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Parental energy-sensing pathways control intergenerational offspring sex determination in the nematode *Auanema freiburgensis*

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Abstract

Background: Environmental stimuli experienced by the parental generation influence the phenotype of subsequent generations (Demoinet et al., *Proc Natl Acad Sci U S A* 114:E2689-E2698, 2017; Burton et al., *Nat Cell Biol* 19:252–257, 2017; Agrawal et al., *Nature* 401:60-63, 1999). The effects of these stimuli on the parental generation may be passed through the germline, but the mechanisms at the basis of this non-Mendelian type of inheritance, their level of conservation, how they lead to adaptive vs non-adaptive, and intergenerational vs transgenerational inheritance are poorly understood. Here we show that modulation of nutrient-sensing pathways in the parental generation of the nematode *Auanema freiburgensis* regulates phenotypic plasticity of its offspring.

Results: In response to con-specific pheromones indicative of stress, AMP-activated protein kinase (AMPK), mechanistic target of rapamycin complex 1 (mTORC1), and insulin signaling regulate stress resistance and sex determination across one generation, and these effects can be mimicked by pathway modulators. The effectors of these pathways are closely associated with the chromatin, and their regulation affects the chromatin acetylation status in the germline.

Conclusion: These results suggest that highly conserved metabolic sensors regulate phenotypic plasticity through regulation of subcellular localization of their effectors, leading to changes in chromatin acetylation and epigenetic status of the germline.

Keywords: Intergenerational inheritance, Transgenerational inheritance, Dauer, Sex determination, nematode, *C. elegans*, *Auanema*, AMPK, Histone acetylation

Background

The phenotype of an individual is the result of the interactions between its genome and the environment. Experiences made by the ancestors may also shape an individual's phenotype. Some of those traits are

transmitted through the germline, including mechanisms that do not involve changes in DNA sequence [1, 2]. Some recent examples of traits that are “epigenetically” inherited include immunity [3, 4], behavior [5, 6], resistance to starvation [7], and osmotic stress [8].

To understand the mechanisms of epigenetically inherited traits, it is important to distinguish between transgenerational and intergenerational inheritance [9]. Transgenerational effects refer to information that is

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passed across multiple generations and that affect the phenotype even when the stimulus is absent. Intergenerational effects refer to parental exposures affecting primarily the phenotype of the immediate generation. These effects can be passed either through the germline or the soma [10]. Recently, the mechanisms underlying gametic transmission of parental effects have been revealed in some animal models. The germ cells of parents fed on specific diets, for example, may undergo chromatin modifications [11], changes in the composition of fragments of tRNAs [12, 13] and yolk content [14, 15] that are consequential for the phenotype of the offspring. While these mechanisms seem passive effects of the parental experiences and may not always be adaptive, there are known examples for intergenerational adaptive responses to the environment [16, 17]. In the crustacean *Daphnia*, for example, chemical sensing of predators by the parents induces the production of predation-resistant offspring [18].

The nematode *Caenorhabditis elegans* has been instrumental in revealing mechanisms of inter- and transgenerational inheritance because of its short generation time, large number of offspring, and availability of genetic resources. While transgenerational effects are superficially mediated by similar mechanisms as for intergenerational effects in this nematode, such as chromatin modifications [19] and small RNAs [20], many questions still remain: what are the mechanisms that determine whether traits are transmitted for either one or multiple generations? How general are these mechanisms across nematodes and the animal kingdom? Are there differences in mechanisms when traits are transmitted from somatic cells to the germline, versus environmental cues that act directly on the germline? Are there differences in mechanisms that result in adaptive versus non-adaptive traits?

To address some of these questions, we have been studying *Auanema* nematodes. Similar to *C. elegans*, these nematodes have a short generation time (~ 4 days) and produce a large number (~ 350) of offspring [21, 22]. Nematodes of this genus have three sexual morphs [23]: males are XO, whereas hermaphrodites and females are XX [24]. In *A. rhodensis*, the maternal environment seems important for the hermaphrodite versus female fate, since most females are produced by young mothers, whereas older mothers produce mostly self-fertilizing hermaphrodites [22]. Although phenotypically almost identical at the adult stage, the larval development is different between *Auanema* hermaphrodites and females: hermaphrodites always develop through a starvation-resistant larval stage named “dauer”. In fact, dauer development is determinant for the sexual morph fate, since larvae initially committed to become females can be

converted to hermaphrodites if forced to undergo dauer formation [25].

