C. elegans AMPKs promote survival and arrest germline development during nutrient stress

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Summary

Mechanisms controlling development, growth, and metabolism are coordinated in response to changes in environmental conditions, enhancing the likelihood of survival to reproductive maturity. Much remains to be learned about the molecular basis underlying environmental influences on these processes. *C. elegans* larvae enter a developmentally dormant state called L1 diapause when hatched into nutrient-poor conditions. The nematode *pten* homologue *daf-18* is essential for maintenance of survival and germline stem cell quiescence during this period (Fukuyama et al., 2006; Sigmond et al., 2008), but the details of the signaling network(s) in which it functions remain to be elucidated. Here, we report that animals lacking both *aak-1* and *aak-2*, which encode the two catalytic α subunits of AMP-activated protein kinase (AMPK), show reduced viability and failure to maintain mitotic quiescence in germline stem cells during L1 diapause. Furthermore, failure to arrest germline proliferation has a long term consequence; *aak* double mutants that have experienced L1 diapause develop into sterile adults when returned to food, whereas their continuously fed siblings are fertile. Both *aak* and *daf-18* appear to maintain germline quiescence by inhibiting activity of the common downstream target, TORC1 (TOR Complex 1). In contrast, rescue of the lethality phenotype indicates that *aak-2* acts not only in the intestine, as does *daf-18*, but also in neurons, likely promoting survival by preventing energy deprivation during L1 diapause. These results not only provide evidence that AMPK contributes to survival during L1 diapause in a manner distinct from that by which it controls dauer diapause, but they also suggest that AMPK suppresses TORC1 activity to maintain stem cell quiescence.

Key words: AMPK, Stem cell, Diapause

Introduction

Juvenile animals coordinate development and growth with nutritional status in order to achieve reproductive maturation. *C. elegans* development shows a clear example of such coordination; when larvae hatch into nutritionally-deficient conditions, they suspend postembryonic development and can survive more than a week, resuming development if ample food is supplied (Johnson et al., 1984). This developmentally dormant state is called "L1 diapause" or "L1 arrest," and it can be simply released by supplying nutrients. Once larval development is initiated, exposure to unfavorable conditions such as lack of nutrients, high population density or high temperature predispose larvae to arrest development in an alternative third larval (L3) stage, called "dauer diapause" (reviewed by Riddle and Albert, 1997). Progression to the L4 stage is then blocked until the environment becomes favorable for growth. Furthermore, once animals have reached the L4 stage, starvation triggers a response that delays reproductive onset and reduces viability of fertilized embryos (Angelo and Van Gilst, 2009; Seidel and Kimble, 2011). The starved animals molt into adults and their oogenic germlines shrink, but can regenerate if the animal survives until re-feeding, indicating the stem cells had entered a quiescent state. Although the significance of these various starvation responses to survival in the wild requires further study, their existence in the life cycle is likely to reflect the importance of coordinating development and growth with nutritional status for optimal fitness and reproduction.

Previous studies have shown that during L1 diapause germline stem cells arrest at the G2 phase of the cell cycle in a manner dependent on *daf-18/pten*, a negative regulator of the insulin/IGF signaling (IIS) pathway (Fig. 1A) (Fukuyama et al., 2006). Interestingly, *daf-16/foxo*, which antagonizes the IIS pathway downstream of *daf-18*, is not required for mitotic quiescence of germline stem cells during L1 diapause, suggesting that *daf-18* suppresses germline proliferation by a *daf-16*-independent pathway (Fukuyama et al., 2006). In contrast, *daf-16* does function downstream of *daf-18* to control dauer larvae formation (Ogg and Ruvkun, 1998). Thus, these observations suggest that developmental arrest during L1 and dauer diapauses is regulated by partly distinct mechanisms.

Another component known to mediate cellular and organismal responses to starvation is AMP-activated kinase (AMPK). In

