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1 # AMPK regulates germline stem cell quiescence and integrity through an
2 # endogenous small RNA pathway

3 **Pratik Kadekar[1] and Richard Roy[1,2]**

4 [1] Department of Biology, McGill University, Montreal, Quebec, Canada H3A 1B1

5 **Keywords:** AMPK, chromatin modifications, small RNAs, dauer, germ line, *C. elegans*

6 [2] to whom correspondence should be addressed
7 Richard ROY
8 Department of Biology,
9 McGill University,
10 1205 avenue Docteur Penfield,
11 Montreal, Quebec, Canada H3A 1B1
12 Email: richard.roy@mcgill.ca

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13 **Abstract**

14 *C. elegans* larvae can undergo a global developmental arrest following the execution of a diapause-
15 like state called 'dauer' in response to unfavourable growth conditions. Survival in this stage
16 surpasses the normal lifespan of reproductive animals quite dramatically, and without any apparent
17 negative impact on their reproductive fitness. During this period, the germ cells become quiescent
18 and must retain their reproductive integrity so the animal can reproduce following recovery. This
19 germline stem cell (GSC) arrest requires the activity of AMP-activated protein kinase (AMPK)
20 and in its absence the germ line undergoes hyperplasia. We show here that AMPK mutant animals
21 exhibit complete sterility after recovery from dauer, suggesting that germ cell integrity is
22 compromised during this stage in the absence of AMPK. These defects correlate with altered
23 abundance and distribution of a number of chromatin modifications that affect gene expression.
24 These aberrant chromatin modifications, along with the supernumerary germ cell divisions and the
25 observed post-dauer sterility, were all corrected by disabling key effectors of the small interfering
26 RNA pathway (*dcr-1* and *rde-4*) and the primary Argonaute protein *ergo-1,* suggesting that AMPK
27 regulates the function of these small RNA pathway components, and in its absence, the pathways
28 become abnormally active. The aberrant regulation of the small RNA pathway components
29 releases the germ cells from quiescence to proliferative state thereby compromising germ cell
30 integrity. Curiously, AMPK expression in either the neurons or the excretory system is sufficient
31 to restore the GSC quiescence and the fertility in the AMPK mutant post-dauer adults, while the
32 fertility of these animals is also partially restored by disabling the dsRNA importer SID-1. Our
33 data suggest that AMPK regulates a small RNA pathway in the soma to establish and/or maintain
34 GSC quiescence and integrity cell non-autonomously in response to the energy stress associated
35 with the dauer stage. Our findings therefore provide a unique model to better understand how the

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36 soma communicates with the germ line to establish the appropriate epigenetic modifications

37 required to adapt to acute environmental challenges.

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38 **Introduction**

39 It is becoming more widely accepted that life history can affect developmental and behavioural
40 outcomes, either in a temporary, or often in a more permanent manner. These modifications can
41 occur downstream of a broad spectrum of environmental factors, including temperature, light,
42 resource availability, population density, and even the presence of predators; all of which can
43 influence gene expression, often with dramatic phenotypic consequences (1, 2). Furthermore, these
44 consequences are not restricted to the generation that experienced the event, but rather, they can
45 be transmitted to subsequent generations.

46 Studies have shown that the molecular record of these events is encoded in the form of epigenetic
47 changes associated with histone modifications, DNA methylation and/or base modification, or
48 alterations in the small RNA repertoire (3). Because the transmission of these molecular memories
49 can span one or several generations, these modifications must impinge in some way upon the germ
50 line, thus providing some adaptive phenotypic change in the unexposed future generations (4-7).
51 These epigenetic modifications in the germ cells can have a significant impact on successive
52 generations, yet the molecular mechanisms through which "experience" is transduced to the
53 genome across several generations remains ill-defined.

54 *C. elegans* has been used successfully to demonstrate how environmental cues can modulate
55 epigenetic change and behaviour (8). Furthermore, a subset of these modifications and associated
56 traits can be transmitted to subsequent generations in a manner dependent on small heritable RNAs
57 (9). Recently, it was shown that acute starvation at the L1 larval stage leads to the generation of
58 small RNA species that are inherited for at least three generations. This heritable pool of RNAs
59 could reflect the adaptive change in expression of genes involved in nutrition and metabolism (6,
60 10).

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61 In addition to the L1 stage, later in development, larvae can execute an alternative developmental
62 program to enhance survival and fitness in response to overcrowding or sub-optimal survival
63 conditions. During this diapause-like state called dauer, they undergo a global, genome-wide,
64 adjustment of chromatin modifications that is accompanied by a significant change in gene
65 expression when compared to the animals that never transited through this stage. These changes
66 in the abundance and distribution of chromatin marks likely contribute to the molecular record of
67 life history and the adaptive adjustment of these chromatin modifications is most probably
68 dependent on the expression of specific endogenous small RNAs (11, 12). Currently, it is still
69 unclear how the physiological stress associated with the dauer stage might impact the population
70 of small RNAs and, in transgenerational contexts, how these changes are transmitted across
71 generations, in light of the erasure of histone marks that normally takes place during each cycle of
72 embryogenesis.
73 Global cell cycle arrest is one of several distinctive features of *C. elegans* dauer larvae. Upon entry
74 into the dauer stage the germline stem cell (GSC) divisions begin to slow to finally establish a state
75 of quiescence, which they maintain until they recover from dauer and resume normal development.
76 Despite potentially long periods in this diapause stage, this cell cycle/developmental quiescence
77 has no impact on their reproductive fitness (11). The activity of the cellular energy sensor AMPK
78 and its upstream kinase LKB1/PAR-4, as well as the activity of tumour suppressor PTEN, are all
79 independently required for the quiescent state of the GSC in response to dauer signalling (13).
80 Maintaining a quiescent state in response to energetic stress may be favourable for survival,
81 presumably because it reduces energy consumption during a period when energy is limited (14,
82 15). Moreover, when quiescence fails to establish and/or maintained during periods of energy
83 stress, germ cell integrity is compromised, resulting in reduced brood sizes (4).

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84 We show here that during the dauer stage, AMPK activity is not only required to block GSC
85 proliferation, but also to maintain GSC integrity to ensure reproductive success following recovery
86 to replete conditions. In the absence of AMPK, several chromatin modifications become
87 misregulated, resulting in inappropriate gene expression that has a detrimental effect on
88 reproductive fitness following exit from the dauer stage. Using genetic analysis, we reveal the
89 importance of the endogenous small RNA pathway and its regulation by AMPK. Moreover, this
90 pathway acts at least partially in a cell non-autonomous manner, to adjust the GSC-specific
91 chromatin landscape in favour of an adaptive gene expression program fine-tuned toward
92 maintaining germ cell integrity during the long-term energy stress typical of the dauer stage.

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93 **Results**

94 **Defects in the dauer germ line result in post-dauer sterility in AMPK mutants.**

95 In *C. elegans,* the decision to execute dauer development is regulated by three independent
96 signalling pathways that converge on a nuclear hormone receptor to ultimately affect multiple
97 developmental and physiological processes (16). Many of these processes involve measures to
98 conserve energy for the duration of the diapause, which are mediated through a significant
99 metabolic adjustment that occurs downstream of all these signalling pathways (17).
100 To conserve the energy, while also ensuring that germ cell cells do not replicate during this period
101 when key cellular building blocks may be limiting, the *C. elegans* orthologues of LKB1 (*par-4*)
102 and the regulatory and catalytic components of AMPK cooperate to establish cell cycle and
103 developmental quiescence in the germline stem cells (GSC). Animals that have reduced, or lack
104 all AMPK activity (*aak(0)*), undergo pronounced germline hyperplasia due to supernumerary cell
105 divisions that occur prior to dauer entry (13). It is unclear, however, if these extra cells retain their
106 germ cell integrity and are competent to yield functional gametes.
107 The dauer larva is remarkable in that it can remain in a quiescent state for months longer than it
108 would normally survive while in its reproductive mode. Nevertheless, it can exit this quiescence
109 upon improvement in growth conditions, to resume reproductive development with no
110 compromise of their reproductive fitness, regardless of the duration of the developmental arrest
111 *per se* (18). The germ cells must therefore retain the appropriate information to maintain their
112 totipotency over lengthy periods so that upon recovery from the diapause the animal can still
113 reproduce without any loss in fitness. Since AMPK and LKB1 are critical to block germ cell
114 divisions during the dauer stage we questioned whether the supernumerary germ cells that are
115 produced in *aak(0)* mutants are indeed competent to generate functional gametes and/or embryos.
116 We therefore quantified the brood size of *daf-2* (control) and *daf-2; aak(0)* animals after allowing

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117 both of these mutants to recover after remaining at least 24h in the dauer stage. In contrast to
118 control *daf-2* animals that recover from dauer with very little to no negative reproductive
119 consequence, AMPK mutant animals that transit through dauer for 24 hours or more exhibit highly
120 penetrant post-dauer (PD) sterility upon recovery (Fig. 1A, Fig. S2). The brood size of *daf-2* PD
121 adults was not significantly different from *daf-2* animals that never transit through dauer
122 suggesting that passage through the dauer stage has no impact on reproductive fitness provided
123 that AMPK signalling is active.
124 To confirm that the AMPK activity is not exclusive to the insulin-like signalling branch involved
125 in dauer formation, but rather, it is required downstream of the other signalling pathways that
126 control dauer formation, we determined if AMPK may play a more general role in PD fertility by
127 testing if it is also required in *daf-7* mutants (TGF-β pathway), or in the *aak(0)* mutants treated
128 with dauer pheromone (16). Similar to what occurs in the insulin-like signalling mutants, inducing
129 the dauer stage through the compromise of the TGF-β pathway, or treatment with dauer
130 pheromone, also results in highly penetrant sterility in PD animals that lack AMPK (Fig. S1). This
131 suggests that the activity of AMPK is critical for PD fertility, and hence the maintenance of germ
132 cell integrity, downstream of the major pathways required for dauer formation.
133 Although most of the *aak(0)* PD animals become sterile, a significant portion of die. Those that
134 survive also show diverse abnormalities in vulva development (Fig. 1B, Table S1). Thus, it is not
135 only the germ line which is compromised in PD animals that lack AMPK, but at least some somatic
136 tissues are also sensitive to loss of AMPK function.

