© 2018. Published by The Company of Biologists Ltd | Development (2018) 145, dev166876. doi:10.1242/dev.166876

RESEARCH ARTICLE

C. elegans PTEN and AMPK block neuroblast divisions by inhibiting a BMP-insulin-PP2A-MAPK pathway

Shanqing Zheng, Zhi Qu, Michael Zanetti, Brandon Lam and Ian Chin-Sang*

ABSTRACT

Caenorhabditis elegans that hatch in the absence of food stop their postembryonic development in a process called L1 arrest. Intriguingly, we find that the postembryonic Q neuroblasts divide and migrate during L1 arrest in mutants that have lost the energy sensor AMP-activated protein kinase (AMPK) or the insulin/IGF-1 signaling (IIS) negative regulator DAF-18/PTEN. We report that DBL-1/BMP works upstream of IIS to promote agonistic insulin-like peptides during L1 arrest. However, the abnormal Q cell divisions that occur during L1 arrest use a novel branch of the IIS pathway that is independent of the terminal transcription factor DAF-16/FOXO. Using genetic epistasis and drug interactions we show that AMPK functions downstream of, or in parallel with DAF-18/PTEN and IIS to inhibit PP2A function. Further, we show that PP2A regulates the abnormal Q cell divisions by activating the MPK-1/ERK signaling pathway via LIN-45/RAF, independently of LET-60/RAS. PP2A acts as a tumor suppressor in many oncogenic signaling cascades. Our work demonstrates a new role for PP2A that is needed to induce neuroblast divisions during starvation and is regulated by both insulin and AMPK.

KEY WORDS: *C. elegans*, Starvation, Q neuroblast divisions, BMP, DAF-18/PTEN, AMPK, PP2A, MPK-1

INTRODUCTION

Nutrition and energy homeostasis are crucial for proper animal development. Studies investigating the effects of nutrients on developmental physiology have made substantial contributions towards improving overall human health. However, how developmental arrest is regulated by nutritional availability and the associated signaling pathways have not been well studied. *Caenorhabditis elegans* that hatch in the absence of food will stop their development until food becomes available; this quiescent state is termed L1 arrest. L1 arrest is a survival strategy and L1-arrested worms can live up to 21 days without food. L1 arrest provides a model in which to study the coordination of postembryonic developmental events and gene regulatory circuits influenced by food (Baugh, 2013; Fukuyama et al., 2015, 2012). The insulin/IGF-1 signaling (IIS) pathway plays a key role in regulating L1 arrest (Fukuyama et al., 2012; Kaplan et al., 2015). The insulin-like peptides (INS) act through the IIS receptor, DAF-2/IR, activating a conserved AGE-1/PI3K-AKT signal transduction to suppress the function of the Forkhead Box O (FOXO) transcription factor DAF-16/FOXO (Ogg et al., 1997). A very important negative regulator of IIS is DAF-18/PTEN. PTEN is a potent human tumor suppressor, and mutations that cause the loss of PTEN result in many cancers (Chalhoub and Baker, 2009). The *C. elegans* PTEN homolog DAF-18 also possesses tumor suppressor-like properties; in this study, we show that the neuronal Q cell lineage continues to divide and move in L1-arrested *daf-18* mutants. We determined that Q cell quiescence requires DAF-18 lipid phosphatase activity, and, surprisingly, the terminal transcription factor in the IIS pathway, DAF-16, is not required to block the abnormal Q cell divisions that occur during L1 arrest. Furthermore, the human PTEN can functionally replace DAF-18 to block Q cell divisions during L1 arrest. The loss of *daf-18* leads to activated IIS and, consistent with this, we showed that the overexpression of agonistic insulin/insulin-like peptides (INS) could cause abnormal Q cell divisions during L1 arrest. We provide evidence that DBL-1, a member of the transforming growth factor β (TGFβ) superfamily (Savage-Dunn, 2005), promotes Q cell divisions upstream of IIS by inducing insulin peptides during L1 arrest.

AMP-activated protein kinase (AMPK) detects shifts in AMP/ATP and ADP/ATP ratios to regulate developmental events (Salminen and Kaarniranta, 2012). Recent studies suggest that AMPK is a tumor suppressor and plays a crucial role in cellular processes (Zadra et al., 2015). Our chemical and genetic epistasis analyses show that AAK-2, which encodes one of two *C. elegans* homologs of the catalytic α-subunit of AMPK, works downstream of or in parallel with DAF-18 and AGE-1 to block Q cell divisions during L1 arrest. Further, we show that protein phosphatase 2A (PP2A) inhibitor and PP2A holoenzyme regulatory subunit mutants can suppress these abnormal cell divisions. Our study demonstrates that the PP2A family is regulated by AMPK and IIS. PP2A is known to regulate the RAF-MEK-ERK/MAPK pathway in mammals and *C. elegans*, and MPK-1 plays an important role in many cellular processes (Lee et al., 2007; Müller et al., 2001; Narbonne et al., 2017; Sundaram, 2013). We report here that in the absence of DAF-18 or AAK-2, L1-arrested animals have abnormal activation of PP2As, which in turn activate MPK-1 via the RAF ortholog LIN-45 in the MAP kinase pathway to promote Q cell divisions during L1 arrest.

RESULTS

Loss of *daf-18* causes Q cells to divide during L1 arrest

During the first larval stage, two embryonic neuroblasts, QL and QR, move and divide to form six neurons, AVM/PVM, SDQR/SDQL and AQR/PQR (Fig. 1A) (Chalfie and Sulston, 1981; Middelkoop and Korswagen, 2014). If *C. elegans* hatch in the absence of food, worms enter an L1-arrested state and do not proceed through postembryonic development (Johnson et al., 1984), and this L1-arrested state can be visualized by observing only two Q cells (QL/QR) (Fig. 1A,E). Using the mechanosensory reporter *Pmec-4::gfp (zdIs5)* (Clark and Chiu, 2003), we found that the Q cell descendants AVM and PVM were consistently present in

Department of Biology, Queen's University, Kingston, ON, Canada K7L 3N6.

*Author for correspondence (chinsang@queensu.ca)

I.C.-S., 0000-0001-7480-9775

Received 23 April 2018; Accepted 16 October 2018

Development (2018) 145, dev166876. doi:10.1242/dev.166876

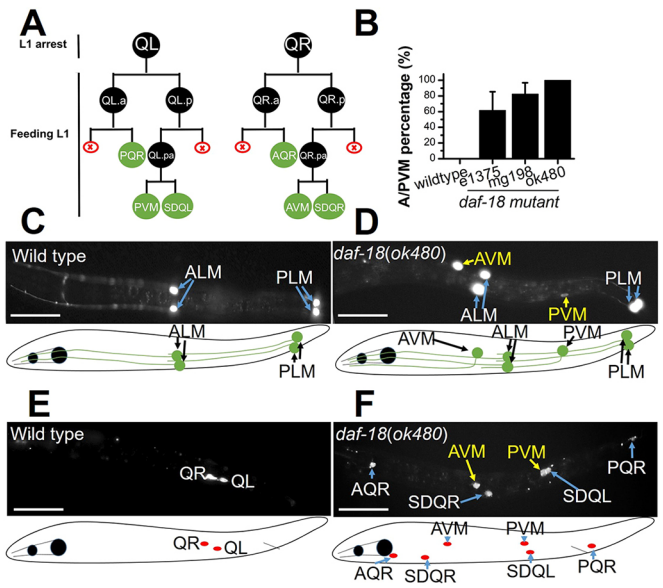

Fig. 1. *daf-18* mutants show Q cell divisions during L1 arrest. (A) The Q neuroblast cell lineage in L1 arrest and under normal feeding conditions. The two Q neuroblasts, QL (left) and QR (right) normally do not divide under L1 starvation arrest (top). During L1 feeding, the Q cells divide and move to form six neurons, AQR/PQR, AVM/PVM and SDQR/SDQL, and four programmed cell deaths, indicated by red crosses. (B) *daf-18* mutants show Q cell descendants AVM/PVM during L1 arrest. Data are the average of at least three independent experiments, with a sample size of at least 70 animals; error bars represent s.d.; also see details in Table S2. (C) Touch neurons (*zdIs5 Pmec-4::GFP*) in L1-arrested wild-type worms show only two ALMs and two PLMs. (D) Touch neurons in L1-arrested *daf-18 (ok480)* mutants show two ALMs, two PLMs and two postembryonic touch neurons, AVM and PVM (Q cell descendants). (E) Q cells (*rvdIs1 Pegl-17::mCherry*) in L1-arrested wild-type worms show only QL and QR. (F) Q cells in L1-arrested *daf-18 (ok480)* mutants show all the Q cell descendants: AQR/PQR, AVM/PVM and SDQR/SDQL. Also see Fig. S2 for AVM/PVM (*zdIs5*) colocalization with Q cell (*rdvIs1*) in L1-arrested *daf-18 (ok480)* worms. Scale bars: 50 µm.

