

Inhibition of germline proliferation during *C. elegans* dauer development requires PTEN, LKB1 and AMPK signalling

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In *C. elegans*, reduced insulin-like signalling induces developmental quiescence, reproductive delay and lifespan extension. We show here that the *C. elegans* orthologues of LKB1 and AMPK cooperate during conditions of reduced insulin-like signalling to establish cell cycle quiescence in the germline stem cell population, in addition to prolonging lifespan. The inactivation of either protein causes aberrant germline proliferation during diapause-like 'dauer' development, whereas the loss of AMPK uncouples developmental arrest from lifespan extension. Reduced TGF- β activity also triggers developmental quiescence independent of the insulin-like pathway. Our data suggest that these two signalling pathways converge on the *C. elegans* PTEN orthologue to coordinate germline proliferation with somatic development during dauer formation, via the regulation of AMPK and its upstream activator LKB1, rather than through the canonical insulin-like signalling cascade. In humans, germline mutations in TGF- β family members, PTEN or LKB1 result in related tumour-predisposing syndromes. Our findings establish a developmental relationship that may underscore their shared, characteristic aetiology.

KEY WORDS: Insulin, TGF- β , AMPK, PTEN, LKB1, Cell cycle, Lifespan, Dauer, Germ line, *C. elegans*

INTRODUCTION

Following embryogenesis in *C. elegans*, the first stage (L1) larvae emerge with a gonad primordium that contains four progenitor cells referred to as Z1, Z2, Z3 and Z4. In the hermaphrodite, the somatic gonad precursors Z1 and Z4 will undergo multiple divisions to give rise to the tissues that will make up the two U-shaped arms of the adult somatic gonad, which are delineated distally by the distal tip cells (DTCs) and proximally by a shared uterus (Hubbard and Greenstein, 2000). The other two cells present in the primordium (Z2, Z3) are the germ cell precursors and, unlike the divisions of the somatic gonadal precursors, which are essentially invariant from one animal to another, Z2 and Z3 do not demonstrate the same stereotypic division pattern and/or division timing as they proliferate to form the germ lineage (Kimble and Hirsh, 1979).

Even though the *C. elegans* germ line develops as a syncytium, individual germline nuclei and their surrounding cytoplasm can be referred to as germ cells (Hubbard and Greenstein, 2000). Sustained proliferation of the germ cell precursors and their daughters during larval development requires signalling through a Notch receptor. The Notch ligand (LAG-2) is expressed in the somatic DTCs, and through the activation of a Notch receptor (GLP-1) present in the germ cells it inhibits them from executing their alternative meiotic pathway, while instructing them to proliferate (Kimble and Crittenden, 2005). Initially, owing to the influence of a nearby DTC, the germline stem cell population expands until the late L3 larval stage. At this point, however, the proximal-most germ cells no longer receive the Notch-dependent proliferative signal provided by the DTCs, and therefore they execute the alternative meiotic pathway. In hermaphrodite animals, a transient period of spermatogenesis is initiated, followed by a switch to oogenesis in the L4 larval stage (Ellis and Schedl, 2006).

Germline proliferation therefore proceeds uninterrupted in the distal germ line during post-embryonic development, but can be pre-empted when environmental conditions are sensed to be inappropriate to sustain reproductive development during the L1 larval stage. Three parallel signals, including reduced insulin-like signalling, can independently generate a neuro-endocrine signal that triggers a developmental switch, instructing the larva to execute an alternative diapause-like stage referred to as 'dauer'. In anticipation of initiating the dauer pathway, the second larval (L2) stage is extended (L2d), allowing the larva to prepare for nutrient deprivation by slowing development and metabolism, while storing energy (Riddle and Albert, 1997). One important feature of this developmental switch is the progressive establishment of a generalized cell cycle arrest that is maintained thereafter, presumably to conserve resources, thus rendering the dauer larva specialized for long-term survival.

The transcriptional targets of the downstream effectors that mediate dauer development identified to date include genes involved primarily in metabolic, antimicrobial and stress-response pathways (McElwee et al., 2003; Murphy et al., 2003; Shostak et al., 2004). Although much interest has been focused on the regulation of adult lifespan by some of these factors, the key to the extreme longevity and developmental quiescence of the dauer juvenile remains largely unclear. Most importantly, how the three parallel signalling pathways (insulin-like, TGF- β , cGMP) that control dauer formation interact and presumably converge on the same downstream targets in order to specify the behavioural, morphological, physiological and metabolic changes associated with this stage is not well understood.

Through our analysis of the mechanisms involved in controlling germ cell divisions during the establishment of developmental quiescence, we found that the independent signals that promote dauer formation converge to control the activity of the tumour suppressor gene *daf-18/PTEN*, an upstream component of the insulin-like signalling pathway. The regulation of germline proliferation, however, diverges from the canonical insulin-like targets Akt/PKB and *daf-16/FOXO*, but requires the tumour

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suppressor *par-4/LKB1* and its downstream target AMP-activated kinase (*aak-1*, *aak-2*/AMPK) to appropriately arrest proliferation of the germline stem cell population in response to the environmental signals that induce dauer development.

MATERIALS AND METHODS

C. elegans genetics

All nematode strains were maintained at 15°C and grown on standard NGM plates seeded with *E. coli* (HB101) (Brenner, 1974), unless otherwise stated. The Bristol (N2) strain was used as wild type throughout. The following alleles, rearrangements and transgenes were used. LGI: *daf-16(mgDf47, mgDf50)*, *rrf-1(pk1417)*, *szT1(lon-2(e678))*. LGIII: *daf-2(e1370)*, *daf-7(e1372)*, *unc-32(e189)*, *glp-1(oz112, q175, e2141)*, *unc-36(e251)*, *pha-1(e2123)*. LGIV: *daf-18(e1375, ok480)*. LGV: *qls56(lag-2p::GFP; unc-119(+))*, *akt-1(mg144)*, *him-5(e1467)*, *par-4(it47, it57)*. LGX: *aak-2(rr48, ok524)*, *unc-1(e719)*, *unc-7(e5)*, *mec-4(u52)*, *lin-15(n765)*, *yDf1*, *szT1*. *qEx308(lag-2p::LAG-2::GFP; rol-6(su1006))*, *arEx645(pha-1(+); lag-2p::CFP::lacZ; celh-22::GFP)* (Horvitz et al., 1979).

aak-2(ok524) is a predicted molecular null allele in which part of the catalytic domain is deleted; this introduces a premature stop codon shortly after the breakpoint, thus eliminating the conserved regulatory domain (Apfeld et al., 2004). The deletion in *daf-18(ok480)* is predicted to remove part of the C-terminal regulatory domain, while introducing a premature stop codon after the breakpoint.