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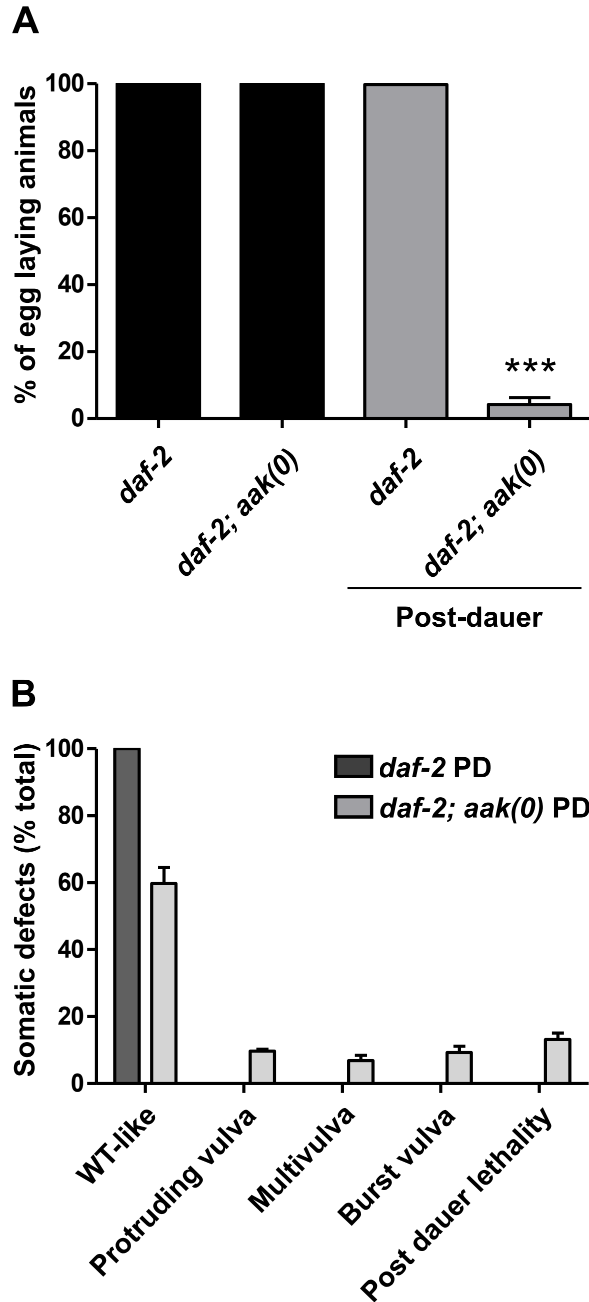

137 **Figure 1. Post-dauer *aak(0)* adults exhibit vulval defects and highly penetrant sterility.**

138 **A)** All adult animals that laid eggs were considered as fertile. Both *daf-2* and *daf-2; aak(0)* animals
139 cultivated under permissive conditions showed no fertility defects compared to wild type. To
140 assess the fertility of the PD adults, animals were maintained in the dauer stage for 24 hours after
141 which they were switched to permissive temperature to resume reproductive development (See
142 materials and methods). Egg laying animals were counted, the means calculated, and the values

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143 are shown with SD. Upon recovery, *daf-2* PD adults were fertile, but *daf-2; aak(0)* PD adults were
144 almost entirely sterile; ***P<0.0001 using Marascuilo procedure. Assays were performed three
145 times and the data represent the mean ±SD; n=50. **B)** In *daf-2; aak(0)* PD animals, the highly
146 penetrant sterility is also associated with vulval defects. A proportion of these animals (16.5±3.5%)
147 prematurely expired during their recovery phase and failed to reach adulthood. Values represent
148 means ±SD; n=50.

149 **AMPK post-dauer gonads are morphologically abnormal and the germ cells fail to exit**
150 **pachytene.**

151 To determine the physiological basis of the observed sterility in the *aak(0)* PD animals, we
152 examined their germline morphology and organization using a germ cell membrane marker (19).
153 We noted that in 95% of the animals, the oocyte morphology appeared abnormal and they also
154 lacked the typical, single-file organization seen in the control *daf-2* PD animals (Fig. 2A, B, Table
155 S2). Also, in 60% of the *aak(0)* PD animals, the general gonad symmetry was abnormal in terms
156 of size and shape of the gonadal arms. (Table S2).

157 We stained the germ lines of PD control and AMPK mutant animals and examined chromosome
158 morphology within the germ cells as they progress through the distinct regions of meiotic prophase
159 to generate fully differentiated oocytes (20). There were no obvious defects in the size of the
160 mitotic zone or the spatio-temporal arrangement of the transition zone. For further characterization
161 we binned the post-transition zone germ cells into 3 different zones: in Zone 1, germ cells enter
162 the pachytene stage; in Zone 2 the cells exit pachytene and initiate the separation of the paired
163 homologous chromosomes (diplotene); in Zone 3 separation of homologues is complete, forming
164 6 tightly condensed DAPI stained bodies representing 6 pairs of homologous chromosomes
165 (diakinesis).

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166 In the *daf-2* PD germline, there were no observed abnormalities as the germ cells complete meiotic
167 progression to eventually give rise to oocytes with 6 condensed DAPI-stained bodies (Fig. 2B).
168 However, in the PD germ lines of *aak(0)* mutants, the germ cells do enter pachytene in Zone 1, but
169 then fail to exit Zone 2. The pachytene arrest persists into Zone 3, as the chromosomes fail to
170 separate and condense (Fig. 2C). This suggests that in *aak(0)* PD animals, the germ cells fail to
171 exit pachytene and thus never undergo diakinesis to produce mature oocytes.

172 To further determine, if the sterility was a result of abnormal sperm formation or function, or
173 whether the defect was associated with the oocytes, we performed reciprocal crosses and
174 monitored the brood size of the resulting cross progeny (Fig. 2E, F). Using an antibody that detects
175 sperm-specific proteins (anti-MSP), we noted that while sperm was present in the *aak(0)* PD
176 hermaphrodites, it may have been produced during dauer stage (13). To determine if the sperm
177 present in the *aak(0)* PD adults is functional, we mated *aak(0)* hermaphrodites that never transited
178 through dauer, with *aak(0)* PD males (a ratio of 20 males per hermaphrodite was maintained). If
179 the mating was successful and the PD males produced functional sperm, we would expect ~50%
180 of the progeny to be male. However, no significant F1 male progeny were observed suggesting
181 that the sperm is defective (Fig. 2E). We cannot rule out however that, despite our monitoring,
182 these mutants could be mating incompetent.

183 Similarly, when we mated PD *aak(0)* hermaphrodites and *aak(0)* males that never transited through
184 dauer, *aak(0)* PD hermaphrodites still exhibited highly penetrant sterility (Fig. 2F) suggesting that
185 integrity of the oocytes is also compromised.

186 These results collectively suggest that germline development is sensitive to periods of energetic
187 stress, and in the absence of AMPK, the germ line becomes severely perturbed; the integrity of
188 both the oocytes and the sperm is affected, ultimately rendering the PD animals sterile.

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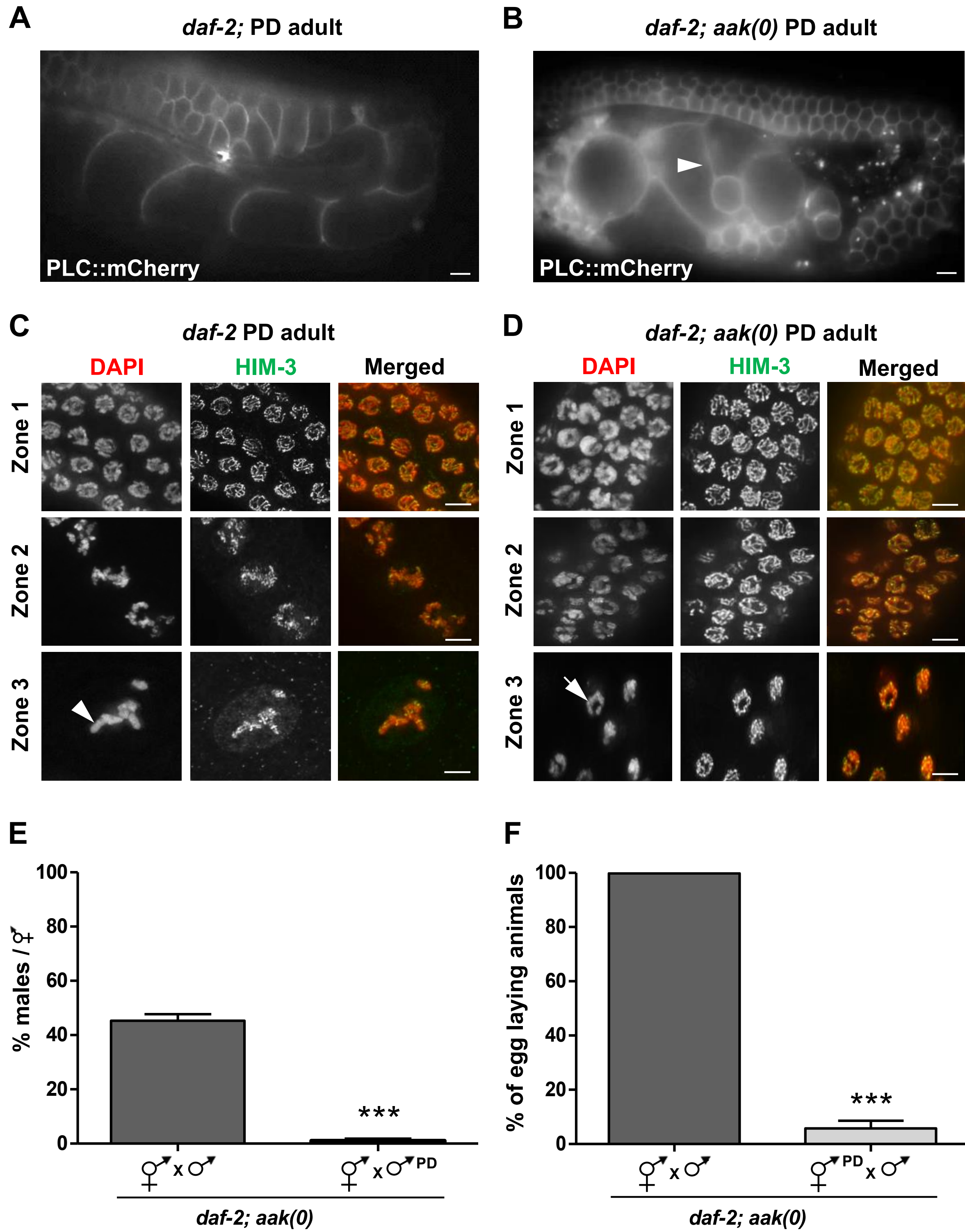

