CORRECTION

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

Dorota Z. Korta, Simon Tuck and E. Jane Albert Hubbard

There were errors published in Development 139, 859-870.

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.
© 2012. Published by The Company of Biologists Ltd

S6K links cell fate, cell cycle and nutrient response in C. elegans germ line/progenitor cells

Dorota Z. Korta¹, Simon Tuck² and E. Jane Albert Hubbard¹,*

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 germ line 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 germ line 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 germ line defects similar to a subset of rsks-1 mutant phenotypes. Consistent with a link between diet and germ line 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 germ line 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 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/P13K 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 germ line proliferation to generate an appropriate progenitor pool for optimal fecundity (Michaelsen 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).

¹Developmental Genetics Program, Helen and Martin Kimmel Center for Stem Cell Biology, Skirball Institute of Biomolecular Medicine, Department of Pathology, New York University School of Medicine, New York, NY 10016, USA. ²Umeå Center for Molecular Medicine, Umeå University, Umeå, SE-901 87, Sweden.

*Author for correspondence (jane.hubbard@med.nyu.edu)

Accepted 24 December 2011
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 anddaf-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-ethyl-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 glp-1 RNAi-induced tumors, N2 and rsks-1(sv31) animals were grown at 20°C, synchronized by hatch-off, fed glp-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 levamasole and mounted on agar pads. Body length was measured on images captured at 50X 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 630X, 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 π r³), with radius extrapolated from the measured area (π r²). 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 1×10⁷ cfu/ml. Bacteria were resuspended in S Basal and diluted (1×10⁸ and 1×10⁷ cfu/ml) or concentrated (1×10⁹ 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 (Stemnagle, 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 1×10⁷ 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 ref-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
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 \textit{rsks-1}(+) solely in the germ line largely rescued the germline progenitor defect (Fig. 1F; supplementary material Table S3).

\textbf{\textit{rsks-1} promotes overall cell cycle progression of larval germ cells}

We examined three possible mechanisms by which \textit{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 \textit{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 \textit{rsks-1} mutant.

When we examined the cell cycle we found that the larval, but not adult, germline progenitor cell cycle was delayed in \textit{rsks-1(sv31)}. Specifically, at the mid-L4 stage, the mitotic and S-phase indexes were significantly reduced in \textit{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

---

**Fig. 1. \textit{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 \textit{rsks-1} (n≥14 for each time point). (C) Average number of proliferative zone nuclei in early adult wild type, \textit{rskn-1}, and two null alleles of \textit{rsks-1}. (D) Representative DAPI-stained mid-L4 and early adult wild-type and \textit{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 \textit{nrf-1} treated with control and \textit{rsks-1} RNAi (E), and wild type, \textit{rsks-1}, \textit{rsks-1; svb64[somatic \textit{rsks-1}(+)]} and \textit{rsks-1; nabs44[pGC520, germline \textit{rsks-1}(+)]} (F). (G) Body length (mm) of wild type, \textit{rsks-1} and \textit{rsks-1; svb64[somatic \textit{rsks-1}(+)]}. **P<0.01, ***P<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.
distributions of rsks-1(sv31) and wild-type germine progenitor cells within each cell cycle phase (Fig. 2C). These results suggest that rsks-1 promotes the larval but not adult germine 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 germine tumors behave like larval germine 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 germine 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 glp-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 germine tumors, supporting the hypothesis that adult proximal germine tumors share features with larval germine 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 germine 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 germine 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<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-I (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

**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<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. 3E,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,
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; *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.

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.
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 $1 \times 10^8$ cfu/ml bacteria displayed 76% fewer progenitors than siblings grown on $1 \times 10^9$ 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 $1 \times 10^8$ cfu/ml contained only 20% fewer germline progenitors than siblings raised on $1 \times 10^9$ 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<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 1×10⁸ than at 1×10⁹ 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 1×10⁸ 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 ortholog of mammalian PEPT1, acts as an 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 elf4E. 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
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

---

**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 x 10⁸, 25 and 3174, and 1 x 10⁹, 20 and 1274. (C) Number of proliferative zone nuclei in wild type, pept-1(g1601), rsks-1, pept-1, glp-1(e2141) and glp-1, pept-1. (D) Mitotic index in mid-L4 stage wild type and pept-1(g1601). 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<0.001, **P<0.01; 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.
reproductive period (Fig. 1A). Genetically, *rsk-1* does not act through *daf-16* (FOXO), whereas *daf-2* does, and the *daf-2 rsk-1* double-mutant phenotype is more severe than either single mutant. *rsk-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 IR. 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 *rsk-1* in germline progenitors does not depend on the same genes that influence *rsk-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 *rsk-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 *rsk-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, *rsk-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 rsk-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, *rsk-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.

**Acknowledgements**

We thank David Michaelson for preliminary data; Josefiv Friberg and Ming Sheng (Umeå, Sweden) for sharing observations concerning the reduced fertility of *rsk-1* mutants; Agneta Rönnlund (Umeå, Sweden) for help in the isolation of *rsk-1* (sv40); Vytas Bindokas (Chicago, IL, USA) for modification of an Imagej plug-in; Di Chen and Pankaj Kapahi (Novato, CA, USA), Richard Roy (Montreal, Quebec, Canada), Brett Keiper (Greenville, NC, USA), Ann Wehman and Jeremy Nance (New York, NY, USA), the *C. elegans* Gene Knockout Consortium, (Oklahoma City, OK, USA and Vancouver, Canada), the Caenorhabditis Genetics Center (CGC, Minneapolis, MN, USA) and Wormbase (www.wormbase.org) for reagents and information; and Robert Schneider and members of the Hubbard and Nance labs for helpful discussions.

**Funding**

Funding was provided by grants ST232GM7308, F30DK089679 and R01GM061706 from the National Institutes of Health, Cancerfonden (090507) and Vetenskapsrådet (K2009-67X-20441-03-3). Deposited in PMC for release after 12 months.

**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/doi/10.1242/dev.074047-fDC1

**References**