Genetic screen for regulators of germ cell proliferation during dauer development

daf-2(e1370) is a temperature-sensitive hypomorphic allele of the *C. elegans* insulin-like receptor, which allows reproductive development at the permissive temperature (15°C), but induces dauer formation constitutively at the restrictive temperature (25°C) (Albert and Riddle, 1988; Kimura et al., 1997). The F1 progeny of EMS mutagenized *daf-2(e1370)*; *qls56* L4 larvae were reared at 15°C until the adult stage, at which point they were distributed five per plate and up-shifted to 25°C. F2 dauer larvae were subsequently screened for gonad enlargement using *lag-2::GFP*, the DTC expression of which delineated the distal extremes of the dauer gonad. Using this approach, we screened 3800 haploid genomes and isolated one mutant (*rr48*) that demonstrated dauer germline hyperplasia and dauer lethality. This mutant was outcrossed five times prior to any subsequent analysis.

Mapping and cloning of *rr48*

Using conventional linkage analysis, followed by three-factor crosses, *rr48* was mapped to the far right of LGX. After crossing *daf-2*; *rr48* males into *daf-2*; *unc-7 mec-4* hermaphrodites, we found 0/59 Mec non-Unc F2 recombinants that produced *rr48* F3 progeny, whereas 15/15 Unc non-Mec produced *rr48* F3 animals, indicating that *rr48* is tightly linked to, or to the right of, *mec-4*, at the far right of LGX. The *rr48* map position was refined using transformation rescue. The YAC clone Y53B5, which is covered by seven overlapping cosmids, rescued *rr48*. The cosmids were injected in three overlapping pools of three, and the second and third pools both rescued *rr48*. The cosmid T01C8 was common to both pools, and contains five open reading frames. T01C8.1, a predicted *C. elegans* orthologue of the mammalian $\alpha 2$ catalytic subunit of AMP-activated kinase (*aak-2*), was an obvious candidate as its yeast and mammalian orthologues have been implicated in cell cycle/growth control (Hardie, 2005). From T01C8, we sub-cloned an 11.1 kb fragment (*NcoI/PstI*) containing *aak-2* into pGEM-T and injected it (10 ng/ μ l) into *daf-2*; *rr48* hermaphrodites, rescuing the dauer lethality phenotype. To identify the molecular lesion in *rr48*, we cloned and sequenced *aak-2* cDNA from mutant animals. In three different clones obtained from two independent RT-PCR reactions, we found a typical EMS-induced G/C to A/T transition at position 1 of the predicted codon 208. This mutation is predicted to substitute a conserved histidine with a tyrosine residue in a highly conserved region of the catalytic domain. Furthermore, *aak-2(ok524)* and *aak-2(RNAi)* phenocopied the dauer germline hyperplasia and dauer lethality of *rr48* mutants, confirming our positional cloning results.

Staining

For whole worm DAPI (4',6-diamidino-2-phenylindole) staining, animals were washed off plates and soaked in Carnoy's solution (60% ethanol, 30% acetic acid, 10% chloroform) on a shaker overnight. Animals were then washed twice in PBST (1×PBS + 0.1% Tween 20), and stained in 0.1 mg/ml DAPI solution for 30 minutes. Finally, larvae were washed four times (20 minutes each) in PBST, and mounted in Vectashield (Vector Laboratories) medium.

For extruded dauer gonad staining, gonads were dissected, fixed and stained as described elsewhere (Arduengo et al., 1998). Primary rabbit polyclonal anti-GLP-1, rabbit polyclonal anti-P-granule (anti-PGL-1) and mouse monoclonal 1CB4, and secondary anti-rabbit FITC and anti-mouse Texas-Red-conjugated (Invitrogen) antibodies were used. DAPI was used as a counterstain.

Germ cell nuclei counts

DAPI staining was performed on a synchronized (by hatching in the absence of food) population of L1 larvae plated and grown at 25°C until the appropriate stage/time was reached, except for RNAi experiments, where adult animals were allowed to lay eggs at 15°C overnight before the plate was transferred to 25°C, and the adults killed. The total number of germ cell nuclei per hermaphrodite gonad was then determined, based on their position and nuclear morphology. Animals in which the proximal germ cells had developed into spermatids were excluded from this analysis.

Dauer longevity assay

Because dauer larvae have a tendency to crawl off the bacterial lawn, and often desiccate on the edges of the plate, we developed a novel method to monitor the survival of individual dauer larva. Briefly, *C. elegans* were synchronized and plated at 25°C. Three days later, ~10 dauer larvae were randomly picked into a 20 μ l drop of double-distilled H₂O suspended under a Petri dish cover. We refer to this system as 'the dauer trap'. A wet tissue was placed in the bottom of the dish to maintain humidity, and the plate was sealed with Parafilm. Dauer longevity was monitored daily, and survival was scored as moving response upon exposure to a focused beam of 425–440 nm light. For the ablation experiments, *unc-1(e719)* was introduced in the background of each strain to prevent dauer larvae from crawling off the agar and survival was scored daily as response to prodding.

Dauer recovery assay

Worms were synchronized and plated at 25°C for ~50 hours. Dauer larvae were then picked to fresh, pre-acclimated plates at 25°C in cohorts of 100 individuals. Recovery was monitored daily. In Fig. 2B, *him-5(e1467)* was in the background of *daf-7(e1372)*; *aak-2(ok524)*.

All other techniques, such as microscopy (Kostic et al., 2003), adult longevity and dauer formation assays (both at 25°C) (Hertweck et al., 2004), laser ablation, *aak-1* RNAi (Kostic et al., 2003), and *aak-2* RNAi (Kamath and Ahringer, 2003), were performed as previously described.

RESULTS

Dauer-dependent germ cell cycle quiescence is mediated by *aak-2*

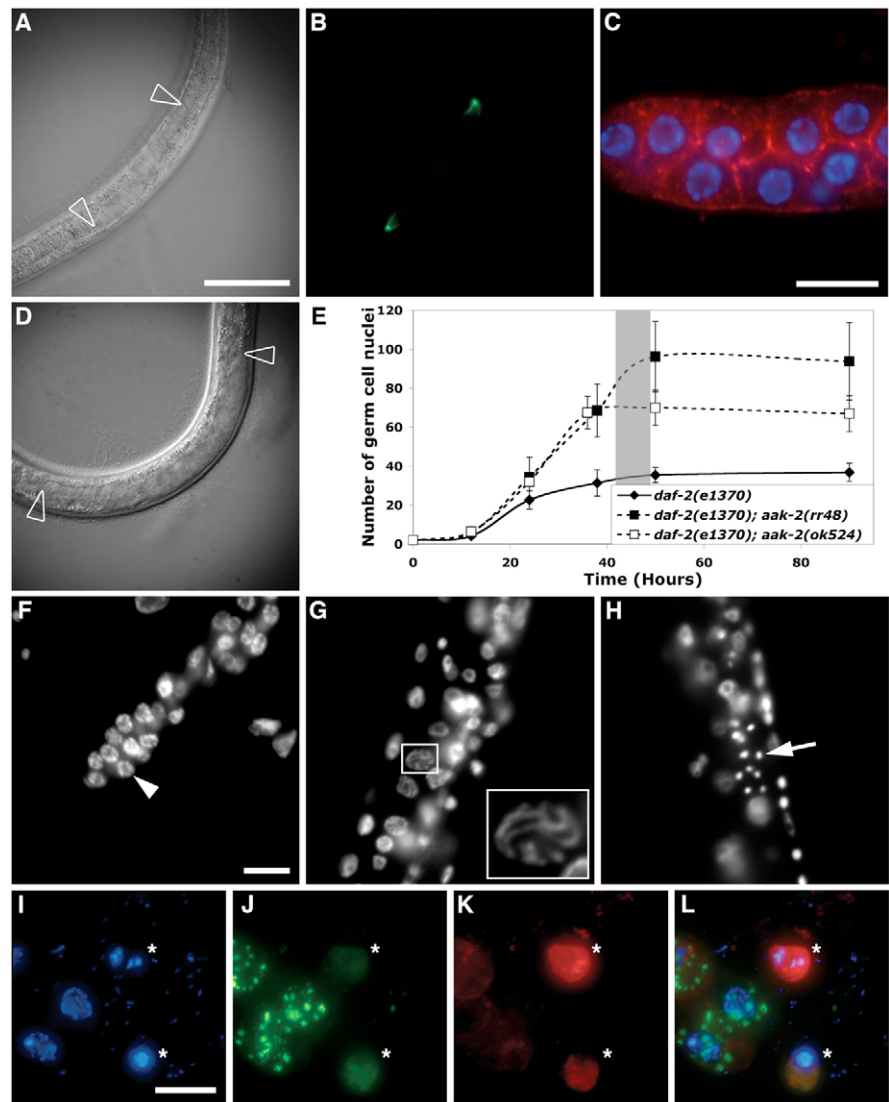
Paradoxically, we noticed that the expression of the Notch ligand remains strong in the DTCs during the dauer stage (Fig. 1A,B), whereas GLP-1 protein is present in the germ line of the dauer larvae (Fig. 1C). Notch should therefore be activated and, with meiosis being repressed, the germ cells would be predicted to still be proliferating during this stage. However, ongoing cellular divisions are never observed in the germ line of dauer larvae, even in *glp-1(oz112)gf* mutants (data not shown) in which Notch signalling is constitutively active (Berry et al., 1997). Hence, we reasoned that mitosis must be inhibited in the germ line downstream of, or in parallel to, *lag-2*, by signals that promote dauer formation.

To better understand how the dauer-associated cell cycle quiescence is established in the germline stem cell population through compromised insulin-like signalling, we performed a genetic screen to isolate mutants defective in regulating germline

Fig. 1. *aak-2* larvae are defective in establishing germline quiescence under compromised insulin-like signalling.

(A) Open arrowheads delineate the distal extremities of the gonad of *daf-2(e1370)* dauer larvae. (B) An integrated *lag-2p::GFP* transgene (*qls56*) is strongly expressed in the DTCs throughout the dauer stage. A *lag-2p::CFP* transgene (*arEx645*) containing the full 6.2 kb promoter (Chen and Greenwald, 2004) was also strongly expressed in the DTCs of dauer larvae (data not shown). We similarly found strong GFP expression in the DTCs of *qEx308* dauer larvae (data not shown), which contain a functional *lag-2p::LAG-2::GFP lag-2(q411)* rescuing construct (S. Crittenden and J. Kimble, personal communication). (C) Extruded *daf-2(e1370)* dauer gonad stained with DAPI (blue) and anti-GLP-1 antibodies (red). (D) Enlarged gonad of *daf-2(e1370); aak-2(rr48)* dauer larvae. (E) *aak-2* is required to progressively suppress germline proliferation during L2d/dauer formation. Grey zone indicates the period during which >90% of the larvae undergo the dauer moult. The dauer moult was delayed by ~1 and ~3 hours in *aak-2(ok524)* and *aak-2(rr48)* mutants, respectively. Error bars represent standard deviation, and the sample size was ≥ 25 for each point. (F) In both *daf-2(e1370)* and *daf-7(e1372)* larvae, germ cells arrest in mitotic interphase during dauer (arrowhead), as shown in a DAPI-stained, extruded *daf-7(e1372)* dauer gonad. (G) In *daf-2(e1370) glp-1(e2141)* dauer larvae, germ cell nuclei arrest in late stage meiotic prophase I, the nuclear size and chromosome morphology being consistent with the pachytene stage (inset). (H) Germ cells often (26/30 animals) progress further through meiosis in *daf-2(e1370) glp-1(e2141); aak-2(rr48)* dauer larvae, and their chromosomal morphology closely resembles that of spermatids (arrow).

Similar defects were observed in *daf-2(e1370) glp-1(e2141); aak-2(ok524)* (19/40 animals) and in *daf-2(e1370) glp-1(e2141); par-4(it57)* (25/30 animals) dauer larvae. (I-L) Extruded *daf-2(e1370) glp-1(e2141); aak-2(rr48)* dauer gonad. Asterisks indicate germ cells undergoing spermatogenesis. Cells were stained with (I) DAPI (blue); (J) anti-P-granule (green), a germ cell marker that is lost during spermatogenesis (Gruidl et al., 1996); (K) anti-membranous organelle (1CB4; red), a sperm marker (Arduengo et al., 1998). A merged image is shown in L. Images in B,C,F-H are single focal planes; condensed z-stacks are shown in I-L. Scale bars: 50 μ m in A,B,D; 10 μ m in C,F-H,I-L.



proliferation during dauer development triggered by reduced insulin-like signalling. We isolated one mutation (*rr48*) that causes pronounced germline hyperplasia in insulin-like receptor (*daf-2*) (Kimura et al., 1997) mutant dauer larvae (Fig. 1D). Although pharyngeal and body radial constriction are partially incomplete, *daf-2; rr48* mutant dauer larvae demonstrate all of the morphological and behavioural characteristics associated with this stage, including SDS resistance (Cassada and Russell, 1975) (data not shown). By contrast, *rr48* animals show no obvious morphological, behavioural or reproductive phenotype during reproductive development (data not shown).

Meiotic mapping, followed by transformation rescue indicated that *rr48* affects an orthologue of the $\alpha 2$ -catalytic subunit of the mammalian AMP-activated kinase (AMPK): *aak-2* (AMP-activated kinase $\alpha 2$) in *C. elegans*. Biochemical studies have shown that AMPK exists as a heterotrimeric complex composed

of one catalytic α -subunit, and two regulatory subunits (Carling, 2004). The EMS-induced point mutation in *aak-2(rr48)* is predicted to disrupt the catalytic activity of the $\alpha 2$ -subunit, without altering the regulatory domain. *aak-2(rr48)* heterozygous animals display a dauer germline phenotype that is intermediate between the wild-type and homozygous mutant situations, and this is not due to haplo-insufficiency (Table 1, rows A-D). Furthermore, *aak-2(RNAi)* partially suppresses the germline phenotype of *aak-2(rr48)* mutants (Table 1, row E). Our analysis therefore suggests that *aak-2(rr48)* behaves semi-dominantly in the germ line because of a dominant-negative effect that most likely arises through interference with the activity of the second *C. elegans* AMPK catalytic subunit: *aak-1* (AMP-activated kinase $\alpha 1$). Consistent with this, our experiments indicate that *aak-1* acts additively with *aak-2* to inhibit germline proliferation during dauer development (Table 2, rows F,J,L).

Table 1. *aak-2(rr48)* acts in a dominant-negative manner in the germ line

Genotype	Number of germ cell nuclei in dauer* (n)
(A) <i>daf-2(e1370)</i>	35.44±3.85 (25)
(B) <i>daf-2(e1370); aak-2(rr48/+)</i> [§]	50.97±5.71 ^{†A} (35)
(C) <i>daf-2(e1370); aak-2(+)/yDf1</i> [¶]	34.93±4.14 ^{†A} (27)
(D) <i>daf-2(e1370); aak-2(rr48)</i>	96.16±18.06 ^{†A} (25)
(E) <i>daf-2(e1370); aak-2(rr48); aak-2(RNAi)</i>	69.35±9.96 ^{†D} (26)

*. Mean values±s.d.

[†]. Statistical significance using the one-tailed t-test with unequal variance ($P \leq 0.0005$), versus row 'X'.

[‡]. As above, but $P=0.64$.

[§]. Genotype includes *qls56/+*.

[¶]. Complete genotype is: *+/szT1[lon-2(e678)] I; daf-2(e1370) III; lin-15(n765)yDf1/szT1 X (yDf1 uncovers *aak-2*)*.

n, sample size.

***aak-2* couples germ cell cycle progression with somatic dauer development**

Dauer germline hyperplasia could result from over-proliferation during dauer formation, and/or from ongoing divisions after the dauer moult. To distinguish between these possibilities, we examined germline proliferation prior to, and during, the dauer stage. In contrast to what we observed in *daf-2* mutants alone, the germline proliferation rate does not decrease appropriately during L2d/dauer formation in *daf-2; aak-2* double mutants (Fig. 1E). Germline proliferation did, however, finally cease in the *daf-2; aak-2* dauer larvae (Fig. 1E), suggesting that additional mechanisms may cooperate with *aak-2* to establish dauer-associated germ cell cycle quiescence. Our data therefore indicate that *aak-2* is required to appropriately decrease the rate of mitotic proliferation in the germ

line during preparation for dauer in response to reduced insulin-like signalling, to coordinate the growth of the germline stem cell population with developmental status.

In both *daf-2* and *daf-7* [TGF- β mutant (Ren et al., 1996)] dauer larvae, germ cells arrest in interphase of the mitotic cell cycle (Fig. 1F). The intensity of DAPI-stained germ cell nuclei in *daf-2* dauer animals is twofold greater than that of the DTC (data not shown), suggesting a 4C DNA content, consistent with a G2/M-phase arrest. Hence, during the dauer stage, germ cells are not only mitotically quiescent, they are also blocked from initiating their alternative meiotic program. During reproductive development, inactivating mutations in the core components of the Notch signalling pathway limit mitotic divisions in the germ line and cause premature meiotic entry, resulting in the production of only a small number of sperm (Austin and Kimble, 1987). To test whether *aak-2* also regulates the execution of the meiotic program, we first examined whether germ cells could undergo meiosis if the Notch-dependent meiotic block was removed from the germ line of dauer larvae. The germ cells of *daf-2 glp-1* larvae enter meiosis prematurely during L2d, but arrest nonetheless in late stage meiotic prophase I in dauer (Fig. 1G), indicating that meiotic progression is blocked independently of Notch signals during this stage. Remarkably, in *daf-2 glp-1; aak-2* mutants, germ cells progress through the entire meiotic program, including both meiotic divisions, during dauer development and in fact, undergo spermatogenesis (Fig. 1H-L). This suggests that under these conditions, *aak-2* ensures that gametogenesis is appropriately coordinated with somatic development, and, in its absence, germ cells can differentiate to sperm prematurely. We therefore conclude that *aak-2* contributes to mitotic and meiotic cell cycle regulation to ensure quiescence of the germ cells under compromised insulin-like signalling conditions during dauer formation.