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189 **Figure 2. AMPK-defective post-dauer adults show abnormal gonadal morphology and the**
190 **germ cells fail to exit pachytene.**
191 All animals analyzed express a *Ppie-1::*PLC::mCherry transgene to monitor germ cell
192 membranes/organization. **A, B**) In *daf-2* PD adults, the gonad and germ cells develop normally
193 and no obvious defects were observed, but *daf-2; aak(0)* PD adults exhibit various defects in gonad
194 development and organization. Oocyte morphology is abnormal (white arrowhead) and they
195 lacked the typical, file-like organization observed in the control *daf-2* PD animals. **C, D)** For
196 further characterization, the post-transition zone germ cells were divided into 3 different
197 subregions. In the first subregion after the transition zone (Zone 1), germ cells enter pachytene
198 stage; in Zone 2, the cells exit pachytene and initiate the separation of the paired chromosomes
199 (diplotene); in Zone 3 separation of the paired chromosomes is complete, forming 6 tightly
200 condensed DAPI-stained bodies representing 6 pairs of homologous chromosomes (diakinesis). In
201 *daf*-2 PD, the germ cells go through all these processes to eventually give rise to 6 condensed
202 DAPI-stained bodies (white arrowhead), but in *daf-2; aak(0)* PD adults, the germ cells enter
203 pachtytene in Zone 1, but fail to completely exit the pachytene stage based on the continued
204 presence of long chromosome tracks (white arrow). A-D) n= 20. Scale bar: 10um in A and B, 4
205 um in C and D. **E, F)** Reciprocal crosses were performed and a ratio of 20 males per hermaphrodite
206 was maintained for all the crosses. *daf-2; aak(0)* PD males were mated with normal *daf-2; aak(0)*
207 15 hermaphrodites and a number of males/hermaphrodite in F1 were counted. Few to no male
208 progeny were identified in the F1 generation of this mating. The mean is shown ± SD. **F)** *daf-2;*
209 *aak(0)* PD hermaphrodites were crossed with normal *daf-2; aak(0)* males. 15 animals were
210 quantified and PD *aak(0)* hermaphrodites exhibited a high frequency of sterility. The mean is
211 represented ± SD. ***P<0.0001 using Marascuilo procedure.

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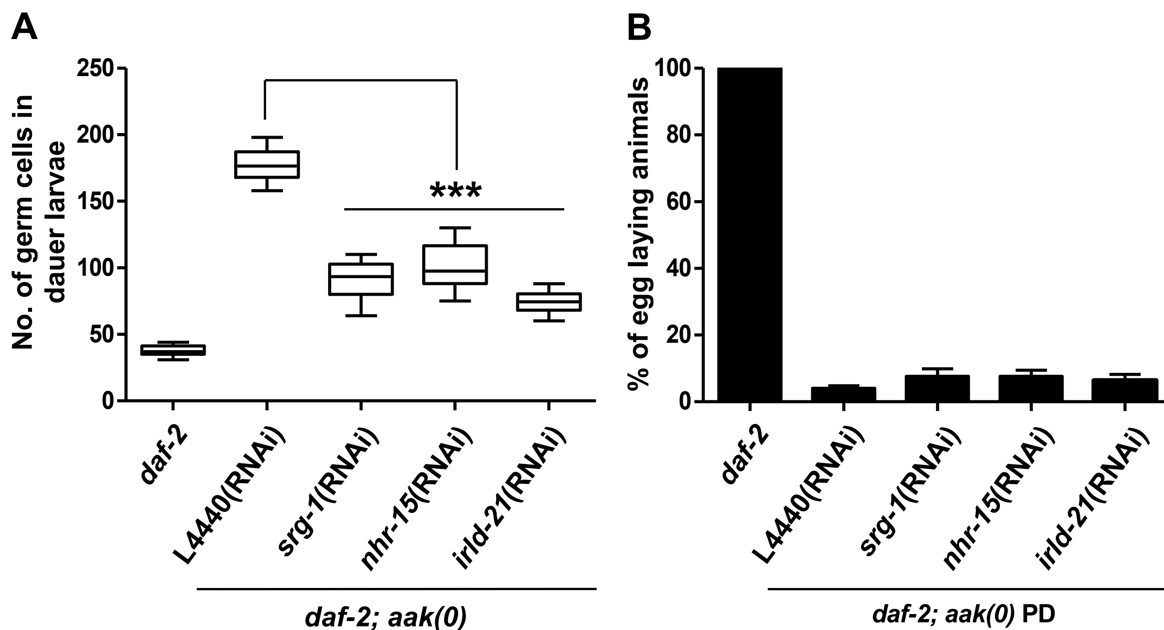
212 **Reducing germline hyperplasia does not restore fertility in post-dauer AMPK mutants.**

213 As *aak(0)* dauer larvae fail to maintain the GSC arrest and exhibit sterility upon exiting the dauer
214 stage, we questioned whether the observed sterility might result from the inappropriate germ cell
215 divisions that occur during the dauer stage. To test this, we performed RNAi on genes that were
216 shown to suppress the supernumerary germ cell divisions in the *aak(0)* dauer larvae (21). We
217 subsequently allowed these animals to recover to form adults, after which we assessed their fertility
218 and brood size. None of the suppressors of dauer germline hyperplasia that we tested were capable
219 of restoring the PD fertility in the *aak(0)* mutants, although the germline hyperplasia typical of
220 *aak(0)* mutant dauer larvae was visibly ameliorated (Fig. 3A, B). Albeit, RNAi of these genes does
221 not completely suppress the germline hyperplasia, leaving the possibility that the extra number of
222 germ cells could be responsible for the PD sterility. Nevertheless, our data suggest that the PD
223 sterility of *aak(0)* animals is not necessarily a direct consequence of the dauer germline
224 hyperplasia, but may involve additional processes that could act in concert with, but possibly
225 independent of, the regulation of cell cycle quiescence during the dauer stage.



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226 **Figure 3. Post-dauer sterility and germline hyperplasia are not obligately interdependent in**
227 **AMPK mutants.**
228 **A, B**) Whole animal DAPI staining was performed to quantify the number of dauer germ cells.
229 The number of egg-laying animals was quantified and the mean is represented ± SD. To test if the
230 germ cell integrity defect results from the dauer-dependent germline hyperplasia, we used RNAi
231 to disrupt three gene products previously found to suppress germline hyperplasia in dauer larvae
232 (21). Larvae were switched to permissive temperature to exit dauer and resume reproductive
233 development. Fertility was assessed 48h after the temperature shift by counting egg-laying adults.
234 L4440 is an empty RNAi vector and is used as a control. ***P<0.0001 when compared with L4440
235 using the two-tailed t-test. n=50.

236 **Many chromatin marks are misregulated both globally and in the germ line in *aak(0)* dauer**
237 **larvae.**
238 *C. elegans* dauer larvae exhibit a significantly different gene expression profile when compared to
239 animals that never transit through the dauer stage (11). Furthermore, these changes in the gene
240 expression persist after the animals exit dauer and become reproductive adults. Thus, a molecular
241 memory of the passage through dauer is recorded, and has been shown to influence fertility in the
242 PD animals (11). The observed changes in gene expression are highly correlated with the changes
243 that occur in the various chromatin marks detected in dauer and in PD larvae compared to controls
244 that never transited through dauer (11).
245 AMPK has been implicated in the regulation of gene expression through its ability to modify
246 chromatin by directly phosphorylating histone H2B to activate stress-promoted transcription (22).
247 Furthermore, recently we showed that AMPK modulates the chromatin landscape to ensure that
248 transcriptional activity is blocked in the primordial germ cells until animals have sufficient cellular

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249 energy levels (4). Since AMPK may directly regulate histone modifying enzymes to bring about
250 changes in gene expression we wondered whether chromatin modification may be perturbed in the
251 dauer germ cells, thereby affecting the adaptive gene expression program that would normally
252 occur in dauer. This inability to appropriately adjust to the energy stress associated with dauer
253 development might explain the loss of integrity in the PD germ cells.
254 We therefore examined the global levels of diverse chromatin marks that were previously found
255 to change following transit through the dauer program. We first performed western blot analysis
256 on whole extracts from *daf-2* and *daf-2; aak(0)* mutant dauer larvae using antibodies specific for
257 histone modifications that are associated both with transcription activation (H3K4me3 and
258 H3K9ac) and repression (H3K9me3 and H3K27me3). Interestingly, all the marks we tested were
259 abnormally high in the absence of AMPK (Fig. 4A). To confirm if the increased level of these
260 chromatin marks was associated with the hyperplasia associated with AMPK mutant dauer larvae,
261 we performed the same experiments in animals that lack *glp-1* (Fig. S3) (20) and quantified the
262 levels of the chromatin marks. The reduction of germ cells significantly decreased the levels of the
263 chromatin marks, suggesting that the germ cells are the major contributors to the global increase
264 in the levels of the chromatin marks in AMPK mutant dauer larvae, although the levels also
265 increase in the soma (Fig. 4B).

