CORRECTION

S6K links cell fate, cell cycle and nutrient response in *C. elegans* germline stem/progenitor cells

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The legend to Fig. 3E should read: Percentage of gonad arms containing meiotic (differentiated) nuclei in *rsks-1*, *glp-1(ar202)* and *glp-1(ar202) rsks-1* (*n*=22, 21, 59) at the indicated stages.

Two strains reported as bearing *daf-16(mu86)* actually carry the *daf-16(m26)* allele. Correct genotypes for these strains listed in Tables S1, S6 and S7 and in experiments reported in Fig. 4A and Fig. 5E, as well as related text and legends are:

GC1238: *rrf-1(pk1417) daf-16(m26)*
VB1040: *daf-16(m26); rsks-1(sv31)*.

The conclusions of the paper are not altered by these errors. The authors apologise to readers for these mistakes.

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S6K links cell fate, cell cycle and nutrient response in *C. elegans* germline stem/progenitor cells

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SUMMARY

Coupling of stem/progenitor cell proliferation and differentiation to organismal physiological demands ensures the proper growth and homeostasis of tissues. However, in vivo mechanisms underlying this control are poorly characterized. We investigated the role of ribosomal protein S6 kinase (S6K) at the intersection of nutrition and the establishment of a stem/progenitor cell population using the *C. elegans* germ line as a model. We find that *rsks-1* (which encodes the worm homolog of mammalian p70S6K) is required germline-autonomously for proper establishment of the germline progenitor pool. In the germ line, *rsks-1* promotes cell cycle progression and inhibits larval progenitor differentiation, promotes growth of adult tumors and requires a conserved TOR phosphorylation site. Loss of *rsks-1* and *ife-1* (eIF4E) together reduces the germline progenitor pool more severely than either single mutant and similarly to reducing the activity of *let-363* (TOR) or *daf-15* (RAPTOR). Moreover, *rsks-1* acts in parallel with the *glp-1* (Notch) and *daf-2* (insulin-IGF receptor) pathways, and does not share the same genetic dependencies with its role in lifespan control. We show that overall dietary restriction and amino acid deprivation cause germline defects similar to a subset of *rsks-1* mutant phenotypes. Consistent with a link between diet and germline proliferation via *rsks-1*, loss of *rsks-1* renders the germ line largely insensitive to the effects of dietary restriction. Our studies establish the *C. elegans* germ line as an in vivo model to understand TOR-S6K signaling in proliferation and differentiation and suggest that this pathway is a key nutrient-responsive regulator of germline progenitors.

KEY WORDS: *C. elegans*, p70S6K, Germ line, TOR, Notch, Insulin

INTRODUCTION

The nutritional status of an animal has many implications. Consequences of poor nutrition include growth and reproductive deficiencies in organisms ranging from *C. elegans* to humans (e.g. Bongaarts, 1980; Greer and Brunet, 2009). Dietary restriction can also extend lifespan and reduce susceptibility to age-related diseases, such as diabetes and certain cancers (Colman et al., 2009; Kritchevsky, 1999; Rous, 1914; Tannenbaum and Silverstone, 1953). Recent studies suggest that specific signaling pathways mediate the cellular effects of changes in diet. For example, although dietary restriction can deter tumor proliferation in some models, tumors with elevated PI3K activity are insensitive to growth-inhibitory effects of dietary restriction (Kalaany and Sabatini, 2009). Therefore, understanding the molecular mechanisms that underlie the effects of diet on development, cell proliferation and reproduction has broad implications.

TOR is a serine/threonine kinase and a conserved regulator of cell growth and proliferation in response to nutritional and growth factor cues (reviewed by Hietakangas and Cohen, 2009; Russell et al., 2011; Wang and Proud, 2006; Wang and Proud, 2009; Wullschleger et al., 2006). Interestingly, although TOR acts downstream of insulin/IGF/PI3K signaling in certain contexts, the two pathways can also have independent functions. TOR participates in a complex (TORC1) with the Regulatory associated protein of TOR (RAPTOR) to promote growth when nutrients are plentiful. Two well-characterized TORC1 targets, p70 ribosomal S6 kinase (p70S6K) and the eukaryotic translation initiation factor (eIF4E)-binding protein 4E-BP1, link TORC1 to translational control. Of these, ribosomal protein S6 kinase (S6K) has been most clearly implicated in cell and organismal growth.

Stem cells are important targets for metabolic control, as they must be tightly regulated to properly establish and maintain stem cell pools and tissue homeostasis in response to changing physiological demands (reviewed by Drummond-Barbosa, 2008). The *C. elegans* germ line is maintained by a pool of proliferating progenitors (stem cells and their progeny) (reviewed by Hansen and Schedl, 2006; Hubbard, 2007; Kimble and Crittenden, 2007). This system offers a genetically tractable framework to study the effects of nutrition on stem cell proliferation and differentiation in the context of a whole animal (Korta and Hubbard, 2010). The somatic distal tip cell (DTC) serves as the niche for germ cells, maintaining the proliferative germ cell fate by producing ligands for the receptor GLP-1 (Notch) on neighboring germ cells. In addition, insulin/IGF-like receptor (IIR) signaling is required for robust larval germline proliferation to generate an appropriate progenitor pool for optimal fecundity (Michaelson et al., 2010). In *C. elegans*, homologs of TOR pathway genes include *let-363* (TOR), *daf-15* (RAPTOR) and *rsks-1* (S6K). Reduction- or loss-of-function of these genes leads to lifespan extension (Jia et al., 2004; Pan et al., 2007; Selman et al., 2009; Vellai et al., 2003). Furthermore, loss of *let-363* or *daf-15* leads to larval developmental arrest (Jia et al., 2004; Long et al., 2002) and loss of *rsks-1* causes reduced body size and smaller broods (Pan et al., 2007; Selman et al., 2009). Obvious sequence homologs of the TOR inhibitors TSC1/2 (Inoki et al., 2002) and of 4E-BP have yet to be identified in the *C. elegans* genome, although five genes (*ife-1-5*) encode eIF4E orthologs (Keiper et al., 2000).

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Here we show that *rsks-1* is required germline-autonomously for the establishment of the proper number of germline progenitors during development and that this role requires a conserved TOR phosphorylation site. We find that *rsks-1* both promotes cell cycle progression and inhibits differentiation. A reduction of TOR or RAPTOR homologs causes a more severe germline defect, and *rsks-1* and *ife-1* appear to mediate the bulk of these effects. Genetic interactions are consistent with *rsks-1* acting similarly to *glp-1* (Notch) in that loss of *rsks-1* enhances and suppresses phenotypes associated with reduced and elevated *glp-1* activity, respectively. Our results are also consistent with *rsks-1* acting in parallel with both *glp-1* and *daf-2* (IIR). Surprisingly, genes that mediate the effects of *rsks-1* on longevity do not similarly affect the germ line. Finally, we find that dietary restriction strongly reduces the number of proliferative germ cells in wild type and in *glp-1* mutants, but not in *rsks-1* mutants.

MATERIALS AND METHODS

Strains and plasmids

Strains (supplementary material Table S1) were derived from N2 wild type (Bristol) and handled using standard methods (Brenner, 1974). Unless otherwise indicated, worms were grown on OP50 at 20°C. For plasmids and construction details, see supplementary material Table S2. Plasmids constructed for this study were pGC479, pGC480, pGC520, pGC609, pGC610.

Analysis of germline proliferative zone and tumors

Synchronization by hatch-off, timecourse analysis and DAPI staining were performed as described (Pepper et al., 2003a). Microscopy, developmental staging, determination of nuclei number in the proliferative zone, apoptosis (SYTO 12) analysis, distance to transition zone, mitotic index, S-phase index by 5-ethynyl-2′deoxyuridine (EdU) labeling, and DNA quantification were as described (Michaelson et al., 2010). In addition, some proliferative zone counts were semi-automated using a modified ImageJ plug-in originally written and subsequently modified at our request by Vytas Bindokas at the University of Chicago Integrated Microscopy Core. For proximal tumors, counts of undifferentiated nuclei proximal to the proximal-most differentiated nuclei were obtained manually. For *gld-1* RNAi-induced tumors, N2 and *rsks-1(sv31)* animals were grown at 20°C, synchronized by hatch-off, fed *gld-1* RNAi-inducing bacteria, and scored as adults (at 48 hours post-mid L4 at 20°C). For *glp-1* proximal tumors, *glp-1(ar202)* and *glp-1(ar202) rsks-1(sv31)* animals were grown at 15°C and synchronized. L1 larvae were moved to 25°C and scored at the early adult stage [defined as just after the L4/adult molt (Michaelson et al., 2010)].

Brood size, egg survival, sperm counts and developmental timing

Brood size and reproductive period were determined as described (Dillin et al., 2002). For embryo survival (hatched larvae/total eggs) the setup was as in the brood size experiment except that the number of eggs on each plate was counted upon adult transfer, and hatchlings were counted after 2 days. For mating experiments, individual *rsks-1(sv31)* hermaphrodites were placed on separate plates with three N2 males, transferred every 24 hours, and progeny counted; older males were replaced with young males every 2 days. For sperm counts, animals were DAPI stained at the adult molt (N2, ~54 hours post-hatch) or as adults [*rsks-1(sv31)*, ~64 hours post-hatch to compensate for later initiation of sperm production; see supplementary material Fig. S1]. Sperm and spermatocytes were counted; each spermatocyte was scored as four sperm. For developmental timing in supplementary material Fig. S1, N2 and *rsks-1(sv31)* were synchronized by a 1-hour hatch-off. Every 4 hours (36-80 hours post-hatch), animals were staged (vulval morphology, DIC microscopy), DAPI stained and scored for germ cells at the stages indicated.

Body length and germ cell size determination

Animals were grown to early adult, immobilized in levimasole and mounted on agar pads. Body length was measured on images captured at 50× magnification. Cell size was determined as described previously (Michaelson et al., 2010), except that *z*-stacks (images captured 0.5 μm apart) were captured at 630×, and germ cell volume was estimated by measuring the largest cell area in the *z*-stack (Zeiss AxioVision) and calculating cell volume as a sphere ($4/3\ \pi\ r^3$), with radius extrapolated from the measured area ($\pi\ r^2$). This method correlated very well with the method described previously.

RNAi

RNAi by bacterial feeding was performed as described (Timmons et al., 2001). The empty RNAi expression vector L4440 in HT115 bacteria served as a negative control. For *let-363*, *daf-15* and *ife-3*, RNAi was performed at 25°C, and progeny from the first ~3 hours of egg laying (collected ~12 hours post-L4) were scored at the early adult stage.

Immunohistochemistry

Immunohistochemical analysis was carried out as described (Pepper et al., 2003b) using rabbit polyclonal anti-GFP (Abcam ab6556, 1:2000 dilution) and Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Molecular Probes A11034, 1:1000).