mutant animals bearing three *daf-18* alleles tested during L1 arrest (Fig. 1B-D). The *daf-18 (ok480)* allele, which is a strong loss-of-function allele (Zheng and Chin-Sang, 2016), showed 100% penetrance for these cell divisions during L1 arrest. Next, we wanted to know whether all Q cell descendants were dividing in *daf-18* mutants during L1 arrest. We used the Q cell lineage marker *Pegl-17::mCherry (rdvIs1)* (Chai et al., 2012). In wild-type L1-arrested worms, only QL/QR are visible (Fig. 1E). In contrast, all six postembryonic Q cells were present and migrated to their correct locations in L1-arrested *daf-18 (ok480)* worms (Fig. 1F). The inappropriate Q cell divisions are postembryonic and divisions occur 12-18 h after egg preparation (Fig. S2E). Thus, *daf-18* mutant worms do not block the Q cell lineage during L1 arrest and these cells divide as if the worms were under fed conditions. For the remainder of our studies, we used the *Pmec-4::gfp (zdIs5)* mechanosensory neuron marker as it allows us to easily visualize AVM and PVM in L1-arrested worms, which are the final descendants of QR and QL, respectively (Fig. 1A,D).

DAF-18 functions cell-autonomously and its lipid phosphatase activity is required for Q cell quiescence

To determine whether *daf-18* regulates the Q cell divisions cell-autonomously or non-cell-autonomously during L1 arrest, we used tissue-specific rescue of *daf-18 (ok480)* mutant worms. We found that the Q cell division phenotype in L1-arrested *daf-18* mutants was rescued by the expression of genomic *daf-18* or *daf-18* cDNA driven by its native promoter, and the human *PTEN* cDNA expressed from a *C. elegans daf-18* promoter could fully rescue the Q cell division phenotype in L1-arrested *daf-18 (ok480)* (Fig. 2A). We show that *daf-18* expressed from the pan-neuronal promoter *Prgef-1* or the Q cell-specific promoter *Pegl-17* could also rescue the Q cell division phenotype in L1-arrested *daf-18 (ok480)*. In contrast, intestinal *Pges-1*, germline *Ppie-1* or muscle-specific *Pmyo-3* did not rescue the Q cell division phenotype in L1-arrested *daf-18 (ok480)* (Fig. 2B). As the lack of rescue is a negative result, we tested the functionality of these constructs and showed that the germ cell-specific *daf-18* transgene can rescue the germ cell proliferation in *daf-18* mutants, the intestine specific *daf-18* can rescue the dauer-defective phenotype, and, more importantly, all these rescue strains could partially rescue the *daf-18* longevity during L1 arrest (data not shown). Thus, we confirm all these rescue strains have DAF-18 functionality and that DAF-18 inhibits Q cell divisions in a cell-autonomous manner.

The DAF-18/PTEN tumor suppressor is best known for its lipid phosphatase activity, which dephosphorylates the 3′ position of phosphatidylinositol 3, 4, 5-trisphosphate (PIP3) (Mihaylova et al., 1999; Ogg and Ruvkun, 1998). However, DAF-18/PTEN has been reported to also have protein phosphatase activity, which can control cell migration and cell development independently of the PI3K/AKT pathway (Brisbin et al., 2009; Dey et al., 2008; Shinde and Maddika, 2016; Stumpf and den Hertog, 2016). To identify which phosphatase activity of DAF-18 was required for blocking Q cell divisions during L1 arrest, we made three variants of DAF-18 that correspond to known human PTEN variants and tested them for their rescuing activity. DAF-18 (D137A) is protein phosphatase defective, DAF-18 (G174E) is lipid phosphatase defective, and DAF-18 (C169S) abolishes both lipid and protein phosphatase activity (Fig. 2C) (Solari et al., 2005; Zheng and Chin-Sang, 2016). We found that only the DAF-18 (D137A) protein phosphatase-deficient variant was able to rescue the Q cell division phenotype (Fig. 2D), whereas DAF-18 (G174E) and DAF-18 (C169S) lost their ability to suppress the abnormal Q cell divisions that occur during L1 arrest (Fig. 2D). These results confirm that the lipid phosphatase activity of DAF-18 plays the primary role in blocking Q cell divisions during L1 arrest.

IIS regulates Q cell divisions independently of DAF-16

Because the loss of DAF-18 leads to activated IIS, we investigated whether we could induce Q cell divisions by overexpressing potential agonistic INS during L1 arrest. The INS are primarily expressed in the nervous system (Pierce et al., 2001), so we overexpressed candidate INS genes in neurons using the *Prgef-1* pan-neuronal promoter (Altun-Gultekin et al., 2001). *ins-3*, *-4*, *-6*, *-9* and *daf-28* have been reported to be strong agonists (Chen and Baugh, 2014; Hung et al., 2014), and overexpressing these INS caused penetrant Q cell divisions during L1 arrest (Fig. 2E).

To identify the role of the IIS pathway in Q cell divisions, we tested all the major components of the IIS pathway. DAF-18/PTEN and AGE-1/PI3K antagonize each other's activity (Morris et al., 1996). We found that *age-1 (m333)* almost fully suppressed the Q cell divisions in L1-arrested *daf-18* mutants (Fig. 2F). We also found that *akt-1/2* and *pdk-1* mutants all significantly suppressed the Q cell divisions in L1-arrested *daf-18 mutants* (Fig. 2F). *C. elegans* has one INS receptor called DAF-2. The *daf-2 (e979)* allele has been reported to be a temperature-sensitive null allele (Patel et al., 2008). We made a *daf-2 (e979); daf-18 (ok480)* double mutant, and showed that *daf-2 (e979)* fully suppressed the Q cell divisions in