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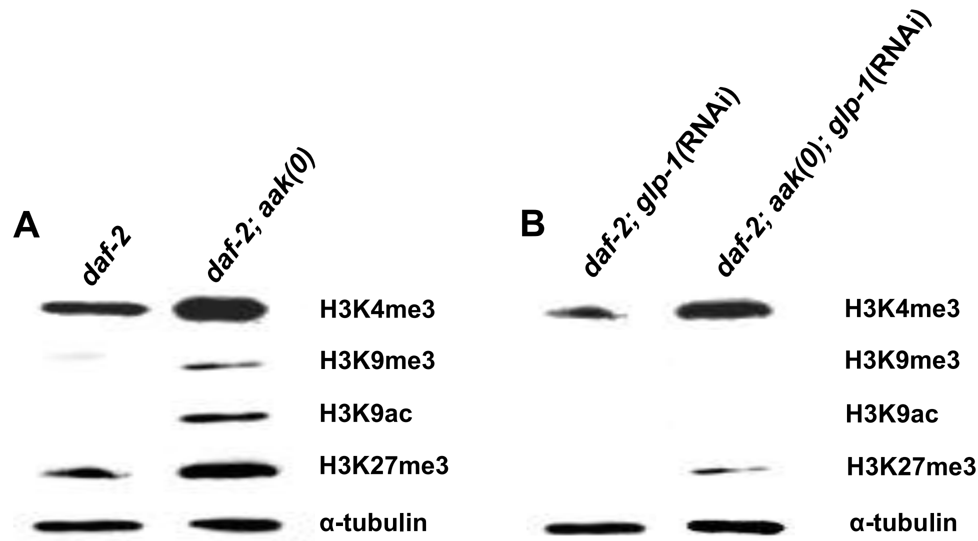

266 **Figure 4. AMPK modulates the abundance of diverse chromatin marks in the soma and the**
267 **germ line during the dauer stage.**
268 **A, B)** Global levels of H3K4me3, H3K9me3, H3K9ac, and H3K27me3 were quantified by
269 performing whole animal western blot analysis of *daf-2* and *daf-2; aak(0)* dauer larvae. *glp-*
270 *1*(RNAi) was performed post-embryonically using dsRNA feeding in order to compromise
271 germline development without affecting early embryogenesis. α-tubulin is used as a loading
272 control.

273 **The distribution of chromatin marks is dramatically altered in *aak(0)* dauer germ cells and**
274 **higher levels of these marks persist in the post-dauer germ line.**
275 From our western analysis we could not discern if the levels in the chromatin marks were abundant
276 simply because of the supernumerary germ cells in the AMPK mutant dauer larvae or whether the
277 levels were higher in each individual nucleus *per se.* We therefore dissected gonads from both *daf-*
278 *2* and *daf-2; aak(0)* dauer larvae and quantified the levels of histone H3 lysine 4 trimethylation
279 (H3K4me3) and histone H3 lysine 9 trimethylation (H3K9me3) to better evaluate their levels per

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280 nucleus and to determine if there were any changes in their proximal-distal distribution through
281 the gonad. In the control *daf-2* dauer germ line, the levels of both H3K4me3 and H3K9me3 are
282 uniform across all nuclei throughout the dauer germ line, but in the AMPK mutant dauer larvae,
283 the pattern of H3K4me3 and H3K9me3 expression in the gonad is altered, while their levels are
284 highly variable in individual nucleus (Fig. 5A, B). Of particular interest, we noted that the
285 expression of H3K4me3 in individual nucleus is comparatively weak in the distal gonad, but
286 gradually increases toward the proximal goal where it is much higher.
287 To test whether the abnormal distribution and abundance of the H3K4me3 and H3K9me3 marks
288 are resolved after the *aak(0)* larvae exit dauer, we examined these marks in PD adult gonads.
289 Interestingly, we noted that higher levels of both H3K4me3 and H3K9me3 persist in the *aak(0)*
290 PD adult germ line when compared to the control *daf-2* PD animals (Fig. 5C, D).
291 Altogether, these data confirm the role of AMPK in the regulation of both transcriptionally
292 activating and repressive chromatin marks in the germ line under energetic stress. In its absence
293 the levels of each mark we tested increased, while the distribution of these marks was dramatically
294 disrupted.

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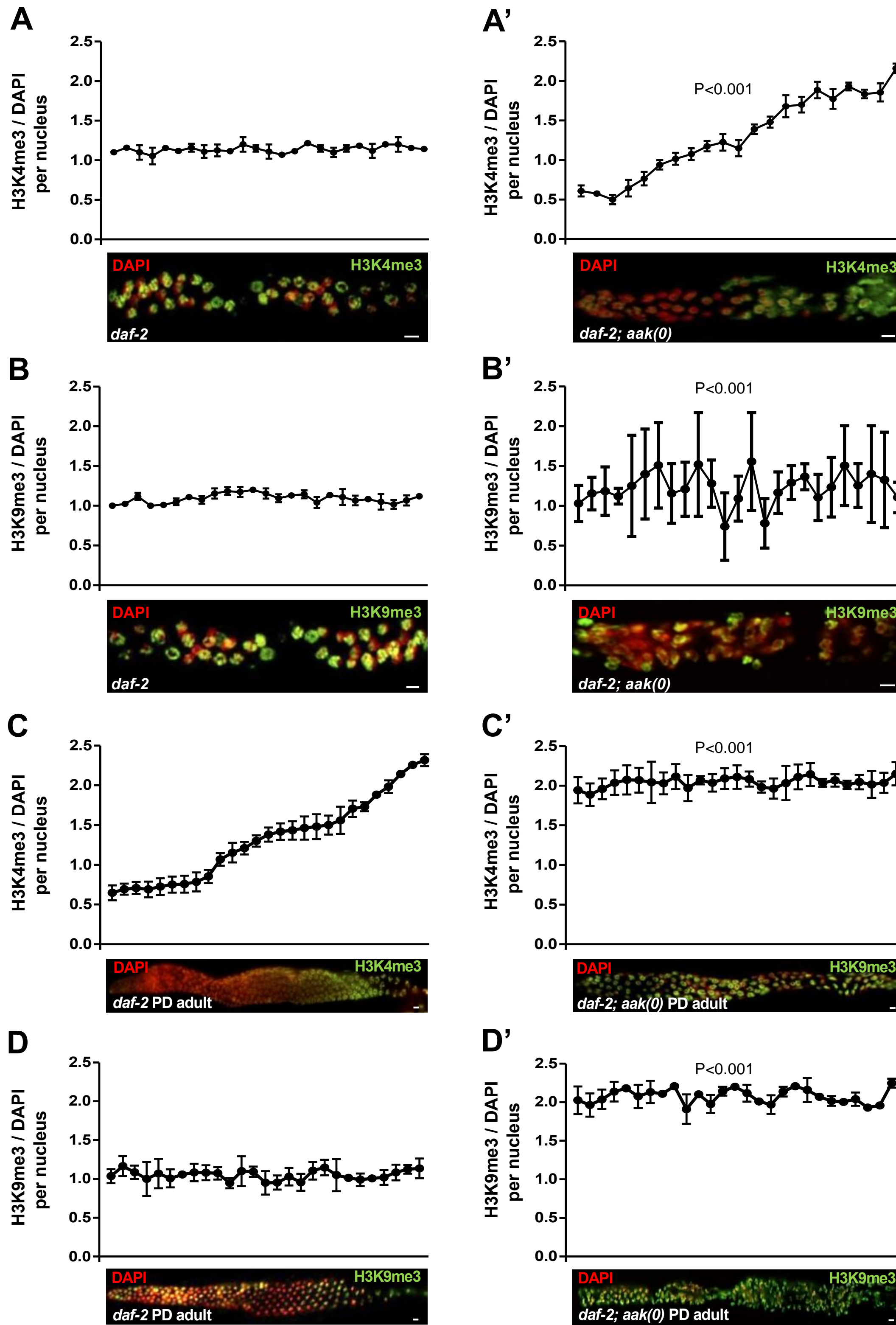

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295 **Figure 5. The distribution and abundance of both activating and repressive chromatin marks**
296 **are dramatically altered in the *aak(0)* dauer and post-dauer germ cells**
297 All images are merged, condensed Z stacks. The graphs represent the average immunofluorescence
298 signal of anti-H3K4me3 and anti-H3K9me3 normalized to DAPI across the dissected germ line.
299 For the micrographs of *daf-2* dauer gonads, the entire dauer gonad was analyzed (distal, proximal,
300 distal). Due to technical difficulties only a single gonadal arm of the *daf-2; aak(0)* gonad was
301 analyzed (distal, proximal). Images in A', B', C, C', D and D' are aligned such that distal is left
302 side and the proximal is right. **A-A')** The left panel (*daf-2*) and right panel (*daf-2; aak(0)*) show
303 H3K4me3 (green), and in **B, B')** H3K9me3 (green) staining merged with DAPI (red). **C-C', D-**
304 **D')** PD *daf-2* and *daf-2; aak(0)* adult gonads were extruded and stained with anti-H3K4me3 and
305 H3K9me3 (green) and signal intensity was quantified throughout the gonad using Image J
306 software. **P<0.001 using the F-test for variance when compared to *daf-2; aak(0)*. Scale bar: 4um
307 n=15 for all the experiments.