Bacterial dilution protocol

OP50 bacteria were grown at 37°C for 16 hours to saturation. The bacterial concentration at saturation was determined by serial dilution on LB plates, and corresponded to 1×10^9 cfu/ml. Bacteria were resuspended in S Basal and diluted (1×10^8 and 1×10^7 cfu/ml) or concentrated (1×10^{10} cfu/ml). Bacteria (400 μl) were seeded onto 60 mm NGM plates containing 60 μg/ml ampicillin. S Basal and NGM media were as described (Stiernagle, 2006). After plates were dried in the hood for ~30 minutes, 6-8 gravid adult worms were spot-bleached in 20% alkaline hypochlorite solution directly on the plates (outside the bacterial lawn) to synchronize progeny and avoid bacterial transfer. Progeny of bleached adults were scored as early adults. At 1×10^7 cfu/ml, animals arrested at the L1 or dauer stages.

RESULTS

rsks-1 is required for proper establishment of the germline stem/progenitor cell pool

rsks-1, which encodes the sole worm homolog of mammalian p70S6K, is required for normal brood size in *C. elegans*, suggesting a role in reproduction (Fig. 1A) (Pan et al., 2007). Since many factors can contribute to brood size, we examined several aspects of reproduction in the *rsks-1(sv31)* null mutant. The most striking phenotype was a failure to establish the proper number of germline progenitors (that is, proliferative germ cells located distal to the position of meiotic entry). Under normal laboratory growth conditions, germline progenitors rapidly accumulate during the third and fourth larval stages (L3 and L4) to establish an adult pool of ~200 progenitors (Killian and Hubbard, 2005). In stage-matched *rsks-1(sv31)* or *rsks-1(ok1255)* mutant animals, this accumulation reached only ~50% of wild-type levels (Fig. 1B-D; supplementary material Fig. S1A, Table S3). By comparison, deletion of *rskn-1*, which encodes the homolog of p90S6K, had no effect (Fig. 1C; supplementary material Table S3). Additionally, *rsks-1* affects sperm production and embryo viability (supplementary material Fig. S1, Table S3). Together with the progenitor defect, these phenotypes are likely to account for the reduced fecundity. Our subsequent experiments focused on the role of *rsks-1* in the regulation of germline progenitors.

rsks-1 acts germline-autonomously to promote expansion of the larval germline progenitor pool

Three independent lines of evidence support the conclusion that *rsks-1* acts within the germ line to control larval stage accumulation of germline progenitors. First, *rsks-1* RNAi caused a reduction in the progenitor pool independent of *rrf-1*, which is required for optimal somatic RNAi (Fig. 1E; supplementary material Table S3) (Sijen et al., 2001). Second, restoring *rsks-1(+)* in somatic cells did

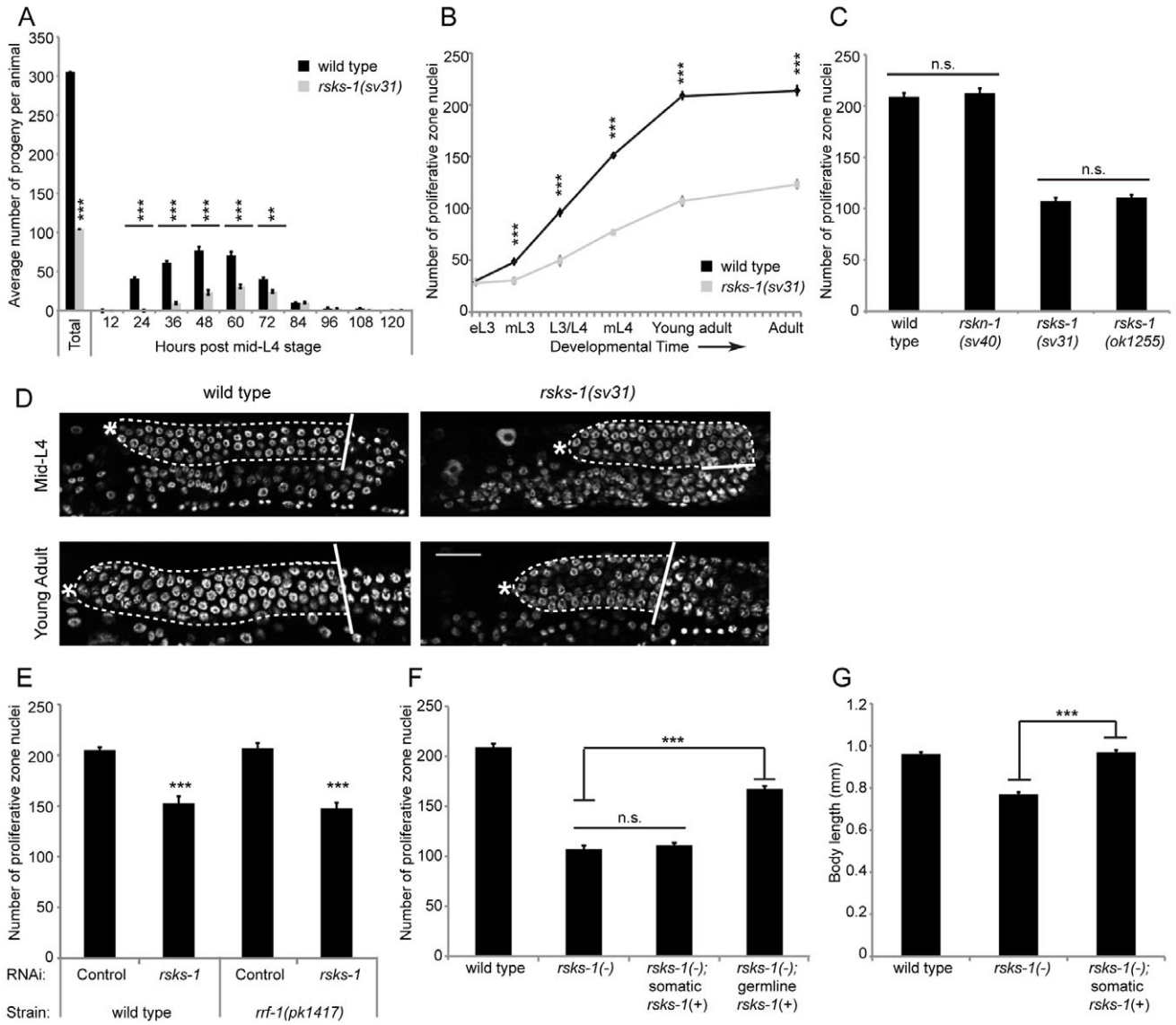


Fig. 1. *rsks-1* (S6K) is required germline-autonomously for normal establishment of the germline progenitor pool and fecundity. (**A**,**B**) Average progeny per animal (A) and timecourse of number of proliferative zone nuclei (B) in wild-type and *rsks-1* ($n \geq 14$ for each time point). (**C**) Average number of proliferative zone nuclei in early adult wild type, *rskn-1*, and two null alleles of *rsks-1*. (**D**) Representative DAPI-stained mid-L4 and early adult wild-type and *rsks-1* mutant animals. Asterisk indicates the distal end of the gonad, the solid line indicates the proliferative zone/transition zone border, and the dashed line outlines the proliferative zone. Scale bar: 20 μm. (**E**,**F**) Number of proliferative zone nuclei in wild type and *rrf-1* treated with control and *rsks-1* RNAi (E), and wild type, *rsks-1*, *rsks-1; svIs64[*somatic *rsks-1(+)]* and *rsks-1; naIs44[pGC520*, germline *rsks-1(+)]* (F). (**G**) Body length (mm) of wild type, *rsks-1* and *rsks-1; svIs64[*somatic *rsks-1(+)]*. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$; n.s., not significant ($P > 0.05$) by two-tailed Student's *t*-test. Error bars indicate s.e.m. See supplementary material Table S3 for data.

not rescue the germline defect (Fig. 1F; supplementary material Table S3), although it did rescue body length (Fig. 1G; supplementary material Table S3). Third, expressing *rsks-1(+)* solely in the germ line largely rescued the germline progenitor defect (Fig. 1F; supplementary material Table S3).

rsks-1 promotes overall cell cycle progression of larval germ cells

We examined three possible mechanisms by which *rsks-1* might promote germline progenitor accumulation: cell survival, cell cycle, and the balance between proliferation and differentiation. We found no SYTO 12-positive progenitor cells ($n > 65$) in the distal germ line of *rsks-1(sv31)* larvae or adults, although apoptotic SYTO 12-positive female germ cells were detected in the loop region, as expected (Gumienny et al., 1999). Nor did we observe debris in the proliferative region that might indicate other forms of cell death. Therefore, inappropriate cell death is unlikely to account for the failure to expand the germline progenitor pool in the *rsks-1* mutant.

When we examined the cell cycle we found that the larval, but not adult, germline progenitor cell cycle was delayed in *rsks-1(sv31)*. Specifically, at the mid-L4 stage, the mitotic and S-phase indexes were significantly reduced in *rsks-1(sv31)* relative to wild type (Fig. 2A,B), whereas both were unchanged in adults (supplementary material Fig. S2A,B). To determine whether these reduced indexes were accompanied by a specific delay in G1 or G2 we measured DNA content, and found no difference between the

distributions of *rsks-1(sv31)* and wild-type germline progenitor cells within each cell cycle phase (Fig. 2C). These results suggest that *rsks-1* promotes the larval but not adult germline progenitor cell cycle.

rsks-1 promotes cell cycle progression in adult germ cell tumors

Our finding that *rsks-1* promotes the larval but not adult germ cell cycle provided an opportunity to further investigate the hypothesis that cells in adult germline tumors behave like larval germline progenitors (Michaelson et al., 2010). If adult tumor cells were sensitive to *rsks-1*, this would support the notion that they are regulated similarly to larval progenitors. First, we looked at tumors caused by the weak gain-of-function (*gf*) allele *glp-1(ar202).* These tumors arise from germ cells that fail to differentiate in a timely manner (McGovern et al., 2009; Pepper et al., 2003a). We found that proximal germline tumors in adult *glp-1(ar202) rsks-1(sv31)* double-mutant animals contained 76% fewer cells (Fig. 2D,E; supplementary material Table S4) and displayed a reduced mitotic index (Fig. 2F) relative to *glp-1(ar202)* tumors, similar to the difference between *rsks-1(sv31)* and wild type (Fig. 2A).