Development (2018) 145, dev166876. doi:10.1242/dev.166876

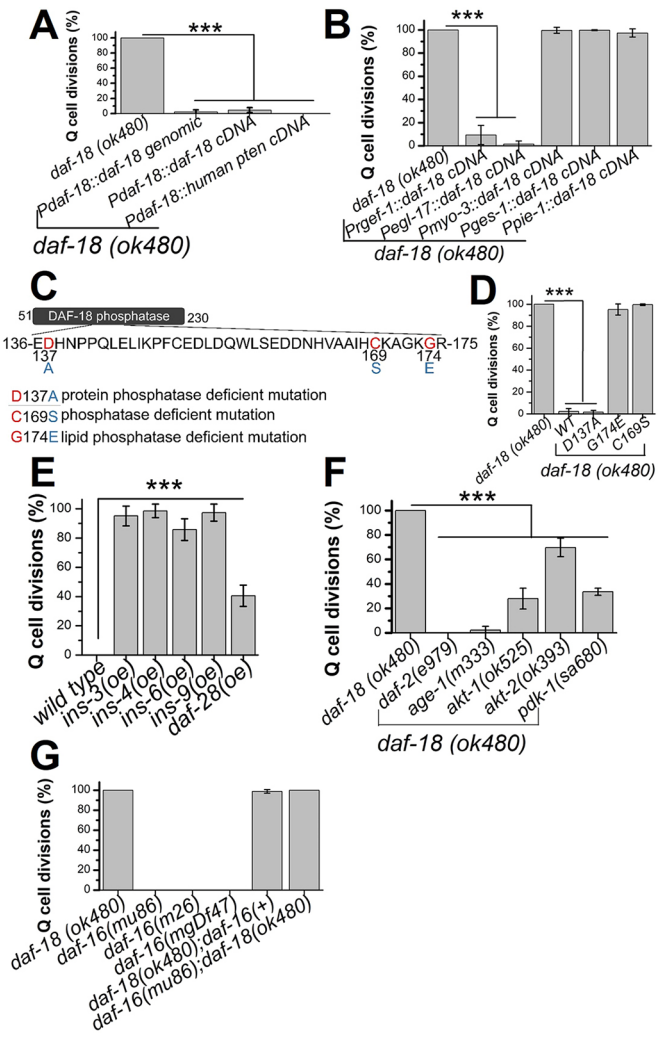

Fig. 2. The DAF-18 lipid phosphatase is required cell-autonomously to keep Q cells from dividing during L1 arrest, and Q cell divisions are independent of DAF-16. (A) Genomic *daf-18*, *daf-18* cDNA and human *PTEN* cDNA rescue the Q cell division phenotype in L1-arrested *daf-18 (ok480)*. (B) *daf-18* functions cell-autonomously in the Q cells. Tissue-specific *daf-18* rescue experiments show only pan-neuronal (*Prgef-1*) and Q cell-specific (*Pegl-17*) *daf-18* expression rescue the Q cell division phenotype in L1-arrested *daf-18 (ok480)*. Muscle (*Pmyo3*), gut (*Pges-1*) or germline (*Ppie-1*) *daf-18* expression show no rescue. (C) The DAF-18 phosphatase domain is encoded by amino acids 51-230. The active site of DAF-18 with three conserved phosphatase loss-of-function mutations are shown. (D) Lipid phosphatase activity is required for DAF-18 Q cell quiescence. Only the protein phosphatase deficient DAF-18 (D137A) rescued the Q cell division phenotype. (E) Pan-neuronal INS expression can induce Q cell divisions during L1 arrest. (F) The major components of IIS are required for Q cell divisions in L1-arrested *daf-18* mutants. *daf-2*, *age-1*, *akt-1/2* and *pdk-1* all suppress the Q cell divisions in L1-arrested *daf-18 (ok480)*. (G) Three *daf-16* alleles were tested and they do not show Q cell divisions during L1 arrest. *daf-18* is epistatic to *daf-16* and overexpression of *daf-16 (+)* does not rescue the *daf-18* Q cell division phenotype. Data were collected from the double mutants and represent the averages of at least three independent experiments. For transgenic worms, data are the average of at least three independent experiments from at least three independent stable transgenic lines for each rescue strain. Error bars represent s.d.; ***P<0.001 (Student's t-test). See details in Tables S1-S3.

L1-arrested *daf-18* (*ok480*) at the restrictive temperature (25°C) (Fig. 2F). These results show that the insulin receptor DAF-2 and downstream components are required for Q cell divisions in L1-arrested *daf-18* mutants.

Because *daf-18* is a negative regulator of IIS, then loss of *daf-18* should cause overactive insulin signaling. Overactive insulin signaling should result in the inhibition of the final transcription factor DAF-16/FOXO (Ogg et al., 1997). Therefore, we predicted *daf-16* mutants should also show abnormal Q cell divisions during L1 arrest. However, we found that three different *daf-16* alleles tested did not display any Q cell divisions during L1 arrest (Fig. 2G). Furthermore, we overexpressed the *daf-16* gene in *daf-18 (ok480)* worms and found that these *daf-18 (ok480); daf-16 (+)* worms still displayed 100% Q cell divisions during L1 arrest (Fig. 2G). We also showed that the *daf-18; daf-16* double mutants behaved like the *daf-18 (ok480)* single mutant (Fig. 2G). Thus, our results demonstrate that hyperactive insulin signaling induces Q cell divisions during L1 arrest, but this pathway branches at AKT-1/2 and is independent of DAF-16/FOXO.

DBL-1/BMP Sma/Mab pathway controls Q cell divisions through regulating insulin peptides

In a candidate gene approach, we found that *daf-4* mutants significantly suppressed the Q cell divisions in L1-arrested *daf-18* mutants. *daf-4* mutants cause a dauer-constitutive phenotype and the *daf-4* gene encodes a type II receptor for two important TGFβ family signaling pathways: DAF-7/TGFβ dauer, which regulates dauer development, and DBL-1/BMP Sma/Mab, which regulates body size and male tail development (Savage-Dunn, 2005).

We showed that mutations in DBL-1 and not DAF-7 suppressed the Q cell divisions in L1-arrested *daf-18 (ok480)*. Genes tested in the DBL-1 pathway included *dbl-1* (BMP ligand), *sma-9* and *lin-31* (transcription factors). Loss-of-function mutants in these genes all significantly suppressed the Q cell divisions in L1-arrested *daf-18 (ok480)*. (Fig. 3A). In contrast, *daf-7* mutants did not show any suppression (Fig. 3A). Furthermore, *daf-3* and *daf-5* mutants, which are predicted to be inhibited by DAF-7 signaling, did not display Q cell divisions during L1 arrest (data not shown).

To assess whether DBL-1 functions upstream or downstream of IIS, we tested whether *dbl-1* mutants could suppress the Q cell divisions in L1-arrested INS overexpression worms. In contrast to suppressing *daf-18 (ok480)* mutants, we found that neither *daf-4* nor *sma-9* mutations could suppress Q cell divisions in our INS-4 overexpressing lines (Fig. 3B). The fact that INS overexpression is epistatic to *daf-4* and *sma-9* is consistent with the INS peptides functioning downstream of DBL-1 signaling pathway (Fig. 3B). Our results suggest that the DBL-1 signaling pathway is upstream of IIS and may regulate the insulins to promote the Q cell divisions in L1-arrested worms. To test this hypothesis, we tested the expression level of two strong agonistic insulin ligands (INS-3 and INS-4) in *dbl-1* and *sma-9* mutants. We found that the transcriptional reporters *Pins-3::gfp* and *Pins-4::gfp* were significantly reduced in these two mutants (Fig. 3C). Furthermore, we predicted that if DBL-1 was inducing INS, overexpression of *dbl-1* may also induce Q cell divisions during L1 arrest. Indeed, when we pan-neuronally overexpressed *dbl-1*, it was sufficient to induce penetrant Q cell divisions, and these cell divisions were dependent on the DAF-2 insulin receptor (Fig. 3D). Our combined results show that DBL-1 induces INS peptides to promote Q cell divisions during L1 arrest.

AAK-2 is required to block Q cell divisions during L1 arrest

Previous studies have reported that loss of AMPK also causes defects in L1 arrest and reduces starvation survival (Baugh and Sternberg, 2006; Fukuyama et al., 2012). The *C. elegans* genome encodes two AMPK catalytic α-subunits. *aak-1; aak-2* double mutants were reported to undergo germ cell divisions during L1 arrest (Demoinet

Development (2018) 145, dev166876. doi:10.1242/dev.166876

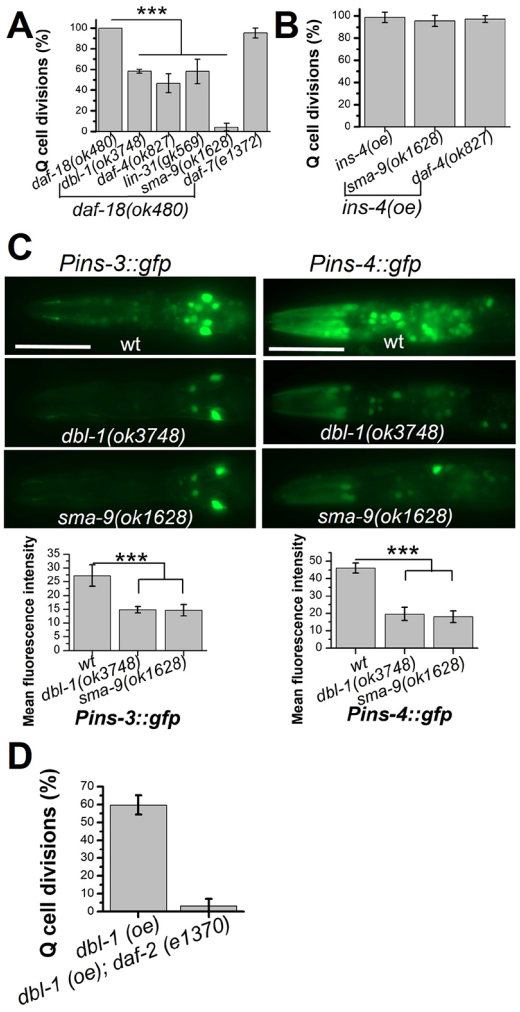

Fig. 3. DBL-1 signaling pathway regulates insulin level to control Q cell divisions. (A) DBL-1 and not DAF-7 mutants suppress the Q cell divisions in L1-arrested *daf-18*. *dbl-1* (BMP), *daf-4* (type II receptor), *lin-31* and *sma-9* (DBL-1 downstream transcription factors) can suppress the Q cell divisions in L1-arrested *daf-18 (ok480)*. In contrast, *daf-7* (TGFβ) does not. (B) INS-4 overexpression mutants are epistatic to *dbl-1*. Although *daf-4* and *sma-9* mutants can suppress *daf-18 (ok480)* mutants, they do not suppress INS-4 overexpression. (C) DBL-1 regulates agonistic insulin level. Two important agonistic insulins, INS-3 and INS-4, are primarily expressed in the head neurons. The expression levels of these two insulin peptides in *dbl-1* and *sma-9* mutants were significantly reduced. (D) Pan-neuronally overexpressed *dbl-1* caused Q cell divisions in L1-arrested worms and was suppressed by the mutation of *daf-2*. Data represent the average of at least three independent experiments for each strain; error bars represent s.d.; ***P<0.001 (Student's *t*-test); see details in Tables S1 and S4. Scale bars: 50 µm.