308 **Gene expression is altered in both the *aak(0)* dauer and post-dauer germ line.**
309 To examine if aberrant chromatin modifications result in abnormal gene expression in animals that
310 lack AMPK signalling, we performed qRT-PCR to quantify the transcript levels of selected
311 germline-specific genes which were found to be significantly altered in dauer and in the PD adults
312 compared to animals that never transited through dauer (11). The abundance of these transcripts
313 was considerably different in *aak(0)* dauer larvae; some of the genes (*prk-2, pmk-1, mek-2)* were
314 present at lower levels, while others (*spe-26, pro-2)* were detected at significantly higher levels
315 when compared to control *daf-2* animals (Fig. 6A). Furthermore, some of these differences in
316 transcript abundances are not resolved in the PD adult germ line of AMPK mutants (Fig. 6B).
317 Therefore, the chromatin modifications that we observed in both the dauer and PD animals that

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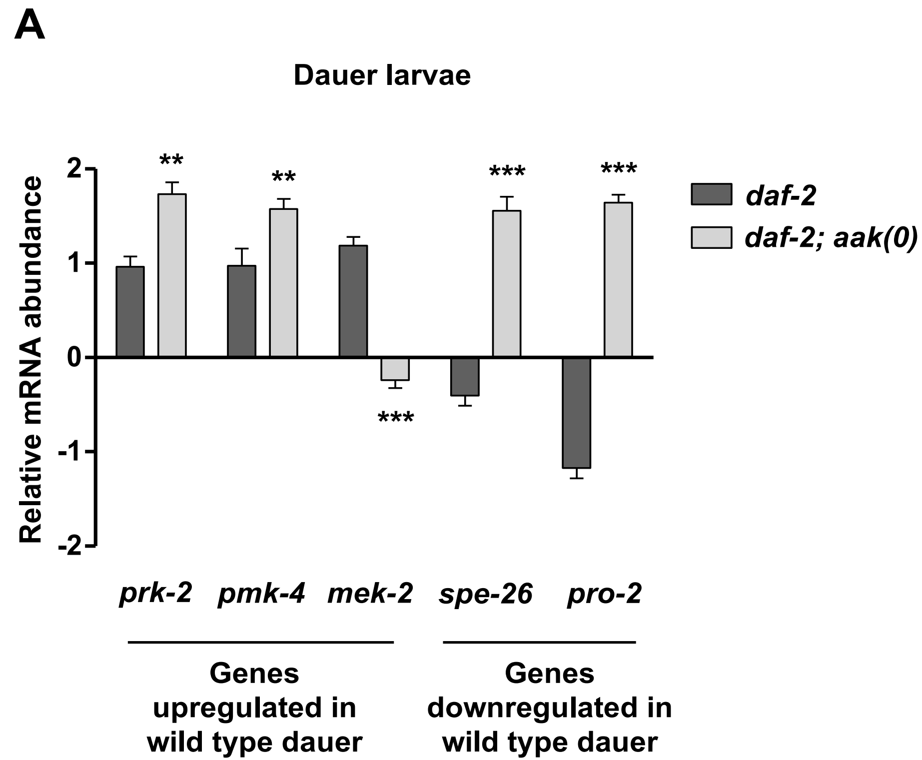
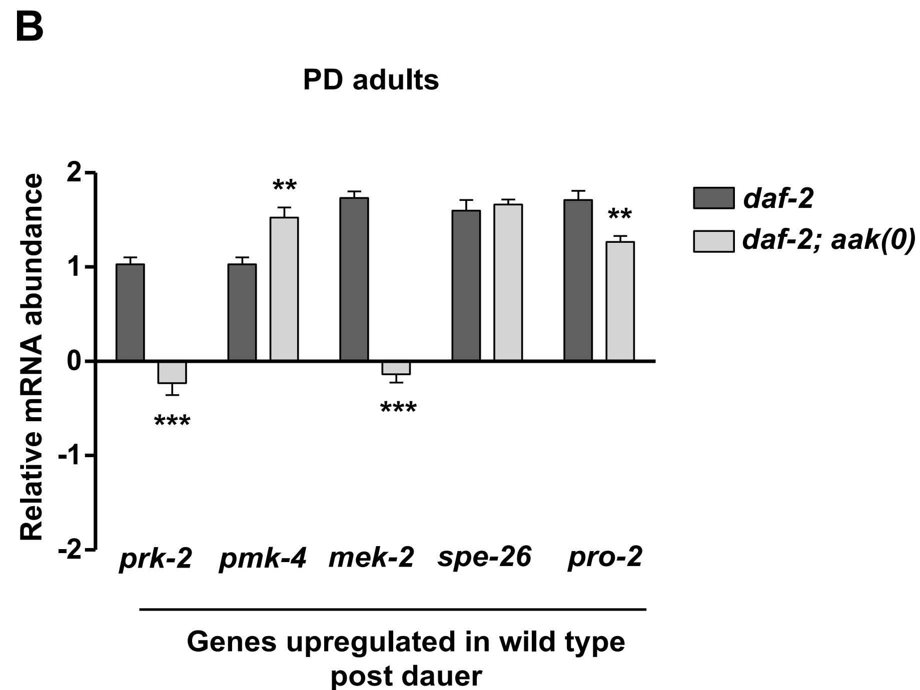
318 lack all AMPK signalling result in dramatic deviation from the gene expression program that
319 would normally occur in the germ line as a result of transit through the dauer stage.





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320 **Figure 6. Gene expression is altered in both the *aak(0)* dauer and post-dauer germ line.**
321 **A, B)** Germline genes which were previously shown to be differentially expressed during and after
322 transit through the dauer stage (Hall, Beverly et al. 2010), were quantified in *daf-2* and *daf-2;*
323 *aak(0)* dauer and PD animals. The relative mRNA levels were analyzed using quantitative real-
324 time PCR in both *daf-2* and *daf-2; aak(0)* dauer and PD adults. The expression of these germline
325 genes was significantly altered in *daf-2; aak(0)* dauer and PD animals, when compared to *daf-2*.
326 Error bars, indicate SD from 3 independent experiments. **P<0.001 using one-way ANOVA when
327 compared to *daf-2*.

328 **Compromise of endogenous siRNA pathway components partially suppresses *aak(0)* post-**
329 **dauer sterility and dauer germline hyperplasia.**
330 Many endogenous small RNAs are critical in distinguishing loci to be targeted by chromatin
331 modifying enzymes, in addition to specifying whether the modification will be active or repressive.
332 They act as mediators of gene expression in order to adapt to cell type information and to varying
333 environmental situations (23). It is therefore not surprising that the small RNA repertoire is
334 dramatically altered in both dauer and PD adults compared to animals that develop in a replete
335 environment (12). We were therefore curious to know whether AMPK might regulate the changes
336 that occur in the suite of small RNA species during dauer owing to its role in regulating chromatin
337 marks in response to energetic stress. To test this possibility, we compromised critical components
338 of the miRNA (*ain-1*), and germ line/nuclear RNAi (*hrde-1, csr-1*) pathways, and some common
339 upstream effectors that impinge on all the small RNA pathways (*dcr-1, rde-4*), to determine if
340 disabling any of these pathways might affect the sterility of the PD AMPK mutant adults.
341 The miRNA pathway is essential for executing dauer entry making it difficult to interpret their
342 potential role in this process (24). On the other hand, we found that the individual disruption of

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343 the RNAse III-like Dicer (*dcr-1*), its accessory factor RDE-4, or the primary Argonaute protein
344 ERGO, could partially rescue the sterility of PD AMPK mutants, the uncontrolled proliferation in
345 the germ line of AMPK mutant dauer larvae, in addition to some of the somatic defects (Fig. 7A,
346 B, Fig. S2, Table S1). In contrast, the compromise of the nuclear Argonaute proteins HRDE-1, or
347 the germline licensing Argonaute CSR-1, had little effect on the hyperplasia or the PD sterility of
348 the AMPK mutants. These data indicate that AMPK must directly, or indirectly, regulate an
349 endogenous small RNA pathway that affects both GSC proliferation and integrity, but that does
350 not include the canonical nuclear Argonaute proteins that have been characterized to regulate
351 germline gene expression.
352 Since both the dauer-associated hyperplasia and the PD sterility of the AMPK mutants correlated
353 with the misregulation of chromatin modifications, we wanted to confirm if the compromise of
354 these various RNAi pathway components might also restore the inappropriate levels and
355 distribution of the chromatin marks in the AMPK mutant germ line. We therefore quantified the
356 global levels of both H3K4me3 and H3K9me3 in *dcr-1*(RNAi) and *rde-4*(RNAi) treated dauer
357 larvae. The levels of both of these marks were significantly reduced in the *dcr-1*(RNAi) AMPK
358 mutant dauer animals, with no effect in control *daf-2* dauer larvae. Surprisingly, only the level of
359 H3K4me3 was significantly reduced in the *rde-4*(RNAi) AMPK mutants (Fig. 7C, D, E), although
360 this may reflect the weak and variable RNAi penetrance typical of *rde-4*. These data confirm that
361 AMPK impinges on the endogenous small RNA pathway to modulate chromatin modifications
362 that affect both GSC proliferation and integrity, although it is currently unclear how AMPK might
363 control these processes, and whether phospho-regulation of key targets might be involved.

