genome was sequenced using an Illumina MiSeq instrument (Illumina Inc.,
479 San Diego, CA, USA) with the paired end application. The sequence reads were assembled de
480 novo using ABYSS software and SPADES, and the resulting contigs were ordered by the
481 Python-based CONTIGuator.py software. The obtained draft genomes were mapped back to
482 verify the read assembly and close gaps. Open reading frames were predicted by GeneMarkS.
483 The tRNA genes were searched using the tRNAscan-SE and ARAGORN software. Predicted
484 proteins of less than 50 amino acids in length were discarded. Gene annotation was performed
485 using Blast2GO software (31). A BLASTp search against the NCBI non-redundant (nr)
486 database was performed, with hits being considered significant if e-values were lower than $1 \times$
487 10^{-3} . A BLASTp search was also performed with the same parameters against the cluster of
488 orthologous groups (COGs) of proteins of the nucleocytoplasmic large DNA viruses (NCVOG)
489 (32). In addition, we searched for conserved domains in different databases using Interproscan
490 implemented in Blast2GO software. The genome annotation and functional classification was
491 then manually revised and curated. Synteny analysis was performed using Mauve software (33).
492 The DNA polymerase tree was constructed using the maximum likelihood evolution method
493 and 1000 replicates, in MEGA 7.0 software (34).

494 **Viral cycle and amoebal encystment characterization**

495 The analysis of the cycle of *F. mariensis* by TEM was performed in the context of asynchronous
496 infection, at an MOI of 0.1. T175 flasks containing 40 million *V. vermiformis* were inoculated
497 with *F. mariensis*, and analyzed 24 hpi by TEM. A total of 250 images were evaluated in order
498 to investigate the major steps of viral cycle.

499 As mentioned, during *F.* replication we observed substantial formation and
500 accumulation of *V. vermiformis* cysts after infection, in especial when cells were infected at
501 high MOIs (1 and 10). Therefore, we verify whether other *V. vermiformis* -infecting viruses
502 would be able to trigger encystment as well. T125 flasks containing 40 million amoebas were

503 inoculated with *F. mariensis*, Tupanvirus or Orpheovirus at MOI of 10, and compared to
504 uninfected cells. After 24 hpi, trophozoites and cysts were quantified in a Neubauer chamber
505 (Kasvi, Brazil) and remaining cells were submitted to TEM to check whether viruses would be
506 imprisoned inside cysts. This experiment was performed three times in triplicates.

507 Considering that only *F. mariensis* were able to remain inside *V. vermiformis* cysts, a
508 new experiment was performed, in order to quantify cysts and trophozoites at different *F.*
509 *mariensis* MOIs: 0.01, 0.1, 1, and 10. A total of 3×10^6 trophozoites were added to T25 flasks
510 with 5 ml of PYG medium and inoculated at MOIs of 0.01, 0.1, 1, or 10. After adsorption, cells
511 were washed and then incubated for 48 hours with PYG medium. Uninfected cells were used as
512 control. Trophozoites and cysts were quantified in a Neubauer chamber (Kasvi, Brazil).

513 The next step was to measure *F. mariensis* replication in *V. vermiformis* inoculated at
514 different MOIs. Therefore, *V. vermiformis* cells were infected with *F. mariensis* at MOIs of
515 0.01, 0.1, 1, and 10, and incubated for 1 week. At this stage, trophozoites were no longer
516 observed, regardless the MOI, since they either were lysed (releasing viruses to supernatant) or
517 were converted to cysts. This longer time of incubation was designed to allow the lysis or
518 encystment of all trophozoites in the system; therefore, at the end of the experiments, we would
519 find viruses only in the supernatant (originated from the lysis of infected trophozoites) or inside
520 cysts. The supernatant and cysts were separated by centrifugation (1200 x g, 10 min), and viral
521 genome load was quantified by qPCR targeting the DNA polymerase gene
522 (5'AAAACGATTCCGTGCGCAA3' and 5'ACTAACATCGGGGCGGTTTT3'). The primers
523 were designed using a freely available primer design tool
524 (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) at the National Center for Biotechnology
525 Information, U.S.A (NCBI).