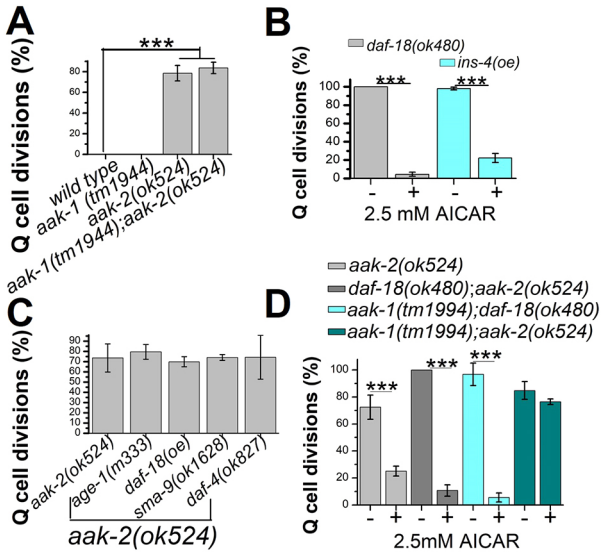

Fig. 4. AMPK is required to block Q cell divisions during L1 arrest. (A) AAK-2 is the main AMPK α subunit required to block Q cell divisions. Q cell divisions in L1-arrested *aak-1*, *aak-2* and *aak-1*; *aak-2* double mutants. (B) AICAR, an AMPK activator, suppresses Q cell divisions in L1-arrested *daf-18* and *ins-4 (oe)* worms. (C) *aak-2* is epistatic to *age-1* (i.e. *age-1* does not suppress *aak-2* Q cell divisions) and *daf-18 (+)* overexpression also does not suppress *aak-2* Q cell divisions. The BMP pathway mutants *sma-9* or *daf-4* failed to suppress *aak-2 (ok524)*, consistent with AAK-2 acting in parallel with or downstream of DBL-1 and IIS. (D) AICAR works on both AAK-1 and AAK-2 to suppress Q cell divisions in *aak-2* and *daf-18* mutants. AICAR can suppress Q cell divisions in L1-arrested *aak-2*, *daf-18; aak-2* or *aak-1; daf-18* mutants but fails to suppress *aak-1; aak-2* double mutants. Data represent the average of at least three independent experiments for each strain and drug treatment; error bars represent s.d.; ***P<0.001 (Student's *t*-test). See details in Tables S4 and S5.

et al., 2017), and AAK-1 is likely to play the primary role in maintaining germ cell quiescence, as *aak-1* but not *aak-2* induce germ cell divisions in L1-arrested and adult worms (Fukuyama et al., 2012; Narbonne et al., 2017). We found that approximately 80% of *aak-2* mutant worms showed Q cell divisions during L1 arrest; in contrast, *aak-1* mutants did not display any Q cell divisions during L1 arrest (Fig. 4A). *aak-1; aak-2* double mutants had a slightly higher frequency of Q cell divisions relative to the *aak-2* single mutants but this was not statistically significant (Fig. 4A). *par-4* encodes a serine-threonine kinase that is homologous to the human LKB1 (STK11) kinase and activates AMPK by phosphorylation (Lee et al., 2008; Narbonne et al., 2010). We tested Q cell divisions in L1-arrested *par-4 (it47)* worms and, surprisingly, *par-4* mutants did not show Q cell divisions (Table S4) (Narbonne et al., 2010). These results suggest that AMPK, like DAF-18, is required in L1-arrested animals to prevent Q cell divisions, and of the two AMPK subunits, AAK-2 plays the main role in blocking Q cell divisions during L1 arrest; however, because *par-4* mutants did not show Q cell divisions during L1 arrest, it suggests that AAK-2 is not regulated by canonical LKB1-AMPK signaling.

AMPK activation can suppress Q cell divisions in the *daf-18* mutant during L1 arrest

AAK-2 and the IIS pathway are reported to regulate the lifespan of *C. elegans* (Apfeld et al., 2004), and AAK-1 is also suggested to act downstream of DAF-18 to regulate germ cell divisions in adult worms (Narbonne et al., 2017). Our results showed that both AAK-2 and DAF-18 are needed during L1 arrest to block Q cell divisions. We provide four experiments to show that AMPK works downstream of, or in parallel with IIS to block Q cell divisions during L1 arrest. First, we predicted that if AMPK works in parallel and acts on the same target with IIS, then activation of the AMPK pathway should bypass the requirement for *daf-18 (ok480)* or suppress the Q cell divisions in L1-arrested INS overexpression worms. To test this, we found that treatment with 2.5 mM 5-aminoimidazole-4-carboxamide ribonucleotide AICAR), an activator of AMPK, significantly suppressed the Q cell divisions in *daf-18* mutants as well as INS overexpression strains (Fig. 4B). Second, if AMPK acts downstream of IIS then IIS mutants, like *age-1* mutants, should not suppress the Q cell divisions in L1-arrested *aak-2* mutants and, indeed we showed

Development (2018) 145, dev166876. doi:10.1242/dev.166876

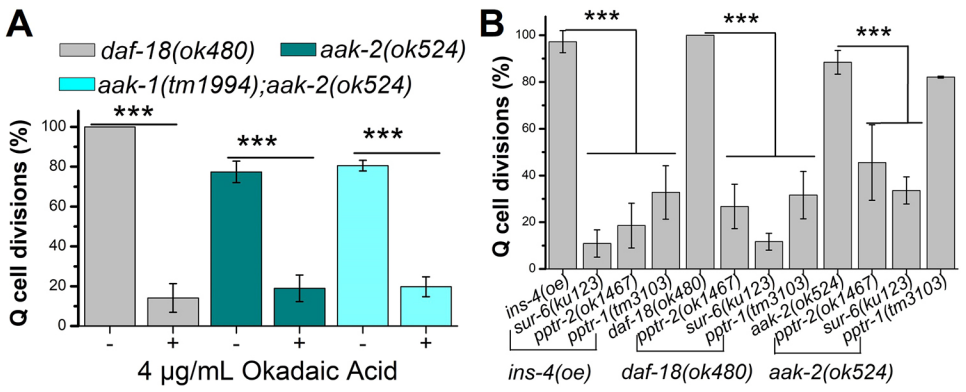

Fig. 5. PP2A functions downstream of AMPK. (A) The PP2A inhibitor okadaic acid suppresses Q cell divisions in L1-arrested *daf-18*, *aak-2* and *aak-1; aak-2* mutants. (B) The PP2As *pptr-1*, *pptr-2* and *sur-6* suppress the Q cell divisions in L1-arrested *ins-4 (oe)*, *daf-18* and *aak-2*. Data represent the average of at least three independent experiments for each strain and drug treatment; error bars represent s.d.; ***P<0.001 (Student's *t*-test); also see detailed data in Tables S6 and S7.

that *aak-2* is epistatic to *age-1* mutants (Fig. 4C). Third, we overexpressed *daf-18 (+)* in an *aak-2* mutant and this was not able to suppress Q cell divisions (Fig. 4C). Finally, we showed DBL-1 mutants could suppress *daf-18* mutants but they failed to suppress *aak-2* mutants (Fig. 3A and Fig. 4C). These results suggest that AMPK functions downstream of DAF-18 or that these two pathways work in parallel and converge on a common pathway to block Q cell divisions during L1 arrest.

Of the two AMPK α subunits in *C. elegans*, only *aak-2* mutants exhibit Q cell divisions during L1 arrest (Fig. 4A). We predicted that AICAR should not suppress *aak-2* mutants, but when we treated with AICAR, we found that AICAR significantly suppressed the Q cell divisions (Fig. 4D). We reasoned that the AICAR could also activate the AAK-1 α subunit and this increased AAK-1 activity was bypassing the requirement for the loss of *aak-2*. This notion was confirmed, as AICAR suppressed Q cell divisions in *aak-1; daf-18* and *aak-2; daf-18* double mutants but failed to suppress *aak-1; aak-2* double mutants (Fig. 4D). Together, our results show that AAK-2 appears to be the main AMPK α subunit responsible for blocking Q cell divisions during L1 arrest; however, AICAR can activate either AAK-1 or AAK-2 to block Q cell divisions (Fig. 4D).