Table 2. Regulation of dauer formation and germline proliferation

Genotype	% Dauer arrest (n)	Number of germ cell nuclei in dauer* (n)
(A) N2	0 (124)	39.92±4.58 (25) [§]
(B) <i>aak-2(ok524)</i>	0 (148)	60.36±9.30 ^{†A} (25) [§]
(C) <i>daf-2(e1370)</i>	100 (293)	35.44±3.85 (25)
(D) <i>daf-2(e1370); aak-2(rr48)</i>	100 (156)	96.16±18.06 ^{†C} (25)
(E) <i>daf-2(e1370); aak-2(ok524)</i>	100 (290)	69.96±9.08 ^{†C} (25)
(F) <i>daf-2(e1370); aak-1(RNAi)</i>	100 (95)	47.77±9.26 ^{†C} (30)
(G) <i>daf-2(e1370); daf-18(e1375)</i>	99.48 (763)	98.17±13.61 ^{†C} (30)
(H) <i>daf-2(e1370); akt-1(mg144)gf</i>	97.7 (607)	50.82±6.57 ^{†C} (28)
(I) <i>daf-2(e1370); akt-1(mg144)gf; aak-2(rr48)</i>	77.4 (1387)	126.60±18.95 ^{†D} (15)
(J) <i>daf-2(e1370); aak-2(rr48); aak-1(RNAi)</i>	ND	165.00±36.13 ^{†D} (28)
(K) <i>daf-2(e1370); akt-1(mg144)gf; aak-2(ok524)</i>	67.7 (653)	85.33±12.40 ^{†E} (15)
(L) <i>daf-2(e1370); aak-2(ok524); aak-1(RNAi)</i>	ND	152.64±32.51 ^{†E} (25)
(M) <i>daf-2(e1370); daf-18(e1375); aak-2(ok524); aak-1(RNAi)</i>	85.13 (316)	189.53±23.71 ^{†L} (15)
(N) <i>daf-7(e1372)</i>	100 (182)	33.19±4.07 (26)
(O) <i>daf-7(e1372); aak-2(rr48)</i>	100 (201)	80.12±6.44 ^{†N} (25)
(P) <i>daf-7(e1372); aak-2(ok524)</i>	100 (153)	65.24±14.85 ^{†N} (25)
(Q) <i>daf-7(e1372); aak-1(RNAi)</i>	100 (311)	44.60±10.68 ^{†N} (25)
(R) <i>daf-7(e1372); daf-18(e1375)</i>	99.8 (577)	87.24±14.41 ^{†N} (25)
(S) <i>daf-7(e1372); daf-18(ok480)</i>	92.8 (376)	177.48±21.48 ^{†N} (25)
(T) <i>daf-7(e1372); akt-1(mg144)gf</i>	100 (423)	32.15±2.60 ^{†N} (26)
(U) <i>daf-16(mgDf50); daf-7(e1372)</i>	98.7 (234)	39.68±4.89 ^{†N} (25)
(V) <i>daf-16(mgDf50); daf-7(e1372); aak-2(rr48)</i>	32.0 (222)	95.24±10.65 ^{†U} (25)
(W) <i>daf-16(mgDf50); daf-7(e1372); aak-2(ok524)</i>	50.5 (497)	82.93±10.14 ^{†U} (15)

*. Mean values±s.d.

[†]. Statistical significance using the one-tailed t-test with unequal variance ($P \leq 0.0005$), versus row 'X'.

[‡]. As above but, $P=0.14$.

[§]. Dauer formation (>90% *daf-c*) was induced using dauer pheromone preparation.

n, sample size.

ND, not determined.

***aak-2* couples developmental arrest to lifespan extension**

In addition to the observed germline hyperplasia, *daf-2*; *aak-2* dauer larvae die within 12–14 days (Fig. 2A), unlike wild-type dauer larvae, which are non-ageing and can survive beyond 70 days (Klass and Hirsh, 1976). The lethality of *daf-2*; *aak-2* dauer larvae was not suppressed by ablation of the germline precursors, or of the somatic

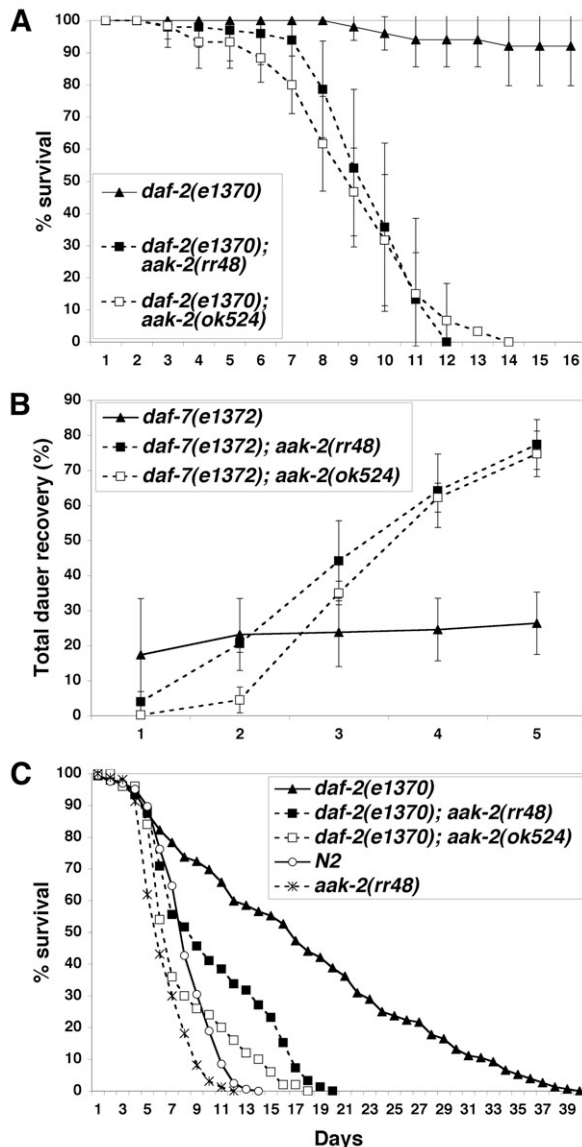


Fig. 2. *aak-2* positively regulates lifespan and dauer maintenance. (A) *daf-2(e1370)* ($n=100$), *daf-2(e1370); aak-2(rr48)* ($n=98$) and *daf-2(e1370); aak-2(ok524)* ($n=60$) individual larvae were assayed for dauer lifespan (days). (B) Whereas most *daf-7(e1372)* ($n=500$) dauer larvae remain at this stage at 25°C, the majority of *daf-7(e1372); aak-2(rr48)* ($n=500$), or *daf-7(e1372); aak-2(ok524)* ($n=400$) dauer larvae recover prematurely within 5 days. (C) *aak-2* is required for the full adult lifespan extension induced by compromised insulin-like signalling. The graph marks the time (day) of the temperature up-shift (corresponding to the L4 larval stage). Each line represents the average of three independent replicate experiments (each with $n=50$), not performed in parallel, except for *daf-2(e1370); aak-2(ok524)*, for which we did one single experiment ($n=50$). Error bars represent standard deviation between cohorts of (A) 10 or (B) 100 individuals.

gonad, or of both (Fig. 3), suggesting that this effect is independent of the germ line defect. Therefore, our data suggest that the extreme lifespan extension characteristic of the *C. elegans* dauer larva does not directly or solely result from the generalized cell cycle/developmental arrest associated with this larval stage, but largely requires *aak-2* activity.

Conversely, compared with *daf-7* mutants, which form dauer larvae constitutively as a result of compromised TGF- β function, *daf-7; aak-2* larvae exhibit a marked increase in recovery from dauer (Fig. 2B). Because *aak-2* dauer larvae display both germ line and dauer longevity defects when induced by either reduced cGMP, TGF- β or insulin-like signalling, or by crowding/starvation, or dauer pheromone (Table 2, rows A-E, N-P; Fig. 2A,B; data not shown), *aak-2* is likely to be required downstream of each of these pathways to ensure appropriate dauer development. Furthermore, because insulin-like signalling is necessary for dauer recovery (Kimura et al., 1997; Ogg et al., 1997), and because reducing *aak-2* function causes premature recovery in *daf-7* dauer larvae, insulin-like signalling may negatively regulate *aak-2*, which would act to promote dauer maintenance. In most organisms examined to date, reduced insulin-like signalling causes adult lifespan extension (Kenyon et al., 1993; Kimura et al., 1997; Lin et al., 1997; Ogg et al., 1997). As the genes involved in the regulation of dauer longevity may additionally affect adult lifespan, we verified whether *aak-2* mutations could also antagonize the extension of adult lifespan in insulin-like mutants. Consistent with this, *aak-2* mutations suppress the lifespan extension of *daf-2* mutants considerably (Fig. 2C), indicating that *aak-2* is required to potentiate the effect of reduced insulin-like signalling on adult lifespan.

TGF- β and insulin-like signalling converge on the tumour suppressor PTEN to mediate germ cell cycle arrest

The severity of the phenotypes that result from reduced insulin-like signalling, such as constitutive dauer formation and increased adult lifespan, are generally dependent on dosage (Kenyon et al., 1993; Kimura et al., 1997; Ogg and Ruvkun, 1998; Paradis and Ruvkun, 1998). To directly test whether the level of insulin-like signalling regulates germline proliferation through *aak-2* in a dose-dependent manner, we verified whether mutations that are known to activate this signalling pathway downstream of the insulin-like receptor (*daf-2*) could phenocopy the effect of *aak-2* mutations on germ cell proliferation at the onset of dauer. We therefore introduced a hypomorphic mutation in the *C. elegans* PTEN orthologue *daf-18* (Ogg and Ruvkun, 1998), or a gain-of-function mutation in an Akt/PKB orthologue, *akt-1* (Paradis and Ruvkun, 1998), to partially upregulate insulin-like signalling in *daf-2* mutant larvae. *daf-2; daf-18* and *daf-2; akt-1(gf)* animals form dauer larvae that have 164.9% and 43.4% more germ cell nuclei than do *daf-2* animals, respectively (Table 2, rows G,H), indicating that efficient inhibition of germline proliferation by reduced insulin-like receptor function occurs via the proper activation of *daf-18*, inactivation of *akt-1*, as well as the activation of *aak-2*. Interestingly, the effects of *daf-18(lf)* or *akt-1(gf)*, and *aak-1/aak-2* inactivation on dauer formation and germline proliferation are additive (Table 2, rows I,K,M), suggesting that these pathways work in parallel, or that other factors, in addition to *aak-1/aak-2*, function downstream of *daf-18* and *akt-1* to regulate dauer formation and insulin-dependent cell cycle quiescence in the germ line.

Activity of the FOXO-like forkhead transcription factor *daf-16*, a direct Akt/PKB target (Lee et al., 2001), is believed to be responsible for all of the phenotypes of reduced insulin-like receptor function

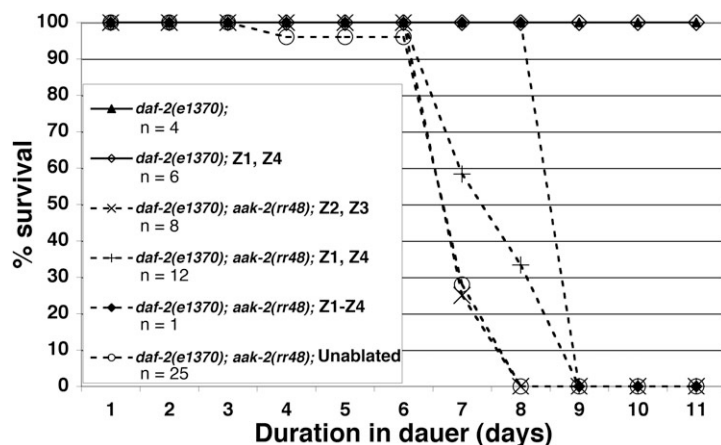


Fig. 3. The dauer lethality in *aak-2(rr48)* mutants is not due to a germ line defect. Precursors of the germ line (Z2, Z3), and/or of the somatic gonad (Z1, Z4), were ablated using laser microsurgery. Larvae were subsequently plated at 25°C and assayed for dauer lifespan. The success of the ablation was monitored 24 hours after surgery, by examining *lag-2::GFP* expression.

(Lin et al., 1997; Ogg et al., 1997). We nonetheless examined *daf-16(0); daf-2* animals to determine whether low insulin-like receptor activity would still result in reduced germ cell proliferation even in the absence of *daf-16*. Although these animals undergo reproductive development seemingly like wild-type animals, we find that germline proliferation is slightly delayed in *daf-16(0); daf-2* young larvae when compared with wild-type or *daf-16(0)* larvae (Fig. 4), suggesting that *daf-16*-independent targets of the insulin-like receptor may also affect germline proliferation. However, because the difference is minor compared with that observed in the presence of a wild-type copy of *daf-16*, such as in *daf-2* mutants alone, our data confirms that the regulation of germline proliferation by compromised insulin-like signalling largely requires the activity of the transcription factor *daf-16*. Therefore, it is likely that the regulation of *aak-2* activity by the insulin-like signalling pathway largely occurs downstream of *daf-16*, although it is impossible for the moment to rule out a parallel mode of action.

However, if *aak-2* activity absolutely depends on compromised insulin-like signalling, mutations that prevent the appropriate downregulation of insulin-like signalling should also mimic the effect of *aak-2* mutations on the germ line during dauer development mediated through the other parallel pathways,

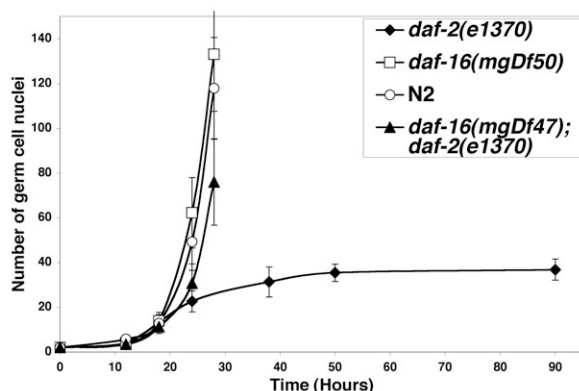


Fig. 4. The inhibition of germline proliferation in *daf-2* mutants largely requires *daf-16* activity. L1 animals were grown at 25°C for the specified times, and the total number of germ cells was determined as described in the Materials and methods. Germline proliferation was also examined in *daf-16(mgDf50); daf-2(e1370)* animals (data not shown), and the results obtained were similar to *daf-16(mgDf47); daf-2(e1370)*. Error bars indicate s.d. Sample size was >10 for each point.

including the TGF- β signalling pathway. We find that the germ line of *daf-7*; *daf-18* dauer larvae is hyperplastic (Table 2, rows R,S). Surprisingly, however, the regulation of germline proliferation is normal during *daf-7*; *akt-1(gf)* dauer development (Table 2, row T). Although the mean number of germ cell nuclei in *daf-16(0); daf-7* dauer larvae is slightly, but significantly, higher than in *daf-7* dauer larvae (Table 2, row U), 72.0% of *daf-16(0); daf-7* dauer larvae had germ cell nuclei counts that were below/equal to the maximum observed in *daf-7* dauer larvae (41), suggesting that *daf-16*, in contrast to *daf-18* and *aak-2*, is not absolutely required for the appropriate downregulation of germline proliferation during dauer formation. Moreover, the effect of *daf-16(0)* on dauer formation and germline proliferation in *daf-7* mutants is enhanced by *aak-2* mutations (Table 2, rows V,W), indicating that *daf-16* is not required for *aak-2* activity during dauer formation triggered by compromised TGF- β signalling. Consistent with previous observations (Ogg et al., 1997), however, several *daf-16(0); daf-7* dauer larvae did not maintain germ cell cycle quiescence and resumed reproductive development (data not shown). Our data therefore suggest that reduced TGF- β signalling downregulates germline proliferation during dauer development primarily through the activation of *daf-18* and *aak-1/aak-2*, whereas the inactivation of *akt-1* and the subsequent activation of *daf-16* appear to be required to ensure the proper maintenance, rather than the establishment, of germ cell cycle quiescence in larvae with compromised TGF- β signalling.

TGF- β and insulin-dependent signals target *aak-2* to regulate proliferation cell autonomously

Because the insulin-like and TGF- β pathways demonstrate both cell autonomous and cell non-autonomous functions (Inoue and Thomas, 2000; Wolkow et al., 2000), we determined whether *aak-2* activity is required for germ cell cycle regulation in the tissues where these signalling pathways function to regulate dauer formation, or within the germ line proper. To address this question, we studied the response of *rrf-1* mutants to *aak-2(RNAi)*. *rrf-1* mutants are resistant to dsRNA-mediated interference in the soma, whereas they remain RNAi-sensitive in the germ line (Sijen et al., 2001). *aak-2(RNAi)* performed in both *rrf-1*; *daf-2* and *rrf-1*; *daf-7* animals recapitulated the germline hyperplasia typical of *daf-2*; *aak-2(RNAi)* and *daf-7*; *aak-2(RNAi)* dauer larvae (Table 3, rows A-H), indicating that *aak-2* is required cell autonomously (within the germ cells) to regulate germline proliferation in response to compromised insulin-like and TGF- β signalling, during dauer development.

Table 3. *aak-2* is required cell autonomously for germ cell cycle regulation

Genotype	Number of germ cell nuclei in dauer* (n)
(A) <i>daf-2(e1370)</i>	35.44±3.85 (25)
(B) <i>daf-2(e1370); aak-2(RNAi)</i>	65.29±8.09 ^{†A} (35)
(C) <i>rrf-1(pk1417); daf-2(e1370)</i>	35.37±3.66 ^{†A} (30)
(D) <i>rrf-1(pk1417); daf-2(e1370); aak-2(RNAi)</i>	55.93±7.25 ^{†C} (28)
(E) <i>daf-7(e1372)</i>	33.19±4.07 (26)
(F) <i>daf-7(e1372); aak-2(RNAi)</i>	66.90±8.02 ^{†E} (10)
(G) <i>rrf-1(pk1417); daf-7(e1372)</i>	34.90±4.36 ^{†E} (10)
(H) <i>rrf-1(pk1417); daf-7(e1372); aak-2(RNAi)</i>	57.40±9.70 ^{†G} (10)

*. Mean values±s.d.; all strains were ~100% dauer constitutive at 25°C.

[†]. Statistical significance using the one-tailed t-test with unequal variance ($P \leq 0.0005$), versus row 'X'.

[‡]. As above, but $P=0.47$.

[§]. As above, but $P=0.15$.

n, sample size.

***par-4* mediates TGF- β and insulin-dependent control of cell cycle**

Recently, it has been shown that AMPK itself is activated by LKB1/STK11, the major AMPK-activating kinase (Hong et al., 2003; Woods et al., 2003). In humans, inactivating mutations in LKB1 are responsible for most cases of Peutz-Jeghers Syndrome, a rare dominantly inherited disorder characterised primarily by predisposition to benign and malignant tumours in many organ systems (Hemminki et al., 1998; Jenne et al., 1998). We reasoned that if LKB1 was required for optimal AMPK function, inactivating mutations in the *C. elegans* LKB1 orthologue *par-4* (Watts et al., 2000) should phenocopy the effect of reduced *aak-1/aak-2* activity. *par-4* mutations indeed phenocopy the dauer germline hyperplasia caused by the inactivation of both *aak* catalytic isoforms (Table 2, rows J,L; Table 4, rows D-G), indicating that *par-4* is required to suppress proliferation of the germline stem cell population in response to reduced insulin-like or TGF- β signalling levels. If the germline hyperplasia in *par-4* mutant dauer larvae results from defective *aak* activation, the loss of *aak-1* and/or *aak-2* activity should not enhance the effect of a strong *par-4(it57)* inactivating mutation (Watts et al., 2000). However, only *aak-1(RNAi)* was not significantly additive to *par-4(it57)* (Table 4, rows I,L). In fact, while *daf-2; par-4* dauer larvae are long-lived when compared with

daf-2; aak-2 animals (Table 4, rows A-E), *par-4* enhances the dauer germline and lifespan phenotypes of *aak-2* mutants (Table 4, rows H,J-K), suggesting that *par-4* and *aak-2* function, at least in part, in an additive manner, and that *aak-2* activity may not absolutely rely on *par-4* to control germ cell cycle and lifespan during compromised insulin-like signalling conditions. Moreover, our data suggest that *par-4* may target other factors along with *aak-1/aak-2* to control germline proliferation under these dauer-promoting conditions.

DISCUSSION

Our analysis of *aak-2* function in *C. elegans* reveals that it is required for the characteristic long-term survival of the developmentally arrested juvenile dauer larva and thereby complements and further supports the recent finding that it is required for the adult lifespan extension associated with reduced insulin-like signalling (Apfeld et al., 2004). In plant, yeast and mammalian systems, the AMPK orthologues act as 'metabolic master switches' to appropriately reset gene expression for survival on alternative energy sources (Carling, 2004; Hardie et al., 1998). The lifespan extension that is associated with developmental arrest and/or compromised insulin signalling in most organisms is therefore likely to require a substantial switch to alternative metabolic pathways, which is triggered predominantly through the activation of AMPK.

We have demonstrated that *daf-18/PTEN*, *par-4/LKB1* and *aak-1, aak-2/AMPK* are crucial factors commonly required for the regulation of germline proliferation in response to the neuro-endocrine signal generated as a result of compromised insulin-like and/or TGF- β signalling (Fig. 5), whereas *daf-16/FOXO* appears, at least in part, dispensable. Our data is therefore consistent with a model in which *par-4* and *aak-1/aak-2* function together with *daf-18*, and partially in parallel with *daf-16*. Namely, the efficient suppression of germ cell cycle progression during early larval development requires either compromised TGF- β signalling or *daf-16* activity. Although we provide evidence that *aak-2* is required in the germ line to control proliferation in response to these signalling pathways, it would be of great interest to determine the cellular requirement of each of the players that link the insulin-like and/or TGF- β -dependent neuro-endocrine signals to the regulation of *aak-2* activity within a target tissue.

Table 4. Regulation of germline proliferation and dauer lifespan

Genotype	Number of germ cell nuclei in dauer* (n)	Dauer lifespan (days)* (n)
(A) <i>daf-2(e1370)</i>	35.44±3.85 (25)	>15 (100)
(B) <i>daf-2(e1370); aak-2(rr48)</i>	96.16±18.06 ^{†A} (25)	8.64±1.77 ^{†A} (98)
(C) <i>daf-2(e1370); aak-2(ok524)</i>	69.96±9.08 ^{†A} (25)	8.18±2.41 ^{†A} (60)
(D) <i>daf-2(e1370); par-4(it47)</i>	109.00±28.42 ^{†A} (25)	>15 (50)
(E) <i>daf-2(e1370); par-4(it57)</i>	148.28±30.90 ^{†A} (25)	>15 (60)
(F) <i>daf-7(e1372)</i>	33.19±4.07 (26)	ND
(G) <i>daf-7(e1372); par-4(it57)</i>	160.20±29.99 ^{†F} (25)	ND
(H) <i>daf-2(e1370); par-4(it47); aak-2(rr48)</i>	187.53±16.60 ^{†D} (15)	6.97±2.12 ^{†B} (60)
(I) <i>daf-2(e1370); par-4(it57); aak-1(RNAi)</i>	155.95±25.21 ^{†E} (20)	ND
(J) <i>daf-2(e1370); par-4(it57); aak-2(rr48)</i>	213.10±38.87 ^{†E} (10)	5.83±1.64 ^{†B} (60)
(K) <i>daf-2(e1370); par-4(it57); aak-2(ok524)</i>	202.65±24.14 ^{†E} (17)	6.85±1.83 ^{†C} (59)
(L) <i>daf-2(e1370); par-4(it57); aak-2(ok524); aak-1(RNAi)</i>	215.87±17.65 ^{†K} (15)	ND

*. Mean values±s.d.; all strains were ~100% dauer constitutive at 25°C.

[†]. Statistical significance using the one-tailed t-test with unequal variance ($P \leq 0.0005$), versus row 'X'.

[‡]. As above, but $P=0.18$.

[§]. As above, but $P=0.035$.

n, sample size.

ND, not determined.

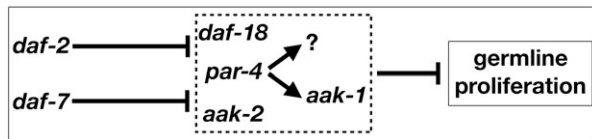


Fig. 5. Regulation of germ cell cycle during dauer development.

Under reduced *daf-2*/insulin-like receptor and/or *daf-7*/TGF- β activity, *daf-18*/PTEN, *par-4*/LKB1 and *aak-1*, *aak-2*/AMPK mediate the downregulation of germline proliferation during dauer development. Although the role of *par-4* in *aak-1* activity is obvious, the relationship between *daf-18*, *par-4* and *aak-2* remains unclear. Arrows and bars represent positive and negative genetic interactions, respectively.

Together, these results complement/modify our current model of cell cycle/growth control, in which growth factors are believed to function through Akt/PKB (which is negatively regulated by PTEN), while intracellular energy stress is thought to work in parallel, activating AMPK through LKB1, then converging on the TSC/mTOR pathway to cell autonomously regulate growth and division (Corradetti et al., 2004; Shaw et al., 2004). Our work indicates that the insulin and TGF- β signalling pathways independently converge on both tumour suppressors, PTEN and LKB1, which then mediate cell cycle control under stress conditions.

Finally, we have shown that inactivating mutations in *par-4*/LKB1 partially phenocopy and enhance the defects associated with reduced *aak-2*/AMPK activity in insulin-like compromised animals, linking metabolic status, through insulin-like signalling, to the regulation of the germline stem cell divisions and lifespan. Previous work has suggested that LKB1 functions as a tumour suppressor through its role in controlling both cell polarity (Boudeau et al., 2003) and AMPK (Corradetti et al., 2004; Shaw et al., 2004); however, evidence that mutations in AMPK recapitulate the proliferative defects associated with LKB1 mutations has up to now been lacking. Our findings provide strong genetic evidence that the aberrant cell cycle regulation associated with Juvenile Polyposis Syndrome, Cowden's Disease and Peutz-Jeghers Syndrome, which are caused by mutations in genes involved in TGF- β signal transduction, in PTEN and in LKB1, respectively (Waite and Eng, 2003; Wirtzfeld et al., 2001), may result from defects at different levels of a single genetic pathway that unifies general growth factor levels to the regulation of cell proliferation via AMPK regulation.

We thank all members of the Roy Laboratory, especially Jimmy Ouellet, for support throughout this work. We are grateful to the *C. elegans* Genetic Centre (CGC) and to the Gene Knockout Consortium for strains; to Judith Kimble, Tim Schedl, Marty Chalfie, Susan Strome, Iva Greenwald and Steve L'Hernault for kindly sharing strains, reagents and discussion; to Victor Ambros and Paul Lasko for their critical reading of the manuscript; and to Beck. This work was supported by a research grant from the Canadian Cancer Society (NCIC). R.R. is a CIHR New Investigator. P.N. is supported by a grant from the Canderel Foundation and NSERC.

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