526 PCR was preceded by DNA extraction using the proteinase K/phenol-chloroform
527 method and used at the concentration of 50 µg/µg as a template for PCR assays. PCR assays
528 were performed using 1 µL of extracted DNA (~50 ng) in an amplification reaction mix

529 containing 5 μ L of SYBR[®] Green Master Mix and 0.4 μ L (10 μ M) of forward and reverse
530 primers. The final volume of the reaction was adjusted with ultrapure water to 10 μ L. The
531 conditions of the StepOne thermal cycler reactions (Applied Biosystem, USA) were: 95°C for
532 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min, which was followed by a
533 final step of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. As negative controls, we used
534 DNA extracted from uninoculated amoebas with purified viruses or samples, and as a positive
535 control, we used DNA from amoebae infected with purified virus. The relative quantification
536 was performed with the delta-Ct method, and results were presented as arbitrary units (log10).
537 This experiment was performed three times in triplicate.

538 **Excystment assays**

539 A stock of uninfected *V. vermiformis* cysts was produced by treating ten T175 flasks containing
540 amoebae with NEFF solution (19) for 3 days. These cysts were quantified and stocked at room
541 temperature. For assays of cysts containing virus, T175 flasks containing 40 million fresh
542 trophozoites of *V. vermiformis* were infected with *F. mariensis* at MOIs of 0.01, 0.1, 1, and 10.
543 One-week pi, remaining cysts were collected and washed 10 times with PBS, which was
544 followed by 1200 x g centrifugation (10 min) to remove external viral particles. Cysts were then
545 quantified in a Neubauer chamber, and then 10⁵ cysts had their excystment competence
546 evaluated after inoculation in T25 flasks containing PYG media with 5% fetal calf serum (FCS).
547 As an excystment control, 10⁵ non-infected cysts were added to a T25 flask containing PYG
548 media with 5% FCS. In parallel, another excystment protocol was tested: the non-nutrient agar
549 plate. Briefly, 1 mL of fresh heat-inactivated (99°C, 2 hours) *Escherichia coli* DH5-alpha (10⁹
550 cells/mL) was added to the surface of 1% non-nutrient agar (Merck, Germany) and spread with a
551 sterile glass Drigalski hook. After drying at room temperature, the bacterial monolayer was
552 inoculated in eleven equidistant spots with *V. vermiformis* cysts produced at different MOIs, for
553 a total of 10⁵ cysts per agar plate. As an excystment control, 10⁵ non-infected cysts were added
554 to a control plate. The excystment rate was calculated after 3 days, by quantifying 1000 cells,
555 three times, in triplicate.

556

557 To investigate whether cells under excystment had viruses inside of them, fresh
558 excysted trophozoites from MOIs of 0.01 and 0.1 (excystment at MOIs of 1 and 10 was not
559 observed) were individually collected from agar plates with sterile tips. The cells were
560 transferred to 200- μ l micro tubes, washed 3 times with 10 μ l PBS, centrifuged at 1200 x g for
561 10 min, and subcultivated onto agar non-nutrient plates containing fresh heat-inactivated (99°C,
562 2 h) *Escherichia coli*. Cells were observed for 5 days to determine the occurrence of any
563 cytopathic effect or encystment. After reaching 80% confluence on the agar plate, trophozoites
564 were transferred to T25 flasks containing PYG medium, and then the presence/replication of the
565 virus was tested by qPCR (targeting the *F. mariensis* DNA polymerase gene) during five
566 subcultivations.

567

568 To investigate whether cells under excystment had *F. mariensis* inside their cytoplasm,
569 T175 flasks containing 40 million fresh trophozoites of *V. vermiformis* were infected with *F.*
570 *mariensis* at MOIs of 0.01, 0.1, 1, and 10 (three flasks per MOI). One-week pi, remaining cysts
571 were collected and washed 10 times with PBS followed by 1200 x g centrifugation (10 min) to
572 remove external viral particles. Cysts were then prepared for TEM and SEM. In parallel, those
573 cysts were inoculated in T175 flasks containing fresh PYG medium supplemented with 5%
574 FCS, and after 24 h, cells were prepared for TEM. The viability of cysts produced at different
575 MOIs was assayed with 0.4% trypan blue (Sigma, U.S.A.), in a Neubauer chamber.