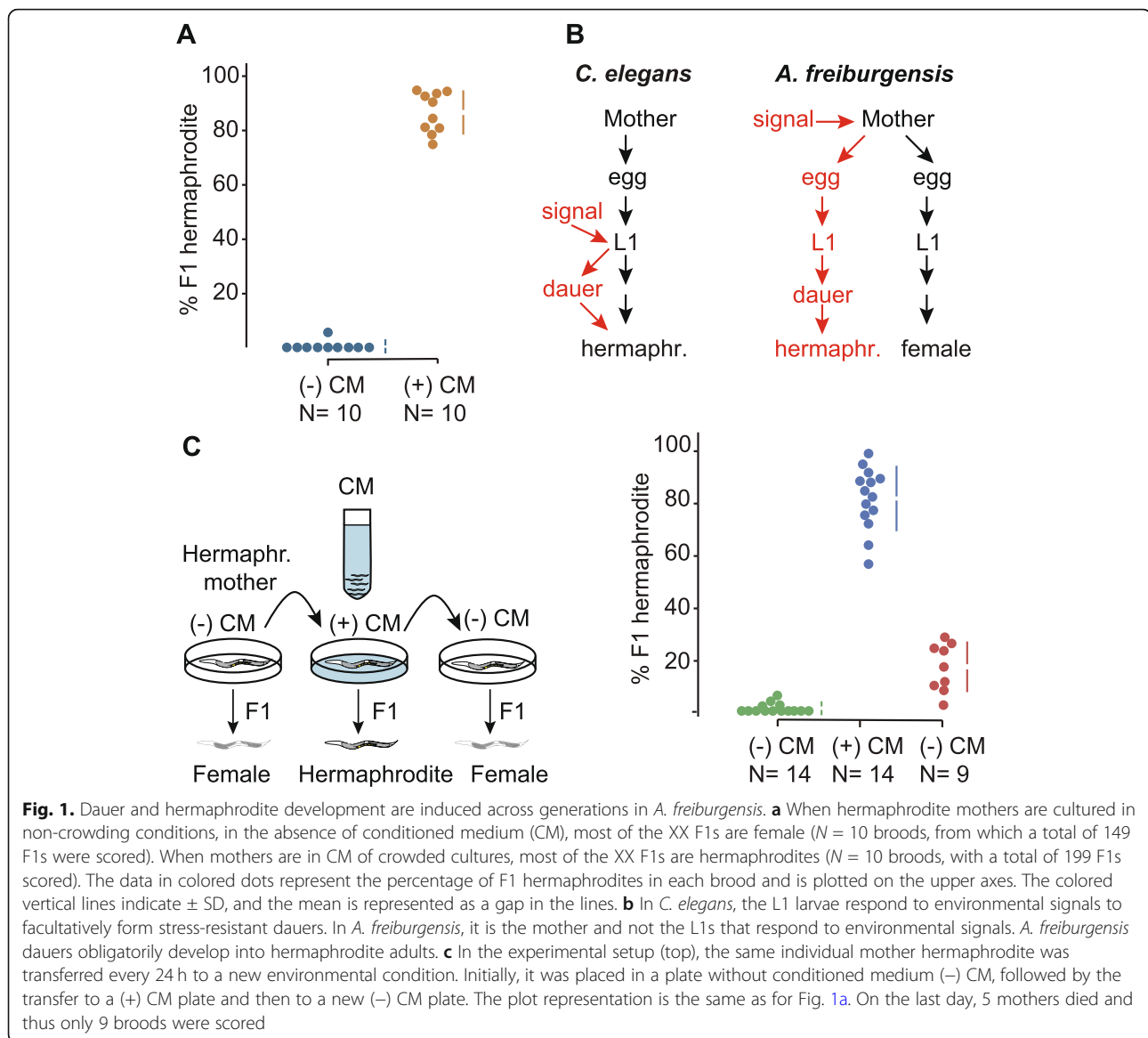
Here we focus on the species *Auanema freiburgensis*, for which the environmental cues experienced by the mother determine dauer formation and sexual morph fate of the offspring [23]. We show that signals indicative of crowding can induce the mother to produce dauers, which become hermaphrodite adults. Both traits (stress resistance and sexual morph fate) are likely to be adaptive and inherited intergenerationally by the transmission of signals sensed by somatic cells to germline. Pharmacological assays indicate that energy-sensing signaling mediated by AMP-activated protein kinase (AMPK), mechanistic target of rapamycin complex 1 (mTORC1), and insulin signaling are involved in intergenerational inheritance in *A. freiburgensis*. The formation of F1 dauers correlates with changes in the germline histone acetylation of the maternal germline.

Results

Individuals of the nematode *A. freiburgensis* produce only sperm (males), only oocytes (females), or both gametes (hermaphrodites) [23]. The hermaphrodite versus female sexual morph is determined by the environment experienced by the mother: hermaphrodite mothers kept in isolation produce mostly female offspring, whereas hermaphrodites exposed to high population density conditions produce mostly hermaphrodite offspring (Fig. 1a).

A crucial factor in the development of *Auanema* hermaphrodites is the passage through the stress-resistant dauer stage [21–23, 25], which has morphological and behavioral adaptations for dispersal. In *A. freiburgensis*, all XX larvae that pass through the dauer stage become hermaphrodites ($N = 96$), whereas XX non-dauer larvae develop into females ($N = 93$). Similar to *A. rhodensis* [25] and other trioecious nematodes [26], we never observed *A. freiburgensis* males to undergo dauer formation. Thus, signals experienced by the maternal generation of *A. freiburgensis* are used to generate non-feeding offspring that can survive starvation conditions and reproduce by self-fertilization once food becomes available. In summary, these results suggest that dauer formation in *A. freiburgensis* is induced across a generation, instead of within the same generation as in *C. elegans* [27] (Fig. 1b).

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 parental generation was exposed to the CM. The induction of dauers through the hermaphrodite mother is limited to one



generation: F1 hermaphrodites derived from mothers in (+) CM plates produce mostly female offspring (99.6% out of 470 F2 offspring, scored from 10 broods). To test if *A. freiburgensis* L1 larvae can also respond to crowding conditions, eggs derived from mothers cultured in isolation were left to hatch and undergo larval development in (+) CM plates until adulthood. 95.7% ($N = 161$) of these L1s developed into females, indicating the effects of crowding are detected only in the parental generation.

To test if the effects of the crowding conditions on the hermaphrodites acted in a reversible manner, the same individual was placed in different conditions on different days. When an individual was in non-crowded conditions, most of the offspring was composed of females (Fig. 1c). When the same individual was placed in plates

with the crowding signal, it produced mostly hermaphrodites. After rinsing it with the M9 buffer and placing the same individual in a plate with no crowding cues, it resumed the production of females. These results suggest that a hermaphrodite adjusts the production of sexual morphs according to the immediate environmental signals.

To determine the minimal population density sufficient for the induction of dauers and hermaphrodites across a generation, we incubated the maternal generation at different densities. When hermaphrodites are cultured for 6 h, a minimum density of 16 adult hermaphrodites per cm^2 is sufficient for the induction of 100% ($N = 295$ F1s) of hermaphrodite offspring. In densities below 10 individuals/ cm^2 , the hermaphrodite

mothers produce only female offspring (10 individuals/cm²: 100% females, $N = 78$ F1s; 6 individuals/cm²: 98.5% F1 female, $N = 66$ F1s). At an intermediate density (13 individuals/cm²), hermaphrodites produce 19% ($N = 126$ F1s) of hermaphrodite offspring.

To investigate if other maternal environmental conditions affect the sexual fate of the F1s, mothers were incubated for 24 h at 25 °C or subjected to diet restriction conditions (no food). Most XX offspring (97%) developed into female adults for both conditions (166 F1s scored from mothers at 25 °C and 146 F1s scored from starving mothers).

Modulation of AMPK signaling changes sexual morph ratios in *A. freiburgensis*

In previous research, energy metabolism has been associated with intergenerational and transgenerational inheritance in *C. elegans* [20, 28, 29]. Although *A. freiburgensis* subjected to diet restriction did not induce dauer formation in F1s, it is possible that the high-crowding conditions experienced by the parental generation are used as indication for imminent lack of food. In eukaryotes, caloric restriction triggers the activation of AMP-activated protein kinase (AMPK) [30], a highly conserved energy sensor [31]. AMPK activity protects cells against the depletion of ATP by stimulating energy-producing pathways and inhibiting energy-consuming processes [32]. In *C. elegans*, AMPK is required for lifespan extension and germline viability when the nematode is in nutrient stress [28, 30, 33, 34]. The full kinase activity of AMPK requires phosphorylation of threonine residue 172 (Thr172) by upstream kinases [30, 35, 36].

