1   **Chromatin-associated effectors of energy-sensing pathways mediate**

2   **intergenerational effects**

3

4   Pedro Robles[1], Anisa Turner[1], Giusy Zuco[1], Panagiota Paganopolou[1], Beth Hill[1],

5   Vikas Kache[2], Christine Bateson[2], Andre Pires-daSilva[*]

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7   [1] School of Life Sciences University of Warwick, Coventry, CV4 7AL, UK

8   [2] Department of Biology, University of Texas at Arlington, Arlington, TX, 76019, USA

9   [*] Corresponding author

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11   **Environmental stimuli experienced by the parental generation influence the**

12   **phenotype of subsequent generations. The effects of these stimuli on the**

13   **parental generation may be passed through the germline, but the mechanisms**

14   **of this non-Mendelian type of inheritance are poorly known. Here we show that**

15   **modulation of nutrient-sensing pathways in the parental generation of a**

16   **nematode (*Auanema freiburgensis*) regulates phenotypic plasticity of its**

17   **offspring. In response to pheromones, AMP-activated protein kinase (AMPK),**

18   **mechanistic target of rapamycin complex 1 (mTORC1) and insulin signaling**

19   **regulate stress resistance and sex determination across a generation. The**

20   **effectors of these pathways are closely associated with the chromatin and**

21   **their regulation affects the acetylation chromatin status in the germline. These**

22   **results suggest that highly conserved metabolic sensors regulate phenotypic**

23   **plasticity by changing the epigenetic status of the germline.**

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25

26   **INTRODUCTION**

27   The phenotype of an individual is the result of the interactions between its genome

28   and the environment. However, the phenotype may also be influenced by

29   experiences of the parents: parental environment, such as diet, may result in

30   epigenetic changes in the germline that cause non-adaptive phenotypes in the

31   offspring (Chen et al., 2016, Sharma et al., 2016). An example case in humans

32 suggests that famine increases the risk of metabolic defects in one or more

33 generations (Kaati et al., 2007).

34

35 However, there are also mechanisms for passing information about the maternal

36 environment to the offspring that increase fitness (Burton et al., 2017, Dantzer et al.,

37 2013, Jablonka, 2013). This is referred to as adaptive phenotypic plasticity, which

38 allows parents to match the phenotype of their offspring to changes in the local

39 environment (West-Eberhard, 2003). For example, by sensing environmental cues,

40 some animals can generate predator-resistant offspring (Agrawal et al., 1999,

41 Gilbert, 2017), or stress-resistant offspring adapted to seasonal conditions

42 (Mousseau and Dingle, 1991). Relatively little is known about mechanisms in which

43 the parental generation senses the environment to induce adaptive phenotypic

44 plasticity across one (intergenerational) or more generations (transgenerational)

45 (Perez and Lehner, 2019).

46

47 Invertebrate model systems, such as the nematode *Caenorhabditis elegans*, have

48 been instrumental in revealing some of the mechanisms of inter- and

49 transgenerational inheritance (Miska and Ferguson-Smith, 2016, Perez and Lehner,

50 2019). The free-living nematode *Auanema freiburgensis* is an attractive new animal

51 model system for studying the mechanisms of inheritance of parental effects

52 (Kanzaki et al., 2017, Zuco et al., 2018, Anderson et al., 2020). This is because the

53 assays for studying the mechanisms of inheritance of parental effects in *A.*

54 *freiburgensis* are fast and easy to perform due its short generation time (~4 days at

55 20 °C) and easy-to-distinguish morphologies in the offspring.

56

57 *A. freiburgensis* produces three sexes, consisting of males, females and

58 hermaphrodites (Kanzaki et al., 2017). The male of *A. freiburgensis* is determined

59 genetically (XO), by mechanisms that will be addressed in a separate report. The

60 hermaphrodite versus female sex (both XX) is determined by the environment

61 experienced by the mother. Hermaphrodite individuals kept in isolation produce

62 mostly female offspring, whereas hermaphrodites exposed to high population density

63 conditions produce mostly hermaphrodite offspring (Fig. 1A).

64

65   Here we show that high-density population conditions experienced by the *A.*

66   *freiburgensis* mother, a signal for imminent starvation, triggers the formation of F1

67   dauer larvae. These dauers develop into hermaphrodite adults, while non-dauer

68   larvae develop into female adults (or males). Pharmacological assays indicate that

69   energy-sensing signaling mediated by AMP-activated protein kinase (AMPK),

70   mechanistic target of rapamycin complex 1 (mTORC1), and insulin signaling are

71   involved in intergenerational inheritance in *A. freiburgensis*. Effectors of these

72   pathways are associated with chromatin, which changes the histone acetylation

73   status in the germline chromatin to produce F1 dauers, which then develop into

74   hermaphrodite adults.

75

76   **RESULTS**

77   A crucial factor in the development of *Auanema* hermaphrodites is the passage

78   through the stress-resistant dauer stage (Félix, 2004, Chaudhuri et al., 2011,

79   Kanzaki et al., 2017, Chaudhuri et al., 2015), which has morphological and

80   behavioral adaptations for dispersal. In *A. freiburgensis*, all XX larvae that pass

81   through the dauer stage become hermaphrodites (N= 96), whereas XX non-dauer

82   larvae develop into females (N= 93). Similar to *A. rhodensis* (Chaudhuri et al., 2011)

83   and other trioecious nematodes (Johnigk and Ehlers, 1999), we never observed *A.*

84   *freiburgensis* males to undergo dauer formation. Thus, environmental stressors

85   experienced by the maternal generation of *A. freiburgensis* are used as a signal to

86   generate non-feeding offspring that can survive starvation conditions and reproduce

87   by self-fertilization once food becomes available. In summary, these results suggest

88   that dauer formation in *A. freiburgensis* is induced across a generation, instead of

89   within the same generation as in *Caenorhabditis elegans* (Cassada and Russell,

90   1975) (Figure 1B).

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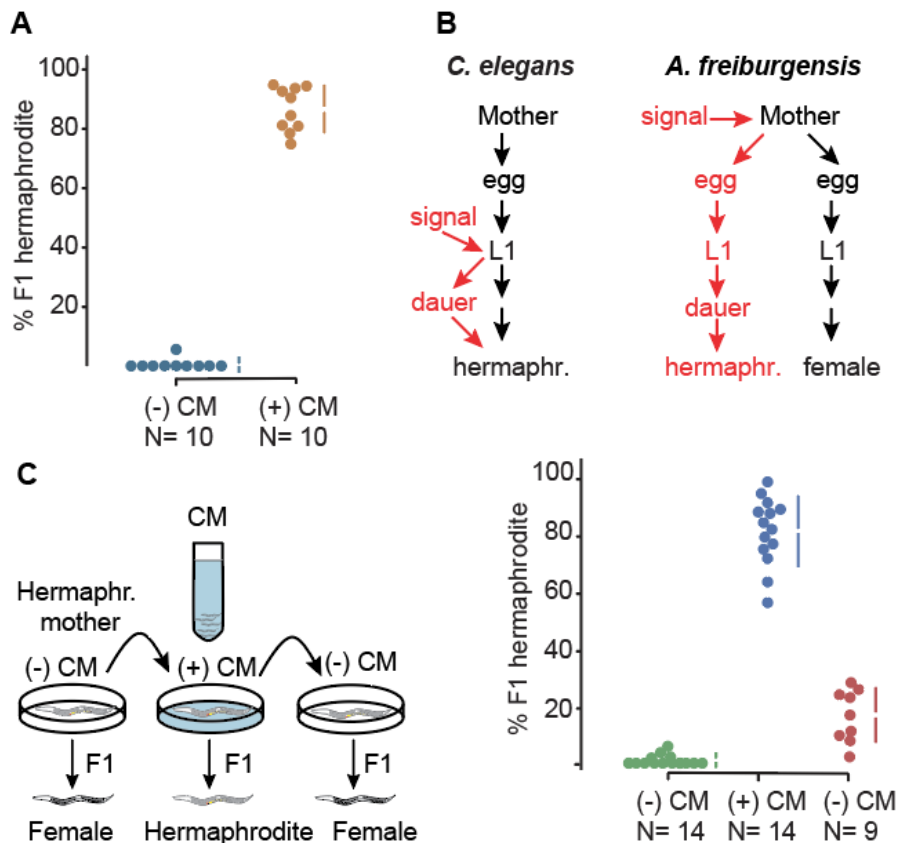
Figure 1. Dauer and hermaphrodite development are induced across generations in *A. freiburgensis*. **A.** When hermaphrodite mothers are cultured in non-crowding conditions ((-) CM), most of the XX F1s are female. (10 broods, from which a total of 149 F1s were scored). When mothers are in crowding conditions ((+) CM), most of the XX F1s are hermaphrodites (10 broods, with a total of 199 F1s scored). The data in colored dots represent the percentage of F1 hermaphrodites in each brood and is plotted on the upper axes. The colored vertical lines indicate ± SD and the mean is represented as a gap in the lines. N= sample size. **B.** In *C. elegans*, the L1 larvae respond to environmental signals to facultatively form stress-resistant dauers. In *A. freiburgensis*, it is the mother and not the L1s that respond to environmental signals. *A. freiburgensis* dauers obligatorily develop into hermaphrodite adults. **C.** In the experimental setup (top), the same individual mother hermaphrodite was transferred every 24 hours to a new environmental condition. Initially, it was placed in a plate without conditioned medium (-) CM, followed by the transfer to a (+) CM plate and then to a new (-) CM plate. The plot representation is the same as for Fig. 1A. On the last day, 5 mothers died and thus only 9 broods were scored.