576

577 For cyst resistance assays, a total of 10^6 cysts produced at MOIs of 1 and 10 were
578 submitted to 4% hydrochloric acid heated (99°C) treatments for 6, 12, 24, 48, 60, and 72 h,
579 followed by 3 cycles of sonication at 50 kHz (30 s each), washed three times with PBS and then
580 quantified in a Neubauer chamber. As a control, we used cysts produced under non-infectious
581 conditions (NEFF). Those samples were also prepared for TEM, with the aim of analyzing the
582 presence and appearance of cyst walls.

583

584 Circumvention of encystment analyses

585 To evaluate whether *F. mariensis* or Tupanvirus were able to circumvent *V. vermiformis*
586 encystment, T25 flasks containing 4 million trophozoites were treated with a 5 ml NEFF
587 solution, and 2 hours later, this solution was removed. The purified viruses (*F. mariensis* or
588 Tupanvirus) were inoculated at a MOI of 10, 5 ml PYG medium were added to the flasks, which
589 were then incubated for 24h at 28°C. Virus genome replication was assayed by qPCR (targeting
590 the DNA polymerase for both viruses) (Tupanvirus primers:
591 5'TCACTGGTTCGTCATGCACT3' and 5'TCGCTTTGAGAGGTTTGGCT3') and cells were
592 analyzed by TEM and optical microscopy. Cell viability was analyzed with 0.4% trypan blue in
593 a Neubauer chamber. Alternatively, we inoculated T25 flasks containing 4 million *V.*
594 *vermiformis* trophozoites with *F. mariensis* or Tupanvirus at a MOI of 10; after 2 h we treated
595 the cells with 5 ml NEFF solution, and then cells were incubated for 24 h, at 28°C.

596

597 Using qPCR, we measured the expression of two serine proteinase mRNA isotypes
598 present in *V. vermiformis* upon infection (MOI of 10) with *F. mariensis* or Tupanvirus or NEFF
599 treatment. T25 flasks containing 4 million trophozoites were inoculated, and 5 h post-infection,
600 cells were collected and submitted to RNA extraction using the RNAeasy Mini Kit (Qiagen).
601 The samples were treated with DNase (Invitrogen) and reverse transcribed using M-MLV
602 Reverse Transcriptase (200 U/L; Thermo Fisher Scientific), according to the manufacturer's
603 instructions. The resulting cDNAs were used as a template in a StepOne thermocycler (Applied
604 Biosystems) quantitative polymerase chain reaction (qPCR) assay to target two *V. vermiformis*
605 serine proteinase mRNA isotypes (5'GACTGGTGGACGAGCTATGG`3 and
606 5'TCTAGGCTCGTCAAGTCCCA`3; 5'GCTAAATCGTTCACCGTGGC`3 and
607 5'CAACGCGTATTCATCGGCTG`3). PCR assays were performed using 1 µL of extracted
608 DNA (50 ng) in an amplification reaction mix containing 5 µL of SYBR® Green Master Mix
609 and 0.4 µL (10 µM) of forward and reverse primers. The final volume of the reaction was
610 adjusted with ultrapure water to 10 µL. The conditions of the StepOne thermal cyclers reactions

611 (Applied Biosystem, USA) were: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and
612 60°C for 1 min, which was followed by a final step of 95°C for 15 s, 60°C for 1 min and 95°C
613 for 15 s. The relative quantification was performed with the delta-Ct method, and results were
614 presented as arbitrary units (log10). This experiment was performed three times in triplicate.

615 **Analyses of secreted factors involved in encystment**

616 To investigate whether *V. vermiformis* cells infected by *F. mariensis* secrete factors were
617 involved with the encystment, the supernatant of a culture infected at a MOI of 10 was collected
618 4 days pi (T175 flask, 40 million trophozoites). The supernatant was filtered with a 0.1-µm filter
619 (Millipore, Brazil) to remove all viral particles and subsequently diluted 2-fold for inoculation
620 in T25 flasks containing 4 million fresh *V. vermiformis* trophozoites. Similarly, supernatants
621 from *V. vermiformis* cells infected by *F. mariensis* at MOIs of 0.01, 0.1, and 1 were also
622 prepared, collected, filtered, and inoculated (undiluted) in T125 flasks containing 40 million
623 fresh *V. vermiformis* trophozoites. To comprehend the nature *V. vermiformis* factors related to
624 encystment, the supernatant was treated with different concentrations of proteinase K (Gibco,
625 U.S.A.) (10, 20, 30, 40 mg/ml, 2h incubation, room temperature) followed by bromelain from
626 pineapple stems (Sigma, U.S.A; 45 mg/ml, 24h incubation, room temperature) and enzyme
627 inactivation. Bovine serum albumin (BSA) was used as the protease k/bromelain activity
628 control. Treated supernatants were then inoculated into T25 flasks containing 4 million fresh *V.*
629 *vermiformis* trophozoites, and their activity was evaluated regarding cyst formation.

630

631 The concentrations of potassium, calcium, and magnesium in the supernatants of cells
632 infected with *F. mariensis*, Tupanvirus, and uninfected cells (control) were also assayed. For
633 this, 96 well plates containing 4×10^4 *V. vermiformis* trophozoites were inoculated, and at 10
634 hpi, the concentrations of potassium, calcium, and magnesium were measured with a Potassium
635 Assay Kit AB102505 (MyBioSource, U.S.A.), a Calcium Detection Assay kit (Abcam, U.S.A.)
636 and a Magnesium Assay Kit (Sigma, U.S.A.), respectively, following manufacturer instructions.
637 To test the potential of Mg^{2+} as an encystment inductor in *V. vermiformis*, a total of 3 nmol of

Mg²⁺ (MgCl₂, Merck, Germany) was put in a culture of 4 x 10⁴ amoeba trophozoites, and the rate of cyst formation from 3–12 h was measured as well as the concentration of Mg²⁺ (using a Magnesium Assay Kit, Sigma, U.S.A.). The effect of ethylenediaminetetraacetic acid (EDTA) (Merck, Germany) on cyst formation during *F. mariensis* infection was evaluated. For this, T25 flasks containing 4 million trophozoites were infected with *F. mariensis* at a MOI of 10, and 2 hours post infection, 10 mM EDTA were added. Flasks were incubated for 24 hours at 28°C, followed by cyst counting and virus quantification by qPCR (targeting the DNA polymerase gene). Results were compared to an EDTA-free control. For evaluate the effect of EDTA on the inhibition of cyst formation induced by the supernatants of pre-infected trophozoites, T25 flasks containing 4 million trophozoites were infected at MOIs of 0.01, 0.1, 1, and 10. After 24 hours, the supernatants were collected, filtered (0.1 µm), and then inoculated into new T25 flasks containing 4 million fresh amoebae. One hour post infection, 10 mM EDTA was added, and at 16 hpi, cysts and trophozoites were quantified in a Neubauer chamber. Results were compared to EDTA-free controls, corresponding to each MOI evaluated.

Accession number

The genome sequence of Fautovirus mariensis has been submitted to Genbank under accession number MK506267.

Acknowledgements

We are grateful to our colleagues from Laboratório de Vírus of Universidade Federal de Minas Gerais. In addition, we thank CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), FAPEMIG (Fundação de Amparo à Pesquisa do estado de Minas Gerais), Ministério da Saúde (MS-DECIT) and the Microscopy Center of UFMG. F.G.F; E.G.K and J.S.A are CNPq researchers. E.G.K.; B.L.S. and J.S.A. are members of a CAPES-COFECUB project.