We hypothesized that AMPK regulates target proteins in the maternal germline to influence the phenotype of the following generation. To functionally test the role of AMPK in mediating intergenerational inheritance in *A. freiburgensis*, we used pharmacological compounds that modulate AMPK activity. We measured the effects of these compounds on intergenerational inheritance by scoring hermaphrodite and female sexual morphs in the offspring. As mentioned previously, high population densities induce the production of dauer larvae in the F1, which mature to become hermaphrodite adults. Consistent with a role of AMPK in mediating this effect, we found that AMPK activators induce the production of hermaphrodites (Fig. 2a) (for a recent review on pharmacological activation of AMPK, see [37]). In some cases, these compounds cause changes in the F1 sex morph ratios when on their own (Additional file 1: Figure S2), but potentiation of their effects was significantly stronger when diluted CM (1:10 CM) was added to the culture medium. This may indicate that synergistic effects of different mechanisms are necessary to fully elicit a robust

response, or that those energy-sensing pathways can be efficiently activated only when upstream events occur first.

Although the mechanisms of action are not clear for all pharmacological compounds, they can be broadly divided into indirect and direct AMPK activators. Any treatments that raise the AMP/ADP:ATP ratios are expected to indirectly activate AMPK. For instance, inhibition of mitochondrial respiration by metformin, phenformin, and rotenone has been implicated in the activation of AMPK [38–45]. Forskolin, an adenylate cyclase activator, activates AMPK by increasing the cytosolic cAMP concentration [46]. Statins, such as fluvastatin [47], have been proposed to activate AMPK. The incubation of mothers with all these compounds resulted in a higher proportion of hermaphrodite progeny (Fig. 2a).

Compounds that are similar to AMP can directly activate AMPK. 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), for example, increases the activity of AMPK after being converted to an AMP analog inside the cell [48], whereas 8-Br-cAMP is a non-hydrolyzable analog of cAMP [49]. Other compounds, such as the plant product salicylate [50], and the synthetic compounds ZLN024 [51] and O-304 [52] bind to AMPK, causing allosteric activation and inhibition of dephosphorylation of the pThr172. All these compounds induced a higher percentage of hermaphrodite offspring than controls (Fig. 2a). When tested at various concentrations, metformin induced hermaphrodites in a dose-dependent manner (Additional file 1: Figure S2B). To inhibit AMPK, we used dorsomorphin [40]. As expected, hermaphrodites in CM with dorsomorphin resulted in a lower proportion of hermaphrodite progeny compared to controls (Fig. 2a).

To examine if levels of AMPK activity in the change according to crowding conditions, we performed antibody staining on dissected gonads and Western blots of whole animals (Fig. 2b–e). Liver Kinase B1 (LKB1), known in *C. elegans* as PAR-4 [36, 53], phosphorylates and activates AMPK in the context of energy stress [54, 55]. LKB1 is part of a complex with two proteins Ste20-related adaptor protein- α (STRAD) [56] and mouse protein 25- α (MO25 α) [57]. Antibody staining against LKB1 and STRAD showed a higher level of staining in the meiotic portion of the germline isolated from animals cultured in crowding conditions (Fig. 2b, d and Additional file 1: Figure S1). Their localization was predominant in the cytoplasm of germline cells (Additional file 1: Figure S1).

To test the levels of AMPK, we used an antibody that detects the active, phosphorylated form of AMPK (AMPK pThr172). Consistent with the higher levels of