PP2A and MPK-1 function downstream of AMPK to promote Q cell divisions during L1 arrest

A previous study indicated that the protein phosphatase 2A (PP2A) regulatory subunit PPTR-1 negatively regulates the IIS pathway through AKT-1 (Padmanabhan et al., 2009). As such, disruption of *pptr-1* was expected to increase IIS signaling and therefore induce Q cell divisions during L1 arrest. However, we found that *pptr-1* mutants showed no Q cell divisions during L1 arrest. We thought this could be due to redundancy as there are several PP2A genes in *C. elegans*. To address this, we used a potent PP2A inhibitor, okadaic acid (Dounay and Forsyth, 2002), to block all PP2A function. Surprisingly, we did not see Q cell divisions, but instead okadaic acid could significantly suppress Q cell divisions in L1-arrested *daf-18*, *aak-2* and *aak-1; aak-2* mutants (Fig. 5A) in a dose-dependent manner (Fig. S4). These results suggested that PP2A may function downstream of DAF-18 and AMPK. To test this, we tested three PP2A mutants: *pptr-1*, *pptr-2* and *sur-6*. All three of these PP2A mutants could suppress Q cell divisions in *daf-18* mutants as well as in agonist INS overexpression lines (Fig. 5B). We also tested functions of these three PP2A mutants on *aak-2* mutants, and found that only *pptr-2* and *sur-6* mutations could significantly suppress Q cell divisions in *aak-2* mutants (Fig. 5B). These results suggest that *aak-2* may not work through *pptr-1*, or that *pptr-1* functions redundantly with other PP2As. Together, our results show that the AMPK pathway and IIS function on a common target and that the PP2A proteins SUR-6 and PPTR-2 function downstream of AMPK to promote Q cell divisions during L1 arrest.

PP2A has been reported to work with the RAF-MEK-ERK/MAPK signal pathway in mammals and *C. elegans* (Galbo et al., 2013; Padmanabhan et al., 2009; Ruvolo, 2016; Sundaram, 2013). In *C. elegans*, the PP2A homolog SUR-6 positively regulates the RAS-ERK/MAPK pathway (Sieburth et al., 1999). A recent study has also suggested that germline divisions in adult *aak-1* and *daf-18* mutants require MPK-1 (Narbonne et al., 2017). Considering these studies and our previous results, we speculated that the Q cell divisions would require MPK-1 in L1-arrested *daf-18* or *aak-2* mutants. Our results showed that mutations in *mpk-1* significantly suppressed the *daf-18* and *aak-2* Q cell division phenotypes (Fig. 6A). Next, we tested the function of major components within the RAS-ERK/MAPK pathway. We found that loss-of-function mutations in *lin-45* and *mek-2* also significantly suppressed the Q cell divisions in *daf-18* and *aak-2* mutants (Fig. 6B). However, *let-23* (EGFR), *egl-15* (FGFR) and *let-60* (RAS) loss-of-function mutations could not suppress *daf-18* and *aak-2* Q cell divisions (Table S8). Our results suggest that Q cell divisions occur via activated MAPK in a RAS-independent manner during L1 arrest. However, because LIN-45 can be activated by LET-60 gain-of-function mutants, and *let-60 (ga89)* has been reported to activate MPK-1 function (Lopez et al., 2013), we tested whether *let-60 (ga89)* gain-of-function mutants displayed Q cell divisions and found that this mutant did show some (20%) Q cell divisions during L1 arrest. Furthermore, the Q cell divisions in L1-arrested *let-60 (ga89)* mutants were not suppressed by AICAR or okadaic acid treatment, consistent with MAPK functioning downstream of AMPK and PP2A (Fig. S5).

Our results suggest that MPK-1 should be activated in *aak-2* and *daf-18* mutants during L1 arrest. To test this, we looked for the activated form of MPK-1, dual phosphorylated MPK-1 (dpMPK-1) (Lopez et al., 2013) in *daf-18* and *aak-2* mutants. We observed that activated dpMPK-1 was present in Q cells of *daf-18* and *aak-2* L1-arrested mutants during migration and division from two cells (QR/QL) to final Q cell lineage (Fig. 6C-F). dpMPK-1 levels in *sur-6; daf-18*, *pptr-2; daf-18* and *sur-6; aak-2* mutants were significantly reduced (Fig. 6F and Table S9). We also found that dpMPK-1 was barely detected in okadaic acid (PP2A inhibitor) and AICAR (AMPK activator) treated *daf-18* and *aak-2* L1-arrested mutants (Fig. 6G). Together, these results support our model in which PP2A and MPK-1 work downstream of AMPK and DAF-18 in the IIS pathway to control Q cell divisions during L1 arrest.

Development (2018) 145, dev166876. doi:10.1242/dev.166876

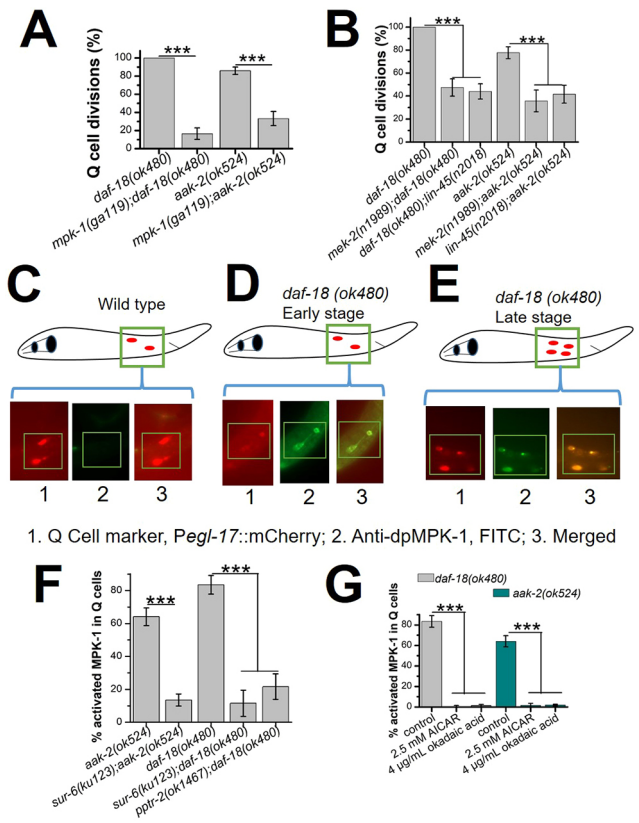

Fig. 6. AMPK and DAF-18 inhibit Q cell divisions by blocking MPK-1 activity during L1 arrest. (A,B) *lin-45*, *mek-2* and *mpk-1* mutants can suppress *daf-18* and *aak-2* Q cell divisions during L1 arrest. (C-E) dpMPK-1 antibody staining in Q cells of L1-arrested worms. (C) Wild-type L1-arrested worms show no activated dpMPK-1. (D) Activated dpMPK-1 is observed in the early Q cell divisions in *daf-18* L1-arrested worms. (E) Activated dpMPK-1 is observed at the four-cell Q cell divisions in *daf-18*. (F) PP2A mutants reduce activated dpMPK-1 in *aak-2* and *daf-18* L1-arrested worms. (G) Activated dpMPK-1 is abolished with AICAR or okadaic acid in L1-arrested *aak-2* and *daf-18* worms. Data represent the average of at least three independent experiments for each strain and drug treatment; error bars represent s.d.; ***P<0.001 (t-test) versus control. Also see detailed data presented in Tables S8 and S9.

DISCUSSION

Our results show that DAF-18 keeps Q cells from dividing in a non-canonical IIS signaling pathway. We provide evidence that the DBL-1 pathway works upstream of DAF-18 to induce agonistic INS ligands. We show that PP2A and MAP kinase act downstream of both IIS and AMPK to promote Q cell divisions during L1 arrest.

Cell quiescence is a survival strategy in L1-arrested worms and requires DAF-18 and AMPK tumor suppressors

What is the consequence of the abnormal cell divisions in L1-arrested worms? *daf-18* and *aak-2* L1-arrested mutants only live for a maximum of about 5-12 days at 20°C compared with 21 days for wild-type worms (Fukuyama et al., 2012). We hypothesize that the inappropriate divisions that occur in *daf-18* mutants may contribute to the shortened L1 survival, as we found that L1 longevity of *daf-18* and *aak-2* mutants could be extended by blocking cell divisions using hydroxyurea (Fig. S8). Thus, DAF-18 and AAK-2 have properties of tumor suppressors in *C. elegans* as they are required to stop unscheduled cell divisions. Germ cell-specific expression of *daf-18* has been reported to suppress the germ cell divisions during L1 arrest; however, is not sufficient to fully rescue the *daf-18* L1 survival (Fukuyama et al., 2012). Similarly, we found that neuronal-specific expression of *daf-18* and Q cell-specific expression of *daf-18* strongly suppressed Q cell divisions in L1-arrested *daf-18* mutants, but did not fully restore the longevity of L1-arrested *daf-18* mutants. It is likely that daf-18 is required both in the soma and germline to fully rescue the L1-arrested *daf-18* longevity.