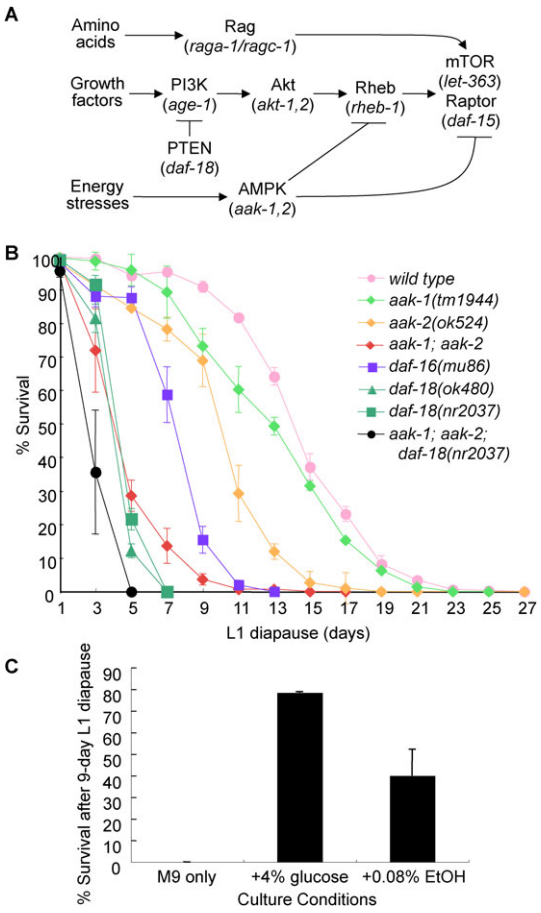

Fig. 1. The *aak* genes promote survival during L1 diapause. (**A**) Simplified signaling network integrating the IIS, AMPK, and TORC1 pathways (reviewed by Zoncu et al., 2011). Gene names of *C. elegans* homologues are indicated in parentheses. (**B**) Survival curves of indicated mutants during L1 diapause in M9 medium. (**C**) Viability of *ampk* mutants in M9 medium with or without the indicated energy source after 9-day L1 diapause. In B and C, the average of at least three independent experiments is reported and error bars indicate s.e.m. >100 animals were scored for each time point per experiment.

mammals, this heterotrimeric serine/threonine kinase is stimulated by AMP and ADP as well as upstream kinases and hormones, and it is best known for its role in maintaining energy balance by directly activating metabolic pathways that generate ATP and inactivating those that consume ATP (reviewed by Hardie, 2011; Kahn et al., 2005). However, recent studies have shown that AMPK also regulates other starvation-responsive processes such as autophagy, mitochondria biogenesis, cell cycle arrest, and food intake (Wang et al., 2001; Zong et al., 2002; Minokoshi et al., 2004; Jones et al., 2005; Narbonne and Roy, 2006; Behrends et al., 2010; Egan et al., 2011; Kim et al., 2011).

Both AMPK and PTEN negatively regulate mechanistic Target of Rapamycin Complex 1 (mTORC1), which consists of several proteins including the atypical serine/threonine kinase mTOR and its scaffold protein Raptor (reviewed by Wullschleger et al., 2006; Shackelford and Shaw, 2009; Laplante and Sabatini, 2012). mTORC1 responds to signals from several environmental cues such as amino acids, growth factors and energy stresses to control diverse cellular processes including growth, autophagy and metabolism (Fig. 1A) (reviewed by Laplante and Sabatini, 2012). In response to energy stresses, AMPK directly and indirectly represses mTORC1 through both Rheb-independent and dependent manners (Inoki et al., 2003b; Gwinn et al., 2008). Growth factors stimulate mTORC1 activity through the PI3K (phosphatidylinositol 3-kinase)-AKT axis (Inoki et al., 2003a; Inoki et al., 2003b; Tee et al., 2003). Thus, deficiency of PTEN, which antagonizes PI3K as a phosphatidylinositol 3-phosphatase (Maehama and Dixon, 1998), results in constitutive activation of mTORC1 (Neshat et al., 2001; Podsypanina et al., 2001).

Similar to its mammalian orthologues, *C. elegans* AMPK is also activated by AMP and energy stresses (Apfeld et al., 2004; Schulz et al., 2007), and it plays an important role in starvation responses, including extension of lifespan by nutritional restriction and maintenance of viability and germline quiescence during dauer diapause (Narbonne and Roy, 2006; Greer et al., 2007; Schulz et al., 2007). Here, we test the significance of AMPK in the regulation of L1 diapause. We find that *aak-1* and *aak-2*, which encode the two catalytic α subunits of AMP-activated protein kinase, act redundantly to arrest germline stem cell proliferation and maintain viability during L1 diapause. Both *aak-1; aak-2* double and *daf-18* single mutants that experience L1 diapause develop into sterile adults when returned to food, indicating the importance of these genes for long term germline viability. Both *daf-18* and the *aak* genes appear to regulate germline quiescence by suppressing activity of TORC1. Previous studies indicate that *aak-2* acts in the hypodermis and excretory cell to maintain viability during dauer diapause (Narbonne and Roy, 2009). In contrast, we find that L1 diapause survival requires *aak*-2 in the intestine and neurons. Together, these results suggest that suppression of TORC1 activity by AMPK maintains germline stem cell quiescence and that AMPK promotes survival during L1 diapause in a manner distinct from that which controls dauer diapause.

Results

The *aak* genes are essential for maintaining survival during L1 diapause

Survival during L1 diapause depends on activity of *aak-2* and *daf-18*/PTEN (Baugh and Sternberg, 2006; Sigmond et al., 2008), but the role of *aak-1*, the sole worm paralogue of *aak-2* (Apfeld et al., 2004) has not been fully investigated. We tested the roles of the *aak* genes in L1 diapause using the putative null alleles, *tm1944* and *ok524*, which result in deletion of a significant portion of the kinase domains of AAK-1 and AAK-2, respectively (Narbonne and Roy, 2006; Narbonne and Roy, 2009). Viability of *aak-1(tm1944)* mutants was reduced during L1 diapause relative to wild-type animals, although the animals survived better than did *aak-2(ok524)* mutants (Fig. 1B). However, animals lacking function of both *aak* genes (hereafter called "*ampk* mutants") displayed a more severe reduction in viability than did either single mutant, indicating that *aak-1* and *aak-2* act redundantly to maintain survival during L1 diapause. The L1 diapause-induced lethality of *ampk* mutants is rescued by an *aak-2* transgene (see below) demonstrating that loss of AMPK activity is responsible for this phenotype. Because L1 diapause is induced by nutritional stress, the observed lethality of *ampk* mutants may be a consequence of carbon and energy deprivation. Consistent with this possibility, supplementation of the starvation medium with glucose or ethanol, compounds known to promote population growth when added to chemically defined media,

significantly increased viability of *ampk* mutants (Fig. 1C) (Lu et al., 1978; Castro et al., 2012).

The *aak* and *daf-18* genes maintain survival in a primarily *daf-16*-independent manner

AAK-2 mediates dietary restriction-induced longevity by acting through the FOXO transcription factor, DAF-16 (Greer et al., 2007). Similarly, *daf-16* also acts downstream of *daf-18* to control lifespan and dauer formation (Ogg and Ruvkun, 1998). These findings raise the possibility that the *aak* and *daf-18* genes could maintain organismal survival during L1 diapause through a *daf-16*-mediated pathway. However, viability of *daf-16* null mutant animals is much greater than that of *ampk* or *daf-18* mutants (Fig. 1B), indicating that loss of *daf-16* function is not solely responsible for the reduced survival in *daf-18* and *ampk* mutants. Furthermore, viability of *daf-18(nr2037); ampk* triple mutants was reduced relative to *daf-18* and *ampk* mutants (Fig. 1B). Because the three alleles used were null (Mihaylova et al., 1999), these findings suggest that the *daf-18* and *aak* genes act in parallel pathways to maintain survival during L1 diapause (also see below).

aak genes are required to maintain germline quiescence

A key feature of L1 diapause is maintenance of quiescent germline stem cells. Intriguingly, loss of *daf-18*, but not *daf-16*, can uncouple these processes (Fukuyama et al., 2006). We tested whether the *aak* genes are also required for maintaining germline quiescence (see Materials and Methods). Animals lacking *aak-1*, but not *aak-2*, occasionally showed ectopic germline proliferation during L1 diapause (Fig. 2A). In contrast, analysis of *ampk* double mutants revealed a striking enhancement; germ cells in all such animals examined (n=150) failed to arrest proliferation during L1 diapause, indicating that the *aak* genes act redundantly to maintain quiescence in the germline (Fig. 2A,B).

L1 diapause causes sterility in *ampk* mutants

When *ampk* and *daf-18* mutants in L1 diapause are cultured in M9 medium containing ethanol, many surviving animals lack the overt tissue degeneration observed in the absence of ethanol. This prompted us to ask whether the germline hyperplasia observed in these L1 animals would have a consequence on subsequent germline development. After 5 days of L1 diapause in M9 containing ethanol, animals were re-fed to allow postembryonic development, and animals that developed to the adult stage were examined. In contrast to wild type, *aak-1* and *aak-2* single mutants, most *ampk* double mutants were sterile when grown to adulthood (Fig. 2C). Microscopic examination revealed variable, disorganized gonad morphologies, ranging from a failure of gonad extension to well-extended gonads containing oocytes (supplementary material Fig. S1). The observed sterility appears to be a consequence of L1 diapause because all continuously fed *ampk* double mutant larvae were fertile (Fig. 2D). Similar but less severe effects of L1 diapause on fertility were also detected in *daf-18* mutants (Fig. 2C,D; supplementary material Fig. S1). Although the cause of the sterility in these mutants requires additional analysis, these observations further illustrate the requirements for *aak* and *daf-18* in ensuring proper germline development in animals that have experienced nutritional stress.