We also investigated the effect of loss of *rsks-1* on *gld-1(RNAi)*-derived tumors. *gld-1* encodes an RNA-binding tumor suppressor protein that is required for proper germ cell differentiation (Francis et al., 1995a; Francis et al., 1995b; Jones and Schedl, 1995). Reduction of *gld-1* causes germ cells to exit the early meiotic pathway and return to the mitotic cell cycle, forming a proximal tumor in adulthood. Despite the difference in tumor etiology, we found that, like *glp-1(ar202)* proximal tumors, loss of *rsks-1* also reduced the number of cells in *gld-1(RNAi)* tumors by 73% (supplementary material Fig. S2C, Table S4). We conclude that *rsks-1* regulates germ cell proliferation in both normal larval germ lines and adult germline tumors, supporting the hypothesis that adult proximal germline tumors share features with larval germline progenitors.

rsks-1 promotes the proliferative (versus differentiated) germ cell fate

In addition to its role in cell cycle progression, we found that *rsks-1* affects the balance between germ cell proliferation and differentiation. We noted that the position of meiotic entry is shifted distally in *rsks-1(sv31)* animals at all stages examined,

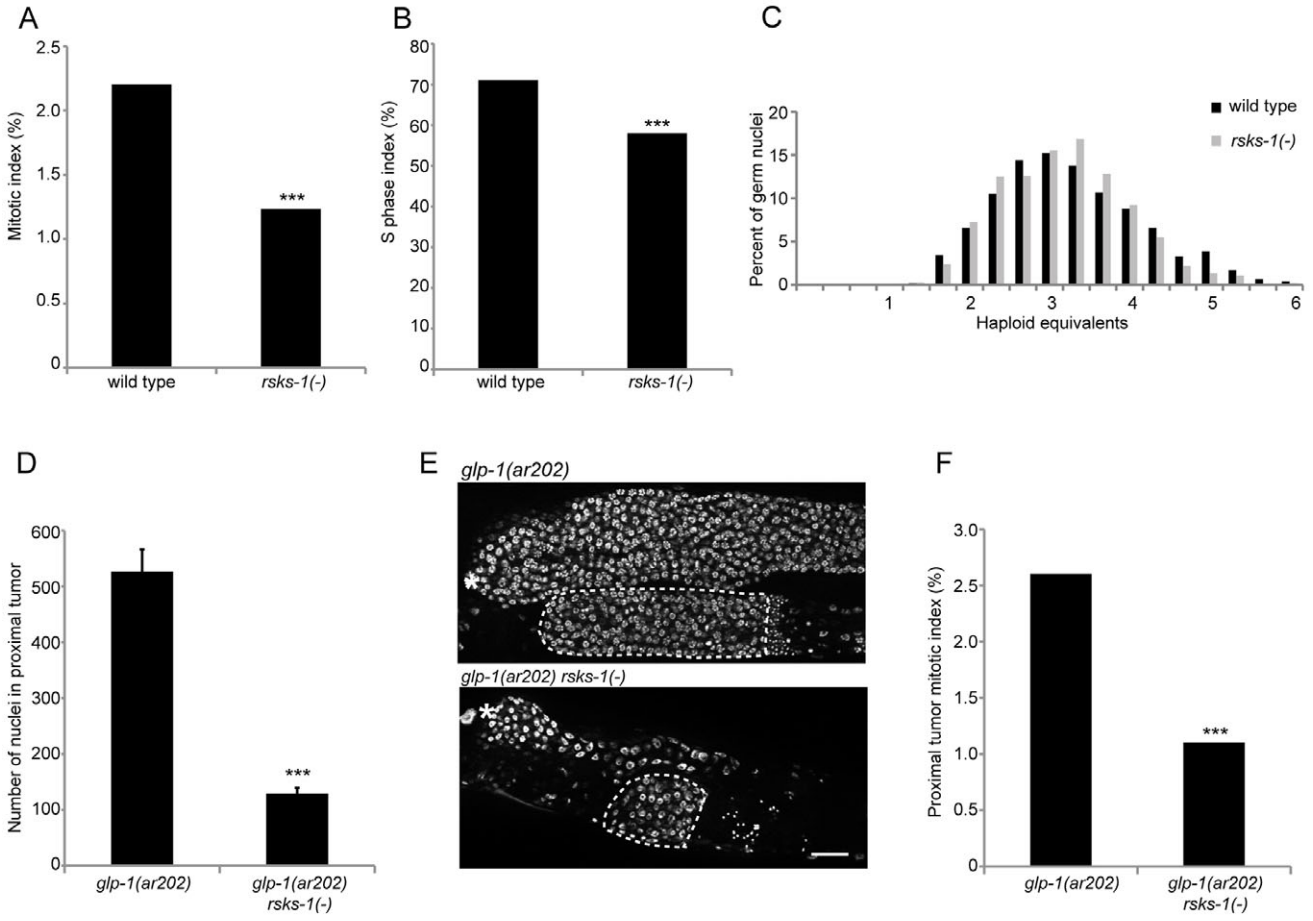
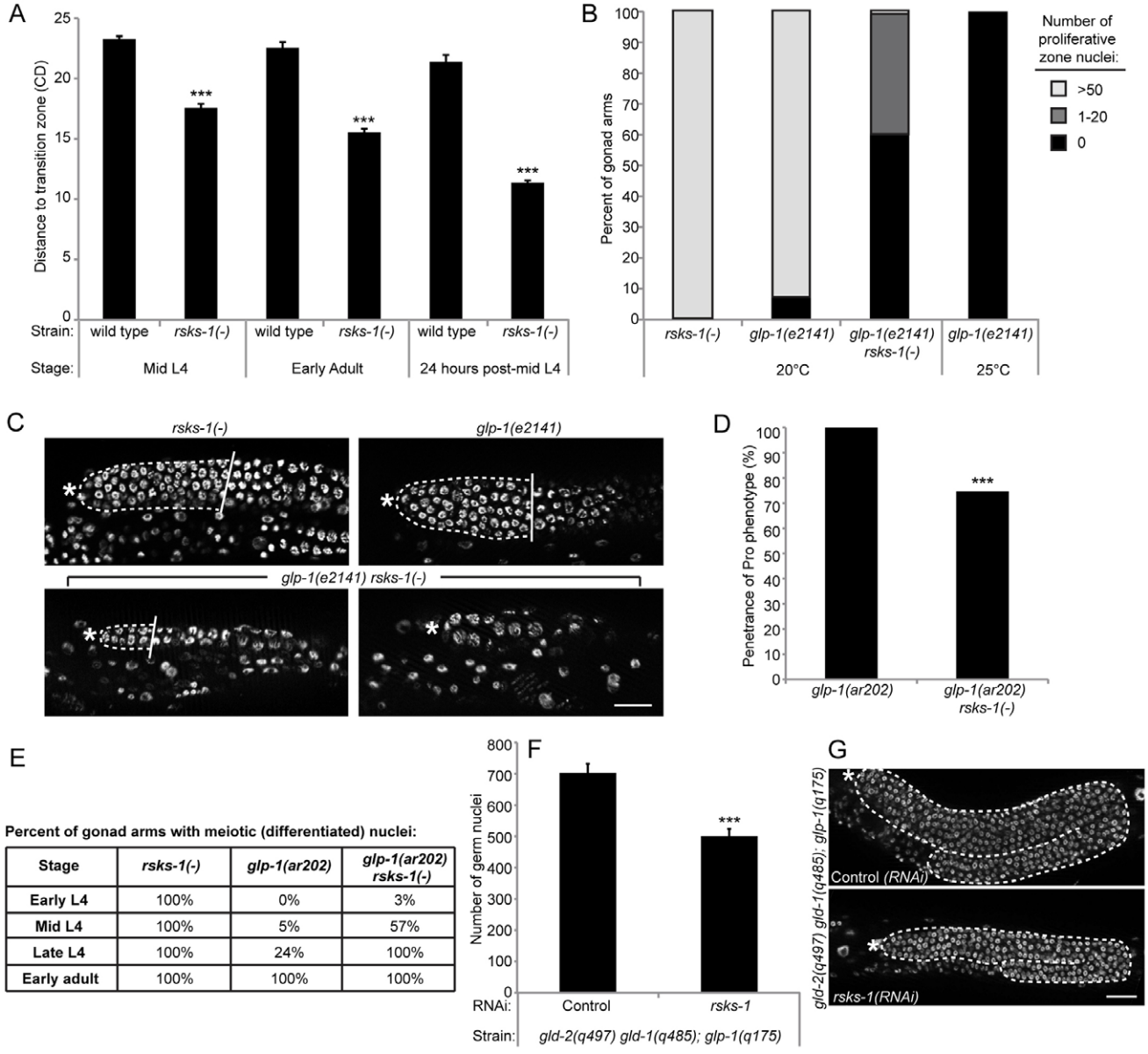


Fig. 2. *rsks-1* promotes germ cell proliferation in both distal larval germ lines and adult proximal germline tumors. (**A-C**) Mid-L4 stage (A) mitotic index, (B) S-phase index and (C) quantification of DNA content in wild-type and *rsks-1* germline proliferative zones. The number of gonad arms and germ cells analyzed: (A) 33 and 4988 for wild type, 29 and 2205 for *rsks-1*; (B) 28 and 4453 for wild type, 27 and 2113 for *rsks-1*; (C) 14 and 1369 for wild type, 15 and 871 for *rsks-1*. (**D**) Number of nuclei in proximal tumors in early adult *glp-1(ar202)* and *glp-1(ar202) rsks-1*. Error bars indicate s.e.m. (**E**,**F**) Representative DAPI-stained germ lines (E) and mitotic index (F) of *glp-1(ar202)* and *glp-1(ar202) rsks-1*. Asterisk indicates the distal end of the gonad and dashed lines outline proximal tumor. Scale bar: 20 μm. (A-F) *rsks-1(–)* is *rsks-1(sv31).* ***$P \leq 0.001$ by Mann-Whitney U test, except for D where two-tailed Student's *t*-test. (C) $P > 0.05$ for wild type versus *rsks-1* by Mann-Whitney U test for each bin. See supplementary material Table S4 for data.

reminiscent of mutants with reduced *glp-1* (Notch) activity (Michaelson et al., 2010). This phenotype becomes most pronounced in *rsks-1* adults (25%, 31% and 47% reduction at the mid-L4, early adult and 24 hours post-L4, respectively; Fig. 3A). This result raised the possibility that, in addition to its role in the cell cycle, *rsks-1* could affect differentiation, similar to the dual role reported for the cyclin E ortholog *cye-1* (Fox et al., 2011).

To explore this hypothesis, we asked whether loss of *rsks-1* enhanced the phenotype of a *glp-1* reduction-of-function (*rf*) mutant to an 'all meiotic' Glp-1 phenotype (in which all germ cells have entered meiosis) characteristic of strong loss of *glp-1* activity. *glp-1(e2141)* is a temperature-sensitive *rf* allele that exhibits reduced germline progenitors at the semi-permissive temperature (20°C; 94.7±3.3 versus 209.0±3.6 in wild type; *n*=22, 33) and a severe Glp-1 phenotype (all germ cells



Stage	*rsks-1(-)*	*glp-1(ar202)*	*glp-1(ar202) rsks-1(-)*
Early L4	100%	0%	3%
Mid L4	100%	5%	57%
Late L4	100%	24%	100%
Early adult	100%	100%	100%

Fig. 3. *rsks-1* promotes proliferation and/or inhibits differentiation in the germ line, similar to *glp-1* (Notch). (**A**) Average distance in cell diameters (CD) from the distal tip to the transition zone in wild type and *rsks-1(sv31)* at the mid-L4, early adult and 24 hours post-mid L4 (*n*=33, 29, 33, 20, 16, 22 gonad arms). (**B**) Percentage of gonad arms scored at the early adult stage containing at least 50, 1-20 or 0 proliferative zone nuclei in *rsks-1*, *glp-1(e2141)*, *glp-1(e2141) rsks-1* at 20°C, and *glp-1(e2141)* at 25°C (*n*=100, 390, 129 and 200 gonad arms; none contained between 21 and 50 cells). The *rsks-1* mutant enhancement of the 'all meiotic' *glp-1(rf)* phenotype includes an 'all sperm' class [24% in the double mutant versus 7% in *glp-1(rf)*] and a 'distal-most pachytene' class (36% versus 0%). (**C**) Representative DAPI-stained germ lines from B at 20°C: *rsks-1*, *glp-1(e2141)* and two classes of *glp-1(e2141) rsks-1* showing 1-20 (left) and 0 (right) mitotic nuclei. (**D**) Percentage of gonad arms displaying a proximal tumor (Pro phenotype) in *glp-1(ar202)* and *glp-1(ar202) rsks-1* (*n*=250, 312 gonad arms). (**E**) Percentage of gonad arms containing meiotic (differentiated) nuclei in *rsks-1*, *glp-1(e2141)* and *glp-1(e2141) rsks-1* (*n*=22, 21, 59) at the indicated stages. (**F**) Average number of germ nuclei in early adult *gld-2(q497) gld-1(q485); glp-1(q175)* animals treated with control or *rsks-1* RNAi (*n*=19, 24). (**G**) Representative DAPI-stained germ lines from F. (C,G) Labels as Fig. 1D. (A-G) *rsks-1(–)* is *rsks-1(sv31)*. ***$P \leq 0.001$ by two-tailed Student's *t*-test, except for D where Mann-Whitney U test. Error bars indicate s.e.m. See supplementary material Table S5 for data.

differentiate) at the restrictive temperature (25°C) (Priess et al., 1987). In the double mutant *glp-1(e2141) rsks-1(sv31)* at 20°C, we found a striking exacerbation of the penetrance of the Glp-1 'all meiotic' phenotype. Whereas virtually all *rsks-1(sv31)* or *glp-1(e2141)* single mutants have at least 50 germline progenitors, 60% of *glp-1(e2141) rsks-1(sv31)* double mutants display the severe Glp-1 phenotype and the remaining 40% contain only 1-20 progenitors (Fig. 3B,C; supplementary material Fig. S3). We also treated *glp-1(e2141)* and *rrf-1(pk1417); glp-1(e2141)* with *rsks-1* RNAi, and found that *rsks-1* RNAi enhanced the Glp-1 phenotype in both strains (supplementary material Fig. S3), suggesting that the effect is germline autonomous. In addition to enhancing the phenotype caused by a reduction of *glp-1* activity, loss of *rsks-1* suppressed the penetrance of a *glp-1(gf)* mutant phenotype: the Pro phenotype (percentage of gonad arms that form proximal tumors at 25°C, as distinct from the number of cells within the tumors, above) was reduced from 100% in *glp-1(ar202)* to 75% in *glp-1(ar202) rsks-1(sv31)* (Fig. 3D). Animals without tumors produced viable progeny. Closer examination of the double mutant revealed a correlation between the delay in differentiation and suppression of tumor initiation (Fig. 3D,E). Thus, reducing *rsks-1* activity enhances phenotypes caused by reduced *glp-1* activity and suppresses phenotypes caused by elevated *glp-1* activity. Taken together, we conclude that *rsks-1* acts similarly to *glp-1* to promote the proliferative fate and/or inhibit the differentiated fate.

rsks-1 acts in parallel with or downstream of *glp-1* signaling in the context of cell cycle control

We reasoned that if *rsks-1* requires the presence of GLP-1 (Notch), then loss of *rsks-1* should have no effect when *glp-1* activity is removed. Conversely, if *rsks-1* does not require GLP-1, then loss of *rsks-1* should still affect the germ line in the absence of GLP-1. We employed a triple null mutant *gld-2(q497) gld-1(q485); glp-1(q175)*, in which the vast majority of germ cells are in the proliferative state in the absence of GLP-1 (Hansen et al., 2004). We observed a 29% reduction (Fig. 3F,G; supplementary material Table S5) in the number of progenitors in *rsks-1(RNAi)* versus control RNAi, which is comparable to the 26% decrease observed in the wild type treated with *rsks-1(RNAi)* (Fig. 1E; supplementary material Table S3). However, this reduction in cell number was not associated with obvious meiotic entry (Fig. 1E; data not shown), suggesting that the cell cycle-promoting role of *rsks-1* acts in parallel with GLP-1 and downstream of GLD-1 and GLD-2. The role of RSKS-1 with respect to meiotic entry is more difficult to place within this pathway. Although *rsks-1(RNAi)* enhancement of the meiotic entry defect of *glp-1(rf)* is likely to be germline autonomous (supplementary material Fig. S3) and is therefore unlikely to be upstream of *glp-1*, it might not cause a sufficiently strong meiotic entry phenotype to reveal a clear epistatic relationship. These results suggest that the two functions of *rsks-1* in the proliferative region, i.e. cell cycle and cell fate control, might be separable.

rsks-1 affects proliferative germ cell number largely independently of *daf-2*

Previously, we showed that *daf-2* (IIR) signaling promotes larval germline proliferation and that this effect depends on the activities of *daf-18* (PTEN) and *daf-16* (FOXO) (Michaelson et al., 2010). We therefore investigated the relationship between the *daf-2* pathway and *rsks-1*. We found that *rsks-1* regulation of germ cell accumulation does not depend on *daf-16* or *daf-18*, as neither *daf-18(RNAi)* nor *daf-16(–)* suppresses the *rsks-1* germline defect (Fig. 4A; supplementary material Fig. S4B, Table S6).

We tested the alternative hypothesis that *daf-16* negatively regulates *rsks-1*. This hypothesis is supported by a report of transcriptional repression of *daf*-15 (RAPTOR) by *daf-16* (Jia et al., 2004). If *daf-16* negatively regulates *daf-15* upstream of *rsks-1*, then eliminating *daf-16* activity should elevate the level of *daf-15* and might ameliorate the effects of a partial loss of *rsks-1*. We tested this possibility using *rsks-1(RNAi)* because it reduces, but does not eliminate, *rsks-1* activity, and we found no difference in the phenotype in the presence or absence of *daf-16* (Fig. 4B; supplementary material Table S6), suggesting that *daf-16* does not negatively regulate *rsks-1* in this context.

We then considered whether *rsks-1* and *daf-2* might affect larval germline progenitor accumulation independently. If so, the *daf-2(e1370) rsks-1(sv31)* double-mutant phenotype should be more severe than that of either single mutant. Indeed, this is what we observed. At both 20°C and after an L3 shift to 25°C, the *daf-2 rsks-1* double mutant contained significantly fewer germline progenitors than either single mutant (Fig. 4C; supplementary material Fig. S4A, Table S6).

Finally, based on studies in *Drosophila* showing that reduction in either insulin signaling or p70S6K activity reduces cell size (reviewed by Hietakangas and Cohen, 2009), we examined germ cell size. We found that whereas cell size was not reduced in the *daf-2* mutant, loss of *rsks-1* led to a significant reduction in germ cell size (Fig. 4D; supplementary material Table S6). We conclude that *rsks-1* and *daf-2* have different and likely independent effects on germ cell progenitors (see Discussion).

The conserved TOR phosphorylation site in RSKS-1 is required for germline progenitor accumulation

In other systems, S6K is a direct substrate for the TOR kinase. TOR phosphorylates a highly conserved threonine residue in *Drosophila* and human S6K (Schalm and Blenis, 2002) (supplementary material Fig. S3A). We generated a transgene that alters this T404 residue. Despite similar germline expression levels between wild-type T404 and the T404A mutant transgenes (supplementary material Fig. S3B,C), the T404A transgene did not rescue the germline phenotype of *rsks-1(sv31)* (Fig. 5A; supplementary material Table S7). Thus, phosphorylation on T404, presumably by LET-363 (TOR), is necessary for the proper establishment of the germline progenitor pool.

rsks-1 partially mediates the effects of *let-363* and *daf-15* on germline progenitors

Animals bearing null mutations in *let-363* (TOR) or *daf-15* (RAPTOR) undergo arrest in L3, precluding direct study of germline progenitor accumulation (Fig. 5B,D). We therefore identified conditions that reduce their activities without interfering with overall development. Although the progeny of mothers fed with either dsRNA starting at mid-L4 undergo arrest (Fig. 5B,D), mothers fed from the L4/adult molt produce 'escapers' that develop into adults with 57% fewer germline progenitors relative to controls (supplementary material Fig. S3D, Table S7). In addition, in the *rrf-1* mutant that attenuates somatic but not germline RNAi, progeny of animals fed *let-363* and *daf-15* RNAi starting in mid/late L4 exhibit sterility with a severe (82%) reduction in the number of germline progenitors (Fig. 5B-E; supplementary material Table S7), while fewer than 5% of these progeny arrest. The *let-363* and *daf-15* RNAi phenotypes are very similar,