High population density conditions were induced by incubating *A. freiburgensis* hermaphrodites with conditioned medium (CM) derived from liquid cultures containing high nematode population densities (see Methods). Importantly, only the

4

112   parental generation was exposed to the CM. The induction of dauers through the

113   hermaphrodite mother is limited to one generation: F1 hermaphrodites derived from

114   mothers in (+) CM plates produce mostly female offspring (99.6% out of 470 F2

115   offspring, scored from 10 broods). To test if *A. freiburgensis* L1 larvae can also

116   respond to crowding conditions, eggs derived from mothers cultured in isolation were

117   left to hatch and undergo larval development in (+) CM plates until adulthood. 95.7%

118   (N= 161) of these L1s developed into females, indicating that larvae do not respond

119   to crowding conditions. To investigate if other maternal environmental conditions

120   affect the sexual fate of the F1s, mothers were incubated for 24-hours to high

121   temperature (25 °C) or starvation. Most XX offspring (97%) developed into female

122   adults for both conditions (166 F1s scored from mothers at 25 °C and 146 F1s

123   scored from starving mothers). These results indicate that the conditioned medium is

124   the only environmental stressor that induces intergenerational polyphenism in *A.*

125   *freiburgensis* on its own.

126

127   To test the minimal population density sufficient for the induction of dauers and

128   hermaphrodites across a generation, we incubated the maternal generation at

129   different densities. When cultured for 6 hours, a minimum density of 16 adult

130   hermaphrodites per cm^2 is sufficient for the induction of 100% (N= 295 F1s) of

131   hermaphrodite offspring. In densities below 10 individuals/cm^2, the hermaphrodite

132   mothers produce only female offspring (10 individuals/cm^2: 100% females, N= 78

133   F1s; 6 individuals/cm^2: 98.5% F1 female, N= 66 F1s). At an intermediate density (13

134   individuals/cm^2), hermaphrodites produce 19% (N= 126 F1s) of hermaphrodite

135   offspring.

136

137   **Modulation of AMPK signaling changes hermaphrodite/female sex ratios in *A.***

138   ***freiburgensis***

139   In eukaryotes, caloric restriction triggers the activation of AMP-activated protein

140   kinase (AMPK) (Apfeld et al., 2004), a highly conserved energy sensor (Hardie et al.,

141   2012). AMPK activity protects cells against the depletion of ATP by stimulating

142   energy-producing pathways and inhibiting energy-consuming processes (Carling,

143   2004). In *C. elegans*, AMPK is required for lifespan extension and germline viability

144   when the nematode is in nutrient stress (Apfeld et al., 2004, Narbonne and Roy,

145   2006, Fukuyama et al., 2012, Demoinet et al., 2017). The full kinase activity of

146   AMPK requires phosphorylation of threonine residue 172 (Thr172) by upstream

147   kinases (Stein et al., 2000, Lee et al., 2008, Apfeld et al., 2004).

148

149   Since high population density is likely to result in imminent food scarcity, we

150   reasoned that the AMPK pathway may be involved in intergenerational inheritance in

151   *A. freiburgensis*. High population density conditions were induced by incubating *A.*

152   *freiburgensis* hermaphrodites with conditioned medium (CM) of high population

153   density liquid cultures (see Methods). We hypothesized that AMPK regulates target

154   proteins in the maternal germline to influence the phenotype of the following

155   generation. To test this hypothesis, we first tested the levels of an enzyme that

156   activates AMPK, Liver Kinase B1 (LKB1). LKB1, known in *C. elegans* as PAR-4

157   (Watts et al., 2000, Lee et al., 2008), phosphorylates and activates AMPK in the

158   context of energy stress (Woods et al., 2003, Hawley et al., 2003). LKB1 is part of a

159   complex with two proteins Ste20-related adaptor protein-alpha (STRAD) (Baas et al.,

160   2003) and mouse protein 25-alpha (MO25alpha) (Boudeau et al., 2003). Antibody

161   staining against LKB1 and STRAD showed a higher level of staining in the meiotic

162   portion of the germline isolated from animals cultured in crowding conditions (Fig.

163   2A, C, Supplemental Figure 1). Their localization was predominant in the cytoplasm

164   of germline cells (Supplemental Figure 1).

165

166   To test the levels of AMPK, we used an antibody that detects the active,

167   phosphorylated form of AMPK (AMPK pThr172). Consistent with the higher levels of

168   LKB1 and STRAD, we also found that the anti-AMPK pThr172 staining was stronger

169   in crowding conditions compared to control animals (Fig. 2 B-D). The difference in

170   the level of staining was restricted to the meiotic region of the germline (Fig. 2, D)

171   and the AMPK staining is closely associated with the chromatin of pachytene cells

172   (Fig. 2D).

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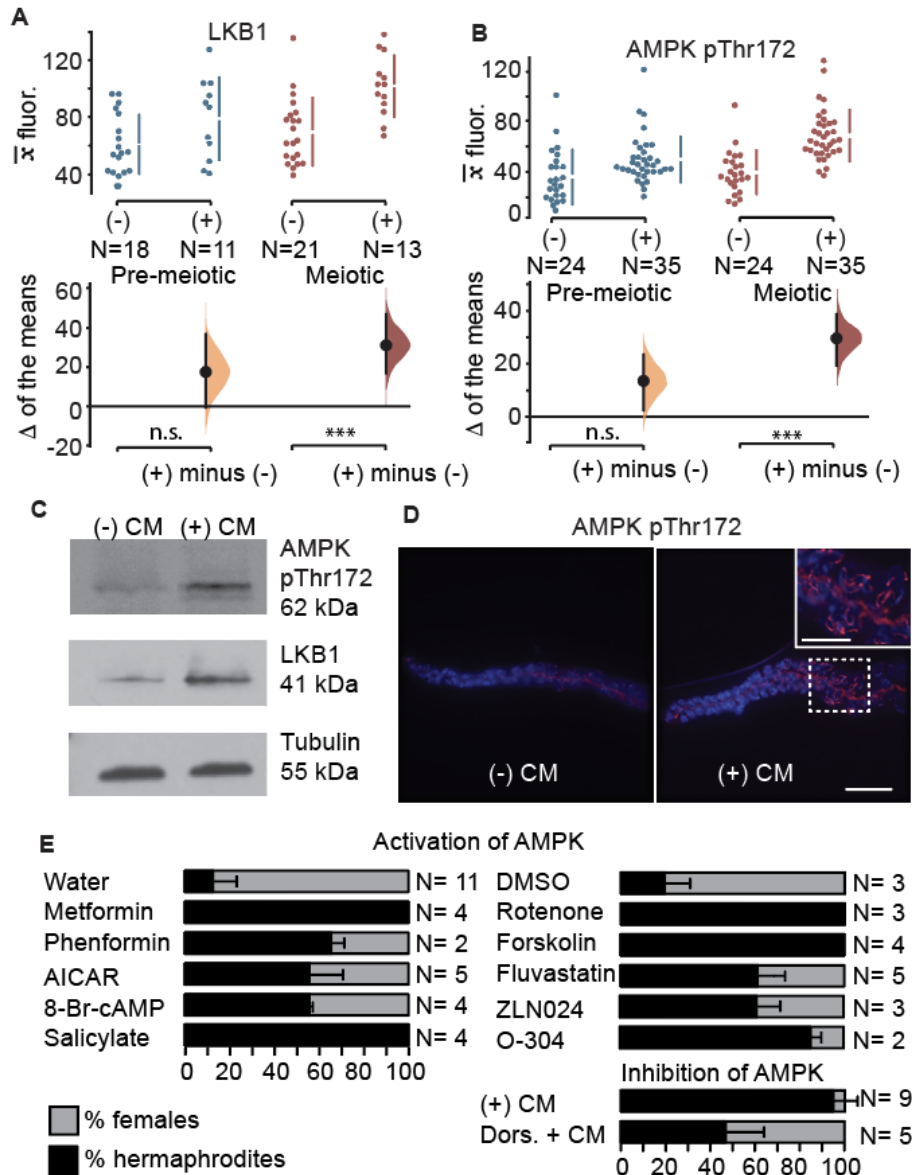
Figure 2. AMPK pathway modulation in the *A. freiburgensis* germline. (A, B) Mean antibody fluorescence ($\bar{x}$ fluor.) in the pre-meiotic (blue) and meiotic portion (red) of the germline, in the absence (-) or presence (+) of conditioned medium. N= sample sizes. The mean difference for the two comparisons is shown as a Gardman-Altman estimation plot. The raw data is plotted on the upper axes, with colored vertical lines indicating ± 95% CI, and the mean is represented as a gap in the lines. Each difference of the means is plotted on the lower axes as a bootstrap sampling distribution. The difference of the means is depicted as a black dot and 95% confidence intervals are indicated by the black vertical error

7

183   bars. n.s., p>0.05; ***= p ≤ 0.001. (**C**) Western blots with proteins derived from

184   hermaphrodites incubated in the absence (-) CM or presence (+) CM of conditioned medium.

185   (**D**) AMPK pThr172 antibody staining of gonads dissected from hermaphrodites incubated in

186   either (-) CM or (+) CM. Bar, 15 µm. Insert in the right picture is a magnification from the

187   region marked with a stippled square. Bar, 7.5 µm. (**E**) Mean percentage and SD of

188   hermaphrodite and female F1 offspring from hermaphrodites treated with chemicals. The

189   control was either using water (left) or DMSO (right), depending on how the chemical

190   compounds were dissolved. Dors.= Dorsomorphin. In all cases, diluted (1:10) CM was

191   added to the medium, with exception to plates with dorsomorphin, which had undiluted CM.

192   N= number of replicates.

193

194   To functionally test the role of AMPK in mediating intergenerational inheritance in *A.*

195   *freiburgensis*, we used pharmacological compounds that modulate AMPK activity.

196   We measured the effects of these compounds on intergenerational inheritance by

197   scoring hermaphrodite and female sexes in the offspring. As mentioned previously,

198   high population densities induce the production of dauer larvae in the F1, which

199   mature to become hermaphrodite adults. Consistent with a role of AMPK in

200   mediating this effect, we found that AMPK activators induce the production of

201   hermaphrodites (Fig. 2E) (for a recent review on pharmacological activation of

202   AMPK, see (Steinberg and Carling, 2019)). In most cases, these compounds cause

203   changes in the F1 sex ratios when on their own (Supplemental Figure 2), but

204   potentiation of their effects was significantly stronger when diluted CM (1:10 CM)

205   was added to the culture medium. This may indicate that synergistic effects of

206   different mechanisms are necessary to fully elicit a robust response, or that those

207   energy-sensing pathways can be efficiently activated only when upstream events

208   occur first.

209

210   Although the mechanisms of action are not clear for all pharmacological compounds,

211   they can be broadly divided into indirect and direct AMPK activators. Any treatments

212   that raise the AMP/ADP:ATP ratios are expected to indirectly activate AMPK. For

213   instance, inhibition of mitochondrial respiration by metformin, phenformin and

214   rotenone have been implicated in the activation of AMPK (El-Mir et al., 2000, Owen

215   et al., 2000, Zhou et al., 2001, Sakamoto et al., 2004, Shaw et al., 2004, Huang et

216   al., 2008, Toyama et al., 2016, Hou et al., 2018). Forskolin, an adenylate cyclase

217   activator, activates AMPK by increasing the cytosolic cAMP concentration (Seamon

218   et al., 1983). Statins, such as fluvastatin (Xenos et al., 2005), have been proposed to

219   activate AMPK. The incubation of mothers with all these compounds resulted in a

220   higher proportion of hermaphrodite progeny (Fig. 2E).

221

222   Compounds that are similar to AMP can activate AMPK directly. 5-Aminoimidazole-

223   4-carboxamide ribonucleotide (AICAR), for example, increases the activity of AMPK

224   after being converted to an AMP analog inside the cell (Corton et al., 1995), whereas

225   8-Br-cAMP is a non-hydrolyzable analog of cAMP (Hussey et al., 2017). Other

226   compounds, such as the plant product salicylate (Hawley et al., 2012), and the

227   synthetic compounds ZLN024 (Zhang et al., 2013) and O-304 (Steneberg et al.,

228   2018) bind to AMPK, causing allosteric activation and inhibition of dephosphorylation

229   of the pThr172. All these compounds induced a higher percentage of hermaphrodite

230   offspring than controls (Fig. 2E). To inhibit AMPK, we used dorsomorphin (Zhou et

231   al., 2001). As expected, hermaphrodites in CM with dorsomorphin resulted in a lower

232   proportion of hermaphrodite progeny compared to controls (Fig. 2E).

233

234   **Maternal inhibition of mTORC1 signaling results in mostly hermaphrodite**

235   **offspring**

236   Since energy-sensing by AMPK induced intergenerational effects in *A. freiburgensis*,

237   we hypothesized that other energy sensors may be involved in the same process.

238   The intracellular nutrient sensor mTORC1 complex is a multisubunit kinase that

239   senses growth signals and stimulates anabolism when nutrients are abundant

240   (Kapahi et al., 2010, Ma and Blenis, 2009, Wullschleger et al., 2006, Zoncu et al.,

241   2011, Laplante and Sabatini, 2012). Therefore, we would predict that in low

242   population densities and readily available nutrients, the mTOR pathway would be

243   active in *A. freiburgensis*. Under these conditions, *A. freiburgensis* produces mostly

244   non-dauer larvae that later become female offspring. To investigate the kinase

245   activity of mTORC1, we examined the expression of a well-characterized target

246   protein, p70 S6K protein kinase (S6K) (Kapahi et al., 2010). Antibody staining

247   against the phosphorylated form of S6K (S6K pThr389) was detected primarily in

248   germline cells isolated from animals grown in low-density conditions (Fig. 3A-C).

249   Most staining was associated with the chromatin, both in mitotic cells (Fig. 3A) and

250   meiotic cells in late pachytene stages (Fig. 3B). Since AMPK and mTORC1 have

251   opposing actions (Hindupur et al., 2015), we hypothesized that treatment of animals

252   with metformin, an activator of AMPK, would inhibit mTORC1 signaling. Consistent

253   with this hypothesis, we found that treatment of animals with metformin resulted in a

254   smaller number of cells stained with SK6 pThr389 (Fig. 3D).

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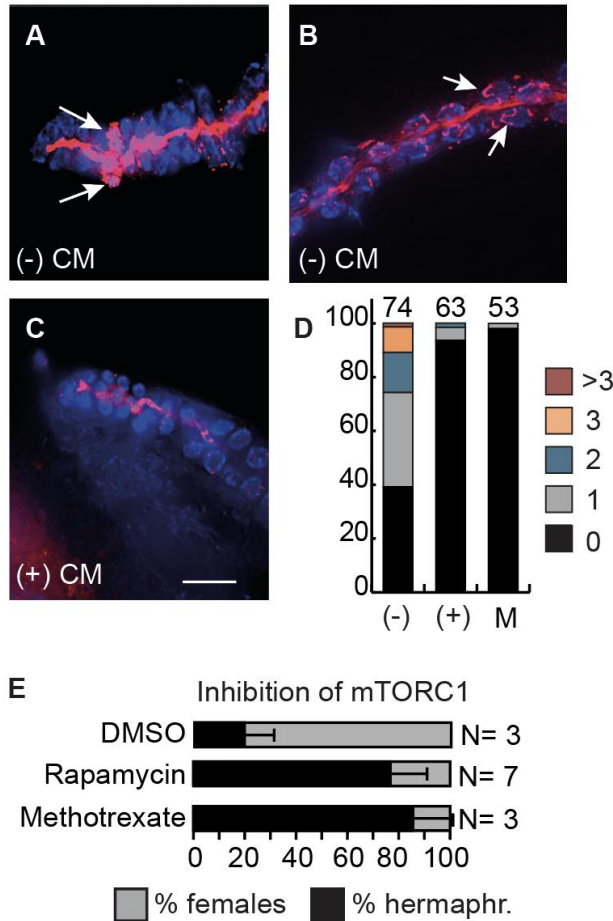
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257   **Figure 3. mTOR signaling modulates intergenerational inheritance in *A. freiburgensis*.**

258   (**A-C**) Staining with S6K pThr389 antibody (red) and DAPI (blue) of dissected gonads from

259   hermaphrodites incubated either in the absence ((-) CM) or in the presence ((+) CM) of

260   conditioned medium. The arrows indicate cells marked with the antibody in the premeiotic

261   region (**A**) and in the meiotic region (**B**), respectively. In (**C**), only the rachis has staining.

262   Bar, 15 μm. (**D**) Percentages of gonads with signal for S6K pThr389 antibody staining. The

263   different colors represent the percentage of gonads with at 0, 1, 2, 3 or more than 3 cells

264   stained in the premeiotic (PM) tip. Quantification was performed from gonads isolated from

265   animals in the absence (-) or in the presence (+) of conditioned medium, and in the presence

266   of metformin (M). The number of gonads analyzed is indicated on the top of the bars. (**E**)

267   Mean percentage and SD of hermaphrodite and female F1 offspring from hermaphrodites

268   incubated with either DMSO or pharmacological compounds, together with some CM (1:10)

269   CM. N= number of replicates.

270

271   To test the effect of modulating mTORC1 activity on sex ratios, we treated mothers

272   with pharmacological compounds. Mothers treated with rapamycin (Heitman et al.,

273   1991, Robida-Stubbs et al., 2012) produced a greater proportion of F1

274   hermaphrodites than control mothers (Fig. 3E). mTORC1 signaling promotes nucleic

275   acid synthesis, as long as nucleotide precursors are available (Hoxhaj et al., 2017).

276   Treatment with methotrexate, a chemical that suppresses the *de novo* purine

277   synthesis enzymes (Rajagopalan et al., 2002), inhibits mTORC1 activity. We found

278   that *A. freiburgensis* hermaphrodites treated with methotrexate generated mostly

279   hermaphrodite offspring (Fig. 3E). Altogether, these results indicate that mTOR

280   signaling is involved in intergenerational inheritance in *A. freiburgensis.*

281

282   **Insulin signaling is downregulated in animals in crowding conditions**

283   The insulin signaling pathway regulates metabolism, development, and lifespan in a

284   wide variety of animals. One of the regulators of the insulin pathway is a conserved

285   phosphatase named PTEN (or DAF-18 in *C. elegans*)(Solari et al., 2005). To

286   examine the regulation of the insulin pathway in *A. freiburgensis*, we used an

287   antibody against PTEN/DAF-18 to stain isolated gonads from hermaphrodites

288   cultured in low- and high- density conditions. We found that the antibody against

289   PTEN/DAF-18 stained more strongly the germline when hermaphrodites were

290   incubated in high-density conditions than in low-density populations (Fig. 4A,

291   Supplemental Figure 3).

292

293   To test if PTEN/DAF-18 mediates the generation of hermaphrodites, we used the

294   PTEN/DAF-18 inhibitors VO-OHpic (Rosivatz et al., 2006) and SF1670 (Li et al.,

295   2011). When in the presence of conditioned medium from high-density populations,

296   hermaphrodites treated with those inhibitors generated mostly female offspring (Fig.

297   4B). Activation of PTEN/DAF-18 in hermaphrodites with the compound Indole-3-

298   Carbinol under low population densities resulted in mostly hermaphrodites (Fig. 4B).
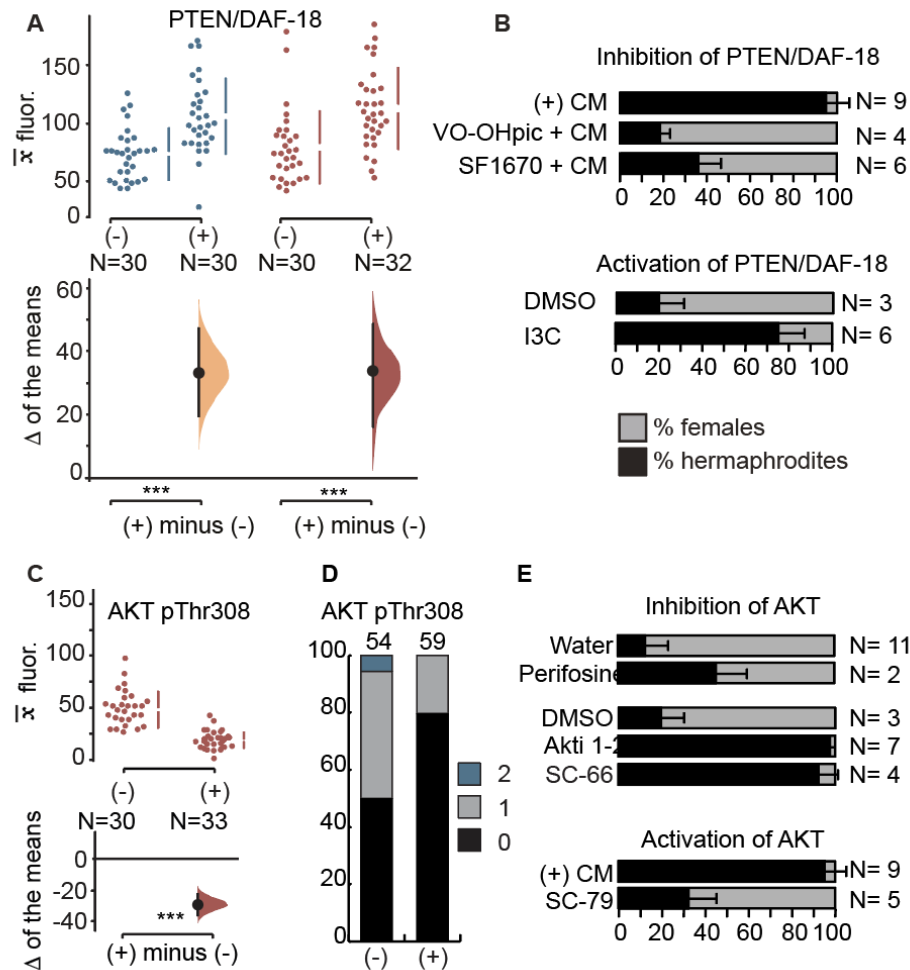
Figure 4. Regulation of PTEN/DAF-18 and AKT. (**A**) Quantification of antibody staining with PTEN/DAF-18 in the maternal gonads. The representation and labeling of graphs are as in Fig. 2A. (**B**) Mean percentage and SD of hermaphrodite and female F1 offspring from hermaphrodites treated with chemicals that activate or inhibit PTEN/DAF-18. (**+**) CM represents undiluted conditioned medium. The DMSO control and Indole-3-Carbinol (I3C) incubations were performed with diluted (1:10) CM. N= number of replicates. (**C**) Quantification of antibody staining for AKT pThr308 in the meiotic portion of the germline, with representation as in (**B**). (**D**) Quantification of meiotic germline cells with staining with an antibody against AKT pThr308, with graphical representation as in Fig. 3D. (**E**) Effect of pharmacological inhibition or activation of AKT on sex ratios in the F1s.

One of the target proteins and effectors for insulin signaling is AKT kinase (also known as PKB) (Paradis and Ruvkun, 1998), which among several anabolic functions, also prevents chromatin condensation (Martelli et al., 2012, Manning and Cantley, 2007, Manning and Toker, 2017). Maximal activation of AKT requires

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315 phosphorylation at residues Thr308 and Ser473 (Alessi et al., 1996). Immunostaining

316 with antibodies against AKT pThr308 (Fig. 4C, D, Supplemental Figure 4) revealed

317 that staining is prominently associated with the chromatin in germline cells of animals

318 grown under non-crowding conditions. No such association is seen when animals

319 are in crowding conditions (Fig. 4C). The same pattern is seen for AKT pSer473

320 (Supplemental Figure 4). Maternal inhibition of AKT with the chemicals perifosine

321 (prevents activation of AKT by affecting its subcellular localization) (Kondapaka et

322 al., 2003), Akti-1/2 (stabilizes the inactive conformation of AKT) (Barnett et al., 2005),

323 and SC-66 (allosteric inhibitor of AKT) (Jo et al., 2011) results in a higher proportion

324 of hermaphrodite progeny (Fig. 4E). On the other hand, activation of AKT with SC-79

325 (Jo et al., 2012) prevents the generation of hermaphrodite progeny when the mother

326 is in crowding conditions (Fig. 4E). Altogether, these results are consistent with the

327 hypothesis that crowding conditions induce a lower insulin signaling, causing the

328 production of hermaphrodite offspring.

329

330 **Changes in the maternal histone acetylation status modulate sex ratios in the**
331 **F1**

332 Energy-sensing pathways have been implicated in the regulation of acetylation of

333 histones, histone modifiers, and cellular proteins (Salminen et al., 2016). To examine

334 if acetylation patterns change in the germline when *A. freiburgensis* is in high

335 population densities, we compared the level of antibody staining in gonads isolated

336 from hermaphrodites cultured in the absence or presence of CM. Antibody staining

337 against acetylated residues on histones 3 and 4 was at higher levels in the germline

338 derived from animals cultured in the presence of CM compared to controls, both for

339 premeiotic and meiotic portions (Fig. 5A-B, Supplemental Figure 5). The same trend

340 was observed when using an antibody that binds to all acetylated proteins (pan-

341 LysAc), although differences were detected only for the premeiotic portion of the

342 germline (Fig. 5C).

343 To test if modulation of acetylation levels causes changes in sex ratios, we induced

344 hyperacetylation by treating *A. freiburgensis* hermaphrodites with the histone

345 deacetylase inhibitors SRT1720 (Zarse et al., 2010, Milne et al., 2007), Trichostatin

346 A (Yoshida et al., 1990), Valproic Acid (Evason et al., 2008, Forthun et al., 2012), D-

13

347 β-hydroxybutyrate (Edwards et al., 2014), Butyrate (Zhang et al., 2009) and EX-527

348 (Solomon et al., 2006). In all cases, more hermaphrodites than females were

349 produced relative to control (Fig. 5D). By contrast, incubating the mothers in high-

350 density conditions together with the inhibitor of acetylation 4-tert-butylbenzoic acid

351 (Chen et al., 2014) resulted in less hermaphrodite offspring (Fig. 5D).
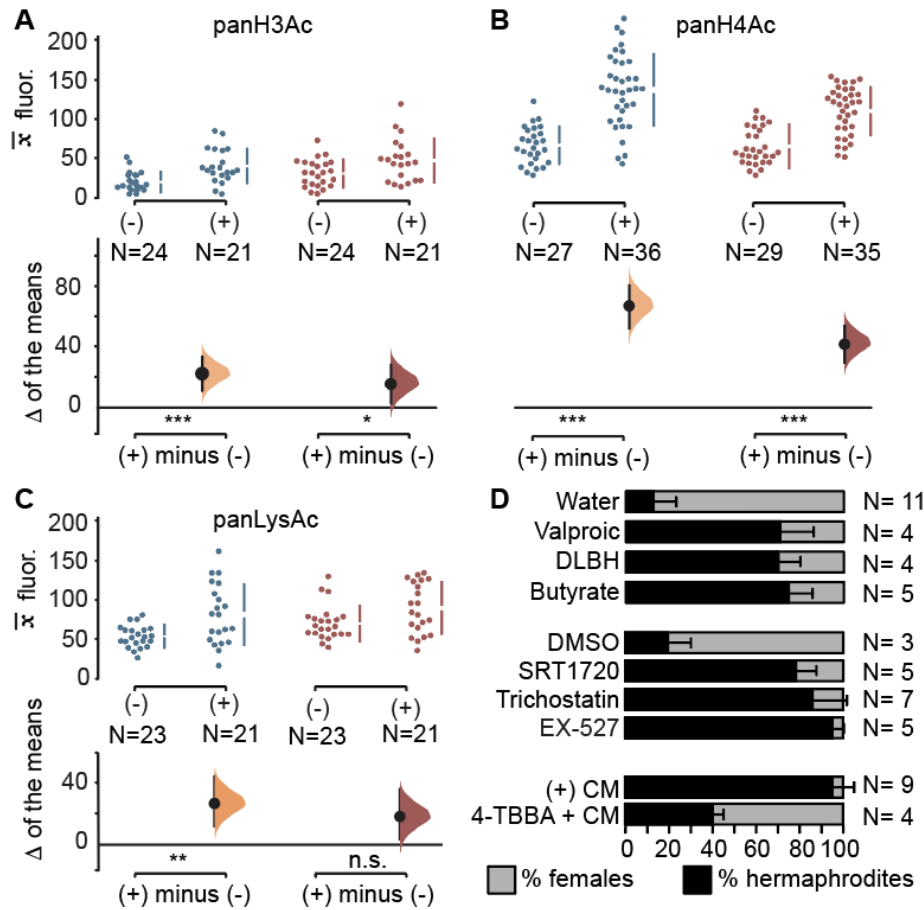
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353 **Figure 5. Regulation of acetylation levels.** Mean antibody fluorescence ($\bar{x}$) for panH3Ac

354 (**A**), panH4Ac (**B**) and panLysAc (**C**) in the pre-meiotic (blue) and meiotic portion (red) of the

355 germline, in the absence (-) or presence (+) of conditioned medium. N= sample sizes. The P

356 values are calculated from a Mann-Whitney test (U): n.s., p>0.05; * = p ≤ 0.05; ** = p ≤ 0.01;

357 ***= p ≤ 0.001. (**D**) Mean percentage and SD of hermaphrodite and female F1 offspring from

358 hermaphrodites treated with chemicals. DLBH: DL-β hydroxybutyrate; TBBA: 4-tert-

359 butylbenzoic acid. (1:10) CM was added to the medium, except plates with 4-TBBA, which

360 had undiluted CM. N= number of replicates.

361

DISCUSSION

Auanema nematodes have been isolated from similar environments as *C. elegans* (Félix and Duveau, 2012), which consists of ephemeral habitats with microbe-rich organic decomposing matter (Schulenburg and Felix, 2017, Kanzaki et al., 2017). Due to rapid population growth and quick depletion of resources, the ecology of these nematodes is characterized by a boom and bust population dynamics. In contrast to *C. elegans*, the developmental and phenotypic response to stress in *A. freiburgensis* occurs across a generation instead of within the same generation: maternal sensing of pheromones secreted by conspecifics induces the production of stress- and starvation-resistant dauer larvae. This indicates that the *A. freiburgensis* mother can predict the environmental conditions to which the offspring is likely to be exposed, and adjusts the F1 phenotype (dauer larvae) to temporarily survive in the absence of food. The *Auanema* dauers have migratory behaviors and always develop into selfing hermaphrodites (Kanzaki et al., 2017). By producing dauers that develop into hermaphrodites, a new population can be established even when the colonizing event is mediated by a single individual (Baker, 1955). This type of intergenerational inheritance, in which parental effects increase the fitness of both offspring and parents, has hallmarks for being adaptive (Uller, 2008).

Here we show that activators of AMPK and insulin signaling activators or mTORC1 inhibitors can mimic the exposure of *A. freiburgensis* to pheromones. These results indicate that highly conserved energy-sensing pathways are involved in mediating intergenerational inheritance in *A. freiburgensis* to generate stress-resistant offspring. How exactly do these energy-sensing pathways regulate phenotypic plasticity in the F1s? One possible mechanism is the direct regulation of the chromatin status in the maternal germline by the energy-sensing enzymes. Thus, activation of transcription of specific genes in the germline may determine the phenotype of the following generation. AMPK, for instance, has been shown to phosphorylate histones, which results in the activation of transcription (Bungard et al., 2010). Consistent with this, we found that protein levels increase for the activated form of AMPK when *A. freiburgensis* is under crowding conditions and is detected in close association with the chromatin of germline cells. By phosphorylating histones,

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395   AMPK has been shown to facilitate histone acetylation (Lo et al., 2001), thus

396   promoting the transcription of a new set of genes (Lee et al., 1993).

397

398   Alternatively, AMPK may indirectly influence the chromatin status via activation of

399   histone acetyltransferases (HATs) or inactivation of histone deacetyltransferases

400   (HDAC), as demonstrated for other model systems (Shimazu et al., 2013, Yang et

401   al., 2001). In *A. freiburgensis*, higher acetylation levels in the chromatin of the

402   germline induced by crowding conditions results in stress-resistant offspring (Fig. 5).

403   It remains to be established whether these acetylation levels are the result of direct

404   phosphorylation of HATs and HDACs by AMPK, or indirectly by natural metabolites.

405   As we show in Fig. 5D, natural metabolites indicative of metabolic stress that inhibit

406   deacetyltransferases, such as D-β-hydroxybutyrate and butyrate (Shimazu et al.,

407   2013), induce the production of stress-resistant offspring.

408

409   The strongest responses to the pharmacological compounds for the production of

410   hermaphrodite progeny occurred when the animals were concomitantly exposed to

411   diluted CM. In the complete absence of CM, only a few compounds elicited a strong

412   response. This may indicate that pheromones in the CM activate more than one

413   pathway and that they have to act in combination to elicit the full effect. Our findings

414   that several energy-sensing pathways are involved in this process in *A.*

415   *freiburgensis*, and that AMPK, insulin and TOR pathways are cross-regulated, are

416   indicative of this hypothesis (González et al., 2020, Ruderman et al., 2010, Banerjee

417   et al., 2016).

418

419   The concentration of the compounds used in our studies are relatively high

420   compared to the ones used in mammalian cells (Burns et al., 2006). This is because

421   nematodes have several physical and physiological adaptations that counteract

422   xenobiotic agents (Burns et al., 2010). Like all pharmacological approaches,

423   interpretation of the results must take into consideration possible lack of specificity

424   (Corton et al., 1995, Longnus et al., 2003, Bain et al., 2007, Pacholec et al., 2010).

425   To ameliorate the possibility of lack of specificity for AMPK activation, for instance,

426   we used compounds that act through several mechanisms (high production of AMP,

427   allosteric binding, protection against dephosphorylation, activation of

16

428   phosphorylation). Genetic approaches using loss- and gain-of-function mutants will

429   help to address some of the above-mentioned concerns (Adams et al., 2019).

430

431   As far as we know, the association of activated AMPK and S6K with the chromatin of

432   germline cells has not been established in other organisms. The presence of AKT in

433   the nucleus of germline cells may be associated with chromatin condensation, which

434   would be reflected in transcription rates (Martelli et al., 2012). Our results indicate

435   that these energy-sensing effectors acquired a new role in intergenerational

436   inheritance in *A. freiburgensis* to regulate gene expression that influences the

437   phenotype of subsequent generations. Given that AMPK, TOR, and insulin pathways

438   are highly conserved in evolution, it is possible that they also mediate non-genetic

439   inheritance via the germline in other organisms in which diet plays a role in

440   determining phenotypic plasticity. Although the epidemiological studies in humans

441   are indicative of diet playing such a role, the mechanisms for this are unknown

442   (Horsthemke, 2018). The findings in this study provide the basis to test such a

443   hypothesis.

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448   **ACKNOWLEDGMENTS**

457

458   **AUTHOR CONTRIBUTIONS**

459   P.R., G.Z., V.K., and A.P.-d.S. designed the study. P.R., A.T., G.Z, V.K., P.P., B.H.,

460   C.B. and B.H. conducted experiments that involved the production of conditioned

461   medium, incubation with chemicals, and sexing offspring. P.R. performed the

462   immunocytochemistry experiments and P. P. performed the Western blots. P.R. and

463   A.P.-d.S. wrote the paper.

464   **AUTHOR INFORMATION**

465   The authors declare no competing interests.

466

467   **MATERIAL AND METHODS**

468   ***Strain and culture***

469   We used the *Caenorhabditis elegans* N2 strain, and the *Auanema freiburgensis*

470   strains SB372 (Kanzaki et al., 2017) and JU1782. The *A. freiburgensis* JU1782 strain

471   was isolated from rotting *Petasites* stems sampled in Ivry, Val-de-Marne, France, in

472   September 2009 by Marie-Anne Félix. Nematodes were cultured at 20 °C on

473   standard Nematode Growth Medium (NGM) (Stiernagle, 2006) plates seeded with

474   *Escherichia coli* OP50-1 strain. NGM medium was supplemented with 25 µg/mL

475   nystatin and 50 µg/mL streptomycin to prevent microbial contamination.

476

477   ***Sexing of progeny***

478   To synchronize the age of the mothers, we collected dauers. *A. freiburgensis* dauers

479   develop into hermaphrodite adults within 24 hours at 20 °C (Kanzaki et al., 2017).

480   Dauer larvae are easily identified by their darker intestine and thinner body

481   compared to similar-sized L3 larvae (which develop into females). Each dauer larva

482   was placed on a 6 cm seeded NGM plate and incubated at 20 °C to develop into

483   adulthood. Each egg laid by the parental (P0) generation was placed into single

484   wells of a 96-well microtiter plate. After 3-5 days, the F1 was scored for their sex:

485   hermaphrodites were identified by their ability to produce offspring in the absence of

486   a mating partner, females by the lack of progeny, and males by their blunt tails

487   (Kanzaki et al., 2017). We calculated sex percentages based only on non-male

488   progeny (hermaphrodites or females). This is because males are not determined by

18

489 environmental cues, but by sex chromosome number. Raw data used to calculate

490 sex percentages are at https://figshare.com/s/48b14ef15a76acc5405d.

491

492 ***Assay with conditioned medium and treatment with pharmacological chemical***

493 ***compounds***

494 To induce *A. freiburgensis* hermaphrodite offspring, the parent hermaphrodites (P0

495 generation) were incubated in the presence of conditioned medium (CM) at 20 °C

496 (Zuco et al., 2018). The CM was derived from 2-3 week old *A. freiburgensis* liquid

497 cultures (M9 medium with *E. coli* OP50-1). Each P0 was placed at the L4 stage onto

498 a 6 cm plate containing NGM and CM. To simulate high-density conditions, 50 mg of

499 lyophilized CM were dissolved in 200 µl of an overnight culture of OP50-1 and

500 spotted onto the plate. F1 eggs were collected for 3-4 days. Each egg was

501 transferred into a single well of a 48-well microtiter plate containing NGM and OP50-

502 1, but no conditioned medium.

503

504 For the pharmacological manipulation of signaling pathways, we added compounds

505 to the NGM and OP50-1. The concentration of the compounds was calculated for the

506 volume of the NGM and OP50-1 used. P0 hermaphrodites were incubated with the

507 compounds for 48-36 h at 20 °C. Information about the providers and catalog

508 number for the compounds used in this study are listed in

509 https://figshare.com/s/48b14ef15a76acc5405d.

510 Chemical compounds were used at the following concentrations: 100 mM Metformin,

511 6 mM Phenformin, 1 µM Rotenone, 5 µM Forskolin, 30 µM Fluvastatin, 0.5 mM

512 AICAR, 0.5 mM 8-Br-cAMP, 5 mM Salicylate, 10 µM ZLN204, 30 µM O-304, 1 µM

513 Dorsomorphin, 100 µM Rapamycin, 100 µM Methotrexate, 100 nM VO-OHpic, 20 µM

514 Indole-3-Carbinol, 10 µM SRT1720, 100 µM Trichostatin A, 4 mM Valproic Acid, 5

515 mM DL-beta hydroxybutyrate, 5 mM sodium butyrate, 100 µM EX-527, 3 mM 4-tert-

516 butylbenzoic acid, 75 nM SC-66, 300 nM Akti-1/2, 20 µM perifosine, and 10 nM SC-

517 79. For nematodes incubated with diluted CM, we used 5 mg of freeze-dried CM

518 dissolved in 200 µl *E. coli* OP50-1.

519

520 ***Inmunohistochemistry***

521 Hermaphrodites were dissected on a slide (Superfrost microscope slide, VWR) in

522 PBS 1X buffer. Dissected gonads were covered by a coverslip and placed on a

523 frozen metal block at -20 °C for at least 10 minutes, and fixed for 2 minutes in a 95%

524 methanol solution at -20 °C. This was followed by 30 minutes in a fixative solution

525 [PBS 1X, 80 mM HEPES (pH= 7.0-7.4), 1.6 mM MgSO4, 0.8 mM EDTA (pH=8.0),

526 4% paraformaldehyde] in a humid chamber at room temperature. Slides were

527 washed twice with PBST (PBS + 0.1% Triton X-100) for 5 minutes and blocked in

528 PBST + 0.5% BSA for 45-60 minutes. The source of primary and secondary

529 antibodies, as well as dilutions used, are listed in

530 https://figshare.com/s/48b14ef15a76acc5405d. All antibodies were diluted in PBST.

531 Incubation with the primary antibodies was performed at 4 °C overnight. Slides were

532 then washed twice in PBST for 10 minutes each and the corresponding secondary

533 antibody was added and incubated for 2 hours at room temperature. Slides were

534 washed in PBST as above to remove the excess of the secondary antibody and then

535 one drop of Fluoroshield Mounting Medium with 4′,6-diamidino-2-phenylindole

536 (DAPI) (Abcam, #ab104139) was added on the immunostained samples.

537

538 Images were taken with a 60X objective in 2.40 μm z-stack intervals (12 sections)

539 with a DeltaVision microscope (Olympus). Acquisition and constrained iterative

540 deconvolution of the images from DeltaVision were processed using the softWoRx

541 software (Applied Precision). The intensity of fluorescence for the secondary

542 antibodies was measured using the ImageJ software (NIH Image, Bethesda, MD).

543

544 ***Western blot***

545 Protein extraction and buffer preparation were performed following the protocol of

546 (Jeong et al., 2018). Six hundred adult hermaphrodites were collected for each

547 sample: control (OP50-1 only) and experimental (50 mg conditioned medium powder

548 per 200 μl of OP50-1) samples. Protein concentration was measured using Bradford

549 assay (Bradford Reagent, Bio-Rad). We loaded approximately 100 μg of protein. The

550 primary antibodies, against Phospho-AMPKα (Thr172) and PAR-4/LKB1, were used

551 at 1:1000 dilution. The source of primary and secondary antibodies, as well as

552 dilutions used for them, are listed in https://figshare.com/s/48b14ef15a76acc5405d.

553 To detect the signal for the antibodies, we used the Amersham™ ECL™ Western

554 Blotting Detection Reagents (RPN2209).

555

Statistical analyses

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Results were presented using the most recent developments in data analysis and

557

presentation (Ho et al., 2019), showing the raw data as 'bee swarm' plots. They

558

summarize the data showing the mean and the 95% confidence interval (CI), as well

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as the sampling error distribution diagrammed as a filled curve. These plots provide

560

transparency of the comparison being made, visual clarity and statistical evaluation

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of the data.

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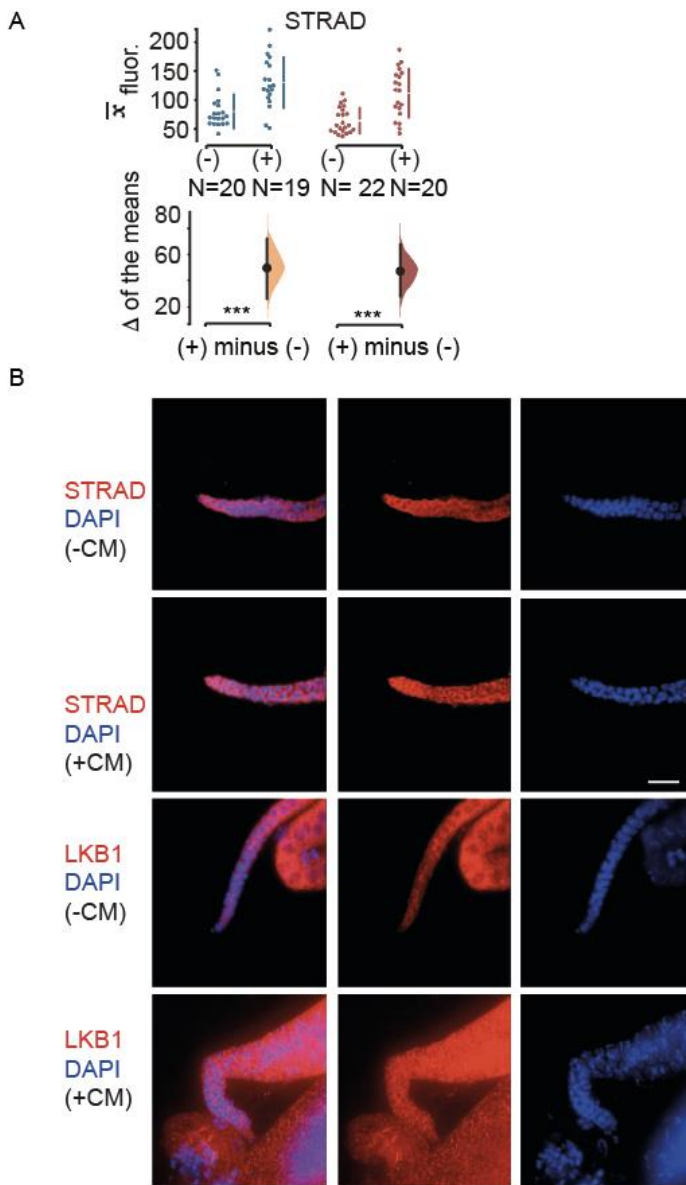
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915 **Supplemental Figures**

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917 **Supplemental Figure 1. STRAD and LKB1 antibody staining is higher in**

918 **animals in crowding conditions. A.** Mean antibody fluorescence ($\bar{x}$) in the pre-

919 meiotic (blue) and meiotic portion (red) of the germline, in the absence (-) or

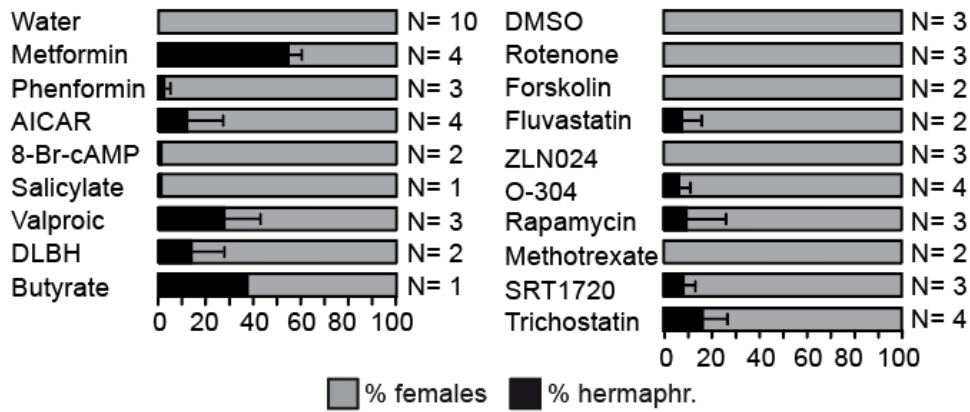920 presence (+) of conditioned medium. N= sample sizes. Graphical representation as

921 Fig. 2, with ***= p ≤ 0.001. **B.** LKB1 and STRAD in the germline. Staining for

922 antibodies (in red) against LKB1 and STRAD of gonads dissected from

923 hermaphrodites incubated in the presence of either (-) CM or (+) CM. The DNA was

924 stained with DAPI (blue). Bar, 15 μm.

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Supplemental Figure 2. Most chemical compounds affect the sex ratios of hermaphrodites in the absence of diluted CM. Mean percentage and SD of hermaphrodite and female F1 offspring from hermaphrodites treated with chemicals. Chemicals were dissolved either in water or in DMSO. N= number of replicates.
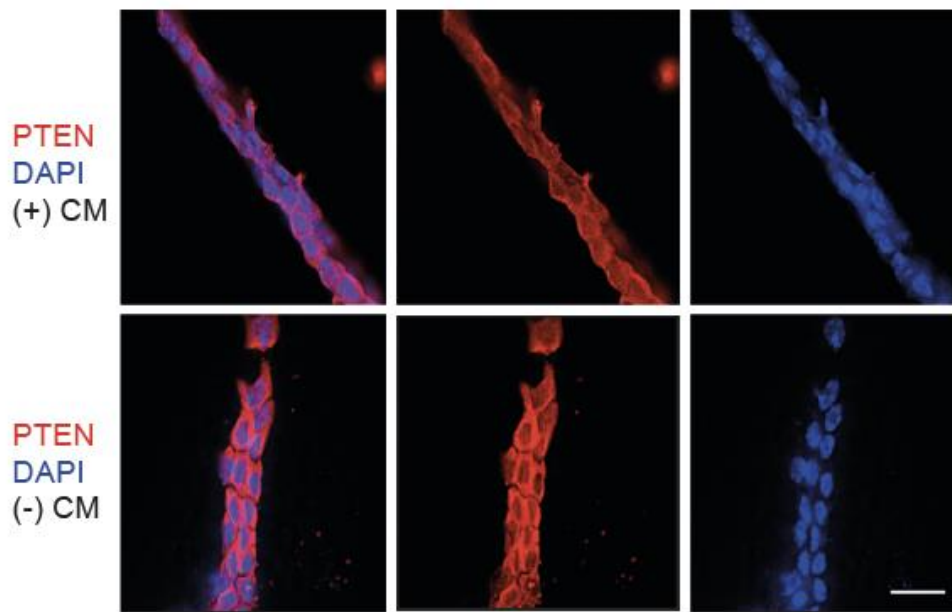
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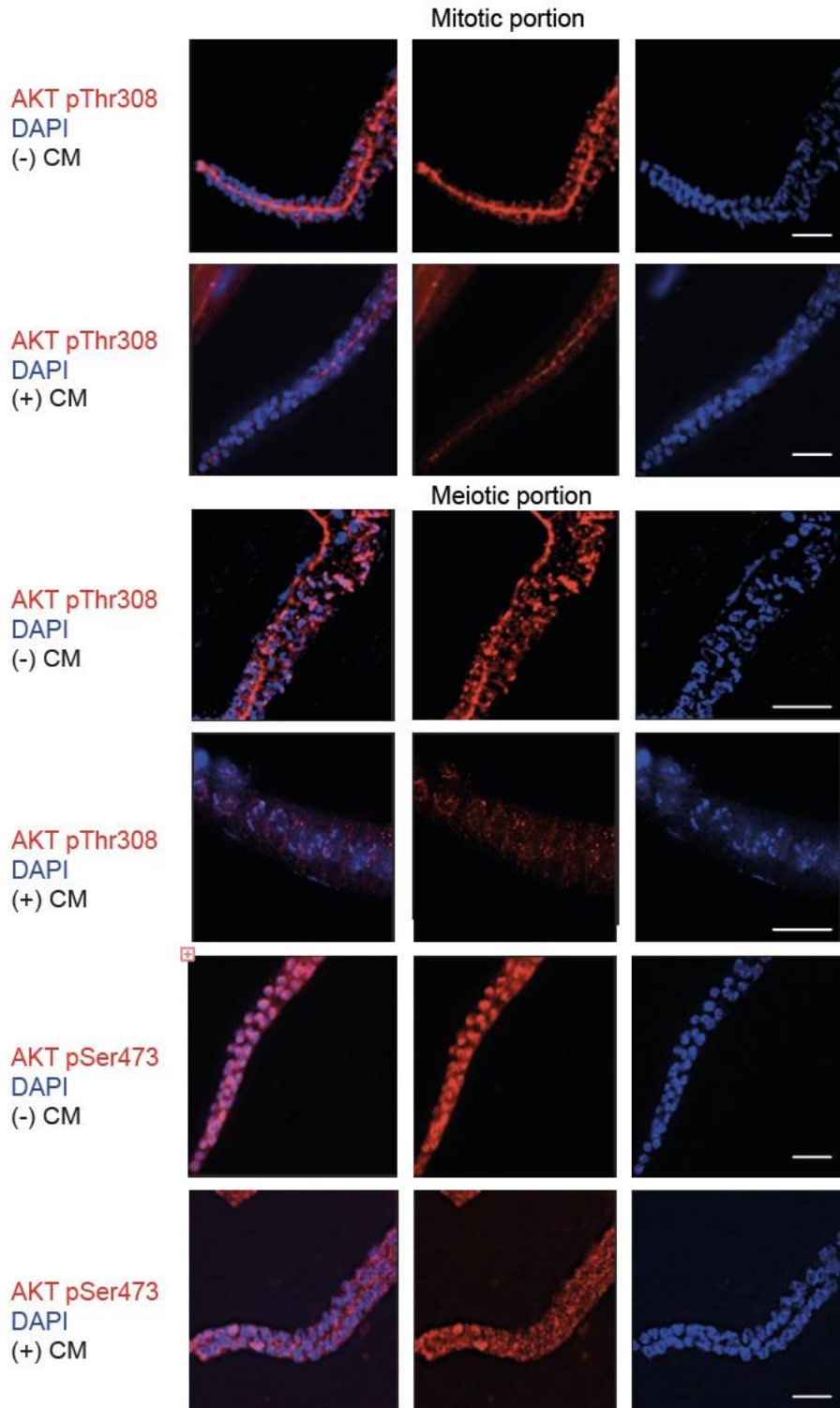
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Supplemental Figure 3. PTEN/DAF-18 in the germline is cytoplasmic and higher in non-crowding conditions. Staining for antibodies (in red) against PTEN/DAF-18 of gonads dissected from hermaphrodites incubated in the presence of either (-) CM or (+) CM. The DNA was stained with DAPI (blue). Bar, 15 µm.

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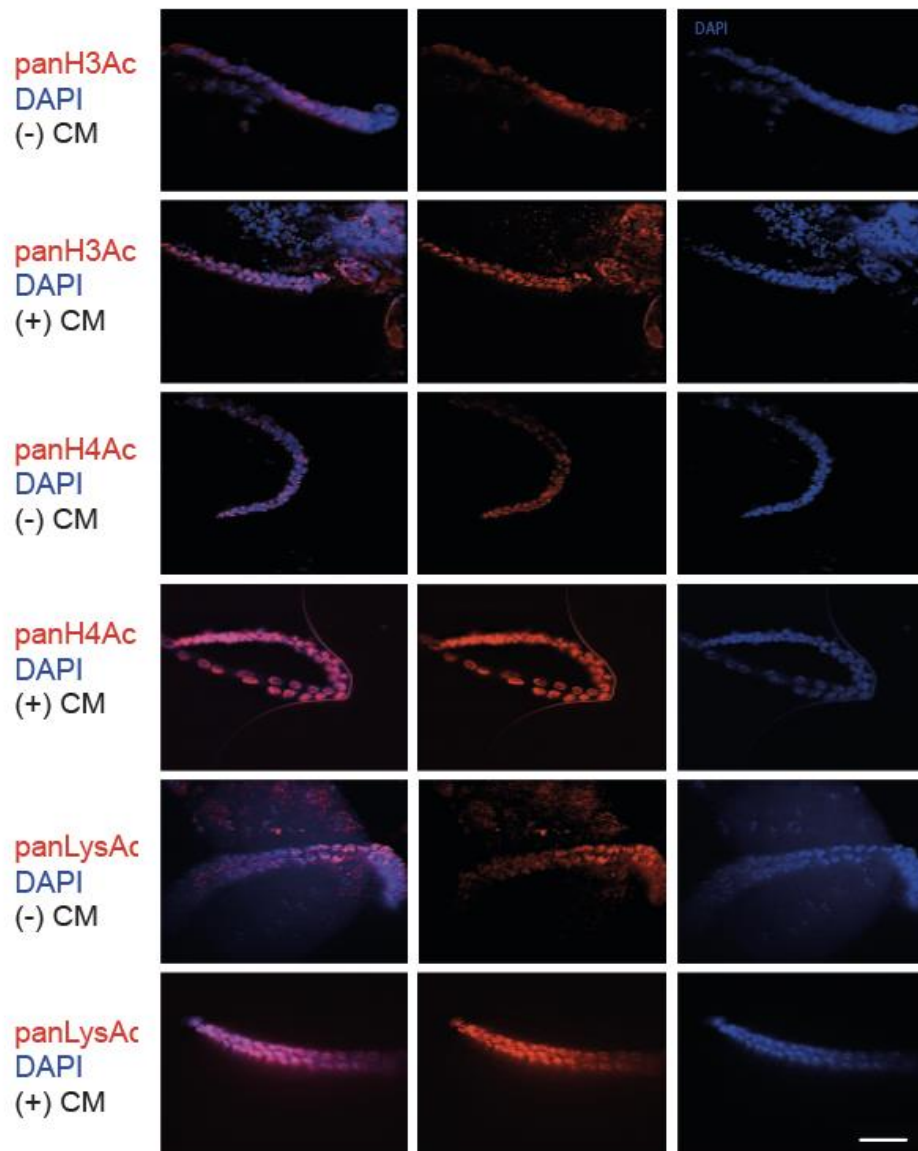**Supplemental Figure 4. AKT pThr308 in the germline is nuclear and higher in crowding conditions.** Staining for antibodies (in red) against AKT pThr308 of gonads dissected from hermaphrodites incubated in the presence of either (-) CM or (+) CM. The DNA was stained with DAPI (blue). Bar, 15 μm.

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Supplemental Figure 5. Acetylation in the germline is nuclear and higher in crowding conditions. Staining for antibodies (in red) against panH3Ac, panH4Ac and panLysAc of gonads dissected from hermaphrodites incubated in the presence of either (-) CM or (+) CM. The DNA was stained with DAPI (blue). Bar, 15 µm.

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