

1 **An intestinally secreted host factor limits bacterial colonization but promotes microsporidia**
2 **invasion of *C. elegans***

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14

15 **Abstract**

16 Microsporidia are ubiquitous obligate intracellular pathogens of animals. These parasites often
17 infect hosts through an oral route, but little is known about the function of host intestinal proteins
18 that facilitate microsporidia invasion. To identify such factors necessary for infection by
19 *Nematocida parisii*, a natural microsporidian pathogen of *Caenorhabditis elegans*, we performed
20 a forward genetic screen to identify mutant animals that have a Fitness Advantage with
21 *Nematocida* (Fawn). We isolated four *fawn* mutants that are resistant to *Nematocida* infection and
22 contain mutations in *T14E8.4*, which we renamed *aaim-1* (Antibacterial and Aids invasion by
23 Microsporidia). Expression of AAIM-1 in the intestine of *aaim-1* animals restores *N. parisii*

24 infectivity and this rescue of infectivity is dependent upon AAIM-1 secretion. *N. parisi* spores in
25 *aaim-1* animals are improperly oriented in the intestinal lumen, leading to reduced levels of
26 parasite invasion. Conversely, *aaim-1* mutants display both increased colonization and
27 susceptibility to the bacterial pathogen *Pseudomonas aeruginosa* and overexpression of *AAIM-1*
28 reduces *P. aeruginosa* colonization. Competitive fitness assays show that *aaim-1* mutants are
29 favoured in the presence of *N. parisi* but disadvantaged on *P. aeruginosa* compared to wild type
30 animals. Together, this work demonstrates how microsporidia exploits an antibacterial immune
31 protein to promote host invasion. Our results also highlight the evolutionary trade-offs that exist
32 to optimizing host defense against multiple classes of pathogens.

33

34 **Introduction**

35

36 Microsporidia are a large group of obligate intracellular parasites that infect most types of
37 animals.¹ These ubiquitous parasites possess the smallest known eukaryotic genome size, and are
38 extremely reliant on their host as a result of the loss of many genes involved in metabolism and
39 energy production.^{2,3} Microsporidia can have a large impact on the evolution of their hosts, as
40 infection with microsporidia often leads to a reduction in host offspring and the effect of this
41 selective pressure has resulted in resistant animals within a population.^{4,5} Microsporidia are
42 currently a major threat to many commercially important species such as honeybees and shrimp.^{6,7}
43 Many species also infect humans and infections in immunocompromised individuals can result in
44 lethality.⁸ Despite their ubiquitous nature, effective treatment strategies are currently lacking for
45 these poorly understood parasites.⁹

46

47 Microsporidia infection begins with invasion of host cells. They possess the most fascinating
48 invasion machinery, a unique structure known as the polar tube.¹⁰ This apparatus, resembling a
49 long thread, is often coiled within a dormant spore. However, once inside of a host, and in
50 proximity to the tissue of interest, the polar tube rapidly emerges or “fires” , releasing its the
51 parasites infectious material (the sporoplasm) which is deposited intracellularly either through
52 direct injection, or through the internalization of the sporoplasm.^{10,11}

53
54 A number of microsporidia proteins have been demonstrated to play important roles during
55 invasion by insect- and human-infecting species of microsporidia.¹⁰ For example, spore wall
56 proteins can interact with host cells through the recognition of sulfated glycosaminoglycans,
57 heparin binding motifs, integrins, and proteins on the cell surface.¹²⁻¹⁷ In *Encephalitozoon* species
58 polar tube proteins (PTP) can mediate interactions with the host. For instance, O-linked
59 mannosylation on PTP1 has been demonstrated to bind mannose binding receptors, whereas PTP4
60 interacts with the transferrin receptor (Trf1).^{11,18-20} Additionally, the sporoplasm surface protein,
61 EhSSP1, binds to an unknown receptor on the cell surface.²¹ These proteins on the spore, polar
62 tube, and sporoplasm have all been shown to promote microsporidia adhesion or invasion of host
63 cells in culture systems, but the role of these proteins during animal infection is unclear.

64
65 The nematode *Caenorhabditis elegans* is infected in its natural habitat by several species of
66 microsporidia, and frequently by *Nematocida parisii*.²²⁻²⁴ This species infects the intestinal cells
67 of *C. elegans*, which possess extreme similarity to those of mammalian cells, making it both a
68 relevant tissue and model to study these infections in vivo.^{24,25} Infection of *C. elegans* by *N. parisii*
69 begins when spores are consumed by the worm, where they then pass through the pharynx into the

70 intestinal lumen and fire, depositing sporoplasms inside of intestinal cells. Within 72 hours the
71 sporoplasm will divide into meronts, which differentiate into spores, that then exit the animal,
72 completing the parasite's life cycle.^{26,27} Infection with *N. parisii* leads to reduced fecundity and
73 premature mortality^{24,26} Several mutants have been shown to affect proliferation and spore exit.^{28,29}
74 Immunity that can either prevent infection or clear the pathogen once infected have also been
75 described.^{4,27,30-32} In contrast, very little is known about how *N. parisii* invades *C. elegans*
76 intestinal cells. Almost all of the microsporidia proteins known to facilitate invasion are not
77 conserved in *N. parisii* and although host invasion factors described in other species are present in
78 *C. elegans*, there is no evidence that they are being used by microsporidia during invasion of *C.*
79 *elegans*.¹¹

80
81 To understand how microsporidia invade animal cells, we performed a forward genetic screen to
82 identify host factors that promote infection. We identified a novel, nematode-specific protein,
83 AAIM-1, whose loss of function confers resistance to microsporidia infection. This protein is
84 expressed in intestinal cells, secreted into the intestinal lumen, and is necessary to ensure proper
85 spore orientation during intestinal cell invasion. In addition, we show that AAIM-1 limits bacterial
86 colonization of pathogenic *Pseudomonas aeruginosa*. Strikingly, T14E8.4 plays opposing roles on
87 host fitness in the face of pathogenesis. The utilization of a host factor critical for bacterial defense
88 reflects a clever strategy to ensuring microsporidia's reproductive success.

89

90 **Results**

91 **A forward genetic screen identifies *aaim-1* as being necessary for *N. parisii* infection.**

92 To identify host factors needed for infection by microsporidia, we carried out a forward genetic
93 screen using a *C. elegans* model of *N. parisii* infection. We took advantage of the previously
94 described phenotypes of *C. elegans* displaying reduced fitness when infected with *N. parisii*,
95 including lower progeny production and stunted development.^{26,27,33} We mutagenized animals and
96 subjected F2 progeny to *N. parisii* infection. After infecting populations for five subsequent
97 generations, we selected individual worms containing embryos, indicating increased fitness in the
98 presence of infection (see Methods). We identified four independent isolates that reproducibly had
99 higher fractions of animals containing embryos compared to wild type (N2). We named these
100 isolates Fitness Advantage With *Nematocida* (*fawn* 1-4) (Figure S1a).

101
102 We first determined if these *fawn* mutants were also resistant to *N. parisii* infection. We grew the
103 three isolates with the strongest phenotype, *fawn* 1-3, in the presence and absence of *N. parisii*,
104 and stained each population of worms with the chitin binding dye, Direct-yellow 96 (DY96), at 72
105 hours post infection (hpi). DY96 allows for the visualization of chitinous microsporidia spores as
106 well as worm embryos (Figure 1a). In the absence of infection, there is no difference in the fraction
107 of *fawn-2* and *fawn-3* animals developing into adults containing embryos (gravid adults), although
108 *fawn-1* has a modest defect. In comparison, all three *fawn* isolates generate significantly more
109 gravid adults than N2 animals in the presence of infection (Figure 1b). We next examined the
110 fraction of animals in each strain containing intracellular microsporidia spores and observed that
111 all three *fawn* isolates display significantly fewer numbers of spore-containing worms (Figure 1c).
112 These results suggest that *fawn* mutants are missing an important factor for efficient microsporidia
113 infection.

114

115 To identify the causal mutations underlying the Fawn phenotype, we used a combination of whole-
116 genome sequencing and genetic mapping. We generated F2 recombinants and performed two
117 rounds of infection with microsporidia, selecting for gravid animals. After each round we used
118 molecular inversion probes to determine the region of the genome linked to the causal mutation.³⁴
119 This revealed strong signatures of selection on the left arm of chromosome X in all three *fawn*
120 isolates absent in N2 (Figure S1b). Analysis of whole genome sequencing showed that all four
121 *fawn* isolates contained different alleles of *T14E8.4*, which we named *aaim-1* (Antibacterial and
122 Aids Invasion by Microsporidia-1) for reasons described below (Figure 1d). We validated the role
123 of *aaim-1* in resistance to infection using several additional alleles: an independent allele RB563
124 (*ok295*), carrying a large gene deletion in both *aaim-1* and *dop-3*, and a CRISPR-Cas9 derived
125 allele, *aaim-1 (kea22)*, that contains a large gene deletion. Both of these alleles displayed a fitness
126 advantage when infected with *N. parisii* (Figure 1d,e, S1c,d). These data demonstrate that *aaim-1*
127 is the causative gene underlying the *fawn* 1-4 infection phenotypes. In subsequent experiments we
128 utilized both *aaim-1 (kea22)*, and *fawn-3 (kea28)*, carrying a 2.2 kb deletion in *aaim-1*, which was
129 outcrossed to N2 six times.

130

131 ***aaim-1* is expressed in the pharynx and intestine, and secretion is important for function.**

132 AAIM-1 is a poorly characterized protein that does not possess any known or conserved domains.
133 Homologs of the protein exist in both free-living and parasitic nematodes (Figure S2). To further
134 characterize the role of AAIM-1 during *N. parisii* infection, we generated transgenic
135 extrachromosomal lines of *C. elegans* carrying a reporter strain of GFP under control of the *aaim-*
136 *1* promoter. GFP fluorescence was observed in the terminal bulb of the pharynx as well as the

137 posterior of the intestine throughout development (Figure 2a). Embryos and L1 animals display
138 additional expression in the arcade cells of the pharynx (Figure 2a, S3a).

139
140 The first 17 amino acids of AAIM-1 are predicted to encode a signal peptide.³⁵ This suggests that
141 AAIM-1 may be secreted into the pharyngeal and intestinal lumen, the extracellular space through
142 which *N. parisii* spores pass before invading intestinal cells. To test where AAIM-1 functions in
143 and if secretion is important for function, we generated a series of transgenic worms expressing
144 extrachromosomal arrays (Supplemental table 1). First, we generated transgenic *aaim-1 (kea22)*
145 animals expressing AAIM-1 tagged on the C-terminus with a 3x Flag epitope. Transgenic animals
146 expressing AAIM-1 under its native promoter complement the ability of *aaim-1 (kea22)* animals
147 to develop into adults in the presence of a high amount of *N. parisii* spores (Figure 2b). A construct
148 expressing GFP or GFP::3xFlag does not influence this phenotype nor does the presence of the
149 epitope tag impair the ability of AAIM-1 to rescue the mutant phenotype (Figure S3b). We next
150 generated a signal peptide mutant allele of AAIM-1 missing the first 17 amino acids (*SPΔaaim-*
151 *1*), which is unable to complement the *aaim-1 N. parisii* infection phenotype. In contrast, AAIM-
152 1 expressed from an intestinal specific promoter can rescue the infection phenotype of *aaim-1*
153 (*kea22*) (Figure 2b).

154
155 To determine where AAIM-1 localizes, we dissected the intestines from transgenic worms and
156 performed immunofluorescence using anti-Flag antibodies. We were unable to detect expression
157 of AAIM-1::3xFlag when expressed from its endogenous promoter. However, we observed protein
158 expression in the intestinal cells of animals expressing AAIM-1::3xFlag from a strong, intestinal
159 specific promoter or when the signal peptide was removed (Figure 2c).³⁶ We did not observe

160 AAIM-1::3xFlag localized in the extracellular space of the intestinal lumen, possibly due to rapid
161 turnover of intestinal contents or due to loss from dissection of the intestines.³⁷ The increased
162 expression in the signal peptide mutant suggests an accumulation of protein that is unable to be
163 secreted. Taken together, these data demonstrate that AAIM-1 is secreted and acts within the
164 intestinal lumen to promote *N. parisii* infection.

165

166 **AAIM-1 is only necessary for microsporidia infection at the earliest larval stage.**

167 *N. parisii* infection of *C. elegans* can occur throughout development, but several forms of
168 immunity towards microsporidia have been shown to be developmentally regulated.^{4,27} To
169 determine if *aaim-1* mutant animals display developmentally restricted resistance to infection, we
170 infected *fawn* 1-3 at the L1 and L3 stage. For these experiments we took advantage of another
171 intestinal-infecting species of microsporidia, *Nematocida ausubeli*, which has a more severe effect
172 on *C. elegans* fecundity, allowing us to determine fitness defects after the L1 stage.^{4,23,26} *fawn*
173 isolates are resistant to *N. ausubeli* as seen by an increase in the fraction of gravid adults in the
174 population after exposure to a medium dose of *N. ausubeli* (Figure 3a). When we initiated
175 infections at the L3 stage of growth, *fawn* isolates do not have increased resistance, and instead
176 exhibit wild type levels of susceptibility (Figure 3b). To rule out the possibility that this L1
177 restricted phenotype was the result of exposure to sodium hypochlorite treatment, used to
178 synchronize worms, we exposed embryos that were naturally laid by adults within a two-hour
179 window to *N. parisii* infection. Animals synchronized in this manner still display a robust resistance
180 to *N. parisii* (Figure S4c). Thus, resistance to infection in *aaim-1* mutants is developmentally
181 restricted and AAIM-1 is utilized by multiple different species of microsporidia.

182

183 **AAIM-1 is needed for efficient invasion of intestinal cells**

184 Resistance to infection could be the result of a block in invasion, proliferation, or through the
185 destruction of the parasite. To test the mechanism of resistance in *aaim-1* mutants, we performed
186 pulse-chase infection assays at the L1 and L3 stage of development.^{4,27} Here, we treated animals
187 with a medium-1 dose of *N. parisii* for 3 hours, washed away any un-ingested spores, and then
188 replated the animals in the absence of spores for an additional 21 hours. We then used an 18S RNA
189 FISH probe to detect *N. parisii* sporoplasms, which is the earliest stage of microsporidia invasion.
190 In our *fawn* 1-3 isolates we detect less invasion at 3hpi compared to N2 (Figure S4a). However,
191 there was no reduction in the number of infected animals between 3hpi and 21 hpi, indicating that
192 pathogen clearance was not occurring. This defect in invasion was not present at the L3 stage,
193 providing further support that resistance is restricted to the L1 stage in *aaim-1* mutants (Figure
194 S4b). A reduction in invasion could be due to a feeding defect, leading to a reduction in spore
195 consumption. To test rates of consumption, we measured the intestinal accumulation of fluorescent
196 beads. We find that *aaim-1* alleles displayed wild-type levels of bead accumulation, unlike the
197 feeding defective strain *eat-2 (ad465)* (Figure S4d).