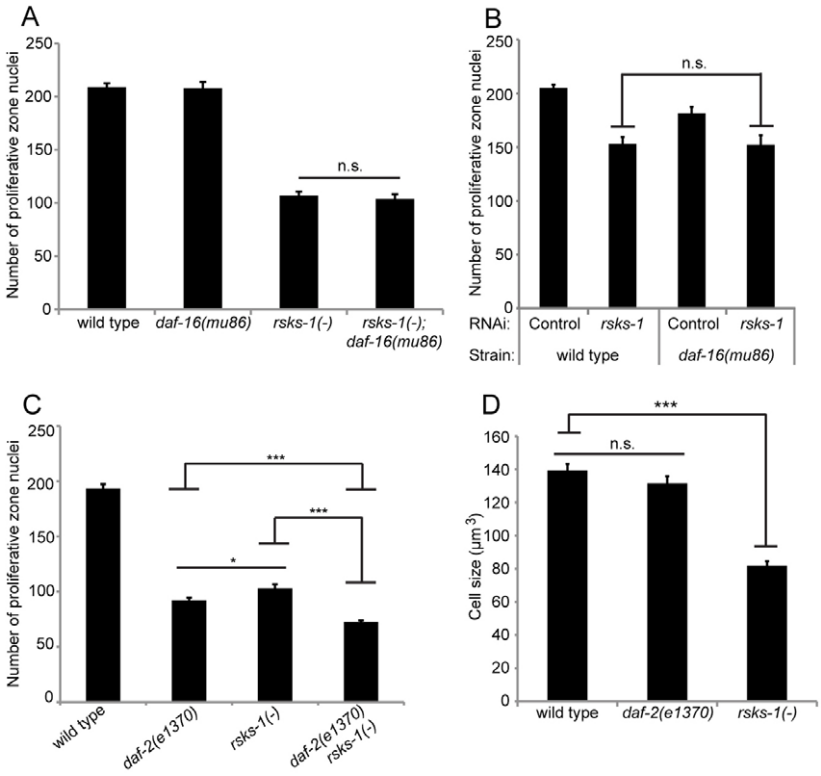


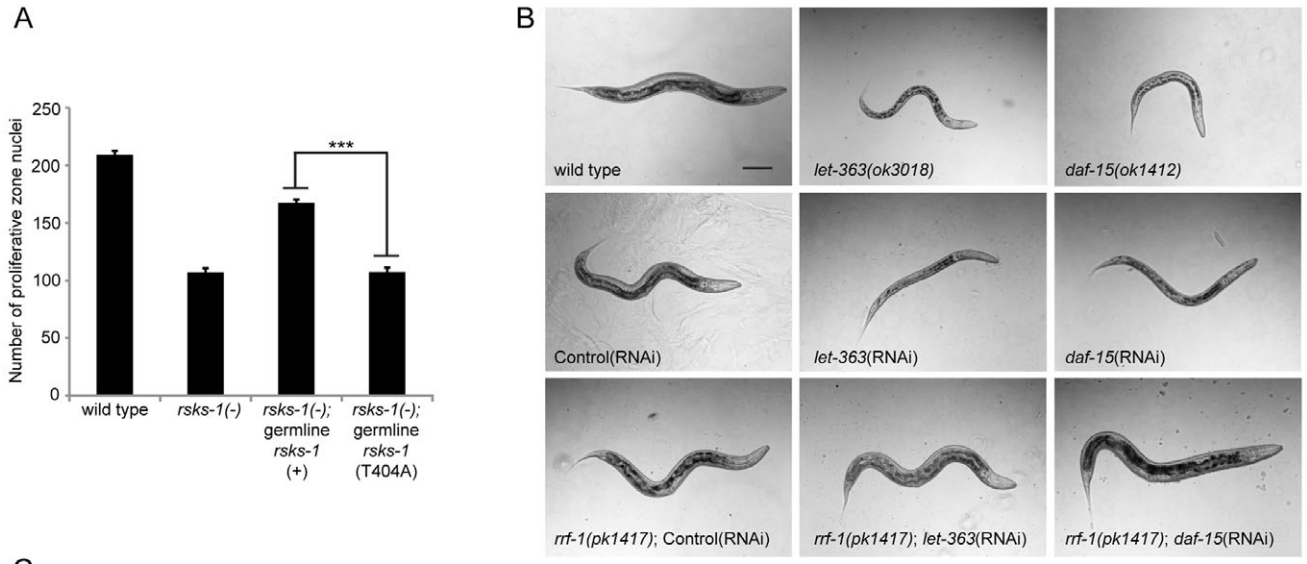
Fig. 4. *rsks-1* acts largely independently of the *daf-2* (insulin receptor) pathway. (**A-C**) Number of proliferative zone nuclei in early adult (A) wild type, *daf-16*, *rsks-1* and *rsks-1; daf-16*, (B) wild type and *daf-16* treated with control or *rsks-1* RNAi and (C) wild type, *daf-2*, *rsks-1* and *daf-2 rsks-1* after shift to 25°C at the mid-L3 stage. (**D**) Average germ cell volume in wild type, *daf-2* and *rsks-1* (n=50, 50, 80 cells from 8-10 animals each). *rsks-1(–)* is *rsks-1(sv31)*. ***P≤0.001, *P≤0.05; n.s., not significant by two-tailed Student's t-test. Error bars indicate s.e.m. See supplementary material Table S6 for data.

consistent with TOR and RAPTOR acting together, as they do in other organisms. Moreover, *let-363(RNAi)* reduced the mitotic index compared with controls (supplementary material Fig. S5E), and caused a statistically significant delay in the G2 phase of the cell cycle (supplementary material Fig. S5F). This G2 delay differs from what we observed in *rsks-1(sv31)*, which slowed the cell cycle but did not alter the distribution of germ cells within each cell cycle phase (Fig. 2C). Since *daf-2* (IIR) signaling causes a delay in G2 in a manner dependent on *daf-16* (FOXO) (Michaelson et al., 2010), we wondered whether the *let-363(RNAi)* phenotype could be partially suppressed by loss of *daf-16*. We found that *rrf-1; let-363* RNAi caused the same defect in the presence or absence of *daf-16*, and loss of *daf-16* did not suppress the developmental arrest phenotype of *let-363* RNAi. Thus, neither the germline nor the somatic arrest phenotypes of *let-363* depend on *daf-16*.

RNAi depletion of *let-363* or *daf-15* caused a more severe defect in the germline progenitor pool than loss of *rsks-1*, suggesting that *rsks-1* mediates most but not all of the effects of TOR/RAPTOR on germline progenitor accumulation. An alternate candidate substrate of TOR is 4E-BP. When phosphorylated by TOR, 4E-BP cannot bind and inhibit the activity of eIF4E (Wang and Proud, 2006). Therefore, TOR activity promotes the activity of eIF4E. In the absence of obvious sequence homologs of 4E-BP in *C. elegans*, we examined five genes that encode eIF4Es (*ife-1-5*) (Keiper et al., 2000). We first assayed the effects of each gene individually on the size of the proliferative germ cell pool, using viable null alleles, with the exception of the essential *ife-3* for which we used RNAi. Loss of *ife-1* strongly reduced the number of proliferative germ cells (supplementary material Fig. S5G, Table S7), consistent with its expression in the larval germline proliferative zone (Amiri et al., 2001). Further, germ lines of *rsks-1(sv31) ife-1(bn127)* double mutants averaged 53 progenitors cells, which is significantly fewer than either single mutant (supplementary material Fig. S5H, Table S7). Since reduction of *let-363* by RNAi results in ~30 proliferative germ cells, *ife-1* and *rsks-1* could account for much of the *let-363* effect on germ cell accumulation. We also saw a small but statistically significant effect from *ife-3* and *ife-4* on the size of the proliferative germ cell pool (supplementary material Fig. S5G), suggesting that these genes contribute as well.

The role of *rsks-1* in the germ line is independent of its role in longevity

In *C. elegans*, lifespan extension conferred by loss of *rsks-1* depends on the activity of *pha-4* (FOXA), *egl-9* and *aak-2* (AMPK) (Chen et al., 2009; Mango et al., 2008; Selman et al., 2009). We tested whether the *rsks-1* defect in the establishment of germline progenitors also depended on these genes, and we found that it did not. Neither *rsks-1* mutant animals subject to *pha-4* RNAi from the L1 stage (to avoid embryonic lethality) nor viable escapers from late L4 maternal RNAi displayed any restoration in the number of proliferative germ cells (Fig. 6A; supplementary material Table S8; for escapers 78.5±4.3 versus 79.3±4.1 in the control; n=10, 13). Similarly, loss of *egl-9*, a dioxygenase that negatively regulates hypoxia inducible factor (HIF-1), did not suppress the *rsks-1* mutant germline defect (Fig. 6B; supplementary material Table S8). Next, we examined the *rsks-1* germline progenitor phenotype in an *aak-2* mutant; *aak-2* encodes a *C. elegans* homolog of the catalytic alpha subunit of AMP-activated protein kinase (AMPK). We saw no suppression of the *rsks-1* phenotype upon reduction of *aak-2* activity (Fig. 6C; supplementary material Table S8, see also Fig. S6A,B for additional combinations with *aak-1*). Finally, we saw no difference in the brood sizes of the *rsks-1(sv31); aak-2(ok524)* double mutant compared with the *rsks-1* single mutant [see correction to Selman et al. (Selman et al., 2009)] (supplementary material Fig. S4B). Taken together, we conclude that unlike its role in lifespan control, *rsks-1* does not act through *pha-4*, *egl-9* or *aak-2* in promoting the accumulation of germline progenitors.



D

Condition	% Arrest	% Sterility
wild type	0	0
let-363(ok3018)	100	N/A
daf-15(ok1412)	100	N/A
Control RNAi (L1)	0	0
let-363 RNAi (L1)	0	0
daf-15 RNAi (L1)	0	0
Control RNAi (M)	0	0
let-363 RNAi (M)	100	N/A
daf-15 RNAi (M)	100	N/A
rrf-1(pk1417)	0	0
rrf-1(pk1417); Control RNAi (M)	0	0
rrf-1(pk1417); let-363 RNAi (M)	4.1	100
rrf-1(pk1417); daf-15 RNAi (M)	1.8	100

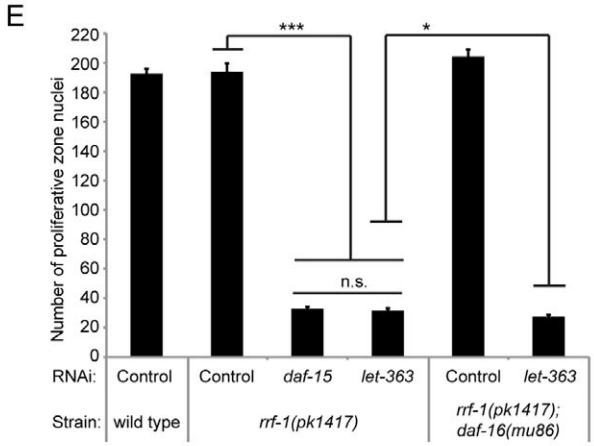


Fig. 5. *rsks-1* acts downstream of *let-363* (TOR). (**A**) Average number of proliferative zone nuclei in gonad arms of early adult wild type, *rsks-1*, *rsks-1; nals44[pGC520*, germline *rsks-1(+)]* and *rsks-1; nals48[pGC609*, germline *rsks-1(T404A)]*; part of Fig. 1F is included for comparison. *rsks-1(–)* is *rsks-1(sv31)*. (**B**) Representative images of early adult or developmentally arrested animals under the indicated conditions. Scale bar: 100 μm. (**C**) Representative DAPI-stained germ lines of early adult wild type treated with control RNAi, and *rrf-1* animals treated with control, *let-363* or *daf-15* RNAi. Labels as Fig. 1D. (**D**) Summary of developmental arrest and sterility under different mutant and RNAi conditions at 25°C (n>120 for each). L1 and M refer to RNAi initiated at the early L1 stage and maternal mid/late L4 stage, respectively. (**E**) Number of proliferative zone nuclei in germ lines of early adult animals raised at 25°C: wild type, *rrf-1* and *rrf-1 daf-16* with control, *daf-15* and *let-363* RNAi. ***P≤0.001,*P≤0.05; n.s., not significant by two-tailed Student's t-test, except for three-sample comparison in E by one-way ANOVA. Error bars indicate s.e.m. See supplementary material Table S7 for data.

rsks-1 mediates the effects of diet on germline proliferation

In cell culture assays, TOR-S6K pathway activity is responsive to nutritional signals, especially intracellular amino acids (Wang and Proud, 2009). To determine whether TOR-S6K pathway activity links nutritional signals to germline progenitors in *C. elegans*, we first examined the effect of dietary restriction (DR) on the germ line in wild type, and then tested its effects in the presence and absence of *rsks-1*. Bacterial dilution had a striking effect on the number of adult germline progenitors: wild-type animals grown on 1×10^8 cfu/ml bacteria displayed 76% fewer progenitors than siblings grown on 1×10^9 cfu/ml (215 versus 52; Fig. 7A; supplementary material Table S9). Consistent with the possibility that *rsks-1* mediates this germline response, *rsks-1* mutants grown on 1×10^8 cfu/ml contained only 20% fewer germline progenitors than siblings raised on 1×10^9 cfu/ml (Fig. 7A; supplementary material Table S9). This attenuated response is not

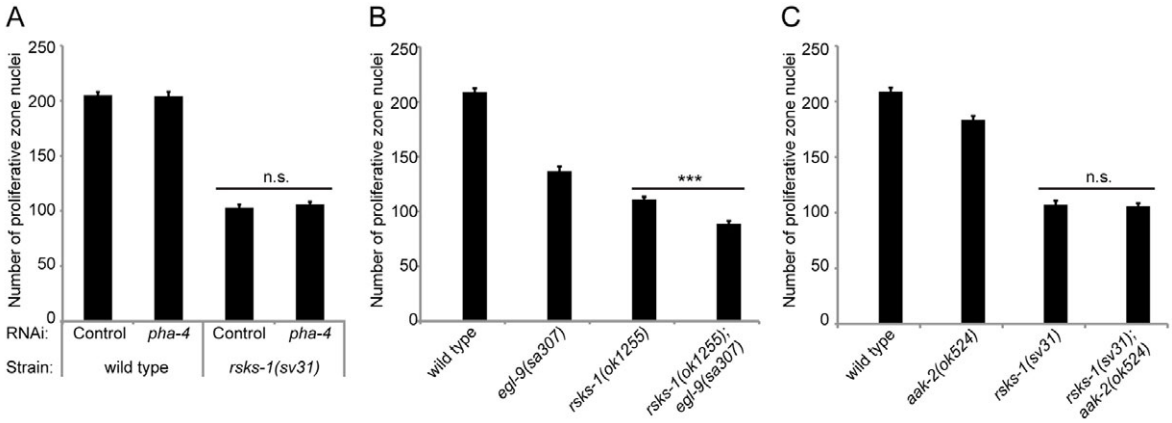


Fig. 6. The *rsks-1* germline and lifespan roles are genetically separable. Number of proliferative zone nuclei in early adult (**A**) wild-type and *rsks-1(sv31)* germ lines in animals treated with control or *pha-4* RNAi, (**B**) wild type, *egl-9(sa307)*, *rsks-1(ok1255)* and *rsks-1; egl-9* and (**C**) wild type, *aak-2(ok524)*, *rsks-1(sv31)* and *rsks-1; aak-2*. Although *rsks-1* and *rsks-1; egl-9* differ significantly, the relevant result is that the *rsks-1(–)* phenotype is not suppressed. Error bars indicate s.e.m. ***$P \leq 0.001$; n.s., not significant by two-tailed Student's *t*-test. See supplementary material Table S8 for data.

simply a consequence of a reduced starting progenitor pool as *glp-1(rf)* animals contain 67% fewer progenitors at 1×10^8 than at 1×10^9 cfu/ml, similar to the wild-type response. We also examined a genetic model of DR, *eat-2(ad1116)*, in which food intake is limited by decreased pharyngeal pumping (Raizen et al., 1995). The size of the germline progenitor pool in the *eat-2* mutant was ~50% of wild type (supplementary material Fig. S7). Again, this was attenuated (14%, as opposed to ~50%) in the absence of *rsks-1* (supplementary material Fig. S7). Together, these data are consistent with a model in which the effect of DR (via general nutrient limitation) on larval germline progenitors is largely mediated by *rsks-1*.

We further examined whether DR by bacterial dilution affects the cell cycle and cell fate in germline progenitors. We saw a significant effect on the mitotic index (Fig. 7B), but we did not see a strong effect on cell fate. Despite a severe reduction in cell numbers (Fig. 7A), none of the *glp-1(rf)* animals raised on 1×10^8 cfu/ml displayed an 'all meiotic' Glp-1 phenotype (n=104 arms). This interaction contrasts with the strong enhancement of this phenotype caused by loss of *rsks-1* (Fig. 3B; supplementary material Fig. S3), and suggests that DR predominately influences the progenitor cell cycle.

To test whether dietary amino acid levels regulate germline progenitors and to what extent this occurs through *rsks-1*, we used a genetic approach. In organisms ranging from humans to worms, intestinal transporters enable amino acid uptake (as either free amino acids or as di-/tri-peptides) from the diet (Daniel et al., 2006). PEPT-1, the *C. elegans* ortholog of mammalian PEPT1, acts as an oligopeptide transporter (Fei et al., 1998; Meissner et al., 2004). We found that adult *pept-1* null mutants contain significantly fewer germline progenitors than wild type. Importantly, similar to DR, additional removal of *rsks-1* does not exacerbate the defect, whereas *glp-1(rf)* does (Fig. 7C; supplementary material Table S9). Further, the mitotic index in *pept-1* was modestly lower than in wild type (1.6% versus 2.2%; Fig. 7D) and, similar to DR, but contrasting with *glp-1(rf); rsks-1*, the severely reduced proliferative zone in *glp-1(rf); pept-1(lg1601)* nevertheless always contained undifferentiated progenitors (n=147). Lastly, unlike the *pept-1* effect on lifespan (Spanier et al., 2010), the *pept-1* germline phenotype is not dependent on the nuclear hormone receptor *daf-12* (Fig. 7E). Consistent with a model in which insulin/IGF-like signaling and S6K act independently in the germ line, reducing *daf-12* also did not suppress the *rsks-1* mutant germline phenotype, although it suppressed the *daf-2* germline phenotype (Fig. 7E; supplementary material Table S9) (D. Dalfó, D. Michaelson and E.J.A.H., unpublished). We conclude that the effect of dietary amino acid uptake as mediated by *pept-1* on the proliferation of germline progenitors primarily influences cell cycle and is likely to act through *rsks-1*.

DISCUSSION

Our studies uncover a prominent role for the TOR-S6K pathway in the relationships between germline development, stem cell behavior and nutrition, and establish the *C. elegans* germ line as a model system with which to further explore these relationships. Our findings indicate that *rsks-1* (S6K) is required germline-autonomously for larval accumulation of germline progenitors, promoting both cell cycle progression and the proliferative fate. Although certain aspects of the *rsks-1* germline function are similar to the *glp-1* (Notch) and the *daf-2* (insulin receptor) pathways, *rsks-1* acts largely in parallel to both. In addition, germline *rsks-1* acts independently of genes known to influence the role of *rsks-1* in lifespan control. We found that *rsks-1* acts downstream of *let-363* (TOR), partially mediating the effects of the TORC1 complex, likely in parallel with eIF4E. Finally, we show that DR, and especially protein deprivation, limits germline progenitor cell accumulation, and that *rsks-1* plays a key role in this response. Fig. 7D presents a model of the influences of nutrition on germline progenitors. We propose that nutrient limitation influences cell cycle through *rsks-1* but that *rsks-1* influences cell fate independently. Distinct phospho substrates of RSKS-1 may mediate these differential effects of *rsks-1*.

Comparison between the influences of the insulin-IGF pathway and S6K in the *C. elegans* germ line

TOR and S6K are responsive to PI3K signaling in other systems, and our previous results implicate the insulin/IGF-like-PI3K signaling pathway in the control of germ cell proliferation in *C. elegans*. Although our *rsks-1* (S6K) studies employed null alleles, and the *daf-2* (IIR) studies utilized strong hypomorphs (Michaelson et al., 2010), our results suggest that while reducing *daf-2* or *rsks-1* leads to fewer adult germline progenitors, the phenotypes are distinct in several

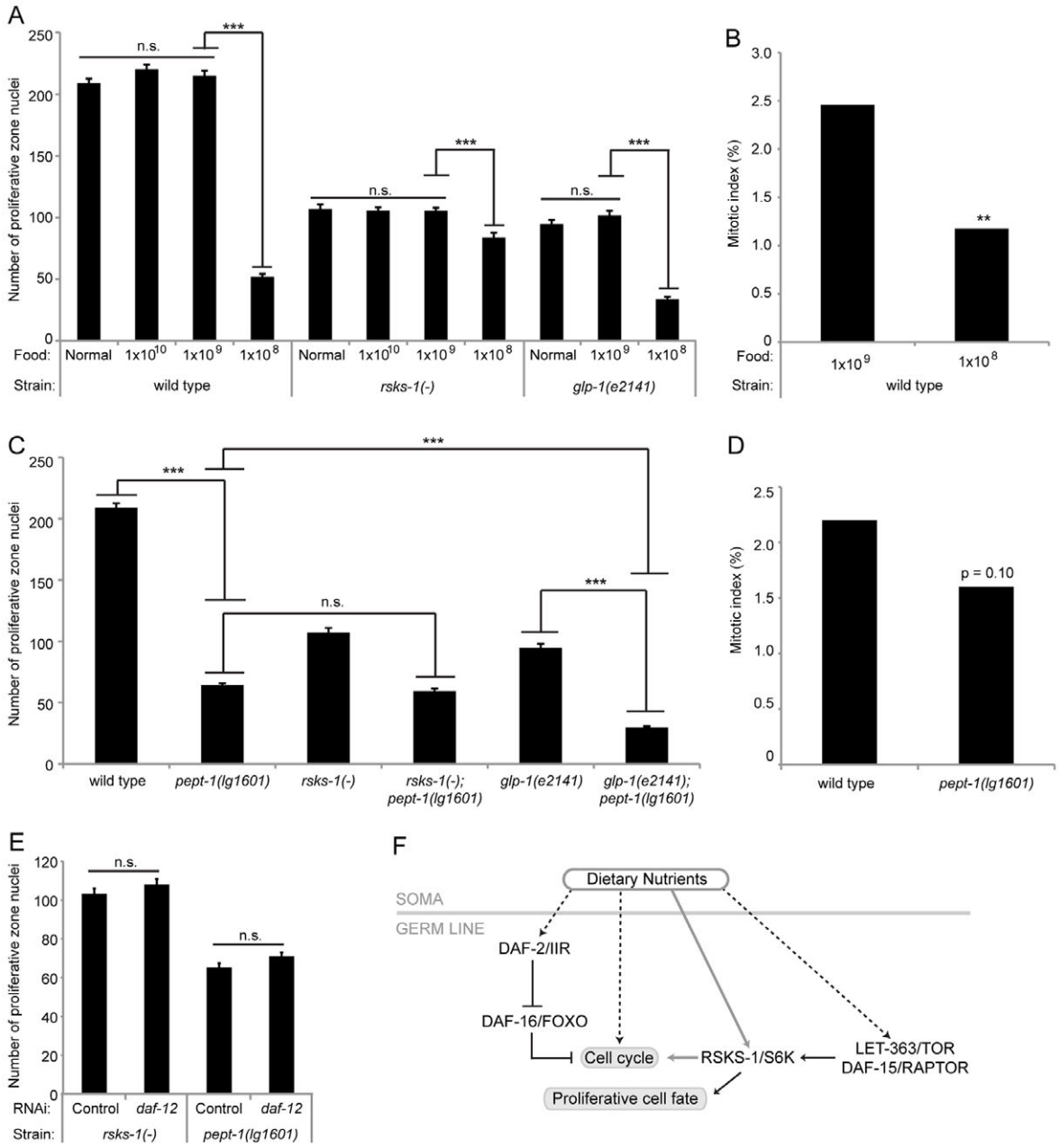


Fig. 7. Dietary restriction and protein deficit affect the germ line largely through *rsks-1*. (**A**) Number of proliferative zone nuclei in wild-type, *rsks-1* and *glp-1(e2141)* animals raised on the indicated bacterial concentrations (cfu/ml). (**B**) Mitotic index in mid-L4 stage gonad arms of the wild type raised on the indicated bacterial concentrations (cfu/ml). The number of gonad arms and germ cells analyzed is 1×10^9, 25 and 3174, and 1×10^8, 20 and 1274. (**C**) Number of proliferative zone nuclei in wild type, *pept-1(lg1601)*, *rsks-1*, *rsks-1; pept-1*, *glp-1(e2141)* and *glp-1; pept-1*. (**D**) Mitotic index in mid-L4 stage wild type and *pept-1(lg1601)*. Number of gonad arms and germ cells analyzed is 33 and 4988 for wild type and 31 and 1567 for *pept-1*. (**E**) *rsks-1* and *pept-1* animals treated with control or *daf-12* RNAi. *rsks-1(–)* refers to *rsks-1(sv31)*. ***$P\leq0.001$, **$P\leq0.01$; n.s., not significant by Student's *t*-test, except for three-sample comparisons in A by one-way ANOVA and in B,D by Mann-Whitney U test. Error bars indicate s.e.m. See supplementary material Table S9 for data. (**F**) Model for the effects of diet, insulin/IGF-like receptor (IIR), TOR/RAPTOR and S6K on larval germline progenitor cells. Solid gray lines depict the influence of diet on the cell cycle through S6K. Dashed lines indicate hypothesized relationships between nutrients, IIR and TOR, as well as the existence of other pathways that account for the residual effects of dietary restriction in *rsks-1(–)*. Other outputs from the TOR complex (not indicated) influence germline progenitor cells, likely through eIF4E.

respects. For example, although both influence the larval but not adult cell cycle, *rsks-1* promotes overall cell cycle progression, whereas *daf-2* is required for appropriate G2 progression. Additionally, loss of *rsks-1* dramatically enhances the penetrance of the 'all meiotic' phenotype of a *glp-1(rf)* mutant, whereas reduced *daf-2* does not, suggesting that S6K (but not IIR) inhibits differentiation. Moreover, *rsks-1* and *daf-2* have distinct roles in reproduction: at the semi-permissive temperature, *daf-2* mutants have normal brood sizes but extended reproductive periods (Dillin et al., 2002), whereas at the restrictive temperature they have severely reduced brood sizes (Michaelson et al., 2010). By contrast, the *rsks-1* mutant (which is not temperature sensitive) has a reduced brood size but a normal

reproductive period (Fig. 1A). Genetically, *rsks-1* does not act through *daf-16* (FOXO), whereas *daf-2* does, and the *daf-2 rsks-1* double-mutant phenotype is more severe than either single mutant. *rsks-1* is required for normal cell size whereas *daf-2* is not. Thus, similar to the insulin pathway-independent role of TOR in *Drosophila* germline stem cells (GSCs) (LaFever et al., 2010) (see below), our data are consistent with independent roles for germline S6K and IIR. These model organism studies underscore the importance of examining relationships between these pathways in vivo.

S6K may influence the germ line and lifespan through distinct downstream effectors in *C. elegans*

We found that the role of *rsks-1* in germline progenitors does not depend on the same genes that influence *rsks-1* in lifespan control. Reduced germline progenitor proliferation has been correlated with extended lifespan (Arantes-Oliveira et al., 2002), and the germline phenotype of the *rsks-1* mutant might influence longevity. Nevertheless, at least some of the genes required to elicit the lifespan-extension phenotype are not involved in the earlier generation of larval germline progenitors. This distinction further suggests that different targets of S6K are relevant in different phenotypic contexts.

A branched pathway downstream of TOR in the *C. elegans* germ line

We found that *rsks-1* (S6K) acts downstream of *let-363* (TOR) and *daf*-15 (RAPTOR) for proper larval expansion of the germline progenitor pool, but does not relay all of the germline functions of the complex. Consistent with a possible role for eIF4E acting downstream of TOR in parallel with S6K, we identified a prominent role for *ife-1*, which encodes an eIF4E paralog, in the regulation of germline progenitor accumulation. Additional studies are required to define this branch of the pathway, the cellular mechanisms by which it influences germline progenitor accumulation, and its precise relationship to TOR.

TOR signaling and the germ line in *C. elegans* and *Drosophila*

Recently, two laboratories demonstrated a role for TOR signaling in GSCs in the *Drosophila* ovary. Taken together with our results, the data suggest functional conservation of TOR-S6K signaling in the regulation of proliferation and differentiation. First, in *Drosophila*, TOR promotes GSC proliferation germline-autonomously, independent of insulin-like signaling through FOXO (LaFever et al., 2010). Similarly, *rsks-1* (S6K) acts within the germ line to promote larval germline cell cycle progression independently of *daf-16* (FOXO). However, whereas reducing TOR in both *C. elegans* and in *Drosophila* GSCs delays G2, loss of *C. elegans rsks-1* does not cause any change in the distribution of cells in different stages of the cell cycle. Our results are more similar to findings in the *Drosophila* wing imaginal disc, where loss of S6K slows overall cell cycle but does not affect the distribution of cells in specific phases of the cell cycle (Montagne et al., 1999). It remains to be determined whether S6K has a similar effect in fly GSCs. Furthermore, *rsks-1* promotes the proliferative (versus differentiated) fate in *C. elegans*. Similarly, TOR signaling influences germ cell differentiation in *Drosophila*, where, surprisingly, either reduced or elevated TOR activity results in GSC loss (LaFever et al., 2010; Sun et al., 2010). We hypothesize that the influence of TOR-S6K signaling on both the germ cell cycle and differentiation might be conserved between worms and flies. However, elevating TOR activity in the *C. elegans* germ line is not currently feasible, and no obvious homologs of TSC1 or TSC2 have yet been identified. Future studies are required to address this hypothesis.

Diet and stem cells

The insulin and TOR pathways have been implicated in the effect of diet on stem cell proliferation in several contexts, including *Drosophila* GSCs (LaFever et al., 2010; Sun et al., 2010), intestinal stem cells (Amcheslavsky et al., 2009; Biteau et al., 2010) and neural stem cells (via relay from TOR in the fat body, which promotes insulin-like peptide production in glial cells to activate neuronal stem cells) (Chell and Brand, 2010; Sousa-Nunes et al., 2011). Thus, although some aspects of the cellular and molecular mechanisms linking diet to stem cells may be context specific, their dependence on the nutrient-responsive insulin-like and TOR signaling pathways is likely to be conserved. These mechanisms are likely to have evolved to link nutritional and metabolic status to stem cell production, which for GSCs ultimately affects fertility and fecundity.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at
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References

Amcheslavsky, A., Jiang, J. and Ip, Y. T. (2009). Tissue damage-induced intestinal stem cell division in Drosophila. *Cell Stem Cell* **4**, 49-61.
Amiri, A., Keiper, B. D., Kawasaki, I., Fan, Y., Kohara, Y., Rhoads, R. E. and Strome, S. (2001). An isoform of eIF4E is a component of germ granules and is required for spermatogenesis in C. elegans. *Development* **128**, 3899-3912.
Arantes-Oliveira, N., Apfeld, J., Dillin, A. and Kenyon, C. (2002). Regulation of life-span by germ-line stem cells in Caenorhabditis elegans. *Science* **295**, 502-505.
Biteau, B., Karpac, J., Supoyo, S., Degennaro, M., Lehmann, R. and Jasper, H. (2010). Lifespan extension by preserving proliferative homeostasis in Drosophila. *PLoS Genet.* **6**, e1001159.
Bongaarts, J. (1980). Does malnutrition affect fecundity – a summary of evidence. *Science* **208**, 564-569.
Brenner, S. (1974). The genetics of Caenorhabditis elegans. *Genetics* **77**, 71-94.
Chell, J. M. and Brand, A. H. (2010). Nutrition-responsive glia control exit of neural stem cells from quiescence. *Cell* **143**, 1161-1173.
Chen, D., Thomas, E. L. and Kapahi, P. (2009). HIF-1 modulates dietary restriction-mediated lifespan extension via IRE-1 in Caenorhabditis elegans. *PLoS Genetics* **5**, e1000486.
Colman, R. J., Anderson, R. M., Johnson, S. C., Kastman, E. K., Kosmatka, K. J., Beasley, T. M., Allison, D. B., Cruzen, C., Simmons, H. A., Kemnitz, J. W. et al. (2009). Caloric restriction delays disease onset and mortality in rhesus monkeys. *Science* **325**, 201-204.
Daniel, H., Spanier, B., Kottra, G. and Weitz, D. (2006). From bacteria to man: archaic proton-dependent peptide transporters at work. *Physiology* **21**, 93-102.
Darby, C., Cosma, C. L., Thomas, J. H. and Manoil, C. (1999). Lethal paralysis of Caenorhabditis elegans by Pseudomonas aeruginosa. *Proc. Natl. Acad. Sci. USA* **96**, 15202-15207.

Dillin, A., Crawford, D. K. and Kenyon, C. (2002). Timing requirements for insulin/IGF-1 signaling in C. elegans. *Science* **298**, 830-834.
Drummond-Barbosa, D. (2008). Stem cells, their niches and the systemic environment: an aging network. *Genetics* **180**, 1787-1797.
Fei, Y. J., Fujita, T., Lapp, D. F., Ganapathy, V. and Leibach, F. H. (1998). Two oligopeptide transporters from Caenorhabditis elegans: molecular cloning and functional expression. *Biochem. J.* **332**, 565-572.
Fox, P. M., Vought, V. E., Hanazawa, M., Lee, M. H., Maine, E. M. and Schedl, T. (2011). Cyclin E and CDK-2 regulate proliferative cell fate and cell cycle progression in the C. elegans germline. *Development* **138**, 2223-2234.
Francis, R., Barton, M. K., Kimble, J. and Schedl, T. (1995a). gld-1, a tumor suppressor gene required for oocyte development in Caenorhabditis elegans. *Genetics* **139**, 579-606.
Francis, R., Maine, E. and Schedl, T. (1995b). Analysis of the multiple roles of gld-1 in germline development: interactions with the sex determination cascade and the glp-1 signaling pathway. *Genetics* **139**, 607-630.
Greer, E. L. and Brunet, A. (2009). Different dietary restriction regimens extend lifespan by both independent and overlapping genetic pathways in C. elegans. *Aging Cell* **8**, 113-127.
Gumienny, T. L., Lambie, E., Hartwieg, E., Horvitz, H. R. and Hengartner, M. O. (1999). Genetic control of programmed cell death in the Caenorhabditis elegans hermaphrodite germline. *Development* **126**, 1011-1022.
Hansen, D. and Schedl, T. (2006). The regulatory network controlling the proliferation-meiotic entry decision in the Caenorhabditis elegans germ line. *Curr. Top. Dev. Biol.* **76**, 185-215.
Hansen, D., Hubbard, E. J. A. and Schedl, T. (2004). Multi-pathway control of the proliferation versus meiotic development decision in the Caenorhabditis elegans germline. *Dev. Biol.* **268**, 342-357.
Hansen, M., Taubert, S., Crawford, D., Libina, N., Lee, S. J. and Kenyon, C. (2007). Lifespan extension by conditions that inhibit translation in Caenorhabditis elegans. *Aging Cell* **6**, 95-110.
Henderson, M. A., Cronland, E., Dunkelbarger, S., Contreras, V., Strome, S. and Keiper, B. D. (2009). A germline-specific isoform of eIF4E (IFE-1) is required for efficient translation of stored mRNAs and maturation of both oocytes and sperm. *J. Cell Sci.* **122**, 1529-1539.
Hietakangas, V. and Cohen, S. M. (2009). Regulation of tissue growth through nutrient sensing. *Annu. Rev. Genet.* **43**, 389-410.
Hubbard, E. J. (2007). Caenorhabditis elegans germ line: a model for stem cell biology. *Dev. Dyn.* **236**, 3343-3357.
Inoki, K., Li, Y., Zhu, T., Wu, J. and Guan, K. L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat. Cell Biol.* **4**, 648-657.
Jia, K., Chen, D. and Riddle, D. L. (2004). The TOR pathway interacts with the insulin signaling pathway to regulate C. elegans larval development, metabolism and life span. *Development* **131**, 3897-3906.
Jones, A. R. and Schedl, T. (1995). Mutations in gld-1, a female germ cell-specific tumor suppressor gene in Caenorhabditis elegans, affect a conserved domain also found in Src-associated protein Sam68. *Genes Dev.* **9**, 1491-1504.
Kalaany, N. Y. and Sabatini, D. M. (2009). Tumours with PI3K activation are resistant to dietary restriction. *Nature* **458**, 725-731.
Kamath, R. S., Fraser, A. G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M. et al. (2003). Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. *Nature* **421**, 231-237.
Keiper, B. D., Lamphear, B. J., Deshpande, A. M., Jankowska-Anyszka, M., Aamodt, E. J., Blumenthal, T. and Rhoads, R. E. (2000). Functional characterization of five eIF4E isoforms in Caenorhabditis elegans. *J. Biol. Chem.* **275**, 10590-10596.
Killian, D. J. and Hubbard, E. J. (2005). Caenorhabditis elegans germline patterning requires coordinated development of the somatic gonadal sheath and the germ line. *Dev. Biol.* **279**, 322-335.
Kimble, J. and Crittenden, S. L. (2007). Controls of germline stem cells, entry into meiosis, and the sperm/oocyte decision in Caenorhabditis elegans. *Annu. Rev. Cell Dev. Biol.* **23**, 405-433.
Korta, D. Z. and Hubbard, E. J. A. (2010). Soma-germline interactions that influence germline proliferation in Caenorhabditis elegans. *Dev. Dyn.* **239**, 1449-1459.
Kritchevsky, D. (1999). Caloric restriction and experimental carcinogenesis. *Toxicol. Sci.* **52**, 13-16.
LaFever, L., Feoktistov, A., Hsu, H. J. and Drummond-Barbosa, D. (2010). Specific roles of Target of rapamycin in the control of stem cells and their progeny in the Drosophila ovary. *Development* **137**, 2117-2126.
Lin, K., Dorman, J. B., Rodan, A. and Kenyon, C. (1997). daf-16: an HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. *Science* **278**, 1319-1322.
Long, X., Spycher, C., Han, Z. S., Rose, A. M., Muller, F. and Avruch, J. (2002). TOR deficiency in C. elegans causes developmental arrest and intestinal atrophy by inhibition of mRNA translation. *Curr. Biol.* **12**, 1448-1461.
Mango, S. E., Sheaffer, K. L. and Updike, D. L. (2008). The Target of Rapamycin pathway antagonizes pha-4/FoxA to control development and aging. *Curr. Biol.* **18**, 1355-1364.
McGovern, M., Voutev, R., Maciejowski, J., Corsi, A. K. and Hubbard, E. J. A. (2009). A 'latent niche' mechanism for tumor initiation. *Proc. Natl. Acad. Sci. USA* **106**, 11617-11622.
Meissner, B., Boll, M., Daniel, H. and Baumeister, R. (2004). Deletion of the intestinal peptide transporter affects insulin and TOR signaling in Caenorhabditis elegans. *J. Biol. Chem.* **279**, 36739-36745.
Michaelson, D., Korta, D. Z., Capua, Y. and Hubbard, E. J. (2010). Insulin signaling promotes germline proliferation in C. elegans. *Development* **137**, 671-680.
Montagne, J., Stewart, M. J., Stocker, H., Hafen, E., Kozma, S. C. and Thomas, G. (1999). Drosophila S6 kinase: a regulator of cell size. *Science* **285**, 2126-2129.
Narbonne, P., Hyenne, V., Li, S., Labbe, J. C. and Roy, R. (2010). Differential requirements for STRAD in LKB1-dependent functions in C. elegans. *Development* **137**, 661-670.
Pan, K. Z., Palter, J. E., Rogers, A. N., Olsen, A., Chen, D., Lithgow, G. J. and Kapahi, P. (2007). Inhibition of mRNA translation extends lifespan in Caenorhabditis elegans. *Aging Cell* **6**, 111-119.
Pepper, A. S. R., Killian, D. J. and Hubbard, E. J. A. (2003a). Genetic analysis of Caenorhabditis elegans glp-1 mutants suggests receptor interaction or competition. *Genetics* **163**, 115-132.
Pepper, A. S. R., Lo, T. W., Killian, D. J., Hall, D. H. and Hubbard, E. J. A. (2003b). The establishment of Caenorhabditis elegans germline pattern is controlled by overlapping proximal and distal somatic gonad signals. *Dev. Biol.* **259**, 336-350.
Praitis, V., Casey, E., Collar, D. and Austin, J. (2001). Creation of low-copy integrated transgenic lines in Caenorhabditis elegans. *Genetics* **157**, 1217-1226.
Priess, J. R., Schnabel, H. and Schnabel, R. (1987). The Glp-1 locus and cellular interactions in early C. elegans embryos. *Cell* **51**, 601-611.
Raizen, D. M., Lee, R. Y. and Avery, L. (1995). Interacting genes required for pharyngeal excitation by motor neuron MC in Caenorhabditis elegans. *Genetics* **141**, 1365-1382.
Riddle, D. L., Swanson, M. M. and Albert, P. S. (1981). Interacting genes in nematode dauer larva formation. *Nature* **290**, 668-671.
Rous, P. (1914). The influence of diet on transplanted and spontaneous mouse tumors. *J. Exp. Med.* **20**, 433-451.
Rual, J. F., Ceron, J., Koreth, J., Hao, T., Nicot, A. S., Hirozane-Kishikawa, T., Vandenhaute, J., Orkin, S. H., Hill, D. E., van den Heuvel, S. et al. (2004). Toward improving Caenorhabditis elegans phenome mapping with an ORFeome-based RNAi library. *Genome Res.* **14**, 2162-2168.
Russell, R. C., Fang, C. and Guan, K. L. (2011). An emerging role for TOR signaling in mammalian tissue and stem cell physiology. *Development* **138**, 3343-3356.
Schalm, S. S. and Blenis, J. (2002). Identification of a conserved motif required for mTOR signaling. *Curr. Biol.* **12**, 632-639.
Selman, C., Tullet, J. M., Wieser, D., Irvine, E., Lingard, S. J., Choudhury, A. I., Claret, M., Al-Qassab, H., Carmignac, D., Ramadani, F. et al. (2009). Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. *Science* **326**, 140-144.
Shi, A., Sun, L., Banerjee, R., Tobin, M., Zhang, Y. and Grant, B. D. (2009). Regulation of endosomal clathrin and retromer-mediated endosome to Golgi retrograde transport by the J-domain protein RME-8. *EMBO J.* **28**, 3290-3302.
Sijen, T., Fleenor, J., Simmer, F., Thijssen, K. L., Parrish, S., Timmons, L., Plasterk, R. H. and Fire, A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* **107**, 465-476.
Sousa-Nunes, R., Yee, L. L. and Gould, A. P. (2011). Fat cells reactivate quiescent neuroblasts via TOR and glial insulin relays in Drosophila. *Nature* **471**, 508-512.
Spanier, B., Rubio-Aliaga, I., Hu, H. and Daniel, H. (2010). Altered signalling from germline to intestine pushes daf-2;pept-1 Caenorhabditis elegans into extreme longevity. *Aging Cell* **9**, 636-646.
Stiernagle, T. (2006). Maintenance of C. elegans. In *WormBook* (ed. The C. elegans Research Community). www.wormbook.org.
Sun, P., Quan, Z. H., Zhang, B. D., Wu, T. Q. and Xi, R. W. (2010). TSC1/2 tumour suppressor complex maintains Drosophila germline stem cells by preventing differentiation. *Development* **137**, 2461-2469.
Tannenbaum, A. and Silverstone, H. (1953). Nutrition in relation to cancer. *Adv. Cancer Res.* **1**, 451-501.
Timmons, L., Court, D. L. and Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. *Gene* **263**, 103-112.
Vellai, T., Takacs-Vellai, K., Zhang, Y., Kovacs, A. L., Orosz, L. and Muller, F. (2003). Genetics: influence of TOR kinase on lifespan in C. elegans. *Nature* **426**, 620.
Wang, X. and Proud, C. G. (2006). The mTOR pathway in the control of protein synthesis. *Physiology (Bethesda)* **21**, 362-369.
Wang, X. and Proud, C. G. (2009). Nutrient control of TORC1, a cell-cycle regulator. *Trends Cell Biol.* **19**, 260-267.
Wullschleger, S., Loewith, R. and Hall, M. N. (2006). TOR signaling in growth and metabolism. *Cell* **124**, 471-484.