Unique roles for AMPK α subunits

Mutations in the *aak-1* AMPK α subunit induces germ cell proliferation, and is suggested to play the main role in keeping germline quiescence (Fukuyama et al., 2012; Narbonne et al., 2017). Our work shows that *aak-2* and not *aak-1* is the main AMPK α subunit controlling Q cell divisions during L1 arrest and, thus, the two AMPKs in *C. elegans* play different roles to block cell divisions in different tissues during L1 arrest. During L1 arrest, AMPK and DAF-18 are required to keep germ cells arrested at the G2/M stage of the cell cycle (Fukuyama et al., 2006, 2012). In contrast, we found that the somatic Q neuroblasts arrested at the G1/S phase of the cell cycle during L1 arrest. If the arrested Q cells had completed S phase then they should be capable of undergoing mitosis when cell division is re-initiated upon feeding, even when DNA replication is halted. Treatment of L1-arrested worms with hydroxyurea, which blocks DNA synthesis (S phase), blocked Q cell divisions when the L1-arrested animals were returned to food, consistent with the Q cells arresting at G1/S stage of the cell cycle (Fig. S6). In addition, AAK-2 is not likely to be regulated by canonical LKB1/AMPK signaling as we did not detect Q cell divisions in *par-4* mutants (Table S4). How does AMPK and DAF-18 control cell divisions differently in different tissues? We show that AAK-2 and DAF-18 regulate PP2A and MAPK in the somatic Q cells, whereas AAK-1 and DAF-18 appear to work through the TOR pathway in germline cells. The TOR pathway is activated in L1-arrested *daf-18* and *aak-1* animals to cause germ cell divisions (Fukuyama et al., 2012). In contrast, our results show that Q cell divisions during L1 arrest are not dependent on the TOR pathway (Fig. S3 and Table S4). We also tested the function of okadaic acid on germ cell divisions and found that okadaic acid could not significantly suppress the germ cell divisions in *daf-18* and *aak-1*; *aak-2* L1-arrested mutants (Fig. S7). Thus, we have identified a new regulatory pathway for somatic Q cell divisions.

The DBL-1 pathway regulates insulin-like peptides

We provide four pieces of evidence that DBL-1 works upstream of IIS to promote Q cell divisions during L1 arrest. First, we show that inhibition of DBL-1 and not DAF-7 can suppress Q cell divisions in L1-arrested *daf-18* mutants, suggesting that DBL-1 activates IIS. Second, the overexpression of INS agonists can bypass the requirement for DBL-1 signaling as INS overexpression caused Q cell divisions in DBL-1 pathway mutant backgrounds (*daf-4* and *sma-9*). Third, DBL-1 mutants regulate INS-3 and INS-4 expression level. Fourth, overexpressed *dbl-1* was sufficient to cause Q cell divisions during L1 arrest. Our work supports a role for BMP regulating insulin signaling and is consistent with mammalian research showing that BMP7 augments insulin signaling (Chattopadhyay et al., 2017). A recent report is also consistent with DBL-1 acting upstream of IIS as Clark et al. (2018) showed that DBL-1 regulates insulin-like peptides in fat accumulation. As with germline quiescence signaling, the regulatory pathways may be specific to different somatic cell lineages as the DBL-1 pathway has been proposed to function downstream of IIS for M cell divisions. Here, the terminal IIS transcription factor DAF-16 inhibits the DBL-1 pathway to keep the M cells quiescent in L1 arrest (Kaplan et al., 2015).

Development (2018) 145, dev166876. doi:10.1242/dev.166876

PP2A functions downstream of AMPK and IIS to regulate LIN-45 in a MAP kinase pathway

PP2A is a heterotrimeric serine/threonine phosphatase composed of catalytic 'C' and structural 'A' subunits and a regulatory 'B' subunit that directs the PP2A complex to different substrates (Janssens and Goris, 2001). There are several B regulatory PP2A genes in *C. elegans* and because some PP2A mutations are synthetically lethal with *daf-18* or *aak-2*, only *pptr-1*, *pptr-2* and *sur-6* were tested in this study. The PP2A inhibitor okadaic acid and PP2A holoenzyme regulatory subunit mutants all suppress the Q cell divisions, suggesting that the PP2A holoenzyme plays an important role in promoting Q cell divisions under starvation. *pptr-2* and *sur-6* but not *pptr-1* mutants partially suppress the Q cell divisions in *aak-2* mutants but not to the same extent as okadaic acid. These results imply that other genes, including the other PP2A genes, may be also regulated by AMPK. Our genetic and drug treatment tests are consistent with PP2A functioning downstream of AMPK and IIS (Fig. 7). Although these interactions are based on genetics, it is plausible that AMPK could negatively regulate PP2A by phosphorylation as protein phosphorylation predictions (Nicholson and Anderson, 2002; Schaffer et al., 2015) show that the three PP2As tested in this work all have several AMPK kinase consensus sequences. However, 'downstream' could also be interpreted as AMPK and PP2A working in parallel and converging on a similar target, in this case, LIN-45. We propose a working model in which AAK-2 inhibits LIN-45 through phosphorylation. This is consistent with a report by Shen et al. (2013) demonstrating that AMPK negatively regulates the RAF-MEK-ERK pathway by direct phosphorylation of BRAF and that this phosphorylation disrupts an RAF/KSR complex leading to attenuation of MEK-ERK signaling. In our model, PP2A can oppose the negative influence of AMPK on LIN-45. Our results are consistent with prior *C. elegans* studies showing that SUR-6 functions with LIN-45 and MEK-2 to control MPK-1 activity (Kao et al., 2004; Sieburth et al., 1999). In addition, molecular data from mammalian PP2A has shown that RAF can be activated by removing inhibitory phosphorylations (Abraham et al., 2000; Dhillon et al., 2002; Jaumot and Hancock, 2001; Kubicek et al., 2002). In our study, *let-23*, *egl-15* and *let-60* mutants did not suppress *daf-18* mutant Q cell divisions, suggesting that Q cell divisions activate MPK-1 independently of LET-60 during L1 arrest. RAS-independent activation of the RAF-MEK-ERK pathway has been shown in *Drosophila* and mammalian systems (Gough, 2012; Hou et al., 1995; Mishra et al., 2005; Schmidt et al., 2000). However, *let-60 (gf)* mutants did show mild Q cell divisions (~20%) on their own. Our results suggest that in the normal context of L1 arrest that the activation of the MAPK pathway for Q cell divisions is independent of receptors such as LET-23/EGFR or EGL-15/FGFR and LET-60/RAS, but that LET-60/RAS gain of function can activate the MAPK pathway to induce some Q cell divisions during L1 arrest. The Q cell divisions in L1-arrested *let-60 (gf)* were resistant to treatment with AICAR and okadaic acid, consistent with MAPK functioning downstream of AMPK and PP2A.

In conclusion, our work identified, for the first time, division of the neuronal Q cell lineage during L1 arrest in *daf-18* and *aak-2* mutants. This is also the first evidence that *daf-18* and *aak-2* function in the neuroblasts to maintain somatic cell divisions during L1 arrest. We demonstrate that DAF-18 functions cell-autonomously to suppress Q cell divisions in a DAF-16-independent manner during L1 arrest. In our working model (Fig. 7) the INS that act on the Q cells are regulated by DBL-1/BMP. More importantly, we show that DAF-18/PTEN and AMPK signaling inhibit PP2As and MAP kinase signaling for Q cell quiescence. Thus, the PP2A proteins play a newly identified role in starved worms, and given that human PTEN can replace the function of DAF-18 in suppressing Q cell divisions during L1 arrest, our work provides valuable new information on how the human PTEN tumor suppressor functions to stop cancer cells from dividing.

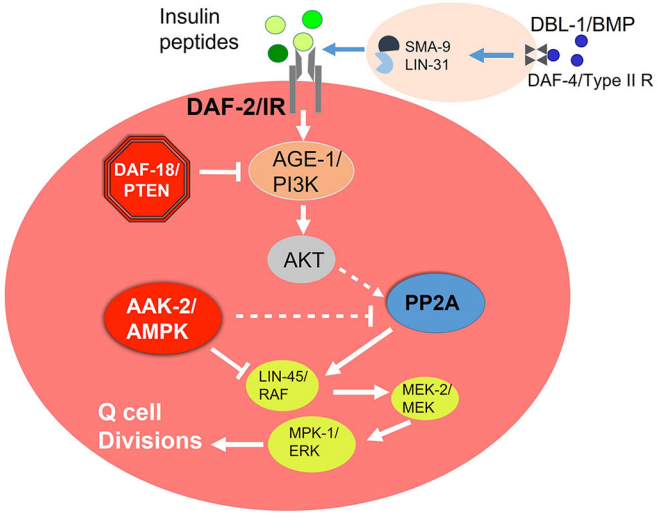

Fig. 7. Working model of the regulation of Q cell divisions during L1 arrest. DBL-1/BMP functions upstream of IIS by producing insulin-like peptides (INS) that act on the Q cells. The INS bind to and activate the DAF-2 insulin receptor. The activated IIS works independently of DAF-16/FOXO and causes Q cell divisions during L1 arrest. DAF-18/PTEN plays an important cell-autonomous role in the Q cells to block IIS. Genetic analysis is consistent with AAK-2/AMPK functioning in parallel with IIS (AKT) to inhibit PP2A (dashed bars). Alternatively, AMPK and PP2A could function in parallel by inhibiting and activating LIN-45/RAF, respectively. In this model, the LIN-45/RAF-MEK-2/MEK-MPK-1/ERK pathway is activated independently of LET-60/RAS to promote Q cell divisions.