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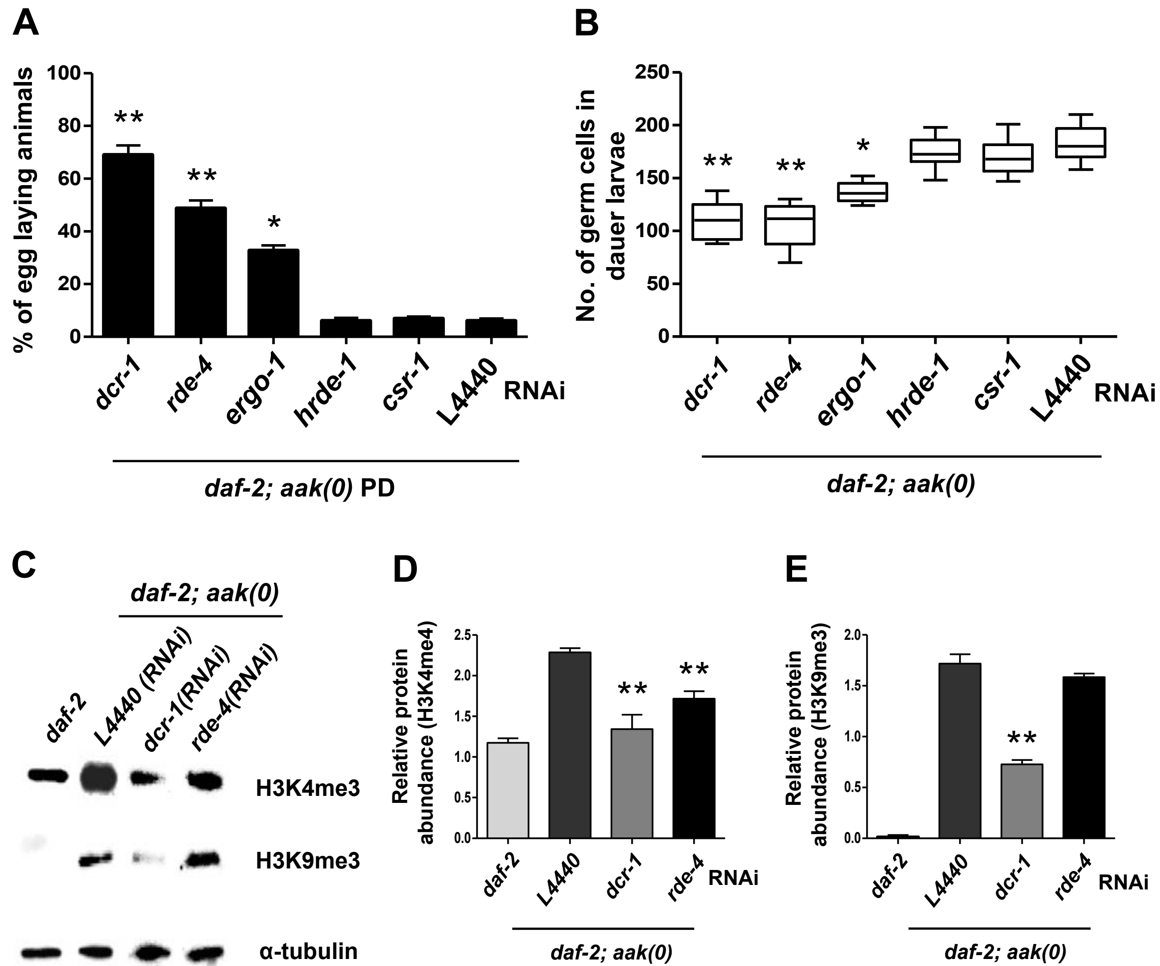

364 **Figure 7. Compromise of small RNA pathway components partially suppresses *aak(0)* post-**
365 **dauer sterility and dauer germline hyperplasia.**

366 To compromise the function of small RNAi pathway, *daf-2; aak(0)* animals were subjected to
367 RNAi by dsRNA feeding against multiple components of the endogenous RNAi pathway. The
368 L4440 empty RNAi vector was used as a control. **A)** The PD sterility observed in the *daf-2; aak(0)*
369 animals was partially rescued by *dcr-1, rde-4* and *ergo-1* RNAi, while RNAi for the germline
370 nuclear Argonautes, *csr-1* and *hrde-1* failed to suppress the observed sterility. **P<0.001 and
371 *P<0.05 using Marascuilo procedure and n=100. **B)** Whole animal DAPI staining was performed
372 to quantify the number of germ cells and the germline hyperplasia in the *daf-2; aak(0)* dauer larvae.

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373 Statistical analysis was performed using the two-tailed t-test when compared to L4440 treated
374 animals where **P0.001 and *P<0.05; n=100. **C, D, E)** Following the RNAi treatment, global
375 levels of H3K4me3 and H3K9me3 were quantified using whole animal western analysis. Global
376 levels of H3K4me3 were significantly decreased in both *dcr-1* and *rde-4* compromised dauer
377 animals.

378 **Somatic AMPK activity is sufficient to regulate dauer germ cell quiescence and integrity**
379 **through small RNA transmission to the germ line.**

380 Recent studies have shown that AMPK can act cell non-autonomously to regulate lifespan, and
381 the L1 survival of AMPK mutants is greatly improved when AMPK is restored in neurons (25,
382 26). To determine if AMPK plays a non-autonomous role in maintaining GSC quiescence and
383 integrity, we expressed the catalytic subunit of AMPK (*aak-2*) ubiquitously in the soma (*sur-5p)*
384 of *aak-2* mutants. Interestingly, ubiquitous somatic expression of *aak-2* restores the fertility in the
385 PD of *aak-2* mutants and also rescues the dauer germline hyperplasia (Fig. 8A, B and Fig. S2).
386 This suggests that AMPK activity in the soma is sufficient to maintain the integrity and quiescence
387 in the germ cells during the dauer stage. To determine in which somatic tissue AMPK function
388 can restore quiescence and integrity to the GSCs during the dauer stage, we used tissue-specific
389 promoters to express *aak-2* and quantified PD sterility and the degree of germline hyperplasia in
390 the AMPK mutants. Using a transgenic strain collection, we generated AMPK null mutants that
391 express *aak-2* exclusively in the neurons (*unc-119)*, the excretory system (*sulp-5)*, the skin (*dpy-*
392 *7*), the gut (*elt-2*), and the muscles (*unc-54*) (27). Surprisingly, only the restoration of AMPK
393 function in the neurons and the excretory system could partially rescue the fertility and GSC
394 quiescence (Fig. 8A, B). These observations suggest that AMPK expression in either (or both) of
395 these two tissues is sufficient to control germ cell homeostasis cell non-autonomously during the
396 energetic stress typical of the dauer stage.

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397 Ubiquitous somatic expression of *aak-2* also restores the normal levels and distribution of
398 H3K4me3 and H3K9me3 marks in the dauer germline (Fig. 8C, D). These data confirm that the
399 somatic function of AMPK can re-establish quiescence and maintain germ cell integrity through
400 its effects on the germline chromatin landscape in dauer larvae that otherwise lack all AMPK
401 signalling.
402 To further investigate if AMPK modulates some aspect of soma to germ line communication
403 through the deployment of somatically-derived small endogenous RNAs, we tested whether the
404 dsRNA importer, *sid-1,* could affect the PD sterility of AMPK mutant by impairing the uptake of
405 these molecules (28). Interestingly, loss of *sid-1* partially restored fertility in AMPK PD animals
406 (Fig. 8E), suggesting that an AMPK-dependent control switch becomes misregulated in AMPK
407 mutants, allowing the transfer of an aberrant population of small endogenous RNAs to the germ
408 line, culminating in the establishment of an inappropriate chromatin landscape. The resultant gene
409 expression may therefore not reflect the necessary metabolic adjustment that must occur during
410 the dauer stage, altering the ability of the germ cells to adapt to the energy stress associated with
411 dauer. This ultimately compromises their integrity and manifests as the consequent PD sterility.

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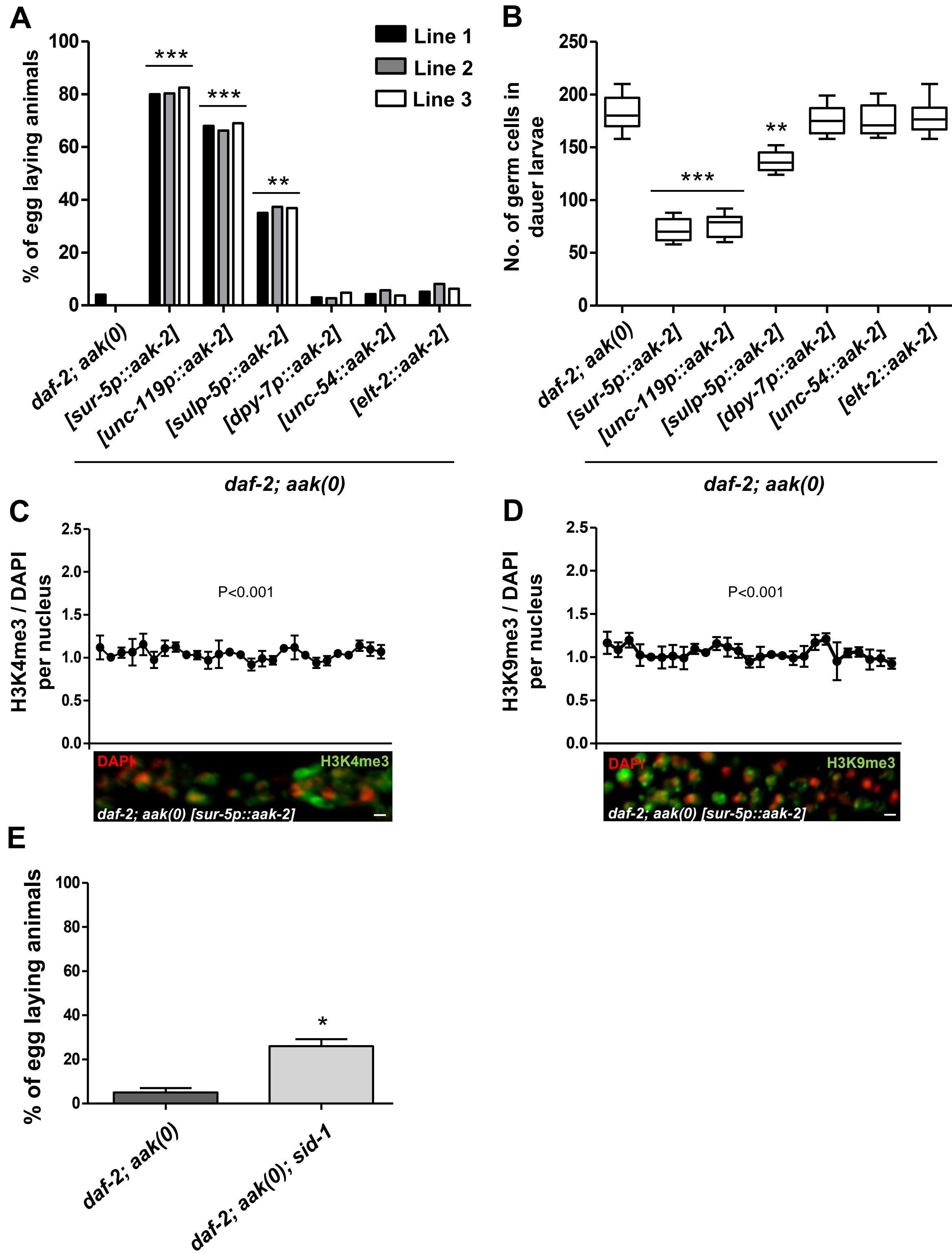

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412 **Figure 8. Somatic AMPK activity is sufficient to restore germ cell quiescence and integrity**
413 **through the transmission of small RNAs in *aak(0)* mutants.**
414 **A)** Plasmid constructs that contain *aak-2* cDNA driven by tissue-specific promoters was injected
415 into *daf-2; aak(0)* mutants and both the dauer-dependent germline hyperplasia and the PD sterility
416 were evaluated for each transgenic strain. All transgenic lines are extrachromosomal and are
417 represented by square brackets, and 3 independently generated lines were used for quantification.
418 PD fertility was assessed 24h following the temperature shift after animals were maintained
419 minimally 24 hours in dauer. ***P<0.0001 and **P<0.001 using Marascuilo procedure when
420 compared to *daf-2; aak(0)* and n=50. **B)** Whole animal DAPI staining was performed to quantify
421 the number of germ cells present in the dauer gonad in the transgenic lines and compared to
422 controls. ***P0.0001 and **P<0.001 using the two-tailed t-test when compared to *daf-2; aak(0)*
423 and n=50. **C, D)** All the analyzed images are merged, condensed Z stacks. The graphs represent
424 the average immunofluorescence for H3K4me3 and H3K9me3 normalized to DAPI across the
425 dissected gonad. **P 0.001 using F-test of variance when compared to *daf-2; aak(0)* and n=10. **E)**
426 Disrupting soma to germline transmission of double-stranded RNA by compromising the function
427 of *sid-1* partially restores fertility in the *daf-2; aak(0)* PD animals. A number of animals laying
428 eggs were counted and the mean is shown ± SD. *P<0.05 using Marascuilo procedure when
429 compared to *daf-2; aak(0)* and n=100.

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430 **Discussion**

431 During periods of energetic stress, *C. elegans* larvae can alter their normal reproductive
432 development and enter a quiescent diapause-like state called dauer. The dauer stage is associated
433 with exceptional stress resistance that is accompanied by a global developmental arrest, including
434 a temporary attenuation of germ cell divisions. One of the predominant cellular energy sensors,
435 AMPK, becomes highly active during this state and regulates the germline quiescence typical of
436 this diapause stage. In the absence of AMPK, or its upstream activating kinase PAR-4/*LKB1,* the
437 dauer germ cells proliferate abnormally, resulting in a dramatic over-proliferation of the germline
438 (13). The consequences of these unscheduled germ cell divisions have never been interrogated. If
439 these cells are competent, the excessive proliferation could result in a significant increase in
440 reproductive fitness. Alternatively, if these supernumerary cells are abnormal it could have
441 detrimental effects on subsequent generations.

442 We show that the supernumerary germ cells that arise during dauer in AMPK mutants are
443 incompetent to generate functional gametes and therefore provide no reproductive advantage.
444 AMPK signalling is therefore critical to coordinate germ cell quiescence and the appropriate
445 metabolic adjustment required to survive the, often lengthy, organismal energy stress associated
446 with dauer. Although both the dauer germline quiescence and the PD fertility are AMPK
447 dependent, our data suggest that the observed sterility may not necessarily be a direct consequence
448 of abnormal cell divisions within the germline stem cells, since the aberrant cell divisions can be
449 suppressed without ameliorating the sterility of the AMPK mutant dauer larvae. The AMPK-
450 dependent processes that are required for PD fertility may be independent of its role in modulating
451 germ cell proliferation (Fig. 3).

452 Following dauer recovery, *daf-2* animals develop normally with no consequence on their
453 reproductive fitness. However, AMPK PD animals show striking defects in germline development

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454 and organization (Fig. 2B, Table S2). Similar to MAPK signalling mutants, the germ cells fail to
455 completely exit from pachytene-like state in PD animals that lack AMPK (Fig.2) (29). Although,
456 morphologically many of the proximal germ cells appear to undergo cellularization and oogenesis,
457 at the nuclear level they fail to complete diakinesis to form 6 condensed nuclear bodies; a hallmark
458 of a mature oocyte and thus fails to produce a functional matured oocyte (30). AMPK has been
459 shown to regulate the MAPK pathway to block the germ cell divisions under nutritional stress
460 (31). Therefore, perhaps during the energy stress associated with the dauer stage, AMPK could
461 regulate key components of the MAPK pathway to modulate meiotic cell cycle progression thereby
462 delaying the production of mature oocytes, and ensuring that reproductive development is
463 coordinated with transit through the dauer stage.

464 This interaction with the MAPK pathway might also account for some of the vulval defects that
465 arise in PD mutant animals, although it is not clear what component is affected in either of these
466 contexts. Curiously, like the germ cells, the vulval precursor cells must also maintain an
467 undifferentiated state during the entire length of dauer, only to re-initiate their divisions and fate
468 specification in response to recovery cues (32, 33). Perhaps AMPK is critical for the appropriate
469 maintenance of developmental plasticity in these somatic cells in a manner that is akin to its role
470 in the germ line. Collectively, these results suggest the role of AMPK activity in establishing GSC
471 cell cycle quiescence, meiotic progression, and maintaining germ cell integrity during periods of
472 extreme energetic stress.

473 But what might constitute germ cell integrity and how might it be maintained by AMPK over the
474 duration of the dauer stage? One possibility might include changes in the gene expression program
475 to mediate the adaptive cellular and metabolic adjustments necessary to endure the energetic stress
476 of the dauer stage, whether it lasts 24h or 6 months. As *C. elegans* larvae enter the dauer stage, the
477 chromatin is concomitantly remodelled, altering gene expression significantly (11, 34). These

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478 modifications are tightly correlated with changes in the small RNA repertoire such that the
479 expression of most endo-siRNAs are affected in both dauer and PD larvae when compared with
480 animals that never transit through the dauer stage. Based on the mechanism of small RNA-
481 mediated changes to the chromatin, these changes in small RNA population likely presage
482 chromatin remodelling, which together provide a molecular memory of this life history event,
483 possibly providing a template for the consequent establishment of distinct adaptive cellular
484 responses or behaviour(s) (11).

485 We show that AMPK is critical to ensure that these global chromatin modifications occur in a
486 regulated manner. In its absence, the abundance of both the transcriptional activating (H3K4me3
487 and H3K9ac) and repressive (H3K9me3 and H3K27me3) marks increase aberrantly in the soma
488 and the germ line of dauer larvae. The normal distribution of the H3K4me3 and H3K9me3
489 chromatin marks become visibly perturbed within the AMPK mutant dauer germline. Moreover,
490 the aberrant marks fail to resolve upon dauer exit, persisting in to the adult PD germline, consistent
491 with AMPK further acting to modulate these modifications upon dauer exit. Although we have not
492 identified the penultimate AMPK target(s) that mediate these changes in chromatin regulation, the
493 relationship between AMPK and chromatin regulators may be akin to its role during the L1
494 diapause, where germ cell integrity is compromised due to irregular chromatin modifications that
495 take place in the primordial germ cells in the absence of AMPK (4).

496 The observed anomalies in both the abundance and the distribution of the activating and repressive
497 marks likely perturbs the coordination of germline gene expression with the energy stress of dauer,
498 which would normally be mediated through a specific chromatin syntax in both dauer and PD
499 AMPK animals. In the absence of AMPK, gene expression may no longer correspond to that of a
500 germ cell, or at least a germ cell that has been subjected to the challenge of surviving severe energy
501 stress. The inability of the gene expression program to adjust to the metabolic challenge is likely

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502 to be responsible for the abnormal gonad and germline development in addition to the somatic
503 defects observed in the AMPK PD adults.

504 The somatic and germ line abnormalities that we observe in AMPK mutants are dependent on
505 various components of a small RNA pathway. Although small RNA pathways have been
506 previously linked to transcriptional and chromatin modification, our data indicate that these
507 regulatory mechanisms are under direct or indirect control of AMPK during periods of energy
508 stress. Although we have not yet identified the key AMPK targets that mediate this small RNA
509 function, it is noteworthy that Dicer contains multiple consensus AMPK phosphorylation sites,
510 while RDE-4 also could be a potential substrate. Alternatively, in addition to the primary
511 ARGONAUTE protein ERGO, a number of ARGONAUTE orthologues remain to be
512 characterized. We cannot rule out that one of these ARGONAUTE family members may somehow
513 respond to AMPK signalling to affect this small RNA-mediated change in the chromatin landscape
514 that occurs during dauer and PD recovery in *C. elegans*.

515 Using *rrf-1* to address where AMPK is required, we initially concluded that AMPK acted in a
516 germline-autonomous manner to maintain GSC quiescence in the dauer larvae (13). The technical
517 shortcomings of this strategy have since been well documented (35), and our recent transgenic
518 experiments confirm that AMPK activity is sufficient in the neurons, or the excretory system, to
519 regulate germ cell quiescence and integrity. Moreover, the somatic expression of *aak-2* also
520 restored the chromatin modifications to wild type levels and a normal pattern of distribution
521 throughout the dauer germline. This neuron-specific function of AMPK may not be a general
522 feature of AMPK signalling, since its neuronal expression failed to suppress the supernumerary
523 germ cell divisions in AMPK mutants during L1 diapause (25). Recently, it was shown that AMPK
524 expression in the neurons can extend lifespan in *C. elegans* under energetic stress (25, 26). The
525 neurons may therefore sense the environment and accordingly signal, perhaps in a neuroendocrine

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526 manner, to other tissues in order to adapt synchronously as an organism. AMPK could be one of
527 the intermediaries in transducing the signals from the neurons, potentially regulating some
528 diffusible molecule, to enhance their survival without any compromise on their fitness. This would
529 place AMPK at a critical position in sensing environmental challenges to ultimately impinge on
530 the germ line to give rise to the observed chromatin-mediated adaptations associated with dauer,
531 and subsequent recovery in replete conditions.

532 But what neuron-derived diffusible signal could affect the chromatin in the germ cells? Like many
533 plants, RNAi is systemic in *C. elegans.* Injection of dsRNA into the somatic tissue can result in
534 RNA-mediated gene silencing in the germ line (28, 36, 37). Furthermore, the endo-siRNA pathway
535 that is active in the somatic tissues can contribute, at least in part, to the changes in germline gene
536 expression and the brood size upon passage through dauer (12). Our results support and extend
537 these findings, as the compromise of the dsRNA importer, *sid-1* partially rescues the AMPK-
538 dependent sterility of PD AMPK mutants, suggesting that the abnormal transfer of small RNAs
539 that occurs in the absence of AMPK is registered in the germline culminating in PD sterility. At
540 present our data cannot discern if AMPK regulates the systemic transfer of small RNAs directly,
541 or whether it is involved in selecting the appropriate sequence-specific small RNAs to ensure the
542 correct adjustment to gene expression is achieved during the dauer stage.

543 The stress associated with the dauer stage is documented molecularly in the chromatin
544 modifications that govern gene expression in PD animals. How this modification in transcriptional
545 output provides some adaptive advantage has not yet been unequivocally determined, but animals
546 that transit through dauer live longer and have a significantly higher brood size (11). These changes
547 may be heritable since animals that remain in dauer for extended periods also show significant
548 alterations in their gene expression, while also demonstrating and enhanced resistance to starvation
549 in subsequent generations (38). Therefore, the dauer larva may be a perfect example of how

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550 perceived environmental duress is transduced to the germ line. Most importantly, this may occur
551 through a somatic sensing mechanism that could include AMPK and its ability to modulate diverse
552 stress-specific epigenetic changes via the endogenous small RNA pathway.
553 At the turn of the last century August Weismann postulated that the germ line was exclusively
554 responsible for the heritable nature of specific traits with little or no impact from the soma.
555 Although this view is widely accepted, diverse situations have been described where the soma acts
556 as a critical regulator of epigenetic change with phenotypic effects that can last for multiple
557 generations (28, 39, 40). AMPK may be one of the critical somatic effectors required to bridge the
558 proposed Weismann Barrier, providing an efficient means of coordinating epigenetic change in
559 the germ line with physiological or environmental cues sensed by the soma. Our findings therefore
560 provide a means of dissecting the mechanisms through which the soma communicates with the
561 germ line in order to adapt to acute environmental challenges, through the generation of a suite of
562 chromatin modifications that confer an epigenetic-based selective advantage to future generations.

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563 # Materials and Methods

564 ## *C. elegans* genetics

565 All *C. elegans* strains were maintained at 15°C and according to standard protocols (41). The
566 strains used for the study include CB1370 [*daf-2(e1370 III*], MR1000 [*daf-2(e1370) aak-*
567 *1(tm1944) III; aak-2(ok523) X*], MR0480 [*daf-7(e1372) III; aak-2(ok523) X*], MR1175 [*aak-*
568 *1(tm1944) III; aak-2(ok523) X*], MR2137 [*daf-2(e1370) aak-1(tm1944) III; aak-2(ok523) X;*
569 *ltIs4[unc-119(+)Ppie1::plc::mCherry]*], MR2138 [*daf-2; ltIs44[unc-*
570 *119(+)Ppie1::plc::mCherry]*], MR1973 [*daf-2(e1370) aak-1(tm1944) III; aak-2(ok523) X; sid-*
571 *1(rr167) V*]. Transgenic lines and compound mutants were created in the laboratory using standard
572 molecular genetic approaches. To create transgenic lines to express tissue-specific *aak-2,* MR1000
573 animals were injected with different constructs as per (27).

574 ## RNAi Feeding

575 Bacterial clones expressing dsRNA from the RNAi library were grown in LB medium with
576 ampicillin at 37°C overnight. The bacterial culture was seeded onto regular NGM plates containing
577 ampicillin and IPTG. Seeded plates were incubated at room temperature for 24 hours to induce
578 dsRNA expression. L4 larvae were fed on the RNAi plates and were allowed to lay eggs at 15°C
579 and then the eggs were switched to 25°C to induce dauer formation.

580 ## DAPI staining and counting germ cell nuclei

581 For whole animal DAPI (4’ ,6-diamidino-2-phenlindole) staining, dauer larvae were washed off
582 plates and soaked in Carnoy’s solution (60% ethanol, 30% acetic acid, 10% chloroform) on a
583 shaker overnight. Animals were washed twice in PBST (1XPBS + 0.1% Tween 20), and stained
584 in 0.1 mg/ml DAPI solution for 30 minutes. Finally, larvae were washed four times (20 minutes

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585 each) in PBST, and mounted in Vectashield medium. The total number of germ cell nuclei per
586 dauer gonad was then determined based on their position and nuclear morphology.

587 **Dauer recovery assay**

588 A population of the genetically identical animals were synchronized, and the resulting embryos
589 were added to normal NGM plates seeded with *E. coli*, and incubated at 25°C for 72 hours in order
590 to induce dauer formation and allow animals to spend at least 24 hours in dauer state. Following
591 this window, dauer larvae were shifted to the permissive temperature of 15°C to allow dauer larvae
592 to recover and initiate regular development. Upon dauer exit, the L4 larvae were individually
593 isolated onto separate plates and were transferred to new plates every 24 hour intervals to quantify
594 their brood size. The brood size of each animal was the sum of non-hatched and hatched progeny.

595 **Immunostaining and quantification**

596 For extruded dauer gonad staining, gonads were dissected, fixed and stained as described
597 elsewhere (42). The following primary antibodies were used: rabbit polyclonal anti-H3K4me3
598 (Abcam, 1:500), anti-H3K9me3 (Cell Signaling Technology, 1:500), rabbit anti-HIM-3 (gift from
599 Zetka lab, 1:200). Secondary antibodies were Alexa Fluor 488-coupled goat anti-rabbit (Life
600 Technologies, 1:500). Microscopy was performed as described in (43). Ratios for the fluorescence
601 intensity across the germ line were determined using Image J.

602 **Western blot**

603 *C. elegans* dauer larvae and PD adults were lysed by sonication in lysis buffer (50mM Hepes
604 pH7.5, 150mM NaCl, 10% glycerol, 1% Triton X-100, 1.5mM $MgCl_2$, 1mM EDTA and protease
605 inhibitors). Protein concentrations were determined using nanodrop 2000c spectrophotometer

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606 (Thermo Scientific). Nitrocellulose membranes were incubated with primary antibodies: rabbit
607 anti-H3K4me3 (Abcam, 1:1000), anti-H3K9me3, anti-H3K27me3, anti-H3K9ac (Cell Signaling
608 Technology, 1:1,000); mouse anti–α-tubulin (1:3,000; Sigma). Proteins were visualized using
609 horseradish peroxidase conjugated anti-rabbit or anti-mouse secondary antibody (Bio-Rad).

610 **RNA Isolation and Real Time PCR**

611 Total RNA was extracted with Trizol (Invitrogen). RNA concentration and purity were determined
612 with a NanoDrop 2000c spectrophotometer. Purified RNA (400 ng) was used to synthesize cDNA.
613 Gene expression levels were determined by real time PCR with the SYBR Green Supermix and
614 BioRad iCycler Real Time PCRSystem (BioRad). Relative gene expression was normalized to
615 *tba-1* which was the loading control.

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621 **Author contributions**

622 Experiments were designed by P.K. and R.R. P.K. performed all the experiments and analyses.

623 The manuscript was written by P.K. and edited by R.R.

624 **Competing interests**

625 The authors declare no competing interest.

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