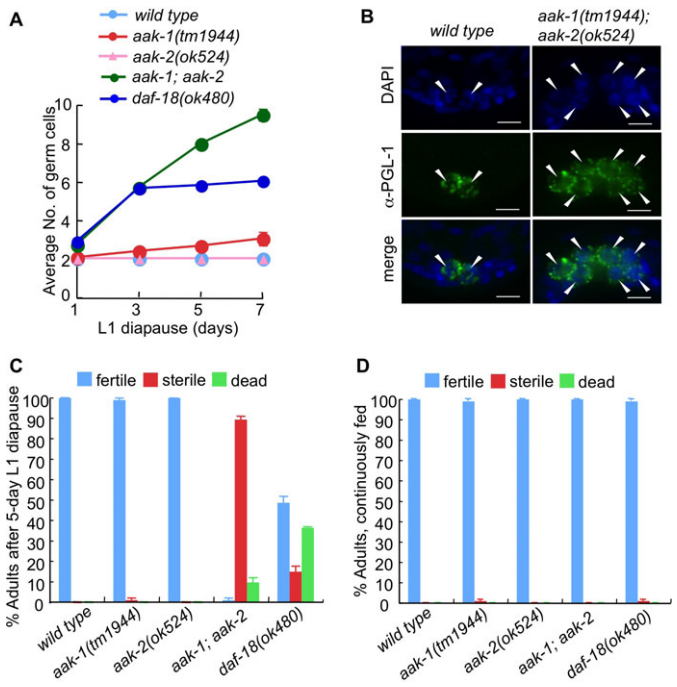

Fig. 2. The *aak* genes act redundantly to maintain germline stem cell quiescence during L1 diapause. (**A**) Time course of germline stem cell proliferation during L1 diapause. Animals were treated as described in Materials and Methods. ⩾50 animals were scored for each time point per experiment. The average number of germ cells was determined for each time point in each of three experiments, and the average of these was plotted in the graph with error bars indicating s.e.m. (**B**) Germline precursors divide in *ampk* mutants during L1 diapause. Fluorescent micrographs of wild-type and *ampk* mutant germlines fixed and stained with DAPI (blue) and anti-PGL-1 (green) polyclonal antibody (Kawasaki et al., 1998) after 7-day L1 diapause. Individual germ cells are indicated by arrowheads. Scale bar, 5 µm. (**C**) L1 diapause causes sterility in *ampk* and *daf-18* mutants. Animals were returned to food following a 5-day diapause and animals were singled when they were late L4s/young adults and their survival and fertility was scored 5 days later. Most *ampk* and about half of *daf-18* mutant adults were sterile. Animals scored as "dead" died without producing progeny, usually by rupturing at the vulva. (**D**) Fertility was not impaired when *ampk* and *daf-18* mutants were grown continuously in nutrient-rich conditions. >35 animals were scored for each time point per experiment. Animals in L1 diapause were cultured in M9 medium plus ethanol. In C and D, the average of three independent experiments is plotted with error bars indicating s.e.m.

aak-2 acts in distinct tissues to maintain viability and germline quiescence during L1 diapause

Although the *aak* and *daf-18* genes are important for maintaining survival and germline quiescence during L1 diapause (Baugh and Sternberg, 2006; Fukuyama et al., 2006; Sigmond et al., 2008; this study), the tissue(s) in which they function to control these processes have not been elucidated. We used a series of *gfp*-tagged reporter genes to identify these tissues (supplementary material Table S1). We first generated reporter fusions for each *aak* gene that contain genomic DNA, spanning the promoter and extending through the full coding region, fused to *gfp* and the *unc-86* 3′-UTR (*Paak-1::aak-1::gfp* and *Paak-2::aak-2::gfp*). Because *Paak-1::aak-1::gfp* expression patterns were weak and variable when expressed from its native promoter (data not shown), *aak-2* was used for these studies. During L1 diapause, *Paak-2::aak-2::gfp* was highly expressed in most tissues including the intestine, excretory canal, pharynx, somatic gonad, neurons, hypodermis, and body wall muscle

(supplementary material Fig. S2; data not shown), a pattern similar to that observed during continuous development (Lee et al., 2008) and dauer diapause (Narbonne and Roy, 2009). The *Paak-2::aak-2::gfp* transgene rescued the lethality and, albeit less effectively, the ectopic germline proliferation phenotype observed in *ampk* mutants during L1 diapause (Fig. 3A,B). Although the construct contains a heterologous 3′-UTR, the significant rescuing activity conferred by *Paak-2::aak-2::gfp* suggests that its expression largely recapitulates that of the endogenous gene.

To determine the tissue(s) in which *aak-2* acts to maintain survival and germline quiescence during L1 diapause, *gfp::aak-2* was expressed in *ampk* mutant animals under the control of various tissue-specific promoters. Intestinal (P*pgp-1*) and pan-neuronal (P*rgef-1*) expression of *gfp::aak-2* significantly restored the survival of *ampk* mutant animals (Fig. 3A), identifying these tissues as foci of *aak* activity relative to survival and indicating that the N-terminal GFP fusion also retains activity. In contrast to their ability to rescue the lethality phenotype, neither of these constructs suppressed the abnormal germline proliferation phenotype of *ampk* mutants during L1 diapause (Fig. 3B). These results suggest that the *aak* genes control germline quiescence in a manner distinct from that which maintains energy homeostasis during L1 diapause.

In contrast to the rescuing activity observed with the intestine and neuronal promoters, *gfp::aak-2* expression in the hypodermis, body wall muscles, or excretory (Exc) cell did not significantly rescue either of the *ampk* mutant phenotypes during L1 diapause (Fig. 3A,B). This result is interesting in light of a previous study that found that *aak-2* acts in the hypodermis and Exc to regulate survival during dauer diapause (Narbonne and Roy, 2009). The lack of rescue observed in our studies was not likely to be due to insufficient expression of the transgenes, since *gfp::aak-2* expression was sufficient to allow transgenic animals to be identified. Thus, these findings illustrate the distinct modes by which *aak-2* functions to control viability during L1 and dauer diapauses.

daf-18 and *aak* differ in their foci of action with respect to maintenance of viability during L1 diapause

We next sought to determine in which tissue(s) *daf-18* functions to maintain survival during L1 diapause. As observed with the *aak* genes, intestinal expression of *daf-18* significantly rescued survival (Fig. 3C). However, in contrast to *aak* function, the hypodermal expression of *daf-18* also rescued survival, whereas neuronal expression did not (Fig. 3C). These observations, together with the genetic analysis already described (Fig. 1B), indicate that the *aak* and *daf-18* genes act by at least partially distinct mechanisms to maintain survival during L1 diapause.

Derepressed TORC1 activity appears to release germline stem cell quiescence in both *ampk* and *daf-18* mutants

Germ cells arrest in the G2 phase of the cell cycle during L1 diapause (Fukuyama et al., 2006). Although AMPK has been proposed to cause G1 cell cycle arrest through regulation of the tumor suppressor p53 and cyclin-dependent kinase inhibitor p27 in mammalian cultured cells (Imamura et al., 2001; Jones et al., 2005; Liang et al., 2007), the mechanism by which AMPK

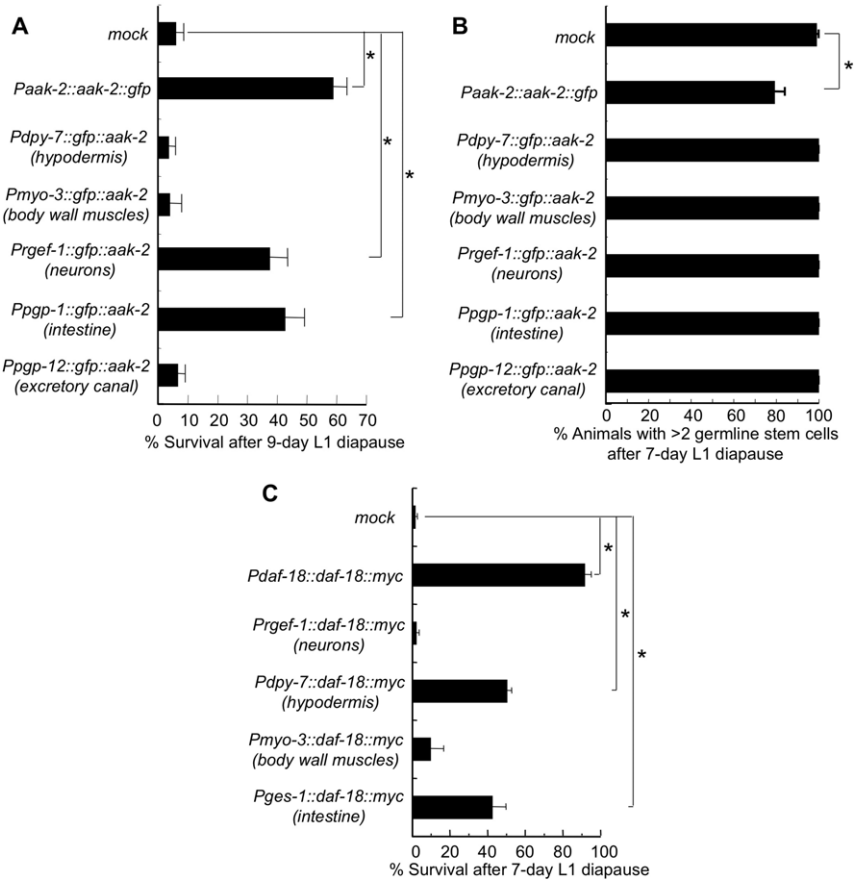

Fig. 3. *aak-2* acts in distinct tissues to regulate survival and germline quiescence during L1 diapause. (**A**) Effects of tissue-specific *aak-2* expression on the survival of *ampk* mutants during L1 diapause. Here and in (**B**) and (**C**), the average of at least three independent experiments ± s.e.m. is plotted. The Y-axis indicates the transgenes tested for rescuing activity, and the tissue in which each promoter drives expression is indicated in parentheses. The animals carrying the transgenes were identified by GFP expression. (B) Effects of tissue-specific *aak-2* expression on germline proliferation of *ampk* mutants during L1 diapause. The average number of germ cells after 7-day L1 diapause is plotted. In (A) and (B), ⩾35 animals were scored for each experiment. (C) Effects of tissue-specific *daf-18* expression on the survival of *daf-18* mutants after 5-day L1 diapause. ⩾100 animals were scored for each experiment. *$P<0.01$ for Student's t-test. "Mock" indicates analysis of animals transgenic for co-injection markers only.

induces G2 arrest is currently unknown. However, studies in multiple organisms including *C. elegans* suggest that TORC1 promotes G2 progression (Nakashima et al., 2008; LaFever et al., 2010; Gaur et al., 2011; Kapoor et al., 2012; Korta et al., 2012). Given that mammalian mTORC1 is negatively regulated by both AMPK and PTEN (reviewed by Wullschleger et al., 2006; Shackelford and Shaw, 2009; also see Introduction), we considered the possibility that the germline proliferation phenotype observed in *ampk* and *daf-18* mutant animals during L1 diapause may be caused by inappropriate activation TORC1. To test this possibility, *C. elegans* TORC1 activity was depleted in *ampk* and *daf-18* mutant animals by RNAi. Key components of TORC1 are the protein kinase TOR and its scaffold protein Raptor, proteins whose worm orthologues are encoded by *let-363* (Long et al., 2002) and *daf-15* (Jia et al., 2004), respectively. RNAi directed against each of these genes significantly suppressed the germline phenotype of *ampk* and *daf-18* mutant L1 larvae, *but did not alter germ cell number during L1 diapause of wild type* (Fig. 4A,B; supplementary material Fig. S3), suggesting that TORC1 functions either in parallel to, or downstream of, the *aak* and *daf-18* genes. Recently, loss of *aak* activity has been shown to partially suppress male tail defects caused by *plx-1* mutation, which significantly reduces TORC1 activity (Nukazuka et al., 2011). Furthermore, the inhibitory AMPK phosphorylation site is conserved between mammalian Raptor and *C. elegans* DAF-15 (Gwinn et al., 2008). Thus, our RNAi results are consistent with a model in which abnormal germline proliferation of *ampk* and *daf-18* mutants during L1 diapause is caused by derepressed activity of TORC1.

Interestingly, neither *let-363(RNAi)* nor *daf-15(RNAi)* significantly restored the viability of *ampk* or *daf-18* mutants during L1 diapause (supplementary material Fig. S4). These observations suggest that *aak* and *daf-18* maintain survival during L1 diapause through TORC1-independent pathways. Alternatively, RNAi against *let-363* and *daf-15* may not be effective enough to confer detectable suppression of this phenotype.

In mammals and *Drosophila*, two G proteins, *Rheb* and *Rag*, mediate growth factor and amino acid signaling in response to TORC1 activation (Fig. 1A) (reviewed by Zoncu et al., 2011). There is a sole *C. elegans* Rheb orthologue, *rheb-1* (Li et al., 2004). Rag proteins are subdivided into two subfamilies, which associate with each other to form heterodimers (Nakashima et al., 1999; Sekiguchi et al., 2001). One subfamily includes *S. cerevisiae* Gtr1p and mammalian RagA and RagB, and the other includes *S. cerevisiae* Gtr2p and mammalian RagC and RagD. Similar to yeast, *C. elegans* possesses only one gene, called *raga-1*, whose translational product belongs to the Gtr1p/RagA/B subfamily (Schreiber et al., 2010). We identified the *ragc-1* gene, which encodes a sole orthologue of Gtr2p, RagC and RagD (supplementary material Fig. S5). As in other organisms (Garami et al., 2003; Saucedo et al., 2003; Stocker et al., 2003; Kim et al., 2008; Sancak et al., 2008), *C. elegans rheb-1*, *raga-1*, and *ragc-1* orthologues are likely to be required for full activation of TORC1. Consistent with this view, inhibition of TORC1 and *ragc-1* results in similar phenotypes including increased autophagy, decreased overall mRNA translation, and enhanced stress tolerance (Robida-Stubbs et al., 2012). If exit from germline quiescence in *ampk* and *daf-18* mutant animals is triggered by derepression of TORC1 activity, then reducing or eliminating the activity of *rheb-1*, *raga-1* or *ragc-1* should suppress these defects. Consistent with this idea, the ectopic germline proliferation observed in both *ampk* and *daf-18* mutants was significantly suppressed by RNAi of *rheb-1* or by making double mutants between these genes and probable null alleles of *raga-1* or *ragc-1* (Fig. 4C,D). One could argue that the observed genetic suppression may simply reflect a requirement for *rheb-1* and the Rag genes during larval cell divisions. However, this is clearly not the case for the Rag genes; in contrast to *let-363* and *daf-15*, neither the *raga-1* nor *ragc-1* mutations result in fully penetrant larval arrest or sterility; both mutants can be maintained as homozygotes (Schreiber et al., 2010; data not shown).

Discussion

For L1 diapause to be advantageous to an animal, the organism must be able to survive an extended period of starvation and resume development when conditions improve. Our studies reveal a role for AMP-activated kinase genes *aak-1* and *aak-2* in

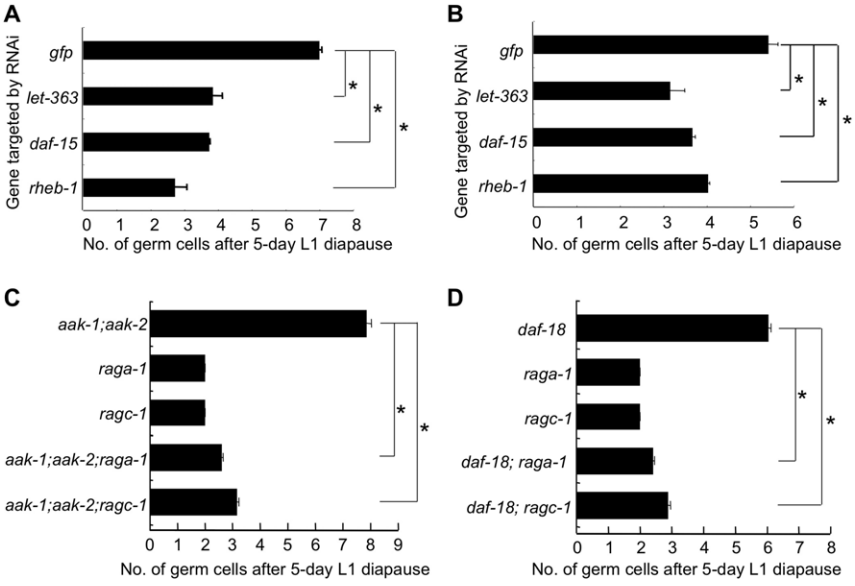

Fig. 4. TORC1 activity mediates ectopic germ cell proliferation in *ampk* and *daf-18* mutants. (**A**,**B**) Effects of TORC1 inhibition on the average number of germ cells in *ampk* (A) and *daf-18* (B) mutants after 5-day L1 diapause. (**C**,**D**) Genetic elimination of Rag activity in *ampk* and *daf-18* mutants. Genotypes are listed on the Y-axis and alleles used were as follows: *aak-1*(*tm1944*), *aak-2*(*ok524*), *raga-1*(*ok386*), *ragc-1*(*tm1974*), and *daf-18(ok480)*. Number of animals scored in each experiment: (A) ⩾24, (B) ⩾10, and (C,D) ⩾35. The average number of germ cells per genotype was determined for each experimental trial, and the average of three trials is plotted in the graph with error bars indicating s.e.m. *$P<0.01$ for Student's t-test.

two key aspects of L1 diapause induced by nutrient deprivation: survival and arrest of germ cell proliferation.

Regulation of survival by AMPK

Loss of AMPK activity dramatically impairs survival during L1 diapause. We speculate that energy deprivation contributes to this lethality for two main reasons. First, the lethality can be partially rescued by supplementation of the medium with glucose or ethanol. Previous studies have shown that glucose and ethanol, as well as other carbohydrates, promote population growth when included in chemically defined media, leading to the conclusion that these compounds were utilized as energy source (Braeckman et al., 2009). Second, tissue-specific expression of *aak-2* in the intestine, an energy storing organ, significantly increases survival of *ampk* mutants. A large body of literature has established that AMPK upregulates catabolic pathways that generate ATP, and downregulates anabolic pathways that consume ATP in energy storing organs such as muscle, liver, and adipose tissue of mammals (Kahn et al., 2005); thus, it is reasonable to hypothesize that multiple metabolic pathways controlled by AMPK contribute to maintenance of survival during L1 diapause. Narbonne and Roy have shown that regulation of a triglyceride lipase, ATGL-1, by AMPK in the hypodermis plays a key role in maintaining lifespan during dauer diapause (Narbonne and Roy, 2009). Because an *atgl-1::gfp* reporter is expressed in the intestine (Zhang et al., 2010), regulation of ATGL-1 by AMPK in this tissue may provide an additional contribution to survival during L1 diapause.

We found that neuronal AMPK activity also plays an important role in maintaining survival during L1 diapause. Recent studies have shown that rodent hypothalamic AMPK affects expression of neuropeptides such as AgRP (Agouti-related peptide), NPY (neuropeptide Y) and POMC (proopioidmelanocortin) to control food intake (Minokoshi et al., 2004). Although *C. elegans* does not possess clear orthologues of these neuropeptides, multiple genes encoding members of neuropeptide Y/RFamide receptor family and RFamides are present in its genome (Li et al., 1999; Hewes and Taghert, 2001). Therefore, there might be an RFamide-receptor pair that functions downstream of AMPK in the *C. elegans* nervous system. Whether loss of AMPKs in *C. elegans* results in abnormal control of food intake, as it does in mice, remains to be examined. However, such a defect is not likely to make a significant contribution to survival during the L1 diapause, which occurs in the absence of food. Thus, roles of AMPKs in the nervous system may not simply be limited to regulation of feeding.

Regulation of germline stem cell proliferation by AMPK

Abnormal germline proliferation during L1 diapause in *ampk* and *daf-18* mutants was significantly suppressed by knockdown or elimination of TORC1 activity or its conserved activators (i.e. *rheb-1*, *raga-1* and *ragc-1*). These results suggest that *aak* and *daf-18* maintain quiescence by suppressing TORC1 activity. Similarly, rapamycin, a potent inhibitor of TORC1, can induce G2 arrest in some types of mammalian cultured cells (Gaur et al., 2011; Kapoor et al., 2012). Genetic inhibition of TOR also results in G2 delay in *Saccharomyces cerevisiae* and adult germline stem cells in *Drosophila melanogaster* and *C. elegans* (Nakashima et al., 2008; LaFever et al., 2010; Korta et al., 2012). Although how TORC1 affects activity of the core cell cycle machinery such as cyclins, cdk (cyclin-dependent kinase), and cdk inhibitors, remains unknown in multicellular animals (Russell et al., 2011), studies in yeast suggest that polo-like kinase CDC5, which is a regulator of the mitotic cdk, Cdc28, mediates the TOR signaling pathway that promotes G2/M transition (Nakashima et al., 2008). Similar mechanisms may function in higher organisms.

One unresolved question is, in which tissue(s) do *aak* and *daf-18* act to regulate germline quiescence? Immunostaining studies showed that cells expressing DAF-18 include the germline precursors Z2 and Z3 (Brisbin et al., 2009), and previous studies have suggested that the *aak* and *daf-18* genes act cell autonomously to regulate germline quiescence during diapause. The germline-cell autonomy conclusions were based on RNAi experiments carried out in *rrf-1* mutants, in which RNAi was thought to be active only in the germline (Sijen et al., 2001; Narbonne and Roy, 2006; Watanabe et al., 2008). Indeed, we also found that when *aak-1; rrf-1* and *aak-2; rrf-1* double mutants were subjected to *aak-2* and *aak-1* RNAi, respectively, each strain exhibited a significant increase in failure to arrest germline proliferation during L1 diapause (data not shown). However, *rrf-1* mutants were recently found to maintain RNAi activity in some somatic tissues, including the gut and hypodermis (Kumsta and Hansen, 2012), leaving the question as yet unanswered. *aak-2::gfp* expressed under the control of its endogenous promoter can weakly rescue the defects in germline quiescence (Fig. 3B), but its expression was not detected in Z2 or Z3 (not shown), typical for expression from extrachromosomal arrays which are often silenced in the germline. Extrachromosomally expressed *daf-18* also significantly suppressed the germline phenotype during L1 diapause (Fukuyama et al., 2006). Thus, one could argue that *aak-2* and *daf-18* act in the soma to regulate germline quiescence. Alternatively, low levels of germline expression from extrachromosomal arrays could be responsible for the rescue; low levels of maternally expressed *lin-35* from the arrays results in substantial suppression of developmental phenotypes in *lin-35; fzr-1* double mutants (Fay et al., 2002). Therefore, it remains possible that *aak* and *daf-18* act in the germline to regulate its quiescence.

Recent studies using lineage-specific *pten* knockout mice have demonstrated that *pten* is required for quiescence of haematopoietic stem cells as well as premature oocytes (Yilmaz et al., 2006; Zhang et al., 2006; Reddy et al., 2008). Thus, one of the physiological roles of *pten* conserved between mammals and worms may be to prevent unwanted exit from quiescence in germline and stem cells. In this respect, it would be of great interest to test whether AMPKs are also essential for maintaining quiescence of stem cells and premature oocytes in mammals.

Materials and Methods

Worm culture and genetics

Worm culture and genetics were conducted according to standard procedures (Brenner, 1974). Cultures were grown at 20℃ unless otherwise noted. All strains used in this study were outcrossed against wild-type (Bristol N2) worms at least twice.

Assessing viability and germline proliferation

Sterilized embryos were hatched in 10 ml of sterile M9 with or without 0.08% ethanol in a 15 ml polypropylene tube, and hatched larvae were continuously cultured in the medium to assess survival or germline proliferation, respectively. L1 larvae were prepared as follows: about 40 to 50 gravid young adults were placed in a 10 cm “4× peptone” plate (standard NGM plus four times more

peptone) seeded with OP50 *E. coli*. For experiments presented in supplementary material Fig. S4, 10 cm 4× peptone plates containing 25 mg/ml carbenicillin and 1 mM IPTG seeded with HT115 harboring RNAi construct were used. Adult F1 animals were washed into a 15 ml tube in M9, gravid adults were allowed to settle, and the supernatant was removed. Embryos were purified by the addition of 5 ml of alkali/bleach solution (0.5 M KOH; 10% sodium hypochlorite solution [Wako Inc, Japan]), washed three times with M9, and incubated in 10 ml of M9 to assess viability or M9 plus 0.08% ethanol to determine the average number of germline precursors. Incubation was at 20℃ with 30 rpm rotation (Rotamix, ATR, Inc.). Germline stem cells and somatic gonadal precursors were identified by inspection (Kimble and Hirsh, 1979) using a Zeiss Axio Imager M1.

To assess the viability for Fig. 1B,C and supplementary material Fig. S4, about 50 µl of culture were placed on a slide, and animals exhibiting any movement were scored as alive, as in previous studies (Baugh and Sternberg, 2006). Animals lacking movement were reassessed with prodding. For the viability assay in Fig. 3A,B, starved animals were mounted on an agar pad, and the transgenic animals were identified by GFP expression. Motility was used as a proxy for viability. Motility and tissue integrity, which would be lost quickly once the animal were starved to death, were well correlated.

Assessing sterility following L1 diapause

About 50 gravid adults were plated onto a 10 cm 4× peptone plates. After 4 days embryos were isolated and incubated 10 ml M9 plus 0.08% ethanol for 5 days. About 1,000 larvae were then plated onto a 100 mm 4× peptone plate and 3 to 4 days later young adults and L4 animals were randomly singled onto seeded NGM plates and fertility and viability were assessed 5 days later. To assess fertility of the continuously-fed animals, L4 larvae were randomly singled onto seeded NGM plates and scored 5 days later.

RNAi for germline defects

Feeding RNAi was conducted according to the standard protocols (Kamath et al., 2003). Sterilized embryos from RNAi-treated animals were treated as already described except that NGM plates seeded with HT115 bacteria harboring the appropriate RNAi vector were used. For RNAi targeting of *rheb-1*, approximately 1000 sterilized embryos were plated onto three 10 cm RNAi plates. For *let-363(RNAi)* and *daf-15(RNAi)*, this resulted in many animals arresting during larval stages. Thus, for these experiments, larvae were synchronously grown on OP50-seeded NGM plates until the L3 to L4 stage and then were transferred to RNAi plates.

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Competing Interests

The authors have no competing interests to declare.

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