198

199 For *N. parisii* to invade host cells, spores must first enter the intestinal lumen and fire their polar
200 tube.²⁷ To test if *aaim-1* mutants have defects in spore entry or spore firing, we infected animals
201 for either 45 minutes or 3 hours, at the L1 and L3 stages. We then fixed and stained animals with
202 both *N. parisii* 18S RNA FISH probe and DY96 and quantified the number of spores present in
203 the intestinal lumen of animals. Here, *aaim-1* animals infected for 45 minutes or 3 hours at L1 or
204 L3 contained similar amounts of spores as N2 animals (Figure 3 c,f, S5a,d). The percentage of
205 fired spores present within these animals is also not significantly different at either developmental

206 stage (Figure 3d,g, S5b,e). We then counted the number of sporoplasms per animal and observed
207 significantly fewer invasion events in *aaim-1* mutant animals infected at L1 (Figure 3e, S5c). In
208 contrast the number of sporoplasms in L3 stage *aaim-1* alleles are similar to that observed in the
209 N2 strain (Figure 3h, S5f). These results demonstrate that the *N. parisii* invasion defect in *aaim-1*
210 mutants is not caused by differences in spore firing or accumulation. Instead, these results suggest
211 that spores are misfiring, leading to unsuccessful invasion.

212

213 **AAIM-1 plays a role in promoting proper spore orientation**

214 To determine how AAIM-1 promotes *N. parisii* invasion, we further examined the invasion
215 process. We pre-stained spores with Calcofluor white (CFW) and assessed their orientation relative
216 to the intestinal apical membrane in L1 worms infected for 45 minutes (Figure 4a). In N2 animals,
217 32.4% of spores are angled relative to the apical membrane. In contrast, spores in *aaim-1* alleles
218 were angled 14.3% of the time (Figure 4b). Other host factors that promote microsporidia invasion
219 cause adherence to host cells.¹¹ To determine if AAIM-1 influences the location of spores relative
220 to intestinal cells in *aaim-1* mutants, we measured the perpendicular distance from the center of a
221 parallel spore to the apical membrane of the intestine. Surprisingly, parallel spores in *aaim-1*
222 alleles were significantly closer to the apical membrane (0.29 μm) than those in N2 (0.34 μm)
223 (Figure 4c). In agreement with resistance being developmentally restricted, *aaim-1* mutants display
224 wild-type spore orientations and distances from the membrane when infections were initiated at
225 L3 (Figure 4d,e). The width of the intestinal lumen at L1 does not differ significantly between N2
226 and *aaim-1* mutants, however, L3 animals generally possess wider intestinal lumens (Figure
227 S5g,h). Thus, taken together these results suggest that AAIM-1 plays a distinct role in the intestinal

228 lumen at L1 to promote proper spore orientation, through maintaining an appropriate distance and
229 angle to the apical membrane, resulting in successful invasion.

230

231 ***AAIM-1* inhibits intestinal colonization by *Pseudomonas aeruginosa***

232 Interestingly, *aaim-1* has been shown to be upregulated by a variety of different fungal and
233 bacterial pathogens, including *P. aeruginosa*.^{38,39} Using our transcriptional reporter strain, we
234 sought to confirm this and determine if microsporidia infection could also induce *aaim-1*
235 transcription. N2 animals carrying a transcriptional reporter (*paaim-1::GFP::3xFlag*) were exposed
236 to *N. parisii*, *P. aeruginosa* PA14, or *E. coli* OP50, and the levels of GFP quantified when grown
237 on these pathogens for 72 hours from the L1 stage, or for 24 hours from the L4 stage. Infection by
238 either *N. parisii* or *P. aeruginosa* PA14 resulted in the upregulation of *aaim-1* as detected by an
239 increase in the GFP signal (Figure 5a, S6e).

240

241 Previously, an *aaim-1* deletion strain, RB563 (*ok295*), was shown to display reduced survival on
242 lawns of *P. aeruginosa* PA14.⁴⁰ The enhanced susceptibility previously reported was attributed to
243 *dop-3*, which is also partially deleted in RB563 (*ok295*).⁴⁰ To determine if *aaim-1* mutants are
244 susceptible to pathogenic bacterial infection, we assayed the survival of L4 stage worms in *P.*
245 *aeruginosa* PA14 slow killing assays was quantified. Here, *aaim-1* alleles displayed reduced
246 survival compared to N2, indicating that *aaim-1* mutants have enhanced susceptibility to PA14
247 (Figure 5b, S6a,b).

248

249 Lethality in slow killing assays is a result of *P. aeruginosa* accumulation within the intestinal
250 lumen.^{41,42} To investigate if *aaim-1* alleles displayed higher levels of bacterial burden, animals

251 grown on lawns of PA14::DsRed at the L1 or L4 stage for 48 hours. *aaim-1* mutant alleles grown
252 on lawns of PA14::DsRed as L4s, but not L1s, displayed higher bacterial burden relative to N2
253 (Figure 5c, S6c,d). To test if intestinal expression of *aaim-1* was sufficient to limit bacterial
254 colonization, transgenic *aaim-1* (*kea22*) overexpressing AAIM-1::3xFlag from the endogenous or
255 an intestinal specific (*spp-5*) promoter were exposed to lawns of PA14::DsRed. When grown for
256 48 hours at the L1 or L4 stage, bacterial burden was significantly reduced, relative to N2 (Figure
257 5d, e). The results indicate that AAIM-1 plays a role in limiting bacterial colonization, and its loss
258 results in reduced survival due to hyper-colonization of the intestinal lumen.

259

260 **Fitness of *aaim-1* animals is dependent upon microbial environment**

261 To investigate how *aaim-1* alleles can influence population structure, we set up competitive fitness
262 assays. A *C. elegans* strain with a fluorescent marker (RFP::ZNFX1) was co-plated with N2 or
263 *aaim-1* mutants on *E. coli* OP50, *N. parisii* or *P. aeruginosa* PA14. Animals were grown for 8
264 days, such that the population was composed of adult F1s and developing F2s. On *E. coli* OP50,
265 there is equal representation of N2 and *aaim-1* mutants in the population (Figure 6a). This is
266 consistent with *aaim-1* mutants not having a developmental delay (Figure 1b) or a decrease in
267 longevity (Figure S7). In contrast, growth on *N. parisii* resulted in *aaim-1* alleles outcompeting
268 the N2 strain. Conversely, *aaim-1* mutants on *P. aeruginosa* PA14 did significantly worse, being
269 underrepresented in the population compared to N2 (Figure 6a). Interestingly, wild isolates of *C.*
270 *elegans* do not carry any obvious loss of function alleles of *aaim-1* suggesting that natural
271 conditions have selected for its retention (Figure S8).⁴³

272

273 Given the opposing fates of *aaim-1* mutants on *N. parisii* and *P. aeruginosa*, we investigated the
274 effects of co-infection. Animals were infected with a maximal dose of *N. parisii* for 3 hours, prior
275 to placement on lawns of PA14. For infections with a single pathogen, we observed similar results
276 as before whereby *aaim-1* mutants have increased fitness in the presence of *N. parisii* and display
277 lower levels of parasite burden but have increased bacterial accumulation when grown on PA14.
278 In the presence of both pathogens, populations of *aaim-1* mutants display fewer gravid adults and
279 increased amounts of *N. parisii* spores. (Figure 6b,c). These results suggests that coinfection with
280 *N. parisii* and *P. aeruginosa* has synergistically negative effects on the fitness of *C. elegans*.

281

282 **Discussion:**

283 To identify host factors needed for microsporidia infection, we isolated mutants from a forward
284 genetic screen that have a fitness advantage when challenged with *N. parisii* infection. This screen
285 identified mutants in the poorly understood protein AAIM-1 (previously T14E8.4). Here, we
286 demonstrate that this protein both promotes microsporidia invasion and limits colonization by
287 pathogenic bacteria. Although we were unable to visualize the localization of secreted AAIM-1,
288 our genetic and infection experiments strongly suggest that this protein acts in the intestinal lumen
289 where both microsporidia invasion and bacterial colonization both take place. The key role that
290 AAIM-1 plays in immunity is further exemplified by its transcriptional regulation in response to
291 infection (Figure 7).

292

293 The processes by which microsporidia invade host cells are poorly understood. We show that *N.*
294 *parisii* spores are often angled in wild-type *C. elegans*, suggesting that successful invasion requires
295 a particular spore orientation. In the absence of AAIM-1, spores are more often parallel to the

296 intestinal lumen, where spores may fire without the successful deposition of the sporoplasm inside
297 an intestinal cell. In contrast to previously described host and microsporidia proteins involved in
298 invasion, AAIM-1 does not appear to be involved in promoting adhesion to the surface of host
299 cells.^{10,11} Instead, AAIM-1 ensures an adequate distance of spores from the intestinal membrane,
300 possibly allowing spores to be able to properly orient themselves to ensure proper host cell
301 invasion. *N. parisii* spores are ~2.2 μm long by ~0.8 μm wide and the average width of the
302 intestinal lumen at the L1 stage is ~0.6 μm ²³. Therefore, at the L1 stage spores may not be able to
303 move freely, but at the L3 stage, where AAIM-1 is not needed for invasion, there is less of a
304 constraint on spore movement as the luminal width increases to ~1.3 μm . Together, our results
305 highlight the power of studying microsporidia invasion in the context of a whole animal model.

306
307 *C. elegans* employs a variety of proteins to protect against bacterial infection. Many of these
308 proteins belong to several classes of antimicrobial effectors used to eliminate and prevent
309 colonization by pathogenic bacteria⁴⁴, are upregulated upon infection and predicted to be
310 secreted.^{45,46} One class of secreted proteins that are known to have immune functions and prevent
311 bacterial adherence are the mucins. These large, glycosylated secreted proteins are upregulated
312 during *C. elegans* infection and their knockdown alters susceptibility to *P. aeruginosa* infection.
313^{47,48} AAIM-1 has many predicted mucin-like O-glycosylation sites on serine and threonine
314 residues.⁴⁹⁻⁵¹ Thus one possibility is that AAIM-1 may be functionally analogous to mucins,
315 preventing the adhesion of microbes to the surface of intestinal cells. As AAIM-1 does not contain
316 any known or conserved domains and further work will be necessary to determine its exact
317 biochemical function.

318

319 *C. elegans* lives in a microbially dense environment containing a wide variety pathogens that *C.*
320 *elegans* has evolved immunity towards.^{23,52–55} Although loss of *aaim-1* provides a fitness
321 advantage to *C. elegans* when grown in the presence of microsporidia, obvious loss of function
322 alleles are not present in wild isolates sequenced thus far. Additionally, *aaim-1* mutants do not
323 have observable defects when grown on non-pathogenic *E. coli*. This is in contrast to mutations in
324 *pals-22* or *lin-35*, which negatively regulate the transcriptional response to infection and provide
325 resistance to microsporidia infection when mutated, but at the cost of reduced reproductive
326 fitness^{27,56}. Loss of *aaim-1* disadvantages *C. elegans* when grown on *P. aeruginosa*, demonstrating
327 that there is a trade-off in host defense between microsporidia and pathogenic bacteria. The
328 opposing functions of *aaim-1* with different pathogens adds to the limited set of known examples
329 of trade-offs that constrain the evolution of host defense to multiple biotic threats^{57,58}

330

331 **Methods**

332

333 **Strain maintenance**

334 *C. elegans* strains were grown at 21°C on nematode growth media (NGM) plates seeded with 10x
335 saturated *Escherichia coli* OP50-1.²⁷ Strains used in this study are listed in Supplemental table 1.
336 For all infection assays, 15-20 L4 staged animals were picked onto 10cm seeded NGM plates 4
337 days prior to sodium hypochlorite/1M NaOH treatment. After 4 days, heavily populated non-starved
338 plates were washed off with 1ml M9, treated twice with 1 ml of sodium hypochlorite/1M NaOH
339 solution, and washed three times in 1 ml M9. Embryos were then resuspended in 5 ml of M9 and
340 left to rock overnight at 21°C. L1's were used in subsequent experiments no later than 20 hours

341 after bleach treatment. All centrifugation steps with live animals/embryos were performed in
342 microcentrifuge tubes at 845xg for 30s.

343
344 Throughout the paper, L1 refers to the stage immediately post hatching or bleach synchronization,
345 L3 refers to 24 hours and L4 refers to 48 hours post plating of bleach synchronized L1's at 21°C.
346 L3 and L4 animals were washed off plates in M9 + 0.1% Tween-20, followed by an additional
347 wash to remove residual bacteria before infection with microsporidia, or plating on PA14.

348

349 **Forward Genetic Screen**

350 6,000 L4 N2 hermaphrodites were mutagenized with a combination of 50 mM EMS and 85.4 mM
351 ENU for 4 hours to achieve a large diversity of mutations within the genome.⁵⁹ P0 animals were
352 then split and placed onto 48 10cm NGM plates, F1s bleached and resulting F2s pooled onto 5
353 separate plates. 180,000 L1 F2 animals were plated onto a 10 cm plate with 10 million *N. parisii*
354 spores and 1 ml 10x saturated OP50-1. Animals were grown for 72 hours, to select for animals
355 that display a fitness advantage phenotype with respect to N2. Each population was bleached and
356 grown in the absence of infection for one generation, in order to prevent the effects of
357 intergenerational immunity²⁷. Two more cycles of infection followed by growing worms in the
358 absence of infection was performed. Populations of bleached L1s were then infected with either
359 20 or 40 million spores and grown for 76 hours. Worms were then washed into 1.5 ml tube and 1
360 ml of stain solution (1x PBS/0.1% Tween-20/2.5 mg/ml DY96/1% SDS) was added. Samples were
361 incubated with rotation for 3.5 hours and then washed 3 times with M9 + 0.1% Tween-20.
362 Individual worms that had embryos, but not spores, were picked to individual plates. Each of the
363 four *fawn* strains was isolated from a different mutant pool.

364

365 **Whole genome sequencing**

366 N2 and *fawn* isolates were each grown on a 10 cm plate until all *E. coli* was consumed. Each worm
367 was washed off with M9 and frozen at -80°C. DNA was extracted using Gentra puregene Tissue
368 Kit (QIAGEN). Samples were sequenced on an Illumina HiSeq 4000, using 100 base paired end
369 reads.