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- 757

758 **Figure legends**

759 **Figure 1: Faustovirus mariensis isolation sites, particle images, and cytopathic effects. (A)**
760 Pampulha Lagoon map with collection sites highlighted (dots). The yellow dot represents where
761 *F. mariensis* were collected, in front of the Pampulha Art Museum (top-right of photo). **(B-D)** *F.*
762 *mariensis* viral particles visualized by scanning **(B and C)** and transmission electron
763 microscopies. **(E-G)** Plaque forming unit (PFU) induced by *F. mariensis* infection in a
764 *Vermamoeba vermiformis* monolayer. In **(F)**, a close-up of a PFU shown in **(E)**, observed 24
765 hours post infection. Forty-eight hours post infection the PFUs expand and coalesce, as shown
766 in **(G)**.

767

768 **Figure 2: Gene categories and phylogeny. (A)** *F. mariensis* gene set classified according gene
769 predicted categories. **(B)** DNA polymerase subunit B tree, constructed using the maximum
770 likelihood evolution method and 1000 replicates. Faustovirus mariensis (pentagon) clusters with
771 other Faustovirus strains. Tree scale represents evolutionary distance.

772 **Figure 3: Electron-lucent viral factory and cytoplasmic modifications induced by**
773 **Faustovirus mariensis. (A-C)** *F. mariensis* presents an electron-lucent viral factory (contoured
774 in red and in detail **(B)**) not easily distinguished from the rest of the cytoplasm and observed at
775 the perinuclear region. It is possible to visualize the abundant presence of mitochondria

surrounding the viral factory (purple highlighted (A and C)). VF: Viral factory. N: Nucleus. Image B was obtained by TEM and graphically highlighted by using IOS image visualization software.

Figure 4: Faustovirus mariensis morphogenesis and particles organization in honeycombs-like structures. (A) *F. mariensis* morphogenesis begins with crescents, open structures of approximately 50 nm, which grow as an electron-dense material of the viral factory fulfills them. Particles of almost 200 nm without genomic content are observed in late phases of morphogenesis, when the genome is incorporated and centralized within several newly formed viral particles. (B-E) *F. mariensis* progeny are organized in a honeycomb fashion inside viral factories. Small honeycombs expand as new mature viruses are formed and coalesce to others in the cytoplasm (B).

Figure 5: Faustovirus mariensis particles and viral factories inside *Vermamoeba vermiformis* cysts. (A) Mature *V. vermiformis* cyst enclosing a large viral factory (highlighted with a red dashed line). In (B), plasma membrane is clearly visible below the thick cyst wall. For this experiment, *V. vermiformis* cells were infected with *F. mariensis* at MOI of 10 and prepared for transmission electron microscopy (TEM) (24 hpi).

Figure 6: *Vermamoeba vermiformis* cysts enclosing Faustovirus mariensis in different stages of viral replication cycle (A-F). The observation of more than one hundred cysts revealed the presence of *F. mariensis* under distinct phases of the replication cycle, including the early viral factory formation (A-B), late morphogenesis/ honeycomb formation/coalescence (C-E) and cytoplasm fulfilled by mature viral particles (F).

Figure 7: Cyst formation and viral replication at different multiplicities of infection (MOI). (A) Cyst and trophozoite quantification 48 hours after the inoculation of *F. mariensis* at MOIs of 0.01, 0.1, 1, and 10. The dashed line represents the input of amoebae in the beginning of the experiment (3×10^6). Uninfected cells had a natural encystment rate of 21.1%. (B) *F.*

803 mariensis genome quantification in the supernatants and cysts of *V. vermiformis* inoculated at
804 different MOIs, 1 week post inoculation. The supernatants and cysts were separated by
805 centrifugation and the viral genome load was quantified by qPCR (DNA polymerase subunit B).
806 Error bars indicate SDs. These experiments were performed three times in triplicate.

807

808 **Figure 8: Excystment assays.** Cysts were produced by the inoculation of *Vermamoeba*
809 *vermiformis* trophozoites with Faustovirus mariensis at MOIs of 0.01, 0.1, 1, and 10, and then
810 their excystment potential was evaluated. (A) Agar plate excystment assay demonstrating that
811 few cysts obtained from infections at MOIs of 0.01 and 0.1 were able to become trophozoites
812 (arrows). Excystment was not observed for cysts obtained from infections at MOIs of 1 and 10.
813 (B) Viability of cysts produced from infections at different MOIs (trypan blue 0.4%). Error bars
814 indicate SDs. (C) Transmission electron microscopy representative image demonstrating that
815 only uninfected cysts are able to excyst. This image corresponds to cysts obtained from
816 infections at a MOI of 0.01, after excystment stimulus. (D-E) Cysts produced after infection can
817 present either a regular shape (D) or reduced diameters and irregular shapes (E). These
818 experiments were performed three times in triplicate.

819

820 **Figure 9: Faustovirus mariensis is not able to circumvent *Vermamoeba vermiformis***
821 **encystment.** (A) Scheme highlighting the experimental strategy and results of an experiment
822 testing the ability of F. mariensis and Tupanvirus to circumvent *V. vermiformis* encystment. In I
823 and III, the encystment solution NEFF was added prior to virus inoculation. In II and IV, the
824 viruses were inoculated before NEFF addition. (B) F. mariensis and (C) Tupanvirus genome
825 quantification, in supernatant and cysts, for each experimental scenario analyzed (I–IV), by
826 qPCR. The relative quantification was performed with the delta-Ct method, and results were
827 presented as arbitrary units (log10). (D) Quantification of the viability of cysts (trypan blue
828 0.4%) produced from infections under different scenarios (I–IV). (E) Relative quantification of

829 the expression of two serine proteinase mRNA isotypes present in *V. vermiformis* upon
830 infection (MOI of 10) with *F. mariensis*, Tupanvirus, or NEFF treatment. The quantification
831 was performed 5 hours post infection or NEFF inoculation. Error bars indicate SDs. The
832 statistical significance was calculated using a two-tailed 2-way ANOVA test and Tukey's range
833 test, using GraphPad Prism. *** $p < 0.001$. These experiments were performed three times in
834 triplicate.

835

836 **Figure 10: Investigation of encystment factors secreted by *Vermamoeba vermiformis*.** (A)

837 Induction of encystment (%) in *V. vermiformis* cells caused by the virus-free supernatant from a
838 previous *F. mariensis* infection of a *V. vermiformis* culture (MOI of 10). Supernatant was
839 diluted two-fold from undiluted to 1/64. (B) Induction of encystment (%) of *V. vermiformis*
840 cells caused by undiluted (pure) supernatant from a previous *F. mariensis* infection in *V.*
841 *vermiformis* culture at different MOIs. (C–E) Quantification of the concentration of (C) K^+ , (D)
842 Ca^{2+} and (E) Mg^{2+} in the supernatants of cells infected (MOI of 10, 10 hpi) with *F. mariensis*,
843 Tupanvirus, and uninfected cells (control). The statistical significance was calculated using a
844 two-tailed 2-way ANOVA test and a Tukey's range test, using GraphPad Prism. *** $p < 0.001$.
845 (F) Evaluation of the potential of Mg^{2+} as an inductor of encystment in *V. vermiformis*. A total
846 of 3 nmol of Mg^{2+} was added to a culture of 4×10^4 amoeba trophozoites, and the concentration
847 of Mg^{2+} was measured, as well as the rate of cyst formation over time. (G and H) Evaluation of
848 EDTA effect on (G) cyst formation and (H) virus genome replication during *F. mariensis*
849 infection, at a MOI of 10. (I) Evaluation of the inhibitory activity of 10 mM EDTA on the
850 encystment process induced by supernatants of *V. vermiformis* infected by *F. mariensis* at
851 different MOIs, 24 hours post inoculation. For all graphs, error bars indicate SDs. These
852 experiments were performed three times in triplicate. (J) Scheme summarizing the phenomenon
853 described in this work. Once infected by *F. mariensis*, a *V. vermiformis* trophozoite can be
854 lysed, as described for other amoebal giant viruses. However, during the first events of infection
855 in a *V. vermiformis* population, encystment factors are released into the supernatant, which can

856 induce the encystment of uninfected cells or even cause the encystment of infected trophozoites.
857 As result, we found cysts under different stages of the replication cycle with no excystment
858 ability. The overall viral load in the supernatant is controlled by *V. vermiformis* trapping a
859 substantial amount of viral progeny inside cysts.

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