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Doxycyclin ameliorates a starvation-induced germline tumor in *C. elegans daf-18*/PTEN mutant background

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ABSTRACT

Managing available resources is a key necessity of each organism to cope with the environment. The nematode *C. elegans* responds to nutritional deprivation or harsh environmental conditions with a multitude of developmental adaptations, among them a starvation-induced quiescence at early larval development (L1). *daf-18*, the *C. elegans* homolog of the human tumor suppressor gene *PTEN*, is essential for the maintenance of survival and germline stem cell arrest during the L1 diapause. We show here that *daf-18* mutants, independently to their failure to maintain G2 arrest of the primordial germ cells, develop a gonad phenotype after refeeding. This highly penetrant gonadal phenotype is further enhanced by a mutation in *shc-1*, encoding a protein homologous to the human adaptor ShcA. Features of this phenotype are a tumor-like phenotype encompassing hyperproliferation of germ cell nuclei and disruption/invasion of the basement membrane surrounding the gonad. The penetrance of this phenotype is reduced by decreasing starvation temperature. In addition, it is also ameliorated in a dose-dependent way by exposure to the antibiotic doxycyclin either during starvation or during subsequent refeeding. Since, in eukaryotic cells, doxycyclin specifically blocks mitochondrial translation, our results suggest that *daf-18* and *shc-1;daf-18* mutants fail to adapt mitochondrial activity to reduced nutritional availability during early larval developing.

1. Introduction

If *C. elegans* embryos hatch in an environment lacking food, they stop development to arrest in a dormant state (L1), which allows them to survive starvation for more than ten days. Once food is replenished, development proceeds normally and the resulting adults appear morphologically similar to animals that were constantly fed. Adult behaviors, including egg laying and adult life span, are unaltered (Johnson et al., 1984).

DAF-18, the *C. elegans* homolog of PTEN (Phosphatase and Tensin homolog (Mihaylova et al., 1999; Li et al., 1997; Solari et al., 2005)), contributes to this starvation response (Fukuyama et al., 2006, 2012). The second most frequently mutated tumor suppressor in human carcinogenesis harbors a dual-specificity phosphatase domain that has been implicated in glioma migration in humans (Dey et al., 2008) and Eph receptor tyrosine kinase signaling in *C. elegans* (Brisbin et al., 2009). In addition, DAF-18 lipid phosphatase activity antagonizes AGE-1/PI3K function by dephosphorylating PI(3,4,5)P_3 to PI(4,5)P_2(Gil et al., 1999; Salmena et al., 2008). A consequence of this activity is that DAF-18 functions as a negative regulator of insulin/IGF-like signaling (IIs). A participation of IIs in the control of L1 diapause was anticipated, since a strong loss of function allele of the insulin receptor (IR)-like gene *daf-2*, *e979*, results in constitutive L1 arrest (Gems et al., 1998).

Mutants of *C. elegans daf-18*/PTEN and *aak-2*/*aak-1*, encoding two subunits of the AMP-activated protein kinase (AMPK), show reduced survival during L1 diapause (Fukuyama et al., 2012; Lee et al., 2012). Supply of the starvation medium with traces of ethanol (EtOH) sufficed to increase survival of wild type animals (Castro et al., 2012) and *aak-2* mutants (Fukuyama et al., 2012). It was shown subsequently that the EtOH supplement in starvation medium is probably used as a carbon source and incorporated into fatty acid synthesis (Castro et al., 2012). In L1-starved *daf-18* mutants maintained in EtOH-supplemented medium, the two primordial germline cells Z2 and Z3 (PGCs) fail to arrest the cell cycle at G2 and further divide, generating up to eight instead of the two PGCs typical for wild type (Fukuyama et al., 2006). It had been suggested that this eventually results in sterility of *daf-18* mutants after refeeding (Fukuyama et al., 2012). Surprisingly, DAF-16/FOXO, the most well-known downstream effector of IIs, which was shown to be required for L1 survival, does not seem to play a role in *daf-18* mediated PGC cell cycle arrest upon starvation (Fukuyama et al., 2006, 2012).

Therefore, besides its roles in aging, stress, neuronal development and glucose metabolism, IIs plays a yet only partially understood role

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in L1 diapause. It has been shown to transmit signals from food sensing organs to remote tissues (Finch and Ruvkun, 2001; Lee et al., 2009; Lehtinen et al., 2006; Michaelson et al., 2010; Song et al., 2003). This cell-nonautonomoussignaling is realized by insulin-like molecules (Hsu and Drummond-Barbosa, 2011; Michaelson et al., 2010). Recently, it was shown that the IIs and Erk signaling pathways couple nutrient availability to meiotic progression (Lopez et al., 2013). Erk signaling itself is regulated by ShcA (a scaffold protein facilitating multiprotein complex formation) via distinct mechanisms (Suen et al., 2013; Sweet and Tzima, 2009). We had shown previously that SHC-1, the *C. elegans* orthologue of p52Shc, inhibits IIs upstream of DAF-2 and indirectly supports DAF-16 nuclear transition via JNK (Neumann-Haefelin et al., 2008; Qi et al., 2012).

Here, we report that the majority (>90%) of *daf-18(e1375)* animals (harboring a truncated version of the PTEN protein), unlike *daf-18(ok480)*, are capable of maintaining cell cycle arrest of the PGCs, when starved in standard medium in the absence of an additional carbon source. Nevertheless, both mutants, after refeeding, show a disrupted gonad. This phenotype is even further enhanced by an additional *shc-1* mutation. Therefore, we suggest that the failure to maintain PGC arrest during L1 starvation and the subsequent development of a gonad phenotype are independent aspects of the *daf-18* phenotype.

We propose that both SHC-1 and DAF-18 functions contribute to the animals' adaptation to starvation, and this modulation is lost in *daf-18* single and *shc-1;daf-18* double mutants. Reducing the growth temperature and application of the antibiotic doxycyclin, known to target mitochondrial translation, suppressed the tumor-like gonad phenotype. Remarkably, the administration of doxycyclin exclusively during starvation or during refeeding sufficed to suppress germline tumors. We hypothesize that failures to synchronize nutrient availability and mitochondrial activity early in development may have deleterious consequences significantly later in life that, however, can be corrected at distinct time points by lowering mitochondrial activity, e.g. by reducing mitochondrial mRNA translation.

2. Materials and methods

2.1. Transgenic and mutant C. elegans strains

C. elegans variety Bristol, strain N2 was used as wild type strain. All strains were maintained and raised at 20 °C on NGM agar seeded with *Escherichia coli* OP50 (Brenner, 1974), unless otherwise indicated. The following strains were obtained from CGC: *shc-1(ok198)I*, *daf-16(mu86)I*, *daf-2(e1370)III*, *daf-18(ok480)IV*, *daf-18(e1375)IV. zcIs13 [Phsp-6::gfp]*, *ife-2(ok306)X*, *pep-2(lg601)X* The following strains were obtained by crossing: *shc-1(ok198)I;daf-18(e1375)IV*, *shc-1(ok198) I; daf-18(ok480)IV*, *shc-1(ok198)I;daf-18(e1375)IV; ltIs37[Ppie-1::mcherry::his-58];qyIs10[Plam-1::lam-1::GFP]*, *shc-1(ok198)I;daf-18 (ok480)IV; ltIs37[Ppie-1::mcherry::his-58];qyIs10[Plam-1::lam-1::GFP]*, *ltIs 37[Ppie-1::mcherry::his-58];qyIs10[Plam-1::lam-1::GFP]*, *shc-1(ok198)I; daf-18(ok480)IV;zhEx343.3. ltIs37[Ppie-1::mcherry::his-58]* was a gift from Karen Oegema, *qyIs10[Plam-1::lam-1::GFP]* was a gift from David Sherwood and *daf-18(ok480)IV;zhEx343.3[Pdaf-18::daf-18::gfp]*was a gift from Alex Hajnal.

2.2. L1 starvation assay

Animals were grown on *E. coli* (OP50) seeded NGM agar plates at 20 °C until they reached adulthood. Gravid adults were washed from the plates in M9 (without $MgSO_4$) and egg preparation was performed. To this end, 0.6% sodium hypochlorite solution was mixed with 5 M potassium hydroxide to a final concentration of 250 mM and added to the worms. Subsequently, tubes were vigorously shaken until all carcasses had been dissolved and the remaining eggs were washed five times with M9 buffer. After washing, eggs were transferred to a 1.5 ml Eppendorf tube and starved at 20 °C, unless otherwise indicated. Standard starvation period for *shc-1(ok198);daf-18(ok480)* was two days (~48 hours), for *shc-1(ok198);daf-18(e1375)* 3 days (~72 hours). For refeeding, animals were placed on OP50 plates for development until adulthood.

2.3. Assessing germline development defects

Young adult animals were immobilized with 20 mM levamisole on a patch of 2% agarose. Microscopy was performed with an Axioimager Z1 compound microscope with an AxioCam MRm3 CCD camera; Axiovision software version 4.8.1 (Carl Zeiss AG, Germany) was used for image analysis. Animals exhibiting germline nuclei- or oocyte-like structures leaking out of the gonad were scored as defective. Especially *daf-18* animals showed uterine tumors without disruption of the gonadal basement membrane. These animals were censored.

2.4. α-PGL-1 antibody staining and PGC counts

The α-PGL-1 antibody was a gift from Susan Strome. After starving the animals at L1 for three days, larvae were immuno-stained with anti-PGL-1 antibody (1:1000) as described (Sweet and Tzima, 2009). PGCs were counted with DIC microscopy on starved L1 larvae. Images were taken with the same microscopic hard- and software as detailed above.

2.5. Quantifications of germline nuclei and fluorescence intensities

Animals were starved as described (see L1 starvation assay, above) and allowed to recover on food. They were collected at late L3 stage to take fluorescent images of the *ltIs37[Ppie-1::mcherry::his-58];* and *qyIs10[Plam-1::lam-1::GFP]* markers. We used the developmental state of the vulva for synchronization of starved and unstarved populations (Sulston and Horvitz, 1977). Micrographs for nuclei counts and GFP intensity were taken using the same imaging equipment as described above. Nuclei were counted with Image J using the Cell Count plugin. GFP intensity was measured using Axiovision software version 4.8.1 (Carl Zeiss AG, Germany).

2.6. Doxycyclin assays

A stock solution containing 32 mg/ml Doxycyclin hyclate in ddH2O was prepared and frozen. Using this stock, a dilution series was freshly produced which was subsequently added to the starvation medium to result in the desired concentrations. In order to prepare the feeding plates, HT115 bacteria containing L4440 empty vector (mediating tetracycline resistance) were spread on standard NGM plates. Thereafter, 200 µl doxycyclin dilution mix was added and plates were left to dry. Starved L1 larvae were added to the plates and let to develop to adulthood (compare L1 starvation assay, above). Phenotypes were scored as described above.

3. Results

3.1. SHC-1 and DAF-18 cooperate to prevent starvation induced germline tumors by inhibiting IIs

When testing mutants in the IIs pathway for modulation of the *daf-18* phenotype, we found that the *shc-1(ok198)* loss of function-mutation enhances *daf-18* gonad disruption (Fig. 1A) from 61% to 91% after two days of starvation, and 85–100% after three days (Fig. 1B), resulting in nearly fully penetrant sterility during adulthood (data not shown). As an additional consequence, *shc-1;daf-18* animals only rarely give progeny during recovery from storage at −80 °C. The standard protocol for storage involves starving large populations of animals in L1, prior to freezing. In contrast, *shc-1(ok198)* single mutants were inconspicuous, even after 48 or 72 hours of starvation, and, thus, behaved like wild type. However,

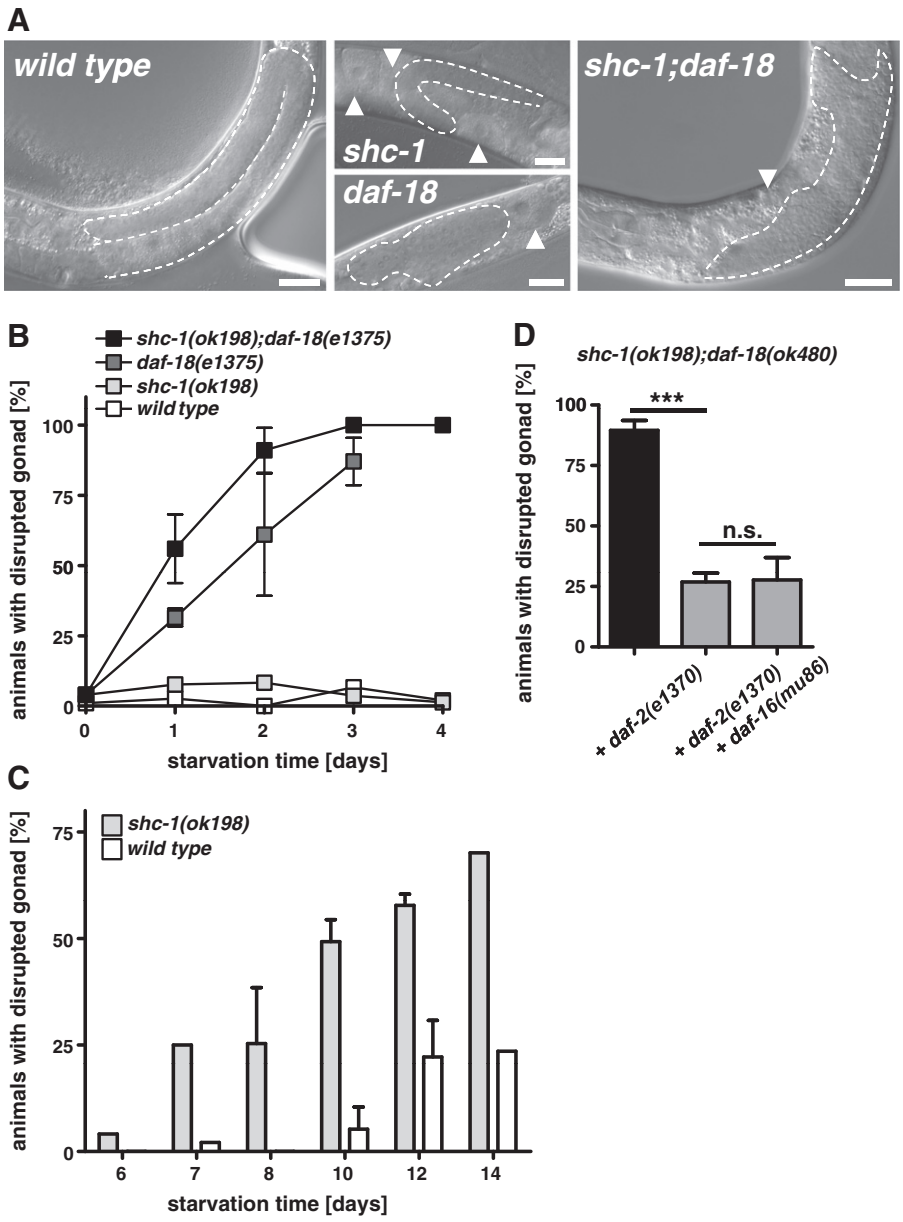

Fig. 1. Starvation induces gonadal damage in *shc-1, daf-18* and *shc-1;daf-18* mutants. **A**. Representative micrographs (DIC) of intact (*wild type*) and damaged gonads in *shc-1(ok198), daf-18(e1375)* single and *shc-1(ok198);daf-18(e1375)* double mutant animals that developed after L1 starvation and refeeding. **B**. *daf-18* and *shc-1;daf-18* exhibited gonadal damage after 1–3 days, whereas *shc-1* behaved like wild type. *shc-1* mutation enhanced the penetrance of the *daf-18* phenotype. Note that *daf-18(e1375)* do not survive longer than 3 days of starvation, more than 100 animals observed per strain and time point **C**. *shc-1* developed a similar gonad defect only after starvation of more than six days. **D**. *daf-2(e1370)* mutation suppresses *shc-1(ok198);daf-18(ok480)* phenotype independent of *daf-16.* Error bars represent standard error of the mean (SEM). Fisher's exact test Scale bar: 25 μm.

after prolonged starvation (seven days) and consecutive refeeding, *shc-1(ok198)* also developed a gonad dysmorphology similar to that of *shc-1;daf-18* (Fig. 1C), albeit at a lower penetrance. The severity of these postembryonic gonad defects was dependent on the length of L1 starvation in all mutant combinations tested, and also occurred infrequently in wild type animals after 12–14 days of starvation (Fig. 1B,C). Therefore, both *shc-1* and *daf-18* mutations contribute to reduce the tolerance of *C. elegans* animals to survive L1 starvation unharmed.

Both DAF-18 and SHC-1 have been identified as negative regulators of insulin/IGF signaling, suggesting that their mutants increase IIs and inactivate DAF-16/FOXO. Consistent with this view, an additional *daf-2* mutation reduces the gonad disruption to levels of unstarved worms (Fig. 1D). Suppression by reduction of IIs does not, however, require active DAF-16, since suppression is also seen in *shc-1;daf-18* mutants in which both *daf-2* and *daf-16* have been inactivated (Fig. 1D). We conclude that *daf-18* and *shc-1* cause starvation induced germline tumors via activating IIs, but using an IIs effector that differs from DAF-16.

3.2. L1 starvation in shc-1;daf-18 animals induces germline hyperproliferation and rupture of the gonadal basement membrane

Sterility as a consequence of extended L1 starvation had been reported before (Fukuyama et al., 2012; Neumann-Haefelin et al., 2008). To characterize gonad dysmorphology after starvation, we employed two fusion constructs staining either the germline nuclei (*Ppie-1::mcherry:* his58) or the basement membrane (*Plam-1::lam-1::*gfp) enclosing the germline. In *shc-1;daf-18* mutants, starving and refeeding increased the number of germline nuclei compared to unstarved animals of the same genotype and to starved wild type (Fig. 2A–C). Increase of germline nuclei is accompanied by rupture of the gonadal basement

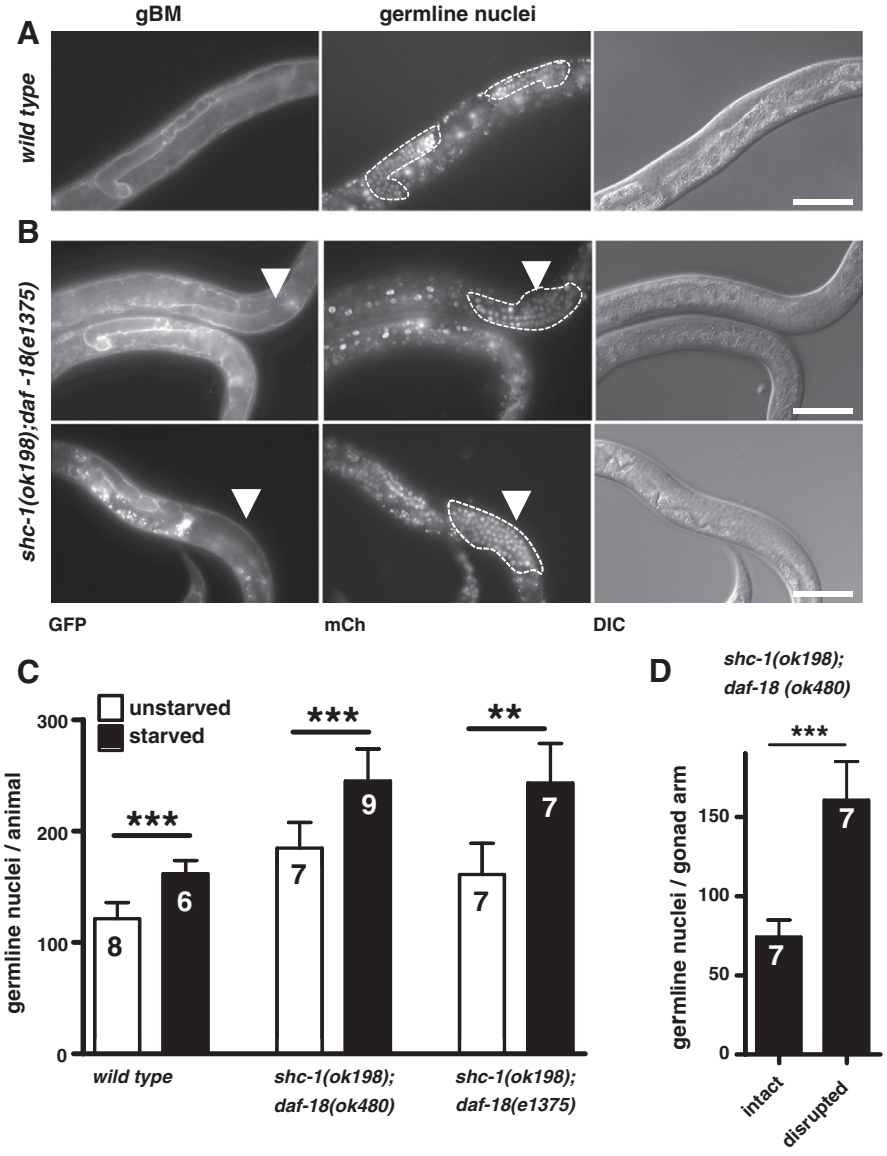

Fig. 2. Germline hyper-proliferation and disruption of the gonadal basement membrane are two aspects of the mutant phenotype. **A**–**B**. *Is[Plam-1::lam-1::gfp]* and *Is[Ppie-1::mcherry::his58]* transgenes were used to stain gonadal basement membrane (gBM) and germline nuclei, respectively. All animals analyzed were at L3 stage. **A**. Wild type. Intact gonadal arms are indicated by dashed line, 72 h starvation. **B**. *shc-1(ok198);daf-18(e1375)*. Upper row, 24 h L1 starvation: Disrupted basement membrane (left picture, arrowhead) results in germline nuclei leaking into the body cavity (central picture, arrowhead, dashed line). Lower row, 72 h L1 starvation: Absence of LAM-1::GFP staining indicates a substantially disintegrated basement membrane (left picture, arrowhead). Hyper-proliferation of germline nuclei (central picture, arrowhead, dashed line). **C**. Animals starved for 72 h (*shc-1(ok198);daf-18(e1375)*), or 48 h (*shc-1(ok198);daf-18(ok480)*) developed hyper-proliferating germlines, indicated by increased numbers of germline nuclei, compared to unstarved animals and wild type. **D**. *shc-1(ok198);daf-18(ok480)* animals with one intact and one disrupted gonad arm showed increased numbers of germline nuclei in the defective gonad arm. Animals were synchronized by their vulval development stages as explained in (Sulston and Horvitz, 1977). mCh: mCherry. Error bars represent standard deviation (SD). ***: $p < 0.001$ **: $p < 0.01$; *: $p < 0.05$. Mann–Whitney test. Scale bar: 25 μm.

membrane in late L3 stage. In severe cases, germline content leaked into the surrounding body cavity, destroying the gonad structure (Figs. 1A and 2B). Depending on the severity of the phenotype, laminin staining sometimes completely faded already in L3, indicating either a total disintegration of the basement membrane and/or the absence of laminin (Fig. 2B lower row).

Two *daf-18* alleles, *ok480* (harboring a deletion of exon 4 and 5, (Fukuyama et al., 2006)), and *e1375* (containing an insertion of a stop codon at exon 4, (Ogg and Ruvkun, 1998)) in *shc-1* background gave comparable results, differing only in the penetrance of their phenotype and total amount of germline nuclei that were lower in the *e1375* allele. Some animals that still showed one intact and one tumorous gonad arm allowed us to investigate cosegregation of germline numbers and gonad disruption within the same animal. Typically, the phenotypically conspicuous gonad arm contained about twice the number of germ cells compared to the intact arm (Fig. 2D). Therefore, both aspects of this phenotype cosegregate.

3.3. Failure to block G2 cell cycle in PGCs and germline tumors are two independent phenotypic consequences of starvation in daf-18 mutants

daf-18(ok480) and *aak-2(ok524)* mutants, during L1 starvation, accumulate up to eight PGCs, and their increase was considered the cause of postembryonic sterility (Fukuyama et al., 2006, 2012). Starvation in these experiments, however, had been conducted in the presence of 0.08% EtOH to extend L1 survival which has later been shown to act as a nutritional carbon source for the animals (Qi et al., 2012). We confirmed, that *daf-18(ok480)* animals indeed comprise a maximum of seven and an average of 5.0 ± 0.8 PGC when starved in the presence of 0.08% EtOH, consistent with a previous report

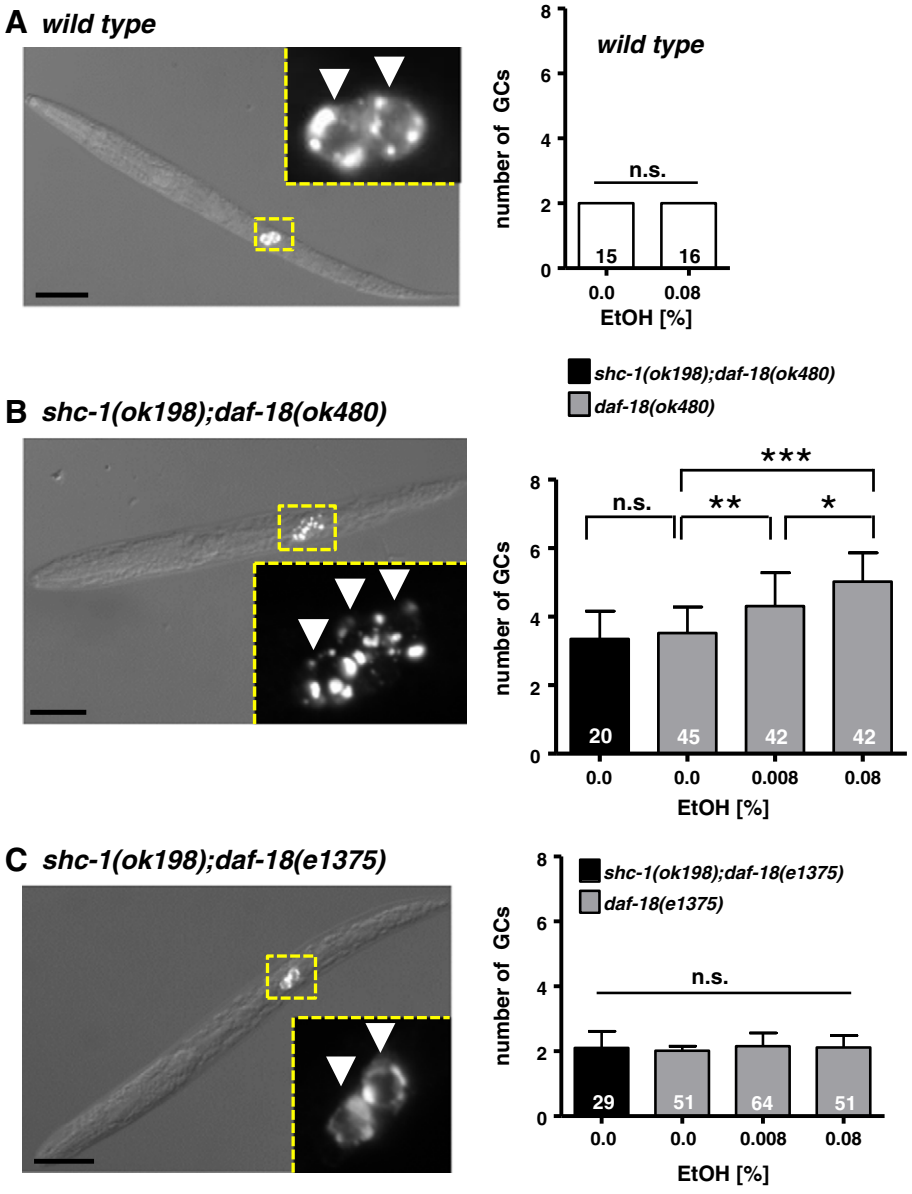

Fig. 3. PGC hyper-proliferation and germline hyperplasia do not cosegregate. **A**. Wild type animals starved with or without ethanol (EtOH) supplement display two primordial germ cells (GCs). **B**. GC number is increased in starved *daf-18(ok480)* and *shc-1(ok198);daf-18(ok480)* animals starved without EtOH. Addition of EtOH further increases GC number in *daf-18(ok480)* (compare (Fukuyama et al., 2006)). Animals were starved for 48 h, since a considerable portion of *daf-18(ok480)* animals did not survive starvation for 72 h without EtOH supplement (data not shown). **C**. In contrast to *daf-18(ok480)*, *daf-18(e1375)* and *shc-1(ok198);daf-18(e1375)* animals did not show increased GC numbers, nor did EtOH addition increase GC number in *daf-18(e1375)*. Representative micrographs show GCs of starved L1 larvae stained with anti-PGL-1 antibody (gift of S. Strome). Insets have been enlarged to show details. Numbers in columns indicate the number of analyzed animals. Error bars represent standard deviation (SD). ***: $p < 0.001$; **: $p < 0.01$; n.s.: $p > 0.05$. Kruskal–Wallis test plus Dunn's Multiple Comparison Test. Scale bar: 25 μm.

(Fukuyama et al., 2006). Numbers were slightly reduced at lower EtOH levels (4.3 ± 1.0, 0.008% EtOH) or in the absence of EtOH supplements (3.5 ± 0.8), but still more than the two PGCs consistently seen in wild type (Fig. 3B). Nevertheless, *daf-18(e1375)* mutants, considered to carry a weaker allele (Lee et al., 1999; Ogg and Ruvkun, 1998), did not show any significant change in PGC numbers, but developed a disrupted gonad, once starved and refed (Fig. 3C, compare Fig. 1B). This suggests, that in the presence of the putative carbon source EtOH, a G2 cell cycle block in the germline is not maintained properly in *daf-18(ok480)* mutants, whereas *daf-18(e1375)* worms maintain enough residual DAF-18 activity to prevent erroneous PGC proliferation. Since both alleles after refeeding later displayed a comparable gonad dysmorphology phenotype, we consider it very unlikely that this germline tumor phenotype is the consequence of PGC number alterations.

3.4. Starvation temperature, a modulator of metabolic rate, alters the penetrance of gonad defects

It had been proposed before that *daf-18* loss-of-function alleles suffer from increased metabolic rate during starvation periods and therefore may consume too much energy when nutrients are scarce (Neumann-Haefelin et al., 2008). One suggested consequence was the observed reduction of L1 survival (Fukuyama et al., 2012; Neumann-Haefelin et al., 2008). Consistent with this hypothesis, it was shown that starved *daf-18(lf)* showed increased levels of oxygen consumption, and L1 survival was partially rescued by administration of glucose or EtOH (Lee et al., 2012).

Metabolic rate and, as a consequence, L1 survival, have been shown to be dependent on growth temperature (Lee et al., 2012). If *daf-18*

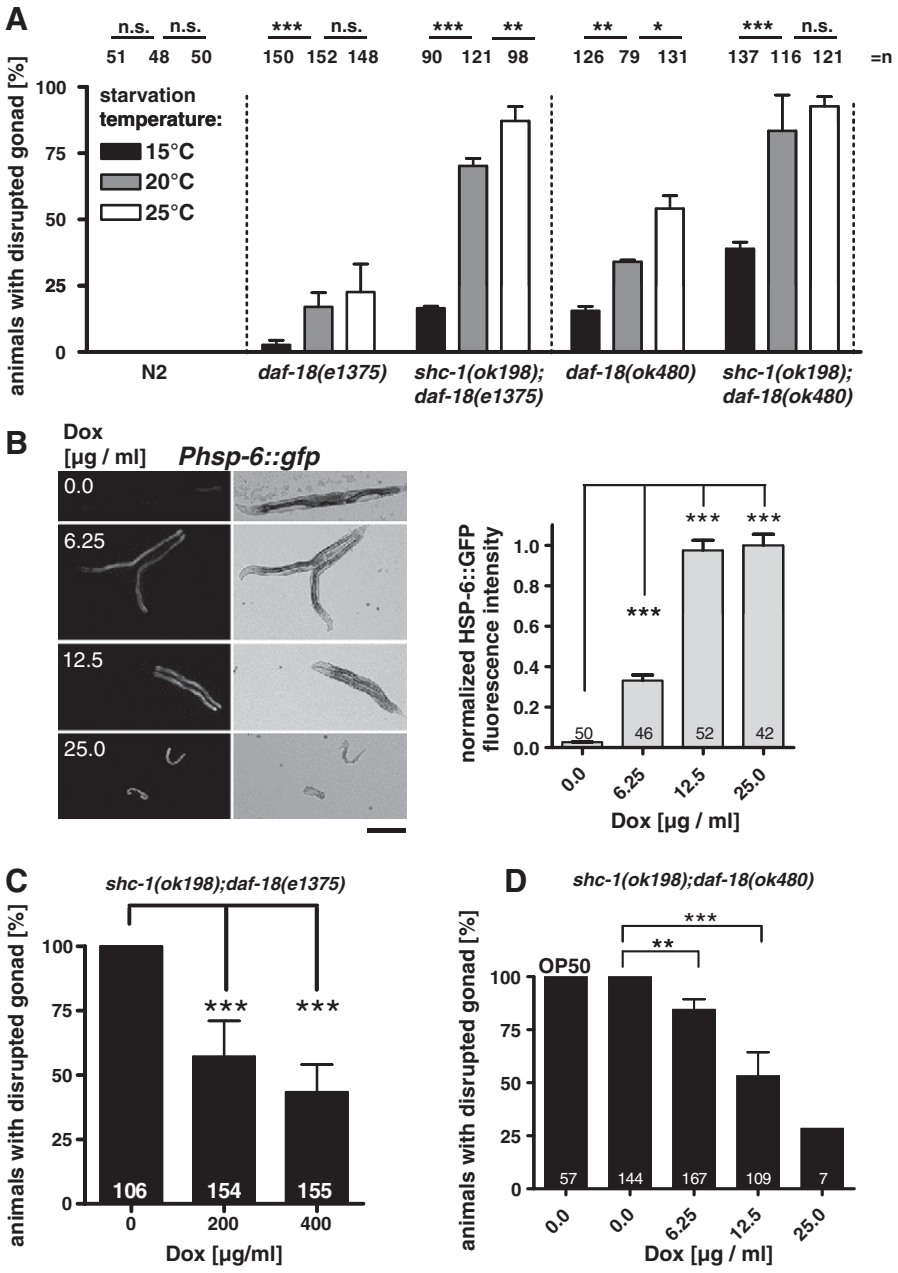

Fig. 4. Temperature decrease and Doxycyclin suppress L1 starvation induced gonad disruption. **A**. Penetrance of the germline phenotype in both *shc-1(ok198);daf-18(e1375)* and *shc-1(ok198); daf-18(ok480)* correlates with temperature (starvation time 24 h). **B**. Doxycyclin (Dox) treatment was controlled with the *Phsp-6::gfp* reporter, as described (Houtkooper et al., 2013; Runkel et al., 2013). Scale bar: 200 µm. **C**, **D**. Application of Dox during L1 starvation (C) or during refeeding (D) decreased the penetrance of the germline phenotype in *shc-1(ok198);daf-18(e1375)* and *shc-1(ok198);daf-18(ok480)* respectively. Error bars represent standard error of the mean ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; n.s.: $p > 0.05$. Fisher's exact test.

animals suffered from failures of their metabolism to adjust to reduced nutrient availability, then the resulting phenotype should be temperature dependent. This was indeed the case. We conducted experiments in the background of the *daf-18(e1375)* allele that does not show elevated PGC numbers, and added *shc-1(ok198)* to enhance tumor penetrance. Refeeding was done at the constant temperature of 20 °C in all experiments. Lowering the temperature during L1 starvation from 20 °C to 15 °C reduced the penetrance of germline tumors in *shc-1(ok198);daf-18(1375)* animals from 68.6% to 16.6%. In contrast, elevated temperature (25 °C) increased penetrance to 87.2% (Fig. 4A). Similar effects were also seen in *shc-1(ok198);daf-18(ok480)* double mutants that, however, displayed a higher penetrant phenotype at 15 °C, consistent with *ok480* being a stronger allele than *e1375*. In summary, these results are consistent with a correlation of increased metabolic rates and oxygen consumption observed in *daf-18* and the appearance of starvation-induced gonad disruption. The experiments also suggest that SHC-1 dysfunction may enhance these defects.

3.5. Doxycyclin, a translational inhibitor of eukaryotic mitochondria, phenocopies low starvation temperature to suppress daf-18 tumorous germline

Due to their central role in ATP production, β-oxidation, and amino acid catabolism, mitochondria are the prime suspects involved in metabolic regulation. Doxycyclin (Dox), a widely used antibiotic that targets prokaryotic ribosomes, has been shown to also interfere with mitochondrial, but not cytosolic ribosomes in eukaryotic cells. Dox reversibly blocks tRNA binding sites in mitochondrial ribosomes to selectively inhibit mitochondrial protein synthesis (Ugalde et al., 2004). Dox was

shown to decrease oxygen consumption, and, thus, metabolism, in *C. elegans* larvae (Houtkooper et al., 2013).

Altering mitochondrial homeostasis, in turn, has been associated with an induction of the mitochondrial stress response UPR^{mt} (mitochondrial unfolded protein response) which can be monitored by the expression of a *Phsp-6::gfp* reporter gene (Runkel et al., 2013; Yoneda et al., 2004). We incubated *C. elegans* with increasing concentrations of Dox and found that the *Phsp-6::gfp* expression is already induced at 6.25 μg/ml Dox, a concentration that had only a mild effect on the duration of postembryonic development, but did not harm *C. elegans* or cause an obvious adult phenotype. Maximal induction of *Phsp-6::gfp* was observed at 12.5 and 25.0 μg/ml Dox (Fig. 4B and data not shown), respectively. These results corroborate a previous finding (Houtkooper et al., 2013) and suggest that we can use these conditions to test whether lowering metabolic rates by Dox treatment has a beneficial effect on starvation recovery in *shc-1;daf-18* mutants.

We reasoned that, if the cause of the *shc-1;daf-18* gonad phenotype is indeed mitochondrial metabolic dysregulation during L1 starvation, then Dox treatment to impair mitochondrial translation and metabolism should be beneficial. Indeed, treating the animals with Dox concentrations of 200 and 400 μg/ml significantly suppressed fully penetrant germline tumors (Fig. 4C). Treatment in post-L1 stages was also sufficient to suppress *shc-1;daf-18* germline defects after L1 starvation, and the effective concentrations were actually substantially lower than those applied during L1 starvation (6.25–25.0 μg/ml Dox, Fig. 4D).

4. Discussion

Previous studies have highlighted that DAF-18, and also the AMPK homologues AAK-1 and AAK-2, are required to adapt metabolism to starvation periods that may occur after hatching, and help to drive the animals into a dormant stage, the L1 diapause. It has been proposed that mutations in these genes fail to maintain a G2 cell cycle arrest of the two primordial germ cells (PGCs), the founder cells of the germline. As a consequence, mutants show PGC hyper-proliferation and, after refeeding, become sterile as adult animals. Sterility, therefore, has been thought to be the consequence of the PGC defects (Fukuyama et al., 2006, 2012; Lee et al., 2012).

In the study reported here, we made several observations that conflict with this simple model. First, we observed that the consequences of L1 starvation in *shc-1;daf-18* animals are not simply sterility, as described previously, but a combination of germ cell hyper-proliferation already at L3, and a profound disintegration of the basement membrane surrounding the gonad. Therefore, this phenotype encompasses two aspects that have been associated with human tumors. We, therefore, suggest that lack of DAF-18 during L1 starvation contributes to a tumor-like phenotype in the developing *C. elegans* germline/gonad. Thus, DAF-18, like its human homolog PTEN, acts as a tumor suppressor.

Second, our results indicate that, unlike what was suggested before (Fukuyama et al., 2006, 2012; Lee et al., 2012), there is no obvious correlation between impaired PGC cell cycle arrest in starved *daf-18* animals during L1 diapause and the germline tumors seen later, after refeeding. Animals harbouring *daf-18(e1375)*, frequently described as a reference allele for *daf-18* (Lee et al., 1999; Ogg and Ruvkun, 1998), rarely developed extra PGCs, but nevertheless displayed a highly penetrant germline phenotype as a consequence of L1 starvation (Fig. 1B). We therefore conclude that at least part of the *daf-18* gonad phenotype may occur independently of the mutant's failure to maintain G2 arrest of PGCs as starved L1 larvae.

We suggest that *daf-18* mutation increases the activity of the IIs pathway. Inactivation of SHC-1, which we identified previously as a protein interactor and negative regulator of the insulin-like receptor DAF-2 (Neumann-Haefelin et al., 2008), enhances this *daf-18* defect. SHC-1 was also shown to activate the JNK pathway in *C. elegans* that, in parallel to IIs, activates DAF-16 by phosphorylation (Neumann-Haefelin et al., 2008). We consider it unlikely that loss of the latter SHC-1 function contributes to the gonad phenotype. Although we found that down-regulation of *daf-2* suppressed the *shc-1* and *daf-18* mediated germline defects, this suppression was independent of DAF-16 (see Fig. 1D), indicating that another, unknown effector of IIs mediates starvation-induced tumorigenesis.

Recent reports suggested, that DAF-18 during L1 diapause is necessary to adapt organisms to nutrient deprivation (Lee et al., 2012). Loss of function mutants of *daf-18* displayed increased oxygen consumption as well as ATP production, an indicator of increased metabolism, and show reduced survival rates during L1 diapause (Goo et al., 2012; Lee et al., 2012). Doxycyclin had been shown before to reduce oxygen consumption and metabolic rates via inhibiting mitochondrial translation (Houtkooper et al., 2013; Ugalde et al., 2004). We demonstrate here that doxycyclin treatment suppressed the germline tumors of *shc-1; daf-18* mutants after L1 starvation. Similarly, modulation of the starvation temperature either increased (25 °C) or decreased (15 °C) the gonad phenotype in these animals. Although we have not directly measured metabolic rates in these mutants during distinct nutritional conditions, the results, together with other reported metabolic alterations of *daf-18* mutants (Lee et al., 2012; Shirwany and Zou, 2014), suggest that both DAF-18 and SHC-1 may be required to adjust mitochondrial metabolism to alterations in nutrient availability. The late manifestation of the phenotype at L3, at a time of massive germline proliferation (Hubbard and Greenstein, 2005), predicts the existence of a monitor that records the energy status during starvation, and a memory of this status later manifests itself during germline development. Dox treatment, and, thus, inhibition of mitochondrial translation, or reducing general metabolism by low temperatures, obviously suffices to correct this monitor, since Dox treatment and 15 °C incubation only during L1 starvation were found to suppress germline tumors in *shc-1;daf-18* mutants. A second model, that is also compatible with the observations made, but does not require the existence of an energy memory, would suggest that *shc-1;daf-18* double mutants fail to down-regulate mitochondrial metabolism when food becomes scarce, but that mitochondria still respond to increasing availability of food (during refeeding) with a further increase of metabolic activity. In favor of this model, Dox treatment, applied exclusively during refeeding, was shown to potently suppress germline hyper-proliferation in these mutants. This indicates a restoration of erroneously increased high mitochondrial activity.

Doxycyclin treatment has recently been shown to induce the mitochondrial UPR^{mt} response, suggesting that inhibition of mitochondrial translation, as proposed via a mito-nuclear protein imbalance (Houtkooper et al., 2013), could suppress *shc-1;daf-18* germline tumors via activating stress resistance. The temperature dependence of the phenotype argues against this hypothesis. Lowering temperature has generally been assumed to reduce mitochondrial activity (Fukuyama et al., 2012), and, thus, generation of reactive oxygen species (ROS) which are a trigger of the UPR^{mt} (Nargund et al., 2012; Runkel et al., 2013) Therefore, high, rather than low, temperatures would induce the UPR^{mt}, suggesting that the suppression of the germline tumors observed at low temperatures does not correlate with an induction of the UPR^{mt}.

In *C. elegans*, both neurons and non-neuronal tissues, including the intestine, have been implicated in monitoring the availability of nutrients and energy (Bishop and Guarente, 2007; Miguel-Aliaga, 2012; Wiederkehr and Wollheim, 2006) and result in adaptation of mitochondrial activity, i.e. metabolism, in a multitude of tissues. Furthermore, mitochondrial function has been shown to control endocrine responses in a variety of species, including *C. elegans* (Billing et al., 2011; Cheetham and Brand, 2013; Hsu and Drummond-Barbosa, 2011; Wiederkehr and Wollheim, 2006), and involve signaling mechanisms that have also been implicated in controlling germline development (Michaelson et al., 2010; Qi et al., 2012). Here, we show that failures to respond to nutrient availability result in a germline phenotype, suggesting that signaling of the metabolic status may derive from the somatic tissues

to affect the germline. Since it is widely accepted that transgenes in *C. elegans* are not expressed in the germline, the potent rescue of *shc-1;daf-18* starvation-induced germline defects by a *daf-18* transgene strongly suggests the existence of such a cell-nonautonomous signaling to the germline (Fig. S1A) (Nakdimon et al., 2012). We and others had reported before that failures in somatic IIs pathways may result in cell-nonautonomous induction of germline tumors (Finch and Ruvkun, 2001; Sweet and Tzima, 2009).

Is IIs the only pathway affected by DAF-18 during L1 starvation? TORC1 is core of a nutrient-sensitive signaling pathway that also controls mitochondrial homeostasis. Amino acids are the most potent activators of mTOR signaling. PEP-2/PEPT-1, a dipeptide transporter at the apical membrane of intestinal cells acts as selective dipeptide/H^+ symporter that triggers enteroendocrine signaling, while its transport capability fuels intracellular amino acid stores to induce mTOR signaling (Matsumura et al., 2005; Meissner et al., 2004). A *pep-2* knock-down suppressed 50% of the germline tumors in *shc-1;daf-18* animals (Fig. S1B), supporting a cell-nonautonomous suppressor activity by the intestine to the germline and suggesting a cross-talk between IIS and mTOR pathways. The suppression observed by inactivating *pep-2*, and, as a consequence, most likely mTOR signaling in this experimental setup, requires further analysis. It cannot be simply explained by down-regulation of general translation, since knock-out of *ife-2*, encoding the *C. elegans* isoform of human eIF4E, a general, somatic regulator of mRNA translation initiation, did not modulate starvation induced tumorigenesis in *shc-1;daf-18* (Fig. S1C).

In summary, our data contribute to a growing awareness, that rather basic environmental changes, here a temporary alteration of nutrient supply, can result in profound negative consequences, if the organism cannot adjust its metabolism, as we see here in mutations of the *C. elegans* homolog of the *PTEN* tumor suppressor gene, *daf-18*. We did not fail to notice that changes in metabolic profiles, hyper-proliferation and membrane disintegration are all aspects commonly observed in tumorigenesis. Our results also suggest that it may be worthwhile to test the efficacy of DOX in treating cell hyper-proliferation, as they occur for example in mammalian tumor models. In this context, it is interesting to note that the use of tetracyclines in tumor treatment has been discussed before (Sagar et al., 2010; Saikali and Singh, 2003), and that osteosarcoma cell cybrids display a hypersensitivity to Dox (Toompuu et al., 1999).

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Conflict of interest

The authors have no conflicts of interests.

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