370

371 **MIP-Map**

372 Molecular inversion probes were used to map the underlying causal mutations in *fawn* isolates as
373 previously described.³⁴ Briefly, *fawn* hermaphrodites were crossed to males of the mapping strain
374 DM7448 (VC20019 Ex[*pmyo3::YFP*]) hereafter referred to as VC20019. Next, 20 F1
375 hermaphrodite cross progeny, identified as those carrying *pmyo3::YFP* were isolated and allowed
376 to self. F2s were then bleached, and 2,500 L1s were exposed to a medium-2 dose of *N. parisii*
377 spores representing the first round of selection. Two plates of 2,500 F3 L1s were set up. The
378 experimental plate was grown in the absence of infection for one generation, to negate
379 intergenerational immunity.²⁷ A second plate of 2,500 L1s was allowed to grow to 72 hours and
380 then frozen in H₂O at -80°C, until used for genomic preparation. The selection and rest steps were
381 repeated once more, and a second frozen sample of worms was taken at the end of the mapping
382 experiment. This process was also performed for a cross between N2 hermaphrodites and males
383 of the mapping strain VC20019, as a negative control to identify non-causal loci that may be
384 selected for reasons other than resistance to infection. Two genomic preparations, corresponding
385 to the two rounds of selection, were used as template for MIP capture, to generate multiplexed
386 libraries for sequencing. An Illumina Mini-seq was used to generate sequencing data that was

387 subjected to demultiplexing via R, and selection intervals were defined as those immediately
388 adjacent to the region on the chromosome carrying the fewest proportion of reads corresponding
389 to the mapping strain, VC20019. This interval was then used to scan for putative causal alleles,
390 resulting in the identification of the four *aaim-1* alleles in the four *fawn* isolates.

391

392 **Identification of causal gene**

393 Variants were identified using a BWA-GATK pipeline. Briefly, sequencing reads were checked
394 for sequence quality using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>)
395 and bases lower than a quality threshold of 30 were trimmed off with Trimmomatic using a sliding
396 window of 4 bases and minimum length of 36 bases.⁶⁰ Reads were aligned to the *C. elegans* N2
397 reference genome (release W220) using BWA-mem.⁶¹ Alignments were sorted by coordinate order
398 and duplicate reads removed using Picard (<https://github.com/broadinstitute/picard>). Prior to
399 variant calling, reads were processed in Genome Analysis Tool Kit (GATK) v3.8.1,⁶² to perform
400 indel realignment and base quality score recalibration using known *C. elegans* variants from
401 dbSNP, build 138 (<http://www.ncbi.nlm.nih.gov/SNP/>). GATK HaplotypeCaller was used to call
402 variants, and results were filtered for a phred-scaled Qscore > 30 and to remove common variants
403 found previously in multiple independent studies. Finally, Annovar⁶³ was used to obtain a list of
404 annotated exonic variants for each sequenced strain.

405

406 **Microsporidia infection assays**

407 *N. parisii* (ERTm1) spores were prepared as described previously.²⁷ All infections were carried
408 out on 6-cm NGM plates, unless otherwise specified by spore dose (see Supplemental table 2), or
409 experimental method. 1,000 bleach-synchronized L1s were added into a microcentrifuge tube

410 containing 400 μ l of 10X *E. coli* OP50, and spores. After pipetting up and down, this mixture was
411 top plated onto an unseeded 6-cm NGM plate, and left to dry in a clean cabinet, prior to incubation
412 at 21°C for 72 hours. Infections set up on 3.5-cm plates used 160 μ l of 10x *E. coli* OP50 and 400
413 L1's.

414

415 **Infection of embryos hatched on plates**

416 Twenty-five 72-hour old synchronized animals of each strain were picked onto 3.5-cm unseeded
417 NGM plates seeded with 16 μ l of 10x *E. coli* OP50. Plates were incubated at 21°C for two hours.
418 Adults were then picked off, and a mixture of 144 μ l of 10x *E. coli* OP50 and a low dose of *N.*
419 *parisii* spores were added to each plate. Animals were fixed and stained after 72 hours.

420

421 **Pulse-chase infection assay**

422 6,000 bleach synchronized animals were exposed to a medium-1 (Figure S4) or medium-3 (Figure
423 3) dose of spores, 10 μ l of 10x *E. coli* OP50 in a total volume of 400 μ l made up with M9. To
424 assay pathogen clearance 3 hpi, animals were washed off in 1 ml M9 + 0.1% Tween-20, and split
425 into two populations. The first was fixed with acetone to represent initial infectious load, while the
426 other half was washed twice in M9 + 0.1% Tween-20 to remove residual spores in the supernatant
427 and prevent additional infection from occurring. These washed worms were then plated on 6-cm
428 unseeded NGM plates with 40 μ l 10x OP50, and 360 μ l M9 and left to incubate at 21°C for 21
429 additional hours before fixation.

430

431 **Spore localization and firing assays**

432 Strains were infected as described for the pulse infection assays for either 45 minutes or 3 hours.
433 Animals were then washed off plates, fixed, and stained with DY96 and *N. parisii* 18S RNA FISH
434 probe. FISH⁺ DY96⁺ events represent unfired spores, FISH⁻ DY96⁺ events represent fired spores
435 and FISH⁺ DY96⁻ events represent sporoplasms. Percentage of fired spores is defined as the
436 number of FISH⁻ DY96⁺ events over the total number of spores.

437

438 To assess spore orientation, the localization of spores relative to the apical membrane of the apical
439 intestine was measured in live anaesthetized animals. To determine if a spore was angled, straight
440 lines were extended from both ends of the spore independently. If either of these two lines crossed
441 the apical membrane, a spore was considered angled. If not, the spore was considered parallel.
442 Distance of spores from the apical membrane was assessed by measuring perpendicular distance
443 from the central edge of a parallel spore to the apical membrane. All measurements were
444 performed on FIJI⁶⁴ using the angle tool or the straight line tool respectively, followed by the
445 Analyze → measure option.

446

447 **Intestinal lumen measurements**

448 Measurements were performed on live anaesthetized worms used for spore localization assays (see
449 above). The width of the lumen was determined by extending a straight line from the apical
450 membrane on one end of the worm to that directly across on the other end, at the midpoint of the
451 intestine, and the distance measured in FIJI, via the straight line tool followed by the Analyze →
452 measure option.

453

454 **Fixation**

455 Worms were washed off infection plates with 700 μ l M9 +0.1% Tween-20 and washed once in
456 1ml M9+0.1%Tween-20. All microsporidia infected samples were fixed in 700 μ l of acetone for
457 2 minutes at room temperature prior to staining. All *P. aeruginosa* PA14::DsRed infected samples
458 were fixed in 500 μ l of 4% paraformaldehyde (PFA) for 30 minutes at room temperature prior to
459 mounting on slides.

460

461 **Live imaging**

462 Animals were mounted on 2% Agarose pads in 10 μ l of 25mM Sodium Azide. This technique was
463 used for spore localization assays, transcriptional reporter imaging, and assessing PA14::DsRed
464 colonization in transgenic animals.

465

466 **Chitin Staining**

467 The chitin binding dye Direct yellow 96 (DY96) was used to assess host fitness (gravidity) as
468 well as parasite burden. 500 μ l of DY96 solution (1 x PBST, 0.1% SDS, 20 μ g/ml DY96) was
469 added to washed worm pellets and left to rock for 20-30 minutes at room temperature. Worms
470 were then resuspended in 20 μ l of EverBrite™ Mounting Medium (Biotium), and 10 μ l mounted
471 on glass slides for imaging.

472

473 To prestain spores prior to infection, 0.5 μ l of Calcofluor white solution (CFW) (Sigma- Aldrich
474 18909) was added per 50 μ l of spores, pipetted up and down gently and left for 2 minutes at room
475 temperature prior to infection.

476

477 **Fluorescence in Situ hybridization-FISH**

478 To quantify the number of sporoplasms in *N. parisii* infected animals, the MicroB FISH probe
479 (ctctcggaactccttctg) labelling *N. parisii* 18S RNA was used. Animals were fixed in acetone,
480 washed twice in 1 ml PBST, and once in 1 ml of hybridization buffer (0.01% SDS, 900 mM NaCl,
481 20 mM TRIS pH 8.0). Samples were then incubated overnight in the dark at 46 °C with 100 µl of
482 hybridization buffer containing 5 ng/µl of the MicroB FISH probe conjugated to Cal Fluor 610
483 (LGC Biosearch Technologies). Samples were then washed in 1ml of wash buffer (Hybridization
484 buffer + 5 mM EDTA), followed by incubation with 500 µl wash buffer at 46 °C in the dark. To
485 visualize sporoplasms and spores simultaneously, the final incubation was replaced with 500 µl
486 DY96 solution and incubated in the dark at room temperature prior to resuspension in 20 µl of
487 EverBrite™ Mounting Medium (Biotium).

488

489 **Microscopy and image quantification**

490 All imaging was performed using an Axio Imager.M2 (Zeiss), except for images of the
491 transcriptional reporter in Figure S6, which were generated using an Axio Zoom V.16 (Zeiss) at a
492 magnification of 45.5x. Images were captured via Zen software and quantified under identical
493 exposure times per experiment. Gravidity is defined as the presence of at least one embryo per
494 worm, and animals were considered infected by 72 hours if clumps of spores were visible in the
495 body of animals as seen by DY96. FISH-stained animals were considered infected if at least one
496 sporoplasm was visible in intestinal cells.

497

498 To quantify fluorescence within animals (Pathogen burden, bead accumulation, GFP), regions of
499 interest were used to outline every individual worm from anterior to posterior, unless otherwise
500 specified in methods. Individual worm fluorescence from variable assays (GFP or dsRed) were

501 subjected to the “threshold” followed by “measure” tools in FIJI.⁶⁴ To assess PA14::DsRed burden
502 in transgenic animals, regions of interest were generated from the beginning of the intestines (int1)
503 to the posterior end of the worm to prevent the *pmyo2::mCherry* co-injection marker signal from
504 interfering with quantifications. When assessing pathogen burden in gravid animals stained with
505 DY96, thresholding was used to quantify spore signal without including signal from embryos.

506

507 ***Pseudomonas aeruginosa* infection experiments**

508 For all *Pseudomonas* assays, a single colony was picked into 3 ml of LB and grown overnight at
509 37°C, 220 rpm for 16-18 hours. 20 µl (for 3.5-cm plate) or 50 µl (for 6-cm plate) of culture was
510 spread onto slow killing (SK) plates to form a full lawn, except in the case of competitive fitness
511 assays (see below). Seeded plates were placed at 37°C for 24 hours, followed by 25°C for 24 hours
512 prior to use. Plates were seeded fresh prior to each experiment. To assess colonization, 1,000
513 synchronized animals were grown on PA14::dsRED for either 24 or 48 hours at 25°C. Animals
514 were washed off with 1ml M9+ 0.1%Tween-20, and washed twice thereafter, prior to fixation.

515

516 To quantify survival of individual strains on PA14, 3.5-cm SK plates were seeded with 20 ul of
517 PA14, to form full lawns. 60 L4s were picked onto each of three, 3.5-cm plates per strain, and 24
518 hours later, 30 animals from each were picked onto a new 3.5-cm plate (T24hrs). Survival was
519 monitored from 24 hours post L4, three times per day. Survival was assessed based on response to
520 touch. Carcasses were removed, and surviving animals were placed onto fresh 3.5-cm plates every
521 24 hours. Animals were grown at 25°C for the duration of the experiment. Technical triplicate data
522 was pooled to represent a single biological replicate. The experiment was carried out until no more

523 worms had survived. Survival curves were generated via GraphPad Prism 9.0, and the Log rank
524 (mantel-cox) test was used to generate P-values.

525

526 **Transgenic strain construction**

527 N2 or *aaim-1* (*kea22*) animals were injected with a 100 ng/μl injection mix composed of
528 50 ng/μl of template, 5 ng/μl of *pmyo2::mCherry*, and 45 ng/μl of pBSK. Three independent lines
529 were generated for each injected construct.

530

531 Gateway BP cloning^{65,66} was performed to insert AAIM-1 and GFP into pDONR221. Around the
532 horn PCR,⁶⁷ was used to insert a 3x Flag sequence at the C-terminus of this construct. Gibson
533 assembly was used to generate different tissue specific clones driving *aaim-1* expression. *Paaaim-*
534 *1*, *aaim-1* and *pspp-5* were cloned from N2 genomic DNA, *pmyo2* was cloned from pCFJ90. GFP
535 and 3x Flag sequences were cloned from pDD282. *SPΔaaim-1* was amplified from *aaim-1::3xFlag*
536 in pDONR221 by omitting the first 17 amino acids, the putative secretion signal as predicted via
537 SignalP 5.0.⁶⁸ All clones possessed an *unc-54* 3' UTR. See Supplemental table 3 for primer
538 sequences.

539

540 **CRISPR-Cas9 mutagenesis**

541 To generate a deletion allele of *aaim-1* via CRISPR-Cas9 mutagenesis, steps were taken as
542 described here.⁶⁹ Briefly, 2 crRNA's were designed using CRISPOR,⁷⁰ near the start and stop sites
543 of *aaim-1* and generated via IDT. A repair template was designed to contain 35 base pairs of
544 homology upstream and downstream of the cut sites. *Streptococcus pyogenes* Cas9 3NLS
545 (10ug/ul) IDT and tracrRNA (IDT #1072532) were utilized. Reaction mixes were prepared as

546 described previously.⁶⁹ pRF4 was co-injected with the Cas9 ribonucleoprotein, and F1 rollers
547 picked. Deletions were identified via PCR primers situated outside the cut sites.

548

549 **Bead-feeding assays**

550 1,000 synchronized L1 animals were mixed with 0.2 μm green fluorescent polystyrene beads
551 (Degradex Phosphorex) at a ratio of 25:1 in a final volume of 400 μl containing 10 μl of 10x *E.*
552 *coli* OP50, 16 μl of beads and up to 400 μl with M9. Animals were incubated with beads for 3
553 hours, washed off with M9 + 0.1% Tween-20 and fixed with 4% PFA for 30 min at room
554 temperature. Bead accumulation was measured as a percentage of the total animal exhibiting
555 fluorescent signal, using FIJI.

556

557 **Lifespan Assays**

558 Lifespan assays were performed as described previously.⁷¹ In brief, 120 synchronized L4 animals
559 were utilized per strain, with 15 animals placed on a single 3.5-cm NGM plate (A total of 8 plates,
560 with 15 animals each per strain). Animals were transferred to a new seeded 3.5-cm NGM plate
561 every 2 days, for a total of 8 days (4 transfers), ensuring no progeny were transferred alongside
562 adults. After day 8, survival was quantified daily, on the same plate, via response to touch. Any
563 animals that exhibited internal hatching, protruding intestines, or were found desiccated on the
564 edges of the plate were censored. Survival curves were generated via GraphPad Prism 9, and the
565 Log rank (mantel-cox) test was used to generate P values.

566

567 **Immunofluorescence (IF)**

568 IF was performed as described previously,⁷² however all steps post-dissection were performed in
569 microcentrifuge tubes, and intestines were pelleted on a mini tabletop microcentrifuge for a few
570 seconds. Briefly, animals were dissected to extrude intestinal tissue. Two 25mm gauge needles on
571 syringes were used to create an incision near the head and/or tail of the animals. Dissections were
572 performed in 5 µl of 10 mM levamisole on glass slides to encourage intestinal protrusion. Fixation,
573 permeabilization and blocking was performed as described previously.⁷² Primary M2 anti-Flag
574 antibody (Sigma F1804) was used at 1:250 overnight at 4°C, and secondary goat anti-mouse Alexa
575 fluor 594 (Thermo Fisher A32742) at 1:300 for 1 hour at room temperature. Animals were mounted
576 in 20 µl of EverBrite™ Mounting Medium (Biotium) and placed on glass slides for imaging.

577

578 **Competitive fitness assays**

579 N2 or *aaim-1* mutants were grown together with RFP::Znfx1 YY1446 (*gg634*), which labels the
580 germ granules and can be observed in all developmental stages⁷³. For *N. parisii* infections, 10-cm
581 NGM plates were seeded with 1 ml of 10xOP50 and a medium-2 dose of spores (no spores were
582 used for uninfected plates). 10 L1s from each strain were picked onto lawns of spores and *E. coli*
583 OP50 immediately after drying, and grown for 8 days at 21°C, washed off with M9 + 0.1% Tween-
584 20, and fixed. For *P. aeruginosa* infections, 3.5-cm SK plates were seeded with a single spot of 20
585 µl of PA14 in the center of the plate. 10 L1s of each strain were placed on plates and grown at
586 21°C for 8 days and then washed off with M9 + 0.1% Tween-20. The percentage of animals that
587 did not display RFP germ granules (i.e. N2 or *aaim-1* mutants) is was determined by quantifying
588 all animals on the plate, including F1 adults and L1/L2 staged F2 animals.

589

590 **Co- infections with *N. parisii* and *P. aeruginosa***

591 Co-infection assays were performed by first pulse infecting co-infection and *N. parisii* single
592 infection groups with an maximal dose of spores for three hours on unseeded 6-cm NGM plates
593 as described above. PA14::DsRed single infections were pulsed with a volume of M9 to match
594 that of the spores. Animals were then washed off in 1ml of M9 + 0.1%Tween-20, followed by 2
595 more washes, prior to placement on full lawns of PA14::DsRed on a 6-cm SK plates prepared as
596 described above. *N. parisii* single infections were placed on a 6-cm NGM plate pre-seeded with
597 200 µl of 10xOP50. Plates were incubated at 21°C.

598

599 **Phylogenetic analysis**

600 Homology between AAIM-1 and other proteins was determined with protein BLAST
601 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) using default parameters. Sequences with less than E-5
602 were aligned using MUSCLE (<https://www.ebi.ac.uk/Tools/msa/muscle/>) using default
603 parameters. Phylogenetic tree of homologs was generated using RAxML BlackBox [https://raxml-](https://raxml-ng.vital-it.ch/#/)
604 [ng.vital-it.ch/#/](https://raxml-ng.vital-it.ch/#/) using default parameters and 100 boot straps. Tree was visualized using FigTree
605 v1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

606

607 **Statistical analysis**

608 All data analysis was performed using GraphPad Prism 9.0. One-way Anova with post hoc (Tukey
609 test) was used for all experiments unless otherwise specified in figure legends. Statistical
610 significance was defined as $p < 0.05$.

611

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619

620 **Author contributions:**

621 **H.T.E.J. and A.W.R.** designed experiments, analyzed results, and co-wrote the paper.

622 **H.T.E.J.** conducted all experiments, except the initial forward genetic screen performed by

623 **A.W.R.**

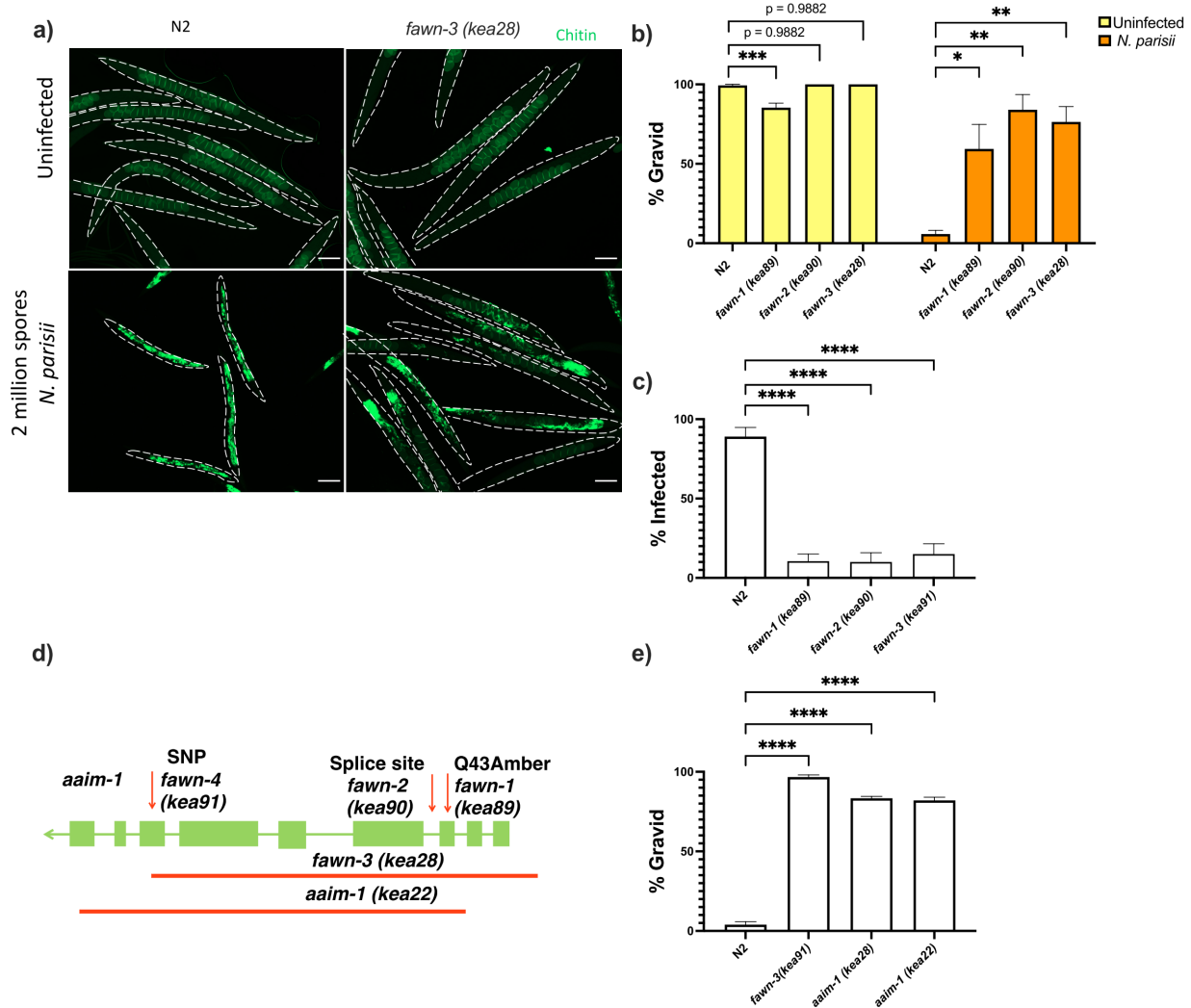
624 **C.M.** designed and performed bioinformatic analysis for the MIP-map experiment.

625 **M.R.S.** analyzed whole genome sequencing to identify causal mutations in *fawn* animals.

626 **E.R.T., A.G.F., and A.W.R.** provided mentorship and acquisition of funding.

627

628 **Competing interests:** The authors declare they have no competing interests.



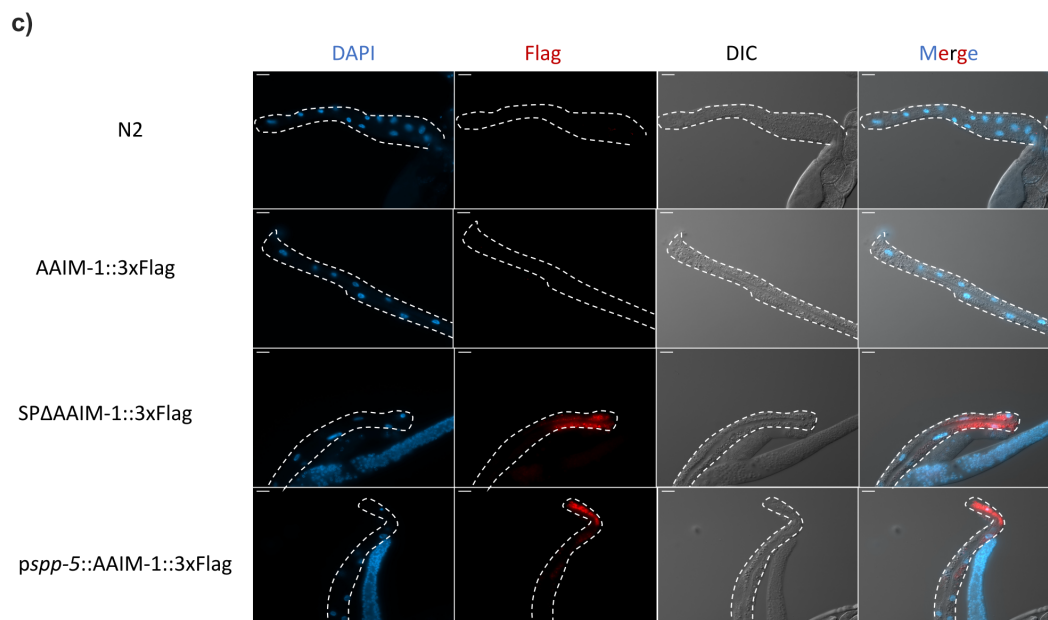
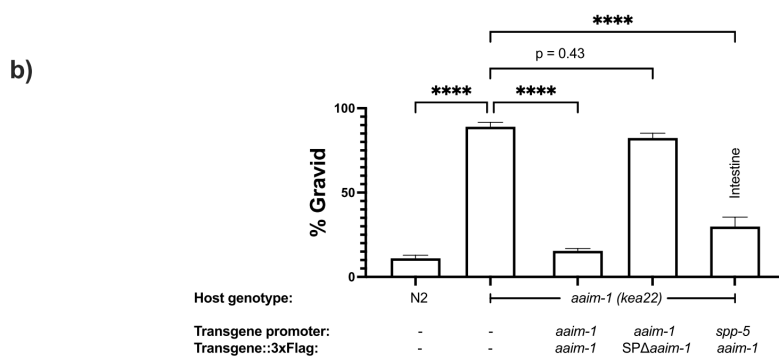
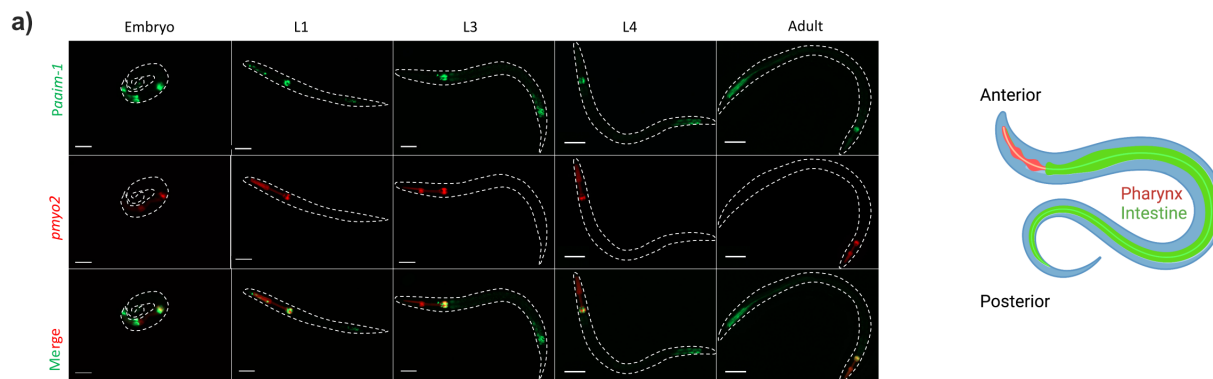
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630 **Figure 1: Mutations in *aaim-1* result in resistance to *N. parisi* infection.**

631 (a-c, and e) L1 stage Wild-type (N2) and *aaim-1* mutant animals were infected with either a high
 632 dose (a, b, and e) or a very lose dose (c) of *N. parisi*, fixed at 72 hours, and stained with direct-
 633 yellow 96 (DY96). (a) Representative images stained with DY96, which stains *C. elegans* embryos
 634 and microsporidia spores. Scale bars, 100 μ m. (b and e) Percentage of worms that are gravid. (c)
 635 Percentage of worms that contain newly formed *N. parisi* spores. (d) Schematic depicting the
 636 nature and location of the different *aaim-1* alleles. Boxes represent exons, and connecting lines
 637 represent introns. Arrows depict point mutations, and the solid red line depicts deletions. *fawn-3*

638 has a 2.2 kb deletion and *aaim-1* (*kea22*) has a 2.3 kb deletion. *fawn-1* carries a C127T , Q43Stop
639 mutation, *fawn-2* carries a G221A splice site mutation and *fawn-4* carries a C1286T, A429V
640 mutation in *aaim-1*.(b,c, and e) Data is from three independent replicates with at least 90 animals
641 counted per replicate. Mean \pm SEM represented by horizontal bars. P-values determined via one-
642 way ANOVA with post hoc. Significance defined as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ****
643 $p < 0.0001$.

644



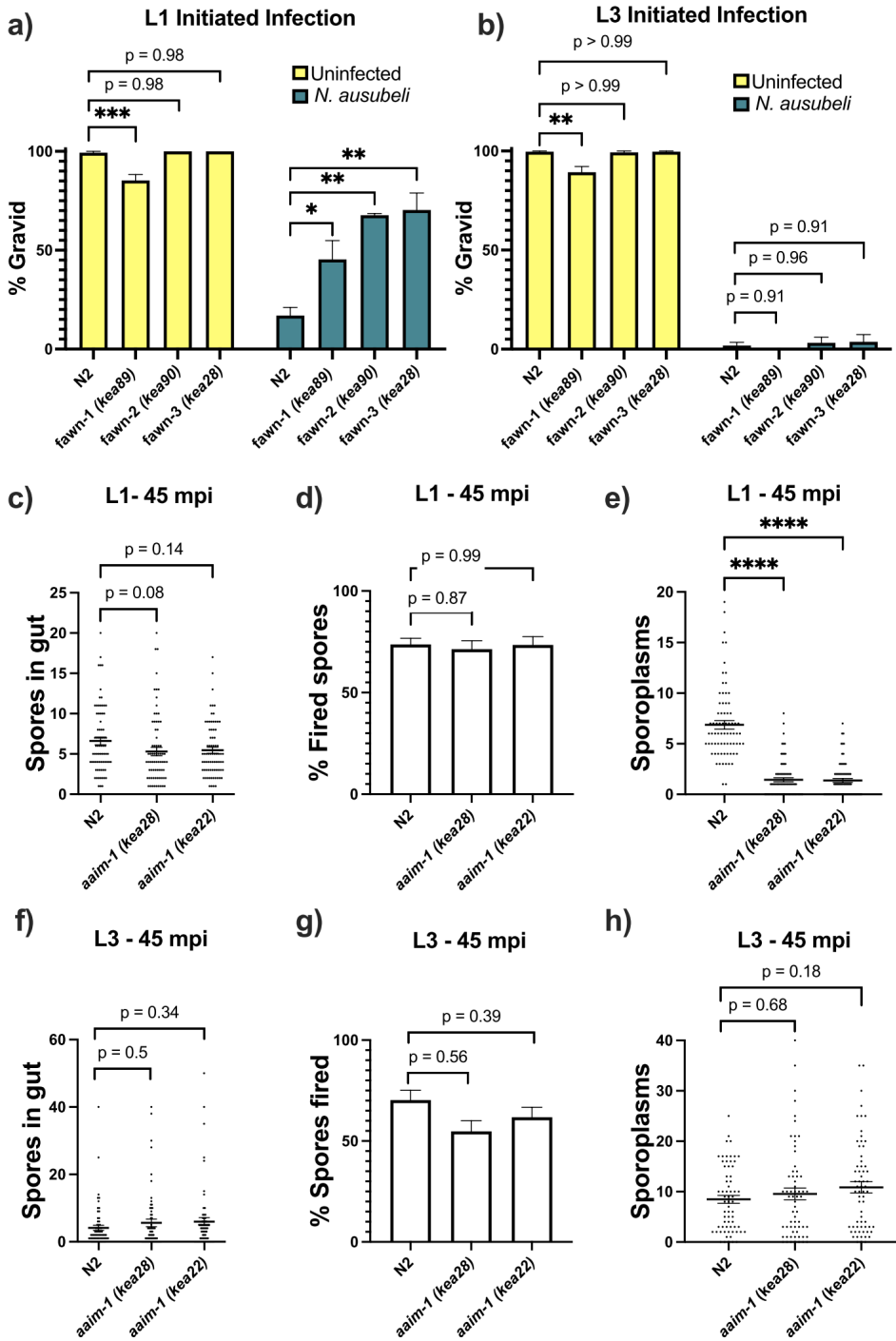
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646

647

648 **Figure 2: AAIM-1 is secreted from intestinal cells.**

649 (a) Wild-type worms containing an extrachromosomal array expressing GFP from the *aaim-1*
650 promoter and mCherry in the pharyngeal muscles were imaged at the embryo, L1, L3, L4, and
651 adult stage. Embryo, L1 and L3 animals were imaged at 40x, scale bar 20 μm and L4 and adult
652 animals were imaged at 20x, scale bar 50 μm . L1 to L4 are oriented anterior to posterior and adult
653 oriented posterior to anterior from left to right. Schematic made with Biorender.com (b) N2, *aaim-1*
654 *1*, and *aaim-1* expressing extrachromosomal arrays were infected with a medium-2 dose of *N.*
655 *parisii*. Percentage of worms that are gravid. Experiment is from three independent replicates with
656 at least 90 worms quantified per replicate. Mean \pm SEM represented by horizontal bars. P-values
657 determined via one-way ANOVA with post hoc. Significance defined as **** p < 0.0001 (c)
658 Intestines (denoted by dashed lines) of 72-hour post-L1 adults were dissected and stained using
659 anti-Flag (red) and DAPI (blue). Images taken at 40x, scale bar 20 μm .



660

661 **Figure 3: *aaim-1* mutants are resistant to microsporidia at the earliest larval stage due to**

662 **spore misfiring. (a-b) N2 and *aaim-1* mutants were infected with a medium dose of *N. ausubeli***

663 **at either the L1 stage for 72 hours (a) or a high doses of *N. ausubeli* at the L3 stage for 48 hours**

664 (b) Percentage of worms that are gravid. (c-f) N2 and *aaim-1* animals were infected with a
665 medium-3 dose of *N. parisii* for 45 minutes at L1 (c-e) or L3 hours (f-h), fixed, and then stained
666 with DY96 and *N. parisii* 18S RNA fish probe. The number of spores per animal (c,f), the
667 percentage of spores fired (d,g), and the number of sporoplasm per worm (e,h) are displayed. (a-
668 b) Experiment is of three replicates of at least 100 animals. (c-h) Experiment is of three replicates
669 of 20-30 animals. (a-h) Mean \pm SEM represented by horizontal bars. P-values determined via one-
670 way ANOVA with post hoc. Significance defined as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ****
671 $p < 0.0001$.

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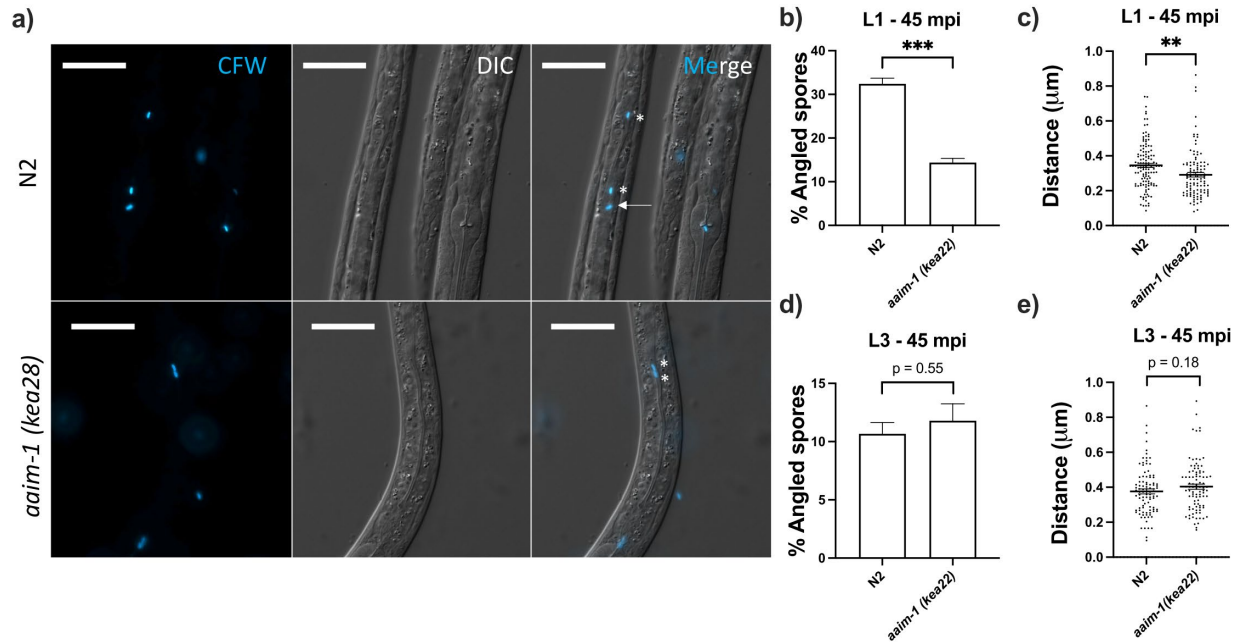
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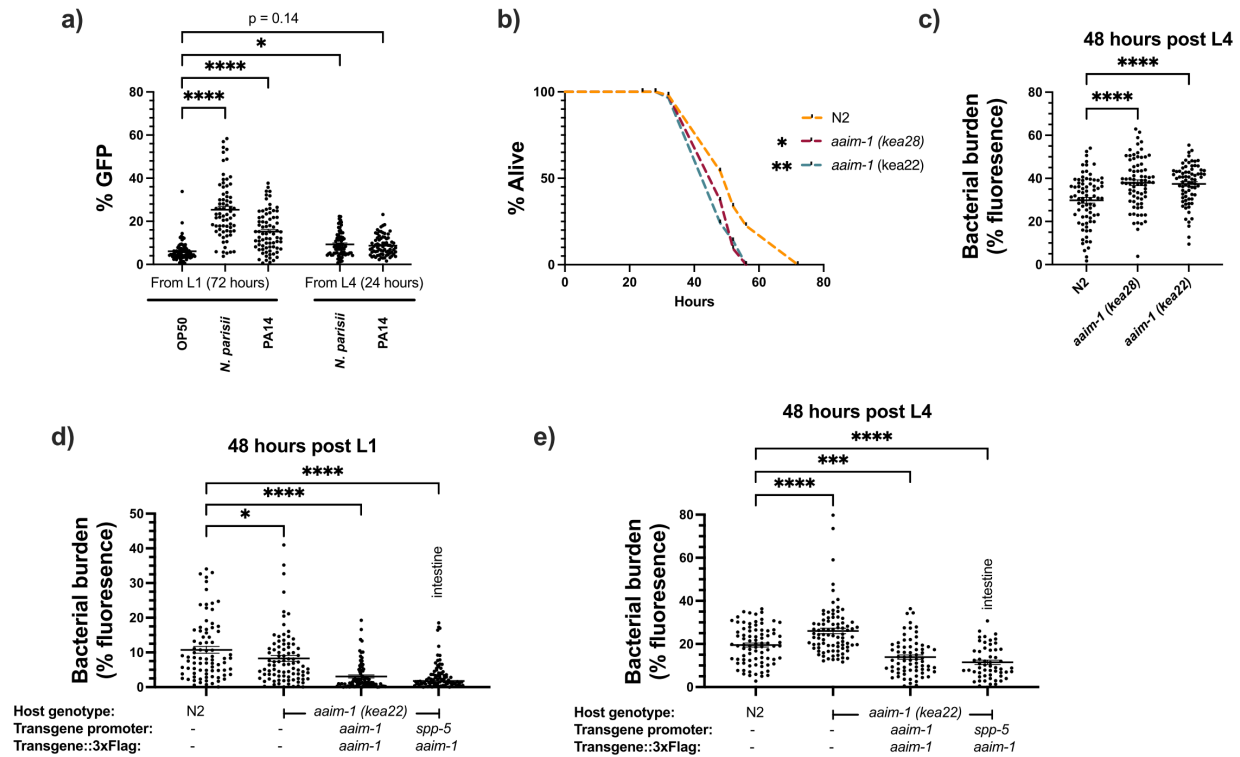
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679

680 **Figure 4: Spores in *aaim-1* mutants display improper orientation and distance to the apical**
681 **intestinal membrane.**

682 (a-e) N2 and *aaim-1(kea22)* animals were infected with a very high dose of Calcofluor white
683 (CFW) pre-stained *N. parisii* spores for 45 minutes at either the L1 stage (a-c) or the L3 stage (d-
684 e). (a) Representative images of live animals containing stained spores (blue). Arrow indicates an
685 example of an angled spore, asterisks indicate parallel spores. Images taken at 40x, scale bar 20
686 μm . (b, d) Percentage of angled spores. The experiment consists of three replicates with at least 90
687 spores per replicate. (c, e) Distance of the center of each spore from the intestinal apical membrane.
688 The experiment consisted of three replicates of at least 25 spores per replicate. Mean \pm SEM
689 represented by horizontal bars. P-values determined via unpaired Student's t-test. Significance
690 defined as ** $p < 0.01$, *** $p < 0.001$.



691

692 **Figure 5: *aaim-1* is upregulated by *N. parisii* and *P. aeruginosa* and *aaim-1* animals are**
 693 **susceptible to infection by *P. aeruginosa*.**

694 (a) Expression of *paaim-1*GFP::3xFlag in response to infection with either PA14 or *N. parisii* for
 695 either 72 hours from L1 or 24 hours from L4. 18-25 animals quantified per replicate. Every point
 696 represents a single worm. Percentage GFP was measured as the percentage of the animal
 697 containing GFP via FIJI. n=3. (b) L4 stage N2 and *aaim-1* were plated on full lawns of *P.*
 698 *aeruginosa* PA14 and the percentage of animals alive was counted over the course of 96 hours.
 699 Three independent replicates were carried out, and a representative replicate is displayed. 40
 700 worms were quantified per strain. P-values determined via Log-rank (Mantel-Cox) test.
 701 Significance defined as * $p < 0.05$, ** $p < 0.01$. (c-e) N2, *aaim-1*, or *aaim-1* with different
 702 extrachromosomal arrays were plated on PA14::DsRed as either L1 stage (d) or L4 stage (c,e) for
 703 48 hours. Bacterial burden was measured as the percentage of the animal containing

704 PA14::dsRED. Three independent replicates carried out, 20-30 worms were quantified per
705 replicate. Every point represents a single worm. Mean \pm SEM represented by horizontal bars. (a,
706 c-e) P- values determined via one-way ANOVA with post hoc. Significance defined as * $p < 0.05$,
707 ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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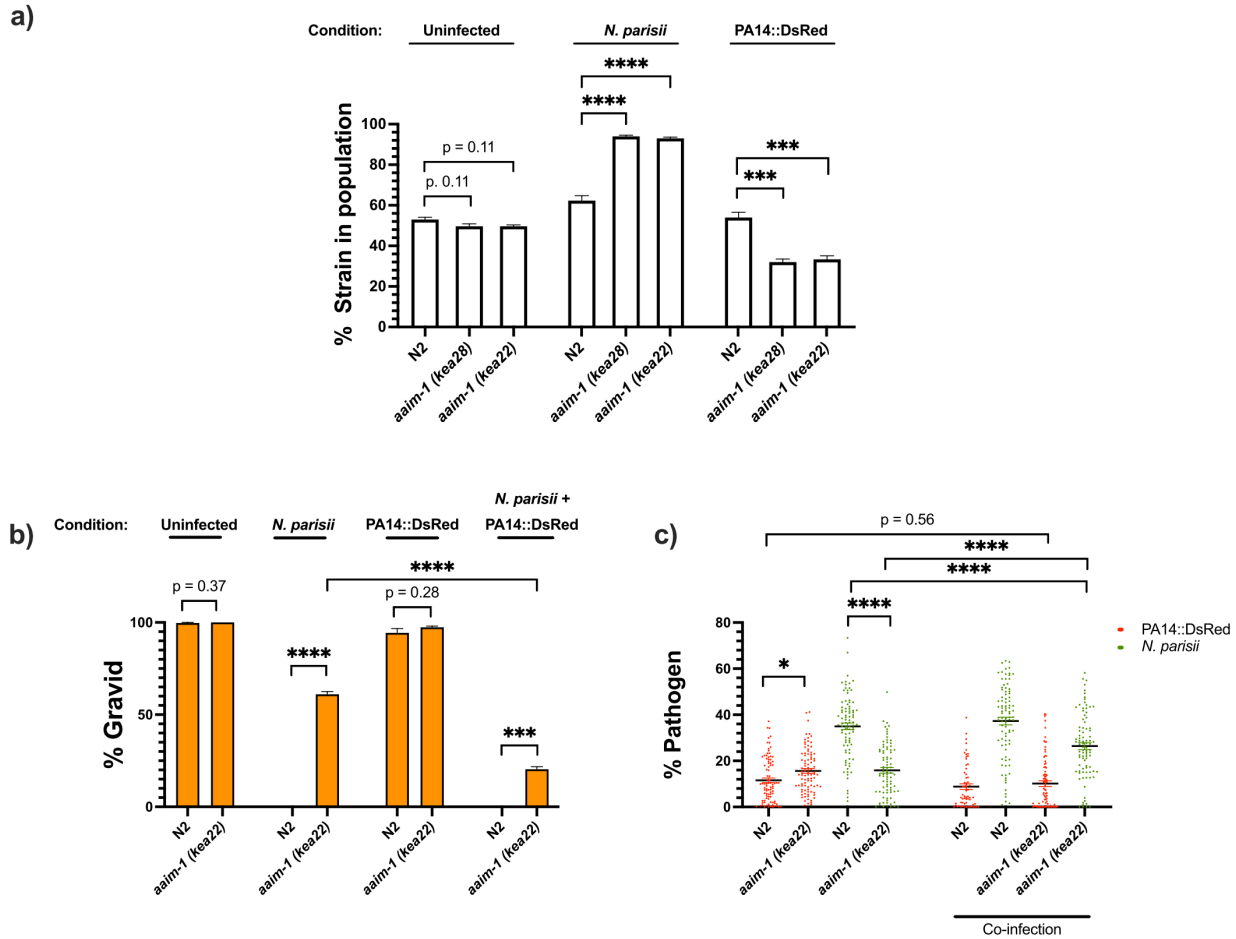
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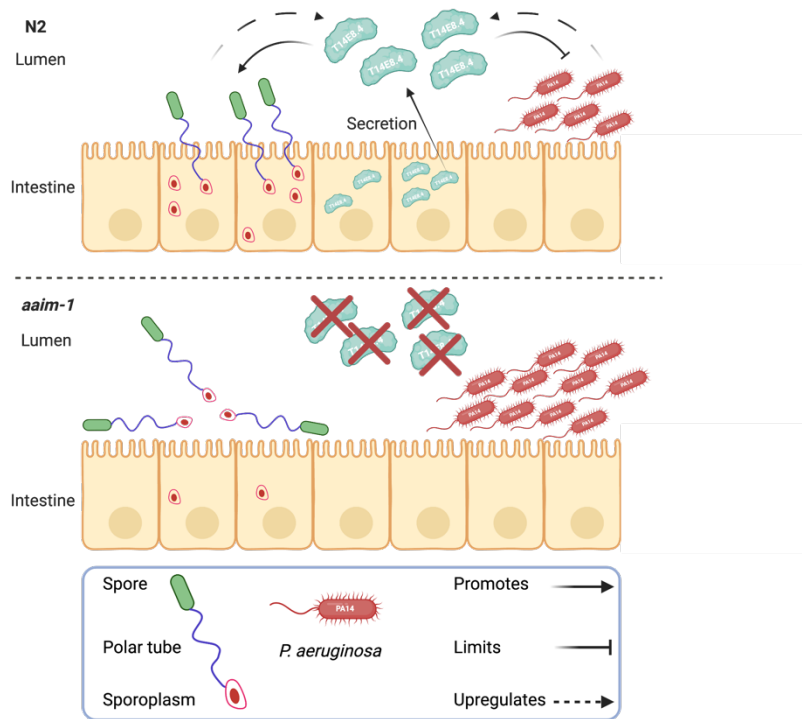


715

716 **Figure 6: *aaim-1* alleles display enhanced fitness on *N. parisii*, but reduced fitness on *P.***
 717 ***aeruginosa*.**

718 (a) Competitive fitness assays performed with a fluorescently marked strain (RFP::ZNF1) mixed
 719 with either N2 or *aaim-1* mutants. These mixed populations of animals were plated at the L1 stage
 720 on either *E. coli*, a medium-2 dose of *N. parisii*, or on *P. aeruginosa*. After 8 days, the fraction of
 721 animals that did not display fluorescent germ granules was counted. Experiment consisted of three
 722 replicates with 20-270 worms quantified per replicate. (b,c) L1 N2 and *aaim-1* animals were either
 723 uninfected or infected with a maximal dose of *N. parisii*. These infected and uninfected population
 724 of animals were then washed and placed on either *E. coli* or PA14::DsRed. After 69 hours, animals
 725 were fixed and stained with DY96. Experiment consisted of three replicates with 60-150 worms

726 quantified per replicate. (b) Percentage of animals that are gravid adults. (c) Quantified amount of
 727 either *N. parisii* (DY96) or *P. aeruginosa* (PA14::DsRed). 12-30 worms were quantified per
 728 replicate. Mean \pm SEM represented by horizontal bars. P-values determined via unpaired Student's
 729 t-test (a) one-way ANOVA with post hoc (b,c). Significance defined as ** $p < 0.01$, *** $p < 0.001$,
 730 ****



731
 732 **Figure 7: Secreted AIM-1 functions in the intestinal lumen to limit bacterial colonization**
 733 **but is exploited by microsporidia to ensure successful invasion of intestinal cells.**
 734 AIM-1 is secreted from intestinal cells, where the protein limits bacterial colonization in the
 735 lumen. Additionally, AIM-1 is parasitized by *N. parisii* spores to ensuring successful orientation
 736 and firing during intestinal cell invasion. Infection by either of these two pathogens results in the
 737 upregulation of AIM-1. Figure made with Biorender.com.

738 **Supplemental material**

739 **Figure S1. Mapping and validation of *aaim-1* as the gene associated with resistance to *N.***
740 ***parisii*.**

741 **Figure S2: AAIM-1 is conserved in both free-living and parasitic nematodes.**

742 **Figure S3: *aaim-1* is expressed in arcade cells and presence of C-terminal 3x Flag tag does**
743 **not disrupt AAIM-1 function.**

744 **Figure S4: *aaim-1* mutants do not clear *N. parisii* and developmentally restricted *N. parisii***
745 **invasion defect is not due to a feeding defect.**

746 **Figure S5: Invasion defects in *aaim-1* only occurs at the L1 stage of development and a**
747 **mutation in *aaim-1* do not alter the width of the intestinal lumen.**

748 **Figure S6: Susceptibility to *P. aeruginosa* PA14 appears at L4.**

749 **Figure S7: A mutation in *aaim-1* does not influence *C. elegans* lifespan.**

750 **Figure S8: List of naturally occurring *aaim-1* variants in wild isolates of *C. elegans*.**

751 **Supplemental table 1: List of strains utilized in this study**

752 **Supplemental table 2: Spore doses utilized in this study.**

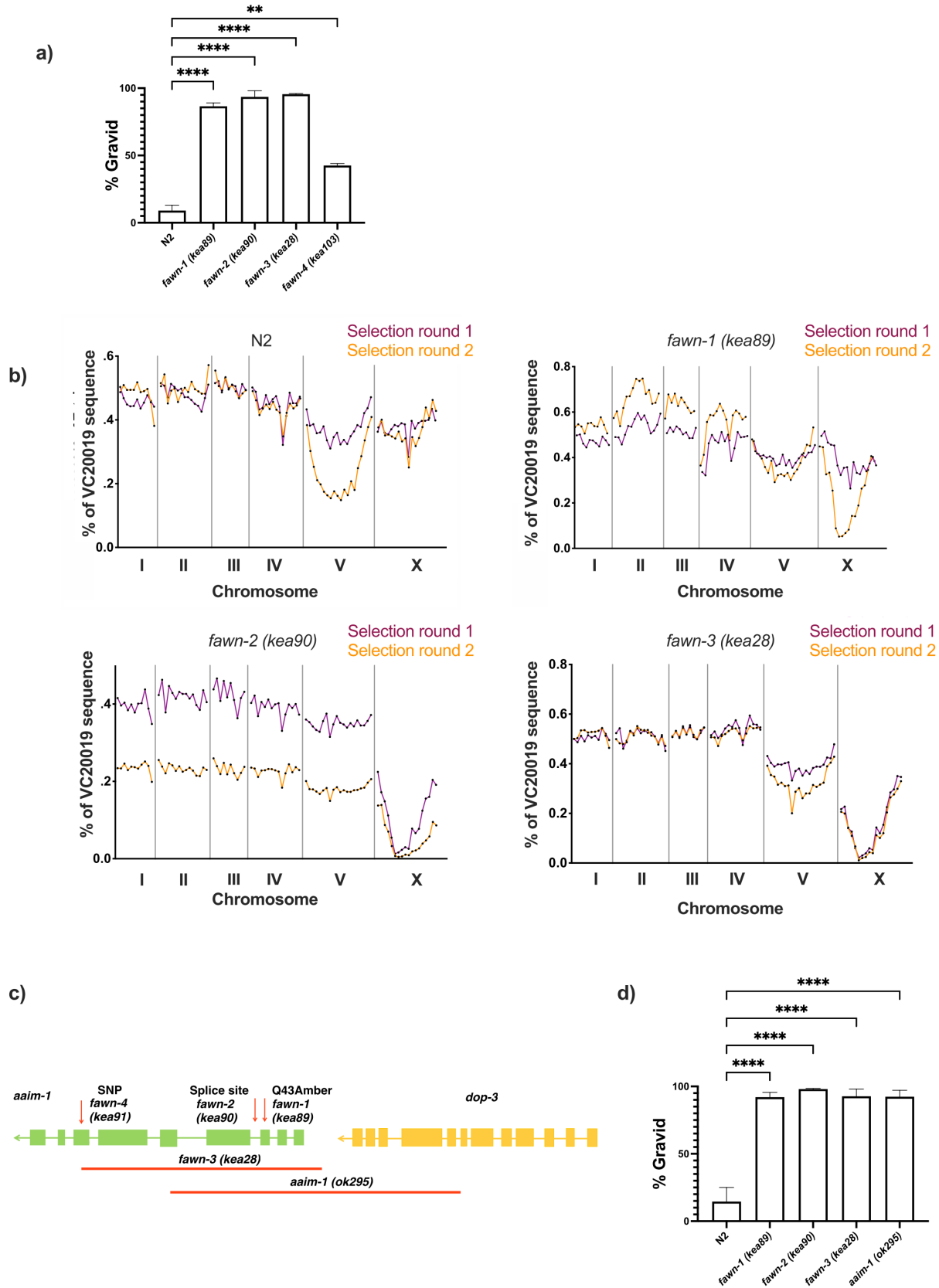
753 **Supplemental table 3: Primer sequences.**

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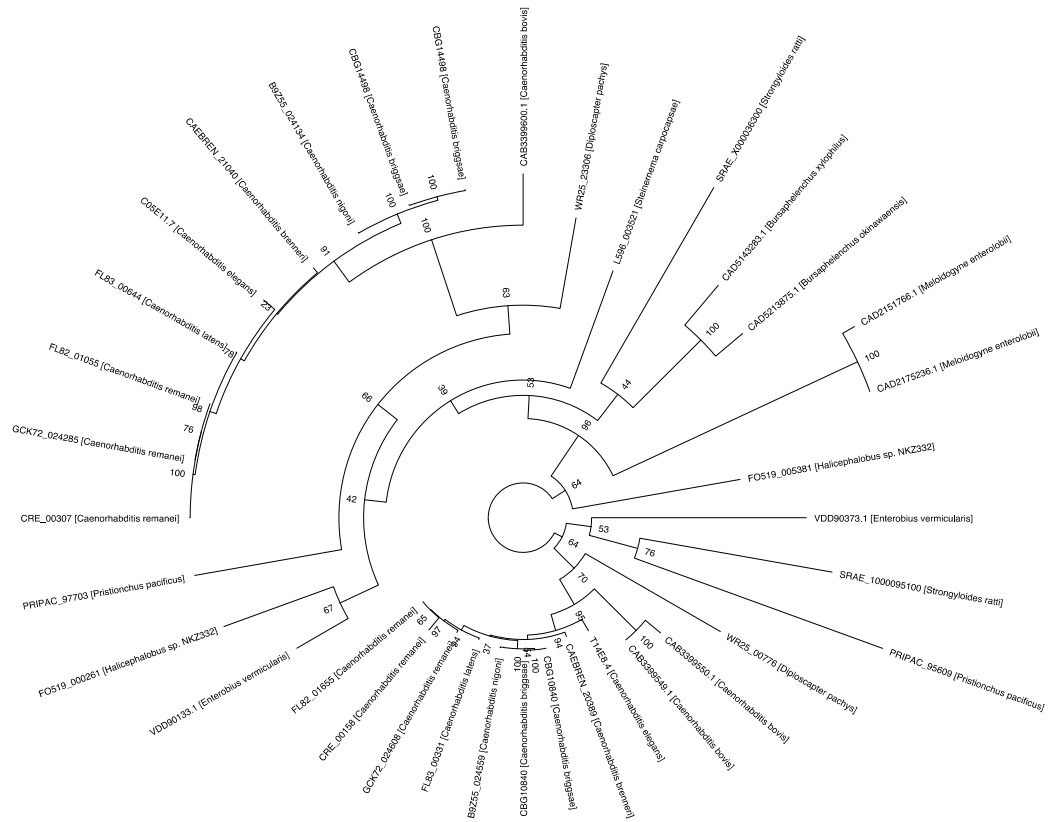


759 **Figure S1. Mapping and validation of *aaim-1* as the gene associated with resistance to *N.***
760 ***parisii*.**

761 (a) N2 or *fawn* animals were infected with a medium-3 dose of *N. parisii* spores on 6-cm plates,
762 fixed at 72 hours, and stained with direct-yellow 96 (DY96). Graph displays percentage of worms
763 that are gravid. Experiment consists of two independent replicates with 66-300 worms quantified
764 per replicate. (b) F2 recombinants between the mapping strain VC20019 and either N2, *fawn-1*,
765 *fawn-2*, or *fawn-3* were infected with a medium-2 dose of *N. parisii*. Two rounds of selection were
766 performed (see methods). The percentage of sequencing reads mapping to the reference strain
767 VC20019 are depicted on the Y axis, and the linkage groups are depicted on the X axis. Sequencing
768 of MIPs resulted in capturing the identity of the genome at 89 distinct regions which are
769 represented as points by their location along the X-axis coordinates. A significantly diminished
770 percentage of VC20019 indicates an enrichment of non-mapping genomic sequence in that region.
771 (c) Schematic representing the location and nature of the different *aaim-1* alleles. Boxes represent
772 exons, and connecting lines represent introns. Arrows represent point mutations and solid red lines
773 represent large deletions. *fawn-3* has a 2.2 kb deletion and *aaim-1 (kea22)* has a 2.3 kb deletion.
774 RB563 (*ok295*) possesses a large deletion overlapping two different genes, *aaim-1* and *dop-3*, the
775 boundaries of which are unclear.^{40,74} (d) L1 stage N2 and *aaim-1* mutant animals were infected
776 with a high dose of *N. parisii*, fixed at 72 hours, and stained with direct-yellow 96 (DY96).
777 Percentage of gravid worms is shown. Experiment consists of three independent replicates with at
778 least 100 worms quantified per replicate. Mean \pm SEM represented by horizontal bars. P-values
779 determined via One-way Anova with post hoc. Significance defined as **** p < 0.0001.