MATERIALS AND METHODS

Strains

Most of the strains used in this study were acquired from the *Caenorhabditis* Genetics Center (CGC) and crossed into *Pmec-4-gfp (zdIs5)* and *Pegl-17-mCherry (rdvIs1)* to study Q cell divisions. All *Pmec-4-gfp (zdIs5)* strains were made in our laboratory and are described in supplementary Materials and Methods and Fig. S1. Standard culture methods were used as previously described (Brenner, 1974). Strains were fed with OP50 *Escherichia coli* and cultured at 20°C unless otherwise indicated. Strains used in this study: CZ10175: *zdIs5*, RDV55: *rdvIs1*, PD4666: *ayIs6*, RB712: *daf-18(ok480)*, RB754: *aak-2(ok524)*, AGD397: *aak-1(tm1944); aak-2(ok524)*, DR722: *age-1(m333)/mnC1* [*dpy-10(e128) unc-52(e444)*], VC546: *daf-4(ok827)*, VC1183: *sma-9(ok1628)*, VC1270: *lin-31(gk569)*, CB1372: *daf-7(e1372)*, JH2787: *pptr-1(tm3110)*, RB1338: *pptr-2(ok1467)*, MH1292: *sur-6(ku123)*, SD420: *mpk-1(ga119)/dpy-17(e164) unc-79(e1068)*, MU48: *lin-45(n2018) dpy-20(e1282)*, MT8666: *mek-2(n1989)*. HT1690: *unc-119(ed3); Pins-3::gfp (wwIs26)*, HT1693: *unc-119(ed3); Pins-4::gfp (wwEx63)*, KK184: *par-4(it47)*, TJ356: *zIs356*. *par-4* mutants were cultured at 15°C and 20°C, then the eggs were collected and cultured at 25°C to perform the L1-arrest assay.

Cell division observations and L1-arrest assays

Well-maintained mixed-staged worms were collected to prepare embryos, as described (Fukuyama et al., 2015). In brief, embryos were maintained and hatched in sterile M9 and incubated at 20°C with low-speed rocking to initiate L1 arrest. Q cell descendants were observed under an Axioplan fluorescent microscope (Zeiss) after 2 days or more in L1 arrest. A total of 50-200 µl M9 containing L1-arrested worms were removed from the culture each time to make sure the sample was larger than 50. The total

Development (2018) 145, dev166876. doi:10.1242/dev.166876

number of worms and the worms with Q cell divisions were counted. A/PVM present was counted as Q cell divided and confirmed by lineage analysis using the Q cell marker *Pegl-17::mCherry (rdvIs1)*. For transgenic strains, only the worms with the injection marker *(odr-1::gfp)* were counted and analyzed.

Antibody staining

Antibody staining was performed as previously described (Chin-Sang et al., 1999). In brief, L1 worms were collected in 100 µl M9, fixed with 200 µl cold 2× witches brew (Bettinger et al., 1996) and 100 µl 10% paraformaldehyde, incubated at 4°C for 30 min to overnight. Worms were washed twice in Tris-Triton buffer [10mM Tris (pH 7.4), 100mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 10% glycerol and 0.1% SDS], incubated in 1% β-mercaptoethanol/Tris-Triton for 1-2 h at 37°C, washed in 1× Borate buffer, incubated in 10 mM DTT/1× Borate buffer for 15 min at room temperature, washed in 1× Borate buffer, incubated in 0.3% H_2O_2/1× Borate buffer 15 min, incubated 15 min in PBST-B [phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA), 0.5% Triton X-100, 5 mM sodium azide and 1 mM EDTA], and then washed with PBST-A (PBS containing 1% BSA, 0.5% Triton X-100, 5 mM sodium azide and 1 mM EDTA). The worms were detected using an Axioplan fluorescent microscope (Zeiss).

For dpMPK-1 observation and antibody staining, the L1-arrested worms were collected at 10, 20, 40 and 48 h after the embryos were prepared. We found that 40 h after the embryos were placed in M9 buffer was the best time to detect dpMPK-1. The control worms with no dpMPK-1 was detected, and the mean dpMPK-1 antibody fluorescence intensity per cell was measured using ImageJ, and was used as a threshold (>5.08) to analyze the samples. The total sample and worms with two or more cells detected with anti-dpMPK-1 were scored and the percentage of the worms detected with dpMPK-1 was calculated. Antibodies used were anti-dpMKP-1 (1:100, M8159, Sigma-Aldrich) and FITCH (1:100, F0257, Sigma-Aldrich).

Fluorescence quantification

The INS and GFP fluorescence were captured at the same setting using an Axioplan fluorescent microscope (Zeiss). For each strain, at least three independent replicate experiments were carried out with at least ten worms of each strain captured in each experiment. The mean fluorescence intensity was calculated using ImageJ.

Transgenic strains

For the INS overexpression strains, insulin genes were amplified from *C. elegans* genomic DNA and were placed under control of the pan-neuronal promoter *Prgef-1*. A plasmid with the injection marker *odr-1::rfp* was injected into *Pmec-4::gfp (zdIs5)* worms using standard microinjection methods (Mello et al., 1991). Each injected strain had at least three stable lines. For the *daf-18*, human *PTEN* and *aak-2* rescue strains, the cDNA and genomic DNA of these genes were cloned into plasmids containing specific promoters as described in this study and supplementary Materials and Methods. Primer sequences can be found in the supplementary Materials and Methods.

Chemical treatments

Drugs used in chemical screening, including AICAR and okadaic acid, were bought from Sigma-Aldrich. AICAR was dissolved in water whereas okadaic acid was dissolved in ethanol. Serial dilutions of each chemical were added into M9 immediately after the embryos were prepared, so the worms were hatched in a drug-treatment environment and influenced by the chemical prior to L1 arrest. Hydroxyurea was dissolved in water to a final working concentration of 30 mM. To test whether hydroxyurea can suppress the Q cell divisions in *daf-18* and *aak-2* mutants, hydroxyurea was added immediately after the embryos were prepared, and thus hatched in the presence of hydroxyurea.

Acknowledgements

We thank William Bendena (Queen's University) for sending us strains and reagents and providing suggestions. We are grateful to *Caenorhabditis* Genomic Center, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440), for providing strains.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.Z.; Methodology: S.Z.; Software: S.Z., Z.Q.; Validation: S.Z.; Formal analysis: S.Z., Z.Q.; Investigation: S.Z., Z.Q., M.Z., B.L.; Resources: S.Z., I.C.-S.; Data curation: S.Z., Z.Q.; Writing - original draft: S.Z.; Writing - review & editing: S.Z., I.C.-S.; Visualization: S.Z., M.Z., B.L.; Supervision: I.C.-S.; Project administration: I.C.-S.; Funding acquisition: I.C.-S.

Funding

The work is supported by grants from the Natural Sciences and Engineering Research Council of Canada (249779) and the Canadian Institutes of Health Research (130541).

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.166876.supplemental

References

Abraham, D., Podar, K., Pacher, M., Kubicek, M., Welzel, N., Hemmings, B. A., Dilworth, S. M., Mischak, H., Kolch, W. and Baccarini, M. (2000). Raf-1-associated protein phosphatase 2A as a positive regulator of kinase activation. *J. Biol. Chem.* **275**, 22300-22304.

Altun-Gultekin, Z., Andachi, Y., Tsalik, E. L., Pilgrim, D., Kohara, Y. and Hobert, O. (2001). A regulatory cascade of three homeobox genes, CEH-10, TTX-3 and CEH-23, controls cell fate specification of a defined interneuron class in C. elegans. *Development* **128**, 1951-1969.

Apfeld, J., O'Connor, G., McDonagh, T., DiStefano, P. S. and Curtis, R. (2004). The AMP-activated protein kinase AAK-2 links energy levels and insulin-like signals to lifespan in C. elegans. *Genes Dev.* **18**, 3004-3009.

Baugh, L. R. (2013). To grow or not to grow: nutritional control of development during Caenorhabditis elegans L1 arrest. *Genetics* **194**, 539-555.