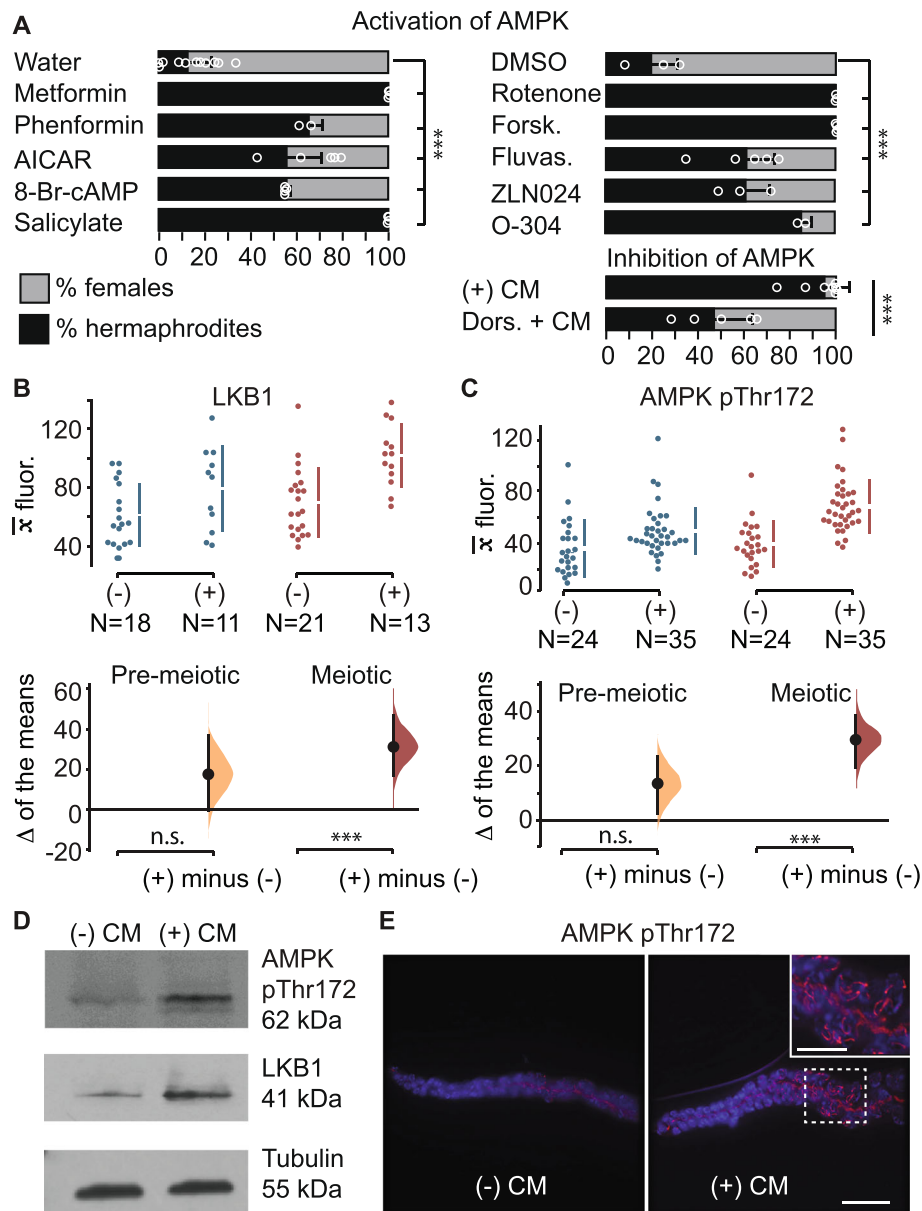


Fig. 2. AMPK pathway modulation in the *A. freiburgensis* germline. **a** Mean percentage of hermaphrodite and female F1 offspring from hermaphrodites treated with chemicals. The control was either water (left) or DMSO (right), depending on how the chemical compounds were dissolved. Error bars represent SD. Dors. = Dorsomorphin. In all cases, diluted (1:10) CM was added to the medium, with exception to plates with dorsomorphin, which had undiluted CM. **b, c** Mean antibody fluorescence (i) in the premeiotic (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 \pm 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 bars. n.s., $p > 0.05$; *** = $p \leq 0.01$. **d** Western blots with proteins derived from hermaphrodites incubated in the absence (-) CM or presence (+) CM of conditioned medium. **e** AMPK pThr172 antibody staining of gonads dissected from hermaphrodites incubated in either (-) CM or (+) CM. Bar, 15 μ m. Insert in the right picture is a magnification from the region marked with a stippled square. Bar, 7.5 μ m. Details for the one-way analysis of variance (ANOVA) followed by a post hoc Holm-Sidak test for multiple comparisons (**a**), *** = $p \leq 0.01$. See "Methods" for sample sizes and *p* values.

LKB1 and STRAD, we also found that the anti-AMPK pThr172 staining was stronger in crowding conditions compared to control animals (Fig. 2c–e). The difference in

the level of staining was restricted to the meiotic region of the germline and the AMPK staining is closely associated with the chromatin of pachytene cells (Fig. 2e).

Maternal inhibition of mTORC1 signaling results in mostly hermaphrodite offspring

Since energy-sensing by AMPK induced intergenerational effects in *A. freiburgensis*, we hypothesized that other energy sensors may be involved in the same process. The intracellular nutrient sensor mTORC1 complex is a multisubunit kinase that senses growth signals and stimulates anabolism when nutrients are abundant [58–62]. Therefore, we would predict that in low population densities and readily available nutrients, the mTOR pathway would be active in *A. freiburgensis*. Under these conditions, *A. freiburgensis* produces mostly non-dauer larvae that later become female offspring.

To test the effect of modulating mTORC1 activity on sexual morph ratios, we treated mothers with pharmacological compounds. Mothers treated with rapamycin [63, 64] produced a greater proportion of F1 hermaphrodites than control mothers (Fig. 3a). mTORC1 signaling promotes nucleic acid synthesis, as long as nucleotide precursors are available [65]. Treatment with methotrexate, a chemical that suppresses the de novo purine synthesis enzymes [66], inhibits mTORC1 activity. We found that *A. freiburgensis* hermaphrodites treated with methotrexate generated mostly hermaphrodite offspring (Fig. 3a).

To investigate the kinase activity of mTORC1, we examined the expression of a well-characterized target protein, p70 S6K protein kinase (S6K) [58]. Antibody staining against the phosphorylated form of S6K (S6K pThr389) was detected in germline cells isolated from animals grown in low-density conditions (Fig. 3b–e). Most staining was associated with the chromatin, both in mitotic cells (Fig. 3b) and meiotic cells in late pachytene stages (Fig. 3c). Since AMPK and mTORC1 have opposing actions [67], we hypothesized that treatment of animals with metformin, an activator of AMPK, would inhibit mTORC1 signaling. Consistent with this hypothesis, we found that treatment of animals with metformin resulted in a smaller number of cells stained with SK6 pThr389 (Fig. 3e). Altogether, these results indicate that mTOR signaling is involved in intergenerational inheritance in *A. freiburgensis*.

Insulin signaling is downregulated in animals in crowding conditions

The insulin signaling pathway regulates metabolism, development, and lifespan in a wide variety of animals. One of the regulators of the insulin pathway is a conserved phosphatase named PTEN (or DAF-18 in *C. elegans*) [68–70]. To test if PTEN/DAF-18 mediates the generation of hermaphrodites, we used the PTEN/DAF-18 inhibitors VO-OHpic [71] and SF1670 [72]. When in the presence of conditioned medium from high-density populations, hermaphrodites treated with those inhibitors generated mostly female

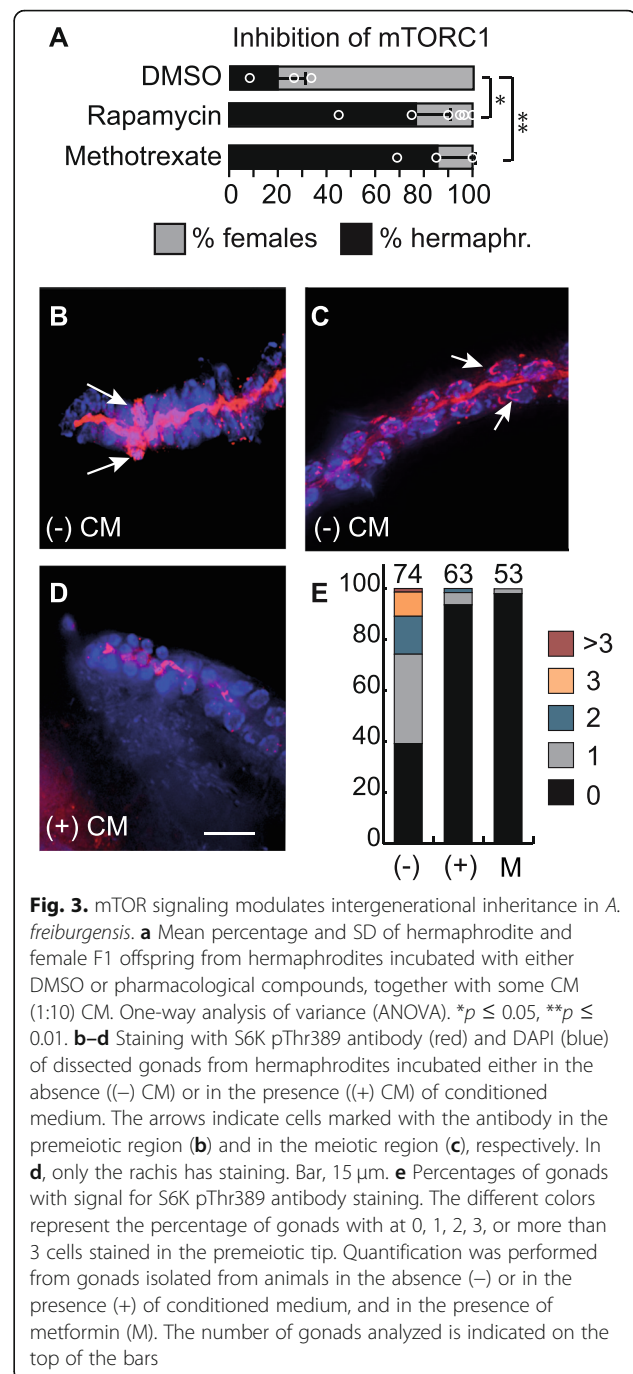


Fig. 3. mTOR signaling modulates intergenerational inheritance in *A. freiburgensis*. **a** Mean percentage and SD of hermaphrodite and female F1 offspring from hermaphrodites incubated with either DMSO or pharmacological compounds, together with some CM (1:10) CM. One-way analysis of variance (ANOVA). * $p \leq 0.05$, ** $p \leq 0.01$. **b–d** Staining with S6K pThr389 antibody (red) and DAPI (blue) of dissected gonads from hermaphrodites incubated either in the absence ((-) CM) or in the presence ((+) CM) of conditioned medium. The arrows indicate cells marked with the antibody in the premeiotic region (**b**) and in the meiotic region (**c**), respectively. In **d**, only the rachis has staining. Bar, 15 μ m. **e** Percentages of gonads with signal for S6K pThr389 antibody staining. The different colors represent the percentage of gonads with at 0, 1, 2, 3, or more than 3 cells stained in the premeiotic tip. Quantification was performed from gonads isolated from animals in the absence (-) or in the presence (+) of conditioned medium, and in the presence of metformin (M). The number of gonads analyzed is indicated on the top of the bars

offspring (Fig. 4a). Activation of PTEN/DAF-18 in hermaphrodites with the compound indole-3-carbinol under low population densities resulted in mostly hermaphrodites (Fig. 4a).

To examine the regulation of the insulin pathway in *A. freiburgensis*, we used an antibody against PTEN/DAF-18 to stain isolated gonads from hermaphrodites cultured in low- and high-density conditions. We found that the antibody against PTEN/DAF-18

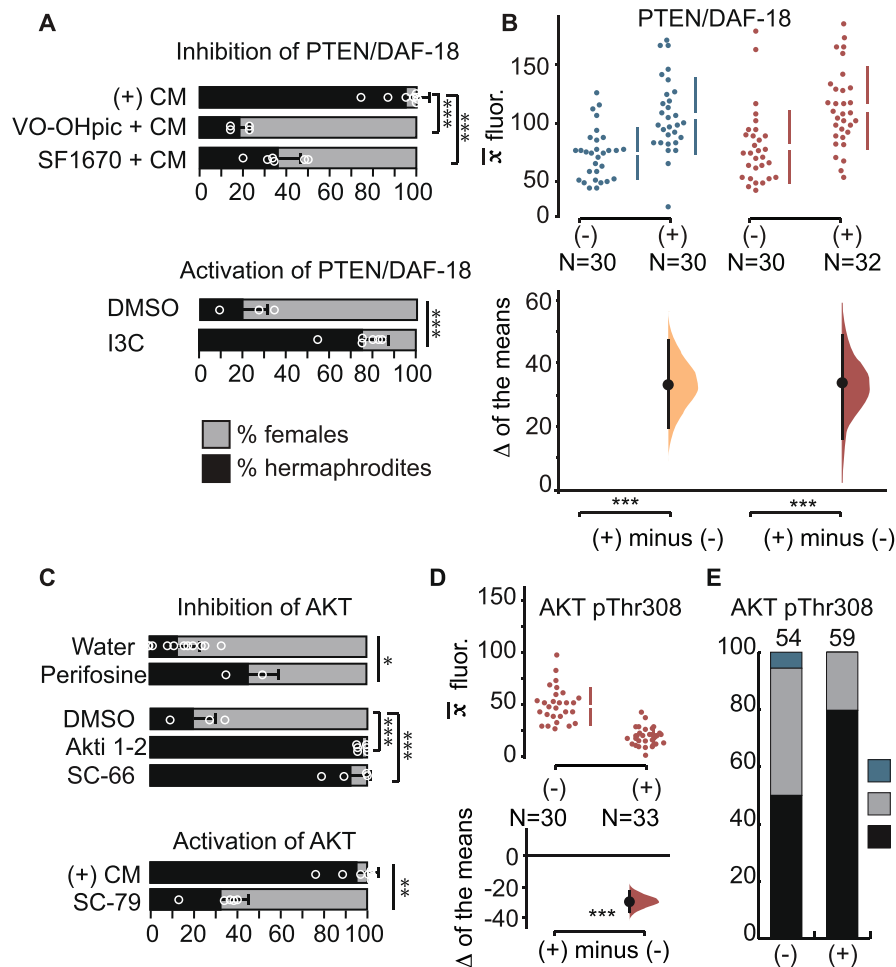


Fig. 4. Regulation of PTEN/DAF-18 and AKT. **a** Mean percentage and SD of hermaphrodite and female F1 offspring from hermaphrodites treated with chemicals that activate or inhibit PTEN/DAF-18. (+) CM represents undiluted conditioned medium. The DMSO control and indole-3-carbinol (I3C) incubations were performed with diluted (1:10) CM. **b** Quantification of antibody staining with PTEN/DAF-18 in the maternal gonads. **c** Effect of pharmacological inhibition or activation of AKT on sexual morph ratios in the F1s. **d** Quantification of antibody staining for AKT pThr308 in the meiotic portion of the germline. **e** Quantification of premeiotic germline cells with staining with an antibody against AKT pThr308. Graphical representation in **b**, **d** as in Fig. 2b, and in **e** as in Fig. 3e. One-way analysis of variance (ANOVA) followed by a post hoc Holm-Sidak test for multiple comparisons (**a** inhibition of PTEN; **c** inhibition of AKT), Student's *t* test (**a** activation of PTEN with I3C; **c** inhibition of AKT with perifosine), Mann-Whitney test (**c** activation of AKT with SC-79). **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001

stained more strongly the germline when hermaphrodites were incubated in high-density conditions than in low-density populations (Fig. 4b and Additional file 1: Figure S3).

One of the target proteins and effectors for insulin signaling is AKT kinase (also known as PKB) [73], which among several anabolic functions, also prevents chromatin condensation [74–76]. Maternal inhibition of AKT with the chemicals perifosine (prevents activation of AKT by affecting its subcellular localization) [77], Akti-1/2 (stabilizes the inactive conformation of AKT) [78], and SC-66 (allosteric inhibitor of AKT) [79] results in a higher proportion of hermaphrodite progeny (Fig. 4c). On the other hand, activation of AKT with SC-79 [80]

prevents the generation of hermaphrodite progeny when the mother is in crowding conditions (Fig. 4c).

Maximal activation of AKT requires phosphorylation at residues Thr308 and Ser473 [81]. Immunostaining with antibodies against AKT pThr308 (Fig. 4d, e and Additional file 1: Figure S4) revealed that staining is prominently associated with the chromatin in germline cells of animals grown under non-crowding conditions. No such association is seen when animals are in crowding conditions (Fig. 4d). The same pattern is seen for AKT pSer473 (Additional file 1: Figure S4). Altogether, these results are consistent with the hypothesis that crowding conditions induce a lower insulin signaling, causing the production of hermaphrodite offspring.

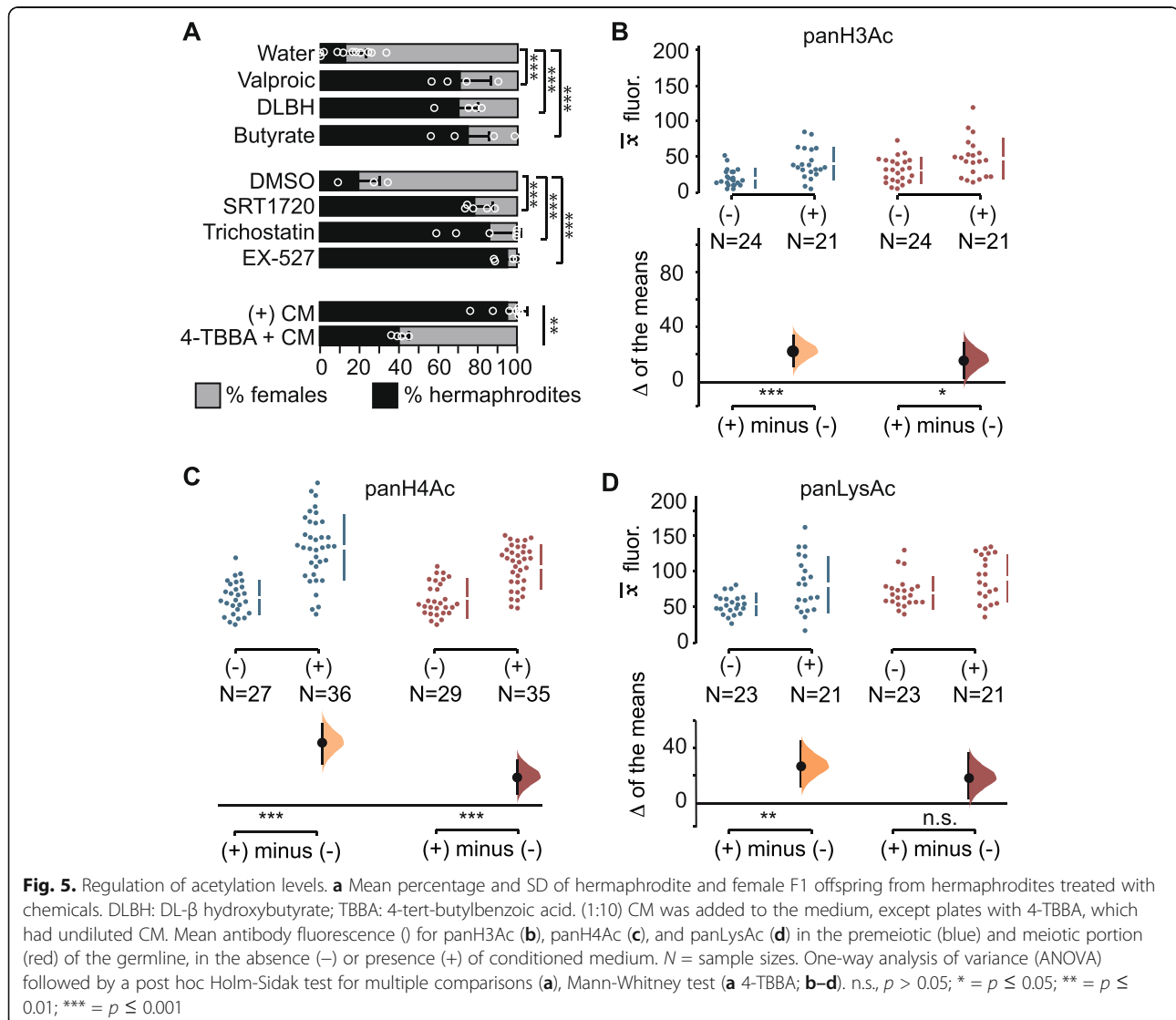
Changes in the maternal histone acetylation status correlate with changes in F1 development and mode of reproduction

Energy-sensing pathways have been implicated in the regulation of acetylation of histones, histone modifiers, and cellular proteins [82]. To test if modulation of acetylation levels causes changes in sexual morph ratios, we induced hyperacetylation by treating *A. freiburgensis* hermaphrodites with the histone deacetylase inhibitors SRT1720 [83, 84], Trichostatin A [85], valproic acid [86, 87], D- β -hydroxybutyrate [88], sodium butyrate [89], and EX-527 [90]. In all cases, more hermaphrodites than females were produced relative to control (Fig. 5a). By contrast, incubating the mothers in high-density conditions together with the inhibitor of acetylation 4-tert-butylbenzoic acid [91] resulted in less hermaphrodite offspring (Fig. 5a).

To examine if acetylation patterns change in the germline when *A. freiburgensis* is in high population densities, we compared the level of antibody staining in gonads isolated from hermaphrodites cultured in the absence or presence of CM. Antibody staining against acetylated residues on histones 3 and 4 was at higher levels in the germline derived from animals cultured in the presence of CM compared to controls, both for premeiotic and meiotic portions (Fig. 5b,c and Additional file 1: Figure S5). The same trend was observed when using an antibody that binds to all acetylated proteins (panLysAc), although differences were detected only for the premeiotic portion of the germline (Fig. 5d).

Discussion

Auanema nematodes have been isolated from similar environments as *C. elegans* [92], which consists of



ephemeral habitats with microbe-rich organic decomposing matter [23, 93]. Due to rapid population growth and quick depletion of resources, the ecology of these nematodes is characterized by a boom-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 [23]. By producing dauers that develop into hermaphrodites, a new population can be established even when the colonizing event is mediated by a single individual [94]. This type of intergenerational inheritance, in which parental effects increase the fitness of both offspring and parents, has hallmarks for being adaptive [95].

Here we show that activators of AMPK and insulin signaling activators or mTORC1 inhibitors intergenerationally induce the formation of dauers and hermaphrodites in *A. freiburgensis*. These results indicate that highly conserved energy-sensing pathways are involved in mediating intergenerational inheritance in *A. freiburgensis* to generate stress-resistant offspring. Consistent with the role of these pathways in dauer formation in *A. freiburgensis*, *C. elegans* integrates nutrient sensing using the same abovementioned signaling pathways to regulate developmental progression through larval stages [96]. How exactly do these energy-sensing pathways regulate phenotypic plasticity in the *A. freiburgensis* 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 [97]. 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, AMPK has been shown to facilitate histone acetylation [98], thus promoting the transcription of a new set of genes [99]. However, AMPK also regulates cytoplasmic proteins responsible for the metabolic status of the cell and interacts with the mTOR, sirtuin, and insulin pathways [100]. Future research will reveal whether the roles of these pathways for intergenerational

inheritance are more important in the cytosolic or nuclear context.

AMPK may also indirectly influence the chromatin status via activation of histone acetyltransferases (HATs) or inactivation of histone deacetyltransferases (HDAC), as demonstrated for other model systems [101, 102]. In *A. freiburgensis*, higher acetylation levels in the chromatin of the germline induced by crowding conditions result in stress-resistant offspring (Fig. 5). It remains to be established whether these acetylation levels are the result of direct phosphorylation of HATs and HDACs by AMPK, or indirectly by natural metabolites. As we show in Fig. 5a, natural metabolites indicative of metabolic stress that inhibit deacetyltransferases, such as D- β -hydroxybutyrate and butyrate [101], induce the production of stress-resistant offspring.

HDACs are subdivided into two protein subfamilies, which differ in protein structure and mechanism of action: the classical HDAC family and the sirtuins family [103]. The pharmacological compounds SRT1720 and EX-527 were designed to inhibit sirtuins [84, 90], whereas sodium butyrate [104], Trichostatin A [85], valproic acid [105], and D- β -hydroxybutyrate [101] inhibit the classic HDACs. Given that the same intergenerational effects were obtained with compounds that interfere with different subfamilies of HDACs, which may also have different target proteins, these results would suggest that the effects are not direct. Therefore, it is still unclear if the intergenerational effects are due to changes in the chromatin acetylation or in other subcellular components.

The strongest responses to the pharmacological compounds for the production of hermaphrodite progeny occurred when the animals were concomitantly exposed to diluted CM. In the complete absence of CM, only a few compounds elicited a strong response. This may indicate that pheromones in the CM activate more than one pathway and that they have to act in combination to elicit the full effect. Our findings that several energy-sensing pathways are involved in this process in *A. freiburgensis*, and that AMPK, insulin, and TOR pathways are cross-regulated, are indicative of this hypothesis [106–108].

The concentration of the compounds used in our studies and in *C. elegans* are relatively high compared to the ones used in mammalian cells [109–111]. This is because nematodes have several physical and physiological adaptations that counteract xenobiotic agents [112]. Like all pharmacological approaches, interpretation of the results must take into consideration possible lack of specificity [48, 113–115]. To ameliorate the possibility of lack of specificity for AMPK activation, for instance, we used compounds that act through several mechanisms (high production of AMP, allosteric binding, protection against

dephosphorylation, activation of phosphorylation). Genetic approaches using loss- and gain-of-function mutants will help to address some of the abovementioned concerns [116].

As far as we know, the association of activated AMPK and S6K with the chromatin of germline cells has not been established in other organisms. The presence of AKT in the nucleus of germline cells may be associated with chromatin condensation, which would be reflected in transcription rates [74].

Conclusions

Our results suggest that these energy-sensing effectors acquired a new role in intergenerational inheritance in *A. freiburgensis* to regulate gene expression that influences the phenotype of subsequent generations. Given that AMPK, TOR, and insulin pathways are highly conserved in evolution, it is possible that they also mediate nongenetic inheritance via the germline in other organisms in which diet plays a role in determining phenotypic plasticity. Although the epidemiological studies in humans are indicative of diet playing such a role, the mechanisms for this are unknown [117]. The findings in this study provide the basis to test such a hypothesis.

Methods

Strain and culture

We used the *Caenorhabditis elegans* N2 strain, and the *Auanema freiburgensis* strains SB372 [23] and JU1782. The *A. freiburgensis* JU1782 strain was isolated from rotting *Petasites* stems sampled in Ivry, Val-de-Marne, France, in September 2009 by Marie-Anne Félix. Nematodes were cultured at 20 °C on standard Nematode Growth Medium (NGM) [118] plates seeded with *Escherichia coli* OP50-1 strain. NGM medium was supplemented with 25 µg/mL nystatin and 50 µg/mL streptomycin to prevent microbial contamination.

Sexing of progeny

To synchronize the age of the mothers, we collected dauers. *A. freiburgensis* dauers develop into hermaphrodite adults within 24 h at 20 °C [23]. Dauer larvae are easily identified by their darker intestine and thinner body compared to similar-sized L3 larvae (which develop into females). Each dauer larva was placed on a 6-cm seeded NGM plate and incubated at 20 °C to develop into adulthood. Each egg laid by the parental (P0) generation was placed into single wells of a 96-well microtiter plate. After 3–5 days, the F1 was scored for their sexual morphology: hermaphrodites were identified by their ability to produce offspring in the absence of a mating partner, females by the lack of progeny, and males by their blunt tails [23]. We calculated sexual morph percentages based only on non-male progeny

(hermaphrodites or females). This is because males are not determined by environmental cues, but by sex chromosome number. Raw data used to calculate sexual morph percentages are in the data repository [119].

Diet restriction experiments

To test the effect of starving the parents in the sexual morph ratios of *A. freiburgensis* hermaphrodites, we followed the diet restriction protocol previously described [120]. Briefly, dauers were collected to plates with *E. coli* OP50 and allowed to mature to adulthood at 20 °C for 24 h. They were then transferred to 6-well tissue culture plates, with each well containing 5 ml of standard NGM medium supplemented with 1 mg/ml erythromycin. Hermaphrodites were left laying eggs in the absence of bacterial food for 2–3 days. F1 eggs or larvae were collected to be sexed as described above.

Assay with conditioned medium and treatment with pharmacological chemical compounds

To induce *A. freiburgensis* hermaphrodite offspring, the parent hermaphrodites (P0 generation) were incubated in the presence of conditioned medium (CM) at 20 °C [121]. The CM was derived from 2- to 3-week-old *A. freiburgensis* liquid cultures (M9 medium with *E. coli* OP50-1). Each P0 was placed at the L4 stage onto a 6-cm plate containing NGM and CM. To simulate high-density conditions, 50 mg of lyophilized CM was dissolved in 200 µl of an overnight culture of OP50-1 and spotted onto the plate. F1 eggs were collected for 3–4 days. Each egg was transferred into a single well of a 48-well microtiter plate containing NGM and OP50-1, but no conditioned medium.

For the pharmacological manipulation of signaling pathways, we added compounds to the NGM and OP50-1. The concentration of the compounds was calculated for the volume of the NGM and OP50-1 used. P0 hermaphrodites were incubated with the compounds for 48–96 h at 20 °C. Information about the providers and catalog number for the compounds used in this study are listed in the data repository figshare [119]. Alignment of the protein sequences of the immunogens used to raise the antibodies and their predicted *A. freiburgensis* orthologs are in Additional file 1: Figure S6.

Chemical compounds were used at the following concentrations: 100 mM metformin, 6 mM phenformin, 1 µM rotenone, 5 µM forskolin, 30 µM fluvastatin, 0.5 mM AICAR, 0.5 mM 8-Br-cAMP, 5 mM salicylate, 10 µM ZLN204, 30 µM O-304, 1 µM dorsomorphin, 100 µM rapamycin, 100 µM methotrexate, 100 nM VO-OHPic, 20 µM indole-3-carbinol, 10 µM SRT1720, 100 µM trichostatin A, 4 mM valproic acid, 5 mM DL-beta-hydroxybutyrate, 5 mM sodium butyrate, 100 µM EX-527, 3 mM 4-tert-butylbenzoic acid, 75 nM SC-66,

300 nM Akti-1/2, 20 μ M perifosine, and 10 nM SC-79. For nematodes incubated with diluted CM, we used 5 mg of freeze-dried CM dissolved in 200 μ l *E. coli* OP50-1.

Immunohistochemistry

To ascertain that antibodies would cross-react with *A. freiburgensis* tissues, tblastn searches [122] were performed using the *A. freiburgensis* transcriptome (details to be published elsewhere). Hermaphrodites were dissected on a slide (Superfrost microscope slide, VWR) in PBS 1 \times buffer. Dissected gonads were covered by a coverslip and placed on a frozen metal block at -20°C for at least 10 min, and fixed for 2 min in a 95% methanol solution at -20°C . This was followed by 30 min in a fixative solution [PBS 1 \times , 80 mM HEPES (pH = 7.0–7.4), 1.6 mM MgSO₄, 0.8 mM EDTA (pH = 8.0), 4% paraformaldehyde] in a humid chamber at room temperature. Slides were washed twice with PBST (PBS + 0.1% Triton X-100) for 5 min and blocked in PBST + 0.5% BSA for 45–60 min. The source of primary and secondary antibodies, as well as dilutions used, is listed in figshare [119]. All antibodies were diluted in PBST. Incubation with the primary antibodies was performed at 4°C overnight. Slides were then washed twice in PBST for 10 min each, and the corresponding secondary antibody was added and incubated for 2 h at room temperature. Slides were washed in PBST as above to remove the excess of the secondary antibody and then one drop of Fluoroshield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Abcam, #ab104139) was added on the immunostained samples.

Images were taken with a $\times 60$ objective in 2.40 μ m z-stack intervals (12 sections) with a DeltaVision microscope (Olympus). Acquisition and constrained iterative deconvolution of the images from DeltaVision were processed using the softWoRx software (Applied Precision). The deconvolved images were processed using restoration methods (instead of deblurring methods) [123]. Restoration methods do not affect the quantitative nature of the final restored product because linearity is better preserved [124]. The intensity of fluorescence for the secondary antibodies was measured in images in the TIFF format, using the ImageJ software (NIH Image, Bethesda, MD). Based on the morphology of the nuclei in DAPI-stained cells, the germline was subdivided into two sections: the premeiotic tip (also known as mitotic region) and the meiotic region. As observed in *C. elegans* [125], cell nuclei between the mitotic and the meiotic region of the *A. freiburgensis* germline have a characteristic crescent-shaped morphology. This region is known as the “transition zone,” with cells in the leptotene and zygotene of the meiotic cycle. For normalization and calculation of fluorescence, we estimated fluorescent signals using drawing tools in ImageJ software and defined “set measurements”

(including areas, integrated intensity, and mean grey value) and then selected “Measure” from the Analyze menu.

Western blot

Protein extraction and buffer preparation were performed following the protocol of [126]. Six hundred adult hermaphrodites were collected for each sample: control (OP50-1 only) and experimental (50 mg conditioned medium powder per 200 μ l of OP50-1) samples. Protein concentration was measured using Bradford assay (Bradford Reagent, Bio-Rad). We loaded approximately 100 μ g of protein. The primary antibodies, against Phospho-AMPK α (Thr172) and PAR-4/LKB1, were used at 1:1000 dilution. The source of primary and secondary antibodies, as well as dilutions used for them, is listed in figshare [119]. To detect the signal for the antibodies, we used the Amersham™ ECL™ Western Blotting Detection Reagents (RPN2209). For *A. freiburgensis* gene_ID, immunogen UniProtKB_ID and percentage identity of the alignment, see in figshare [119]. Uncropped images for Western blots are in Additional file 1: Figure S7.

Statistical analyses

Results were presented using the most recent developments in data analysis and presentation [127], showing the raw data as “bee swarm” plots. They summarize the data showing the mean and the 95% confidence interval (CI), as well as the sampling error distribution diagrammed as a filled curve. These plots provide transparency of the comparison being made, visual clarity, and statistical evaluation of the data. For the effect of pharmacological compounds on sex ratios, Student's *t* test, one-way analysis of variance (ANOVA) followed by the Holm-Sidak method for multiple comparisons or Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn's method were performed. We used the software package SigmaPlot 12.0 (Systat Software, Inc., San Jose, CA, USA). Details of sample sizes, statistical tests and *p* values are in the data repository figshare [119].

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12915-021-01032-1>.

Additional file 1: Figure S1. STRAD and LKB1 antibody staining are higher in animals in crowding conditions. **Figure S2.** Metformin and trichostatin affect the F1 sex ratios in the absence of diluted CM. **Figure S3.** PTEN/DAF-18 in the germline is cytoplasmic and higher in non-crowding conditions. **Figure S4.** AKT pThr308 in the germline is nuclear and higher in crowding conditions. **Figure S5.** Acetylation in the germline is nuclear and higher in crowding conditions. **Figure S6.** Alignment of the protein sequences of the immunogens used to raise the antibodies and their predicted *A. freiburgensis* orthologs. **Figure S7.** Uncropped images of Western blots.

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Authors' contributions

P.R., G.Z., V.K., and A.P.-d.S. designed the study. P.R., A.T., G.Z., V.K., M.W., P.P., B.H., C.B., and B.H. conducted experiments that involved the production of conditioned medium, incubation with chemicals, and sexing offspring. S.A. performed the transcriptome analysis to find gene orthologs. P.R. performed the immunocytochemistry experiments and P. P. performed the Western blots. P.R. and A.P.-d.S. wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

Correspondence and requests should be addressed to A.P.-d.S. All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

No ethics approval or consent to participate was required for this study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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