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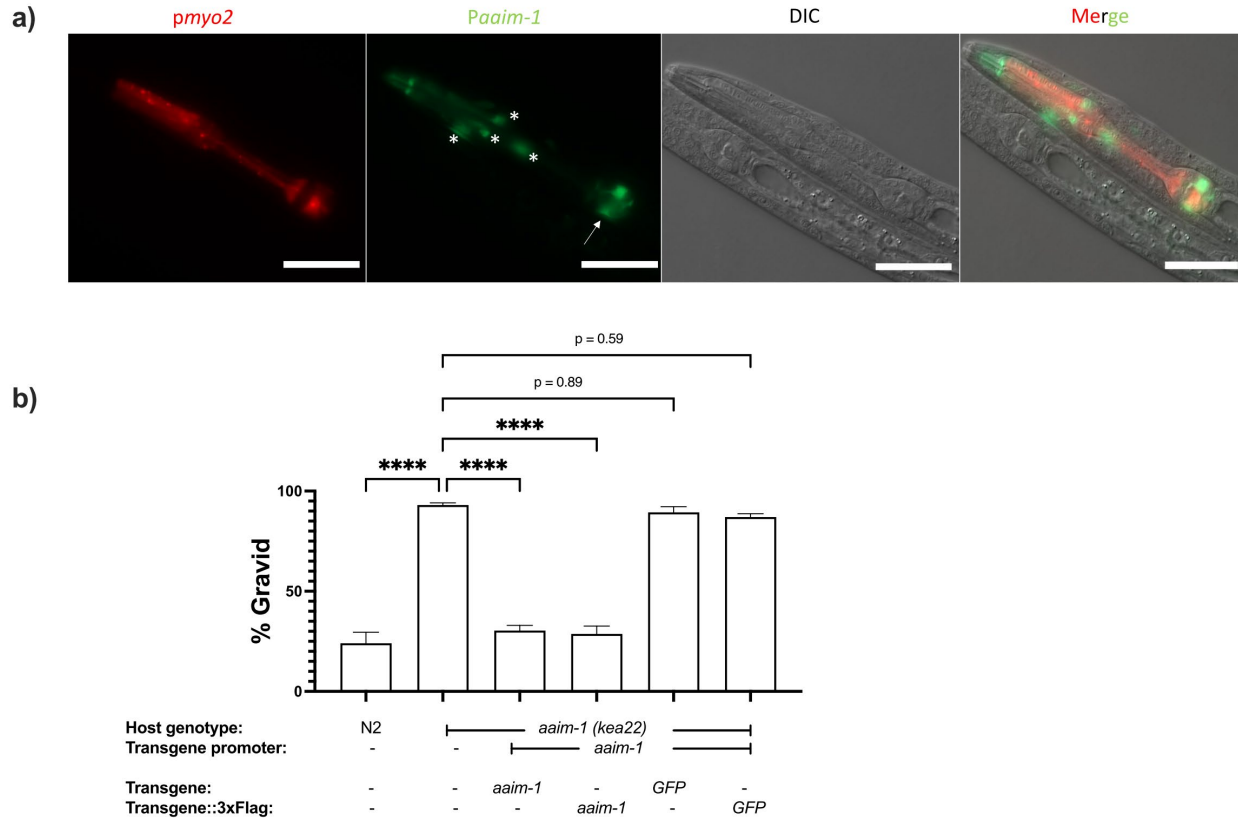
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Figure S2: Homologs of AAIM-1 are present in both free-living and parasitic nematodes. Phylogenetic tree of AAIM-1 homologs. Bootstrap values are shown at the nodes.



791

792 **Figure S3: *aaim-1* is expressed in arcade cells and presence of C-terminal 3x Flag tag does**
 793 **not disrupt AAIM-1 function.**

794 (a) N2 containing an extrachromosomal array expressing GFP from the *aaim-1* promoter and
 795 mCherry in the pharyngeal muscles were imaged at the L1 stage at 40x. Scale bar 20 μ m. Arrow

796 indicates terminal bulb, and asterisks represent arcade cells. (b) N2, *aaim-1*, and *aaim-1* expressing

797 extrachromosomal arrays of wild-type or 3x Flag tagged constructs were infected with a medium-

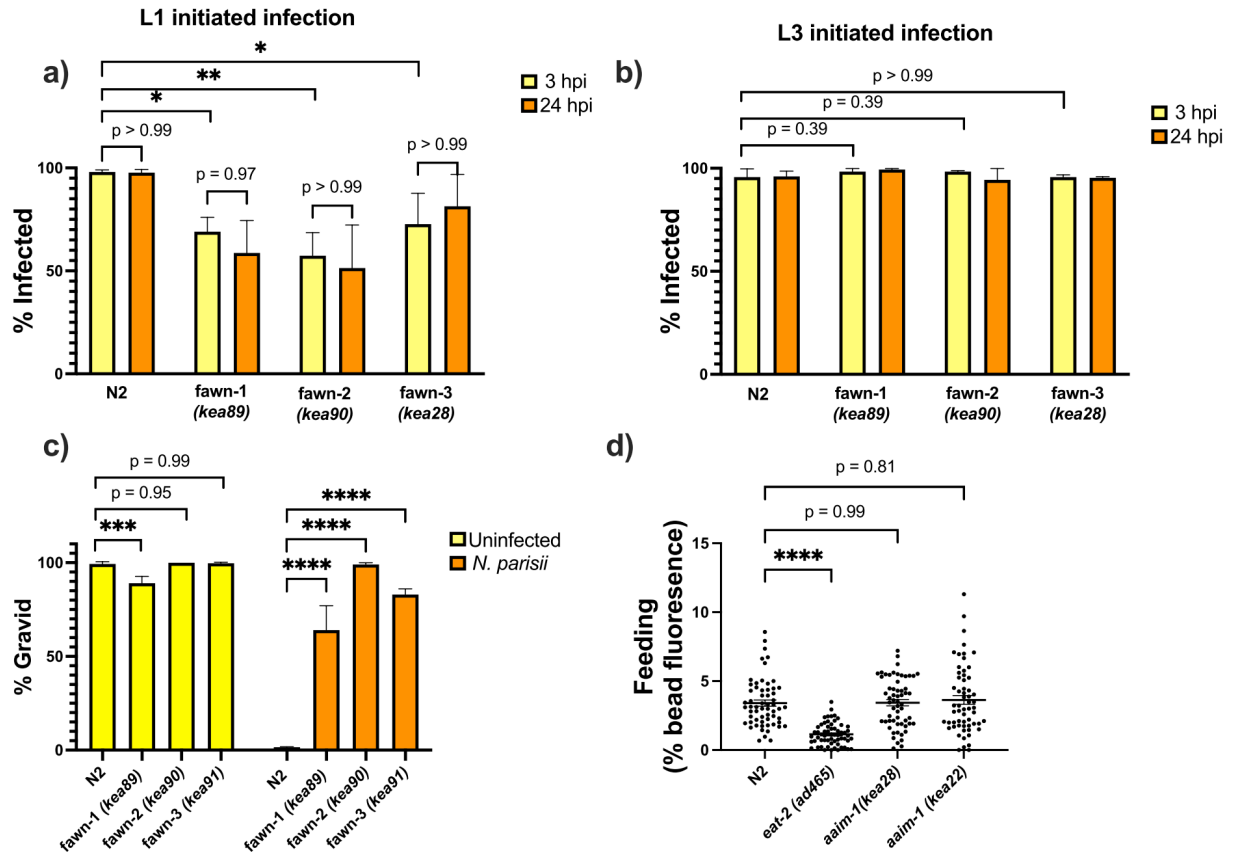
798 2 dose of *N. parisii*, fixed at 72 hours, and stained with direct-yellow 96 (DY96). Percentage of

799 gravid worms is shown. Experiment is of three independent replicates of at least 100 animals each.

800 Mean \pm SEM represented by horizontal bars. P-values determined via one-way ANOVA with post

801 hoc. Significance defined as **** $p < 0.0001$.

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803

804 **Figure S4: *aaim-1* mutants do not clear *N. parisii* and developmentally restricted *N. parisii***

805 **invasion defect is not due to a feeding defect.** (a-b) N2 and *aaim-1* mutants were infected at

806 either the L1 stage (a) or the L3 stage (b) with a medium-1 dose of *N. parisii* spores for 3 hours.

807 Animals were then washed to remove spores and re-plated for an additional 21 hours. Worms were

808 fixed at both the 3 hour and 24 hour timepoints and stained with an *N. parisii* 18S RNA fish probe.

809 Worms containing either sporoplasm or meronts were counted as infected. (c) N2 and *aaim-1*

810 adults were allowed to lay embryos on plates. Adults were removed and a low dose of *N. parisii*

811 was added to the plate. Animals were fixed at 72 hours and stained with direct-yellow 96 (DY96).

812 Percentage of gravid worms is shown. (d) N2 and *aaim-1* mutants were fed fluorescent beads for

813 3 hours. Quantitation of percentage of worm with bead fluorescence. Three replicates were

814 performed experiment with at least 100 worms (a-c) or 20-30 worms (d) quantified per replicate.

815 Mean \pm SEM represented by horizontal bars. P-values determined via one-way ANOVA with post
816 hoc. Significance defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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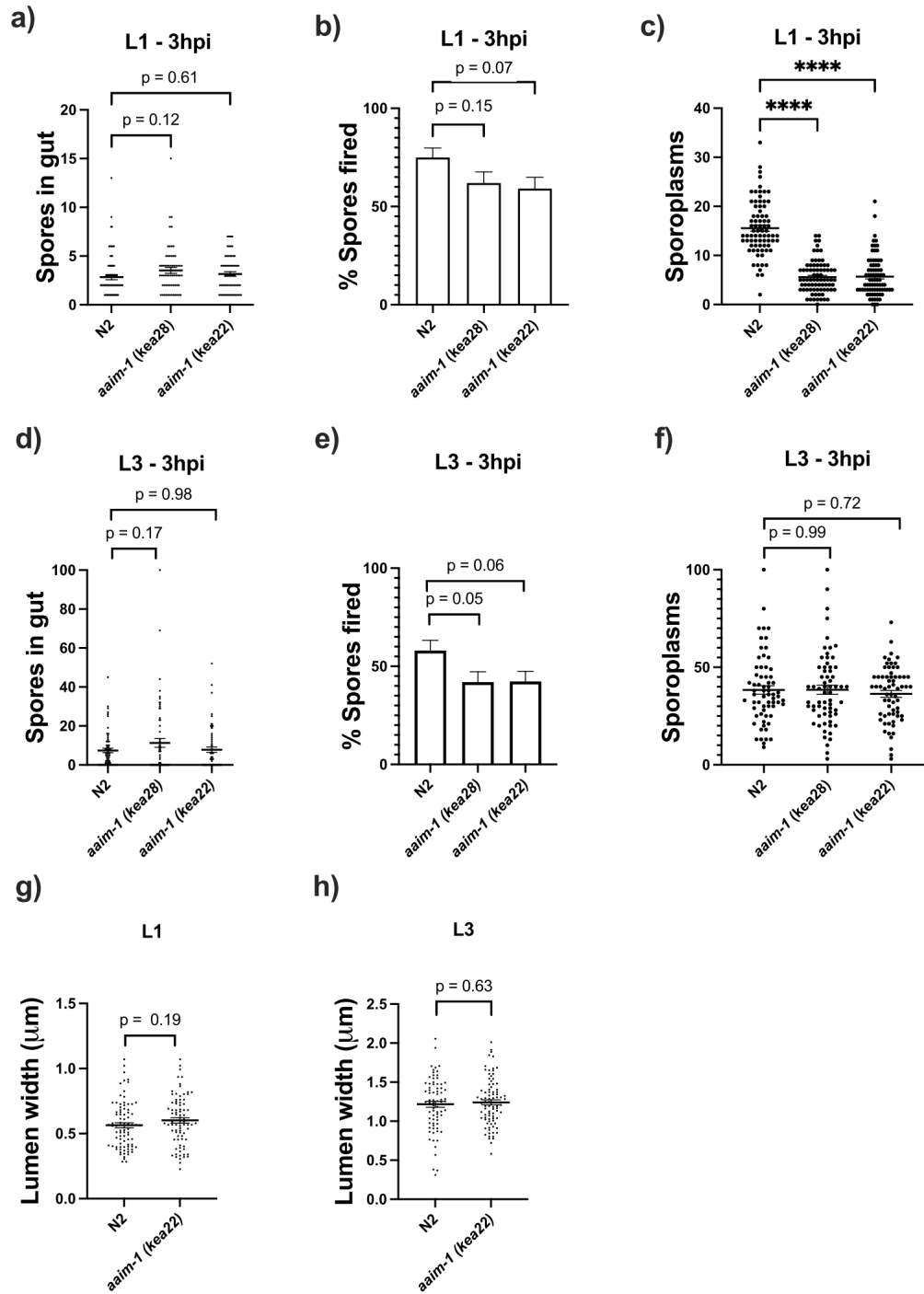
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827 **Figure S5: Invasion defects in *aaim-1* only occurs at the L1 stage of development and a**

828 **mutation in *aaim-1* do not alter the width of the intestinal lumen.**

829 (a-f) N2 and *aaim-1* animals were infected for 3 hours at L1 (a-c) or L3 (d-f), fixed, and then
830 stained with DY96 and an *N. parisii* 18S RNA fish probe. The number of spores per animal (a,d)
831 the percentage of spores fired (b,e) and the number of sporoplasm per worm (c,f) are displayed.
832 (g,h) The width of the intestinal lumen was measured in L1 (g) or L3 (h) wild-type or *aaim-1*
833 animals. (a-h) Experiment is of 3 replicates of 16-30 animals each. Mean \pm SEM represented by
834 horizontal bars. P-values determined via one-way ANOVA with post hoc (a-f) or Unpaired
835 Student's t-test (g,h). Significance defined as **** $p < 0.0001$.

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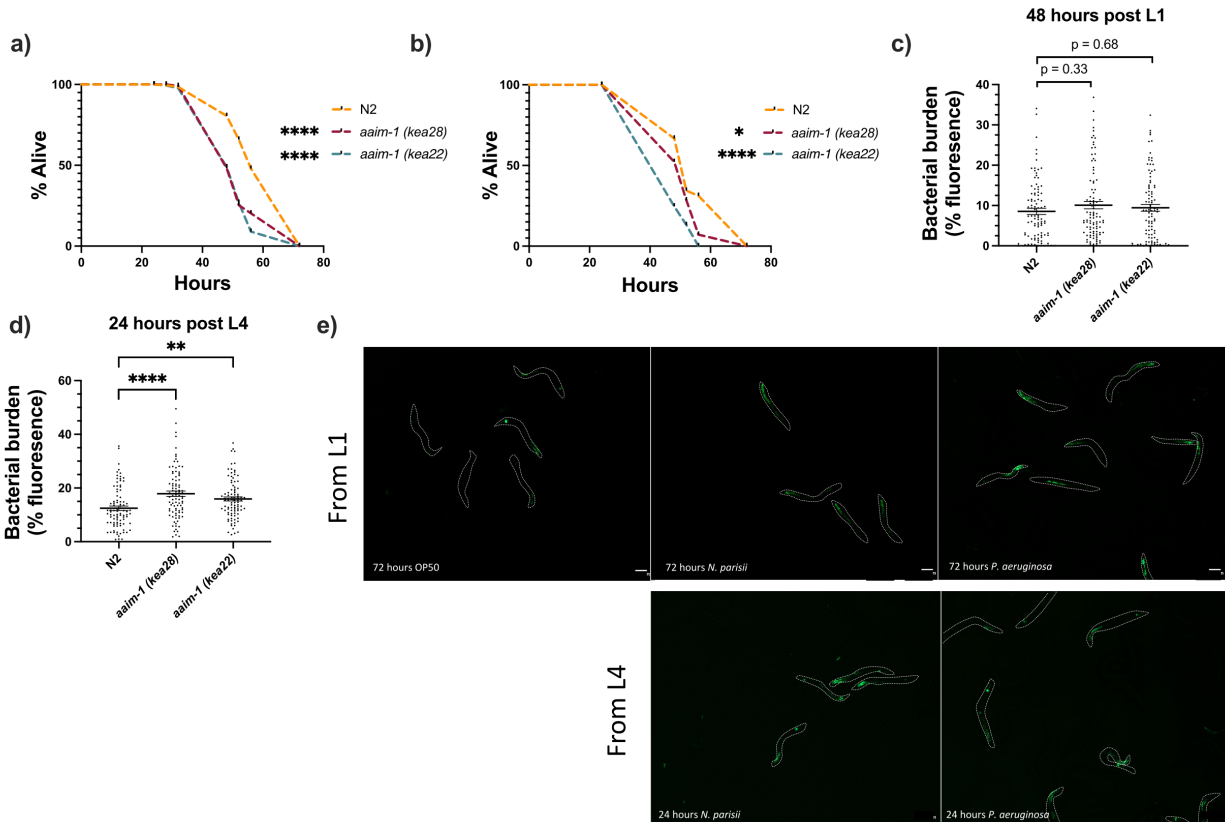
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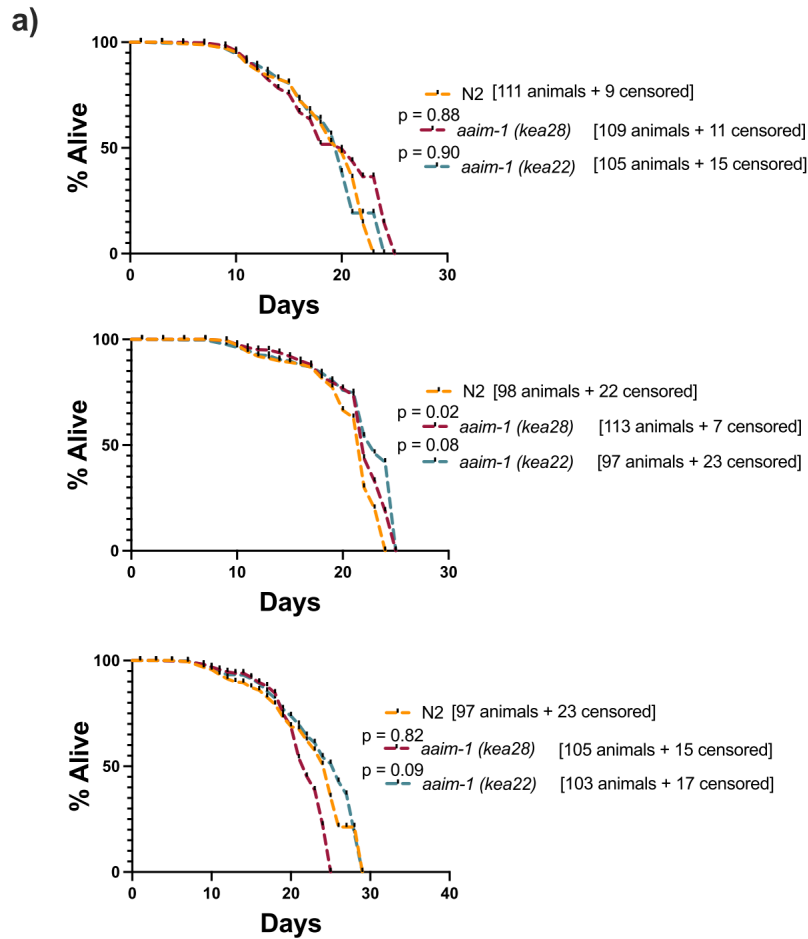
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843 **Figure S6: Susceptibility to *P. aeruginosa* PA14 appears at L4.**

844 (a,b) Additional replicates of survival assays of animals grown on full lawns of PA14 as in Figure
 845 5a. (c-d) N2 and *aaim-1* mutants were grown on PA14::DsRed 48 hours post L1 (c) or 24 hours
 846 post L4 (d). Experiment is of three replicates, with 20-30 worms were quantified per replicate via
 847 FIJI. Every point represents a single worm. Bacterial burden was measured as the percentage of
 848 the animal containing PA14::dsRED via FIJI. Mean \pm SEM represented by horizontal bars. (e)
 849 *paaim-1::GFP::3xFlag* were exposed to either PA14 or *N. parisii* 72 hours post L1 or 24 hours
 850 post L4. Animals were imaged at 45.5x, scale bar 500 μ m. P-values determined via one-way
 851 ANOVA with post hoc. Significance defined as ** $p < 0.01$, **** $p < 0.0001$.



852

853 **Figure S7: A mutation in *aaim-1* does not influence *C. elegans* lifespan.**

854 N2 and *aaim-1* mutants were grown on *E. coli* OP50 for one month, and survival measured as
855 number of animals responsive to touch. The number of animals quantified, as well as those
856 censored are denoted on the graph. Three independent survival assays are displayed. P-values
857 determined via Log-rank (Mantel-Cox) test.

T14E8.4 (X-6559570-6562366)

CHROM	POS	REF	ALT	AF	allele	effect	impact	gene_name	gene_id	feature_id	transcript_biotype	nt_change	aa_change
X	6559573	A	G	0	G	synonymous_variant	LOW	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.1545T>C	p.Asn515Asn
X	6560363	A	G	0	G	synonymous_variant	LOW	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.1284T>C	p.Ser428Ser
X	6560426	A	G	0	G	synonymous_variant	LOW	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.1221T>C	p.Tyr407Tyr
X	6560445	T	TA	0	TA	splice_region_variant&intron_variant	LOW	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.1204-3_1204-2insT	NA
X	6560485	A	G	0.77	G	splice_region_variant&intron_variant	LOW	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.1203+7T>C	NA
X	6560524	C	G	0	G	missense_variant	MODERATE	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.1171G>C	p.Val391Leu
X	6560647	C	A	0.02	A	missense_variant	MODERATE	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.1048G>T	p.Ala350Ser
X	6560670	A	G	0	G	missense_variant&splice_region_variant	MODERATE	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.1025T>C	p.Val342Ala
X	6560810	T	A	0.01	A	missense_variant	MODERATE	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.940A>T	p.Ile314Phe
X	6560841	G	A	0.01	A	synonymous_variant	LOW	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.909C>T	p.Thr303Thr
X	6560869	A	T	0.02	T	missense_variant	MODERATE	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.881T>A	p.Val294Glu
X	6560873	C	T	0.01	T	missense_variant	MODERATE	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.877G>A	p.Val293Ile
X	6560876	T	C	0	C	missense_variant	MODERATE	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.874A>G	p.Lys292Glu
X	6560955	C	T	0	T	synonymous_variant	LOW	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.795G>A	p.Ser265Ser
X	6561004	C	T	0.09	T	missense_variant	MODERATE	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.746G>A	p.Arg249Lys
X	6561012	G	T	0.07	T	synonymous_variant	LOW	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.738C>A	p.Ile246Ile
X	6561013	A	T	0.01	T	missense_variant	MODERATE	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.737T>A	p.Ile246Asn
X	6561030	C	T	0.54	T	synonymous_variant	LOW	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.720G>A	p.Thr240Thr
X	6561062	C	T	0	T	missense_variant	MODERATE	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.688G>A	p.Ala230Thr
X	6561122	T	A	0	A	missense_variant	MODERATE	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.628A>T	p.Ile210Phe
X	6561199	G	T	0.03	T	missense_variant	MODERATE	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.551C>A	p.Thr184Asn
X	6561201	T	A	0.02	A	missense_variant	MODERATE	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.549A>T	p.Leu183Phe
X	6561212	A	G	0	G	splice_region_variant&intron_variant	LOW	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.544-6T>C	NA
X	6561830	G	A	0	A	synonymous_variant	LOW	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.403C>T	p.Leu135Leu
X	6562016	G	C	0.01	C	missense_variant	MODERATE	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.217C>G	p.Gln73Glu
X	6562145	A	T	0	T	missense_variant	MODERATE	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.138T>A	p.Asn46Lys
X	6562349	A	T	0	T	missense_variant	MODERATE	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.18T>A	p.Phe6Leu
X	6562355	T	A	0.05	A	missense_variant	MODERATE	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.12A>T	p.Leu4Phe
X	6562361	C	G	0.01	G	missense_variant	MODERATE	T14E8.4	WBGene00043981	T14E8.4.1	protein_coding	c.6G>C	p.Arg2Ser

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859 **Figure S8: List of naturally occurring *aaim-1* variants in wild isolates of *C. elegans*.**

860 This table represents a list of *T14E8.4* coding variants found to naturally occur in wild isolates of

861 *C. elegans* generated by the CeNDR variant browser.⁴³ The reference allele (REF) as well as the

862 alternate variant (ALT) and the allele frequency (AF) are displayed for various sites (POS) across

863 *T14E8.4*. The nature (effect) and impact of these variants are depicted as well as the nucleotide

864 changes (nt_change) and the corresponding amino acid change (aa_change). *T14E8.4* does not

865 possess any variants predicted to have a high impact, implying that there are no obvious loss of

866 function alleles and that its retention in the wild is advantageous.

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869 Supplemental table 1: List of strains utilized in this study

Strain name	Genotype	Source
N2	Wild-type , Bristol strain	Caenorhabditis genetics center (CGC)
<i>fawn-1</i> (AWR 05)	<i>aaim-1 (kea89)</i> X C127T , Q43Stop	This study
<i>fawn-2</i> (AWR 11)	<i>aaim-1 (kea90)</i> X G221A splice site mutation	This study
<i>fawn-3</i> (AWR 17)	<i>aaim-1 (kea28)</i> X 2.2 kb deletion	This study
<i>fawn-4</i> (AWR03)	<i>aaim-1 (kea91)</i> X C1286T, A429V	This study
DM7448	VC20019 Ex[<i>Pmyo-3::YFP</i>]	Mok et al. (2020) ⁷⁵
RB563	<i>aaim-1 (ok295)</i> X	CGC
AWR 73	<i>aaim-1 (kea22)</i> X	This study
AWR 83	<i>aaim-1 (kea28)</i> X	This study
DA465	<i>eat-2 (ad465)</i> II	CGC
AWR 131	N2Ex[<i>pmyo2::mCherry::Unc54, paaaim-1::GFP::3xFlag::Unc54</i>]	This study
AWR 125	<i>aaim-1 (kea28)</i> Ex[<i>pmyo2::mCherry::Unc54, paaaim-1::GFP::3xFlag::Unc54</i>]	This study
AWR 122	<i>aaim-1 (kea28)</i> Ex[<i>pmyo3::mCherry::Unc54, paaaim-1::GFP::Unc54</i>]	This study
AWR 115	<i>aaim-1 (kea28)</i> Ex[<i>pmyo2::mCherry::Unc54, paaaim-1::aaim-1::Unc54</i>]	This study

AWR119	<i>aaim-1</i> (<i>kea28</i> <i>Expmyo2::mCherry::Unc54</i> , <i>paaim-1::aaim-1::3xFlag::Unc54</i>)	This study
AWR 127	<i>aaim-1</i> (<i>kea28Expmyo2::mCherry::Unc54</i> , <i>paaim-1::SPΔ aaim-1::3xFlag::Unc54</i>)	This study
AWR129	<i>aaim-1</i> (<i>kea28</i> <i>Expmyo2::mCherry::Unc54</i> , <i>pspp-5:: aaim-1::3xFlag::Unc54</i>)	This study
YY1446	<i>znfx-1</i> (<i>gg634</i> [<i>HA::tagRFP::znfx-1</i>]) II.	CGC

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886 **Supplemental table 2: Spore doses utilized in this study.**

Species	Dose	Plate concentration (spores/cm ²)	Total spores on assay plate (Millions) Plate size: *3.5cm, **6cm, ***10cm
<i>N. parisii</i>	Very low	25,984	0.25*
	low	207,875	2.0*
	Medium-1	1,247,232	3.0*
	Medium-2	115,050	3.25
	Medium-2	41,380	3.25**
	Medium-3	141,600	4.0
	High	194,700	5.50
	Very high	318,600	9.0
	Maximal	637,200	18.0
<i>N. ausubeli</i>	Medium	103,936	1.0*
	High	519,680	5.0*

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896 **Supplemental table 3: Primer sequences.**

Primer description	Sequence
Forward primer to amplify <i>aaim-1</i>	5'- atgaggttattatTTTTTcagcat -3'
Reverse primer to amplify <i>aaim-1</i>	5'-ttaatTTTTTgctggtgagg-3'
Forward primer to generate <i>SPΔaaim-1</i>	5'-atgctaaaggattcttgcctg-3'
Forward primer to amplify <i>paaim-1</i>	5'-ttagttggaaatgcacaaaaaactgatctct-3'
Reverse primer to amplify <i>paaim-1</i>	5-cagtggtctctgcttataaaatgacttc-3'
Forward primer to amplify <i>pmyo2</i>	5'-cattttatatctgagtagtatccttgccttaaatgtcc-3'
Reverse primer to amplify <i>pmyo2</i>	5'- gcatttctgtgtctgacgat-3'
Forward primer to amplify <i>pspp5</i>	5'-aaagcaaaatatcattattgggaaaatc-3'
Reverse primer to amplify <i>pspp5</i>	5'-tctgtaataaaataaattgaaatgaaacac-3'
Forward primer to amplify GFP from pDD282	5'-atgagtaaaggagaagaattgttact-3'
Reverse primer to amplify GFP from pDD282	5'-ttactgtagagctcgtccattccg-3'
Forward ultramer to add a Gly Ala Gly Ser linker and <u>3x Flag</u> with <i>stop codon</i> to C-Terminal end of constructs in pDDONR221 via round the horn PCR. ⁶⁷	5'- <u>ggagccggatctgattataaagacgatgacgataagcgtgactacaaggacgacgacgaca</u> <u>agcgtgattacaaggatgacgatgacaagagataaacccagcttctgtacaaagttg-3'</u>

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898 **References:**

- 899
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901 Analysis of the Extensive Ecological and Phenotypic Diversity of Microsporidia. *mBio* **0**,
902 e01490-21.
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904 Horizontal Gene Transfers. *Annu. Rev. Microbiol.* **69**, 167–183 (2015).
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915 wall, virulence protein from the shrimp microsporidian, Enterocytozoon hepatopenaei
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