Baugh, L. R. and Sternberg, P. W. (2006). DAF-16/FOXO regulates transcription of CKI-1/CIP/KIP and repression of lin-4 during C. elegans L1 arrest. *Curr. Biol.* **16**, 780-785.

Bettinger, J. C., Lee, K. and Rougvie, A. E. (1996). Stage-specific accumulation of the terminal differentiation factor LIN-29 during Caenorhabditis elegans development. *Development* **122**, 2517-2527.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. *Genetics* **77**, 71-94.

Brisbin, S., Liu, J., Boudreau, J., Peng, J., Evangelista, M. and Chin-Sang, I. (2009). A role for C. elegans Eph RTK signaling in PTEN regulation. *Dev. Cell* **17**, 459-469.

Chai, Y., Li, W., Feng, G., Yang, Y., Wang, X. and Ou, G. (2012). Live imaging of cellular dynamics during Caenorhabditis elegans postembryonic development. *Nat. Protoc.* **7**, 2090-2102.

Chalfie, M. and Sulston, J. (1981). Developmental genetics of the mechanosensory neurons of Caenorhabditis elegans. *Dev. Biol.* **82**, 358-370.

Chalhoub, N. and Baker, S. J. (2009). PTEN and the PI3-kinase pathway in cancer. *Annu. Rev. Pathol.* **4**, 127-150.

Chattopadhyay, T., Singh, R. R., Gupta, S. and Surolia, A. (2017). Bone morphogenetic protein-7 (BMP-7) augments insulin sensitivity in mice with type II diabetes mellitus by potentiating PI3K/AKT pathway. *Biofactors* **43**, 195-209.

Chen, Y. and Baugh, L. R. (2014). INS-4 and DAF-28 function redundantly to regulate C. elegans L1 arrest. *Dev. Biol.* **394**, 314-326.

Chin-Sang, I. D., George, S. E., Ding, M., Moseley, S. L., Lynch, A. S. and Chisholm, A. D. (1999). The ephrin VAB-2/EFN-1 functions in neuronal signaling to regulate epidermal morphogenesis in C. elegans. *Cell* **99**, 781-790.

Clark, S. G. and Chiu, C. (2003). C. elegans ZAG-1, a Zn-finger-homeodomain protein, regulates axonal development and neuronal differentiation. *Development* **130**, 3781-3794.

Clark, J. F., Meade, M., Ranepura, G., Hall, D. H. and Savage-Dunn, C. (2018). Caenorhabditis elegans DBL-1/BMP regulates lipid accumulation via interaction with insulin signaling. *G3* **8**, 343-351.

Demoinet, E., Li, S. and Roy, R. (2017). AMPK blocks starvation-inducible transgenerational defects in Caenorhabditis elegans. *Proc. Natl. Acad. Sci. USA* **114**, E2689-E2698.

Dey, N., Crosswell, H. E., De, P., Parsons, R., Peng, Q., Su, J. D. and Durden, D. L. (2008). The protein phosphatase activity of PTEN regulates SRC family kinases and controls glioma migration. *Cancer Res.* **68**, 1862-1871.

Dhillon, A. S., Meikle, S., Yazici, Z., Eulitz, M. and Kolch, W. (2002). Regulation of Raf-1 activation and signalling by dephosphorylation. *EMBO J.* **21**, 64-71.

Dounay, A. B. and Forsyth, C. J. (2002). Okadaic acid: the archetypal serine/threonine protein phosphatase inhibitor. *Curr. Med. Chem.* **9**, 1939-1980.

Fukuyama, M., Rougvie, A. E. and Rothman, J. H. (2006). C. elegans DAF-18/PTEN mediates nutrient-dependent arrest of cell cycle and growth in the germline. *Curr. Biol.* **16**, 773-779.
Fukuyama, M., Sakuma, K., Park, R., Kasuga, H., Nagaya, R., Atsumi, Y., Shimomura, Y., Takahashi, S., Kajiho, H., Rougvie, A. et al. (2012). C. elegans AMPKs promote survival and arrest germline development during nutrient stress. *Biol. Open* **1**, 929-936.
Fukuyama, M., Kontani, K., Katada, T. and Rougvie, A. E. (2015). The C. elegans hypodermis couples progenitor cell quiescence to the dietary state. *Curr. Biol.* **25**, 1241-1248.
Galbo, T., Perry, R. J., Nishimura, E., Samuel, V. T., Quistorff, B. and Shulman, G. I. (2013). PP2A inhibition results in hepatic insulin resistance despite Akt2 activation. *Aging* **5**, 770-781.
Gough, N. R. (2012). ERK activation without Ras. *Sci. Signal.* **5**, ec278.
Hou, X. S., Chou, T.-B., Melnick, M. B. and Perrimon, N. (1995). The torso receptor tyrosine kinase can activate Raf in a Ras-independent pathway. *Cell* **81**, 63-71.
Hung, W. L., Wang, Y., Chitturi, J. and Zhen, M. (2014). A Caenorhabditis elegans developmental decision requires insulin signaling-mediated neuron-intestine communication. *Development* **141**, 1767-1779.
Janssens, V. and Goris, J. (2001). Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem. J.* **353**, 417-439.
Jaumot, M. and Hancock, J. F. (2001). Protein phosphatases 1 and 2A promote Raf-1 activation by regulating 14-3-3 interactions. *Oncogene* **20**, 3949-3958.
Johnson, T. E., Mitchell, D. H., Kline, S., Kemal, R. and Foy, J. (1984). Arresting development arrests aging in the nematode Caenorhabditis elegans. *Mech. Ageing Dev.* **28**, 23-40.
Kao, G., Tuck, S., Baillie, D. and Sundaram, M. V. (2004). C. elegans SUR-6/PR55 cooperates with LET-92/protein phosphatase 2A and promotes Raf activity independently of inhibitory Akt phosphorylation sites. *Development* **131**, 755-765.
Kaplan, R. E. W., Chen, Y., Moore, B. T., Jordan, J. M., Maxwell, C. S., Schindler, A. J. and Baugh, L. R. (2015). dbl-1/TGF-beta and daf-12/NHR signaling mediate cell-nonautonomous effects of DAF-16/FOXO on starvation-induced developmental arrest. *PLoS Genet.* **11**, e1005731.
Kubicek, M., Pacher, M., Abraham, D., Podar, K., Eulitz, M. and Baccarini, M. (2002). Dephosphorylation of Ser-259 regulates Raf-1 membrane association. *J. Biol. Chem.* **277**, 7913-7919.
Lee, M.-H., Ohmachi, M., Arur, S., Nayak, S., Francis, R., Church, D., Lambie, E. and Schedl, T. (2007). Multiple functions and dynamic activation of MPK-1 extracellular signal-regulated kinase signaling in Caenorhabditis elegans germline development. *Genetics* **177**, 2039-2062.
Lee, H., Cho, J. S., Lambacher, N., Lee, J., Lee, S.-J., Lee, T. H., Gartner, A. and Koo, H.-S. (2008). The Caenorhabditis elegans AMP-activated protein kinase AAK-2 is phosphorylated by LKB1 and is required for resistance to oxidative stress and for normal motility and foraging behavior. *J. Biol. Chem.* **283**, 14988-14993.
Lopez, A. L., III, Chen, J., Joo, H.-J., Drake, M., Shidate, M., Kseib, C. and Arur, S. (2013). DAF-2 and ERK couple nutrient availability to meiotic progression during Caenorhabditis elegans oogenesis. *Dev. Cell* **27**, 227-240.
Mello, C. C., Kramer, J. M., Stinchcomb, D. and Ambros, V. (1991). Efficient gene transfer in C.elegans: extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* **10**, 3959-3970.
Middelkoop, T. C. and Korswagen, H. C. (2014). Development and migration of the C. elegans Q neuroblasts and their descendants. *WormBook : the online review of C. elegans biology*, 1-23.
Mihaylova, V. T., Borland, C. Z., Manjarrez, L., Stern, M. J. and Sun, H. (1999). The PTEN tumor suppressor homolog in Caenorhabditis elegans regulates longevity and dauer formation in an insulin receptor-like signaling pathway. *Proc. Natl. Acad. Sci. USA* **96**, 7427-7432.
Mishra, S., Smolik, S. M., Forte, M. A. and Stork, P. J. S. (2005). Ras-independent activation of ERK signaling via the torso receptor tyrosine kinase is mediated by Rap1. *Curr. Biol.* **15**, 366-370.
Morris, J. Z., Tissenbaum, H. A. and Ruvkun, G. (1996). A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in Caenorhabditis elegans. *Nature* **382**, 536-539.
Müller, J., Ory, S., Copeland, T., Piwnica-Worms, H. and Morrison, D. K. (2001). C-TAK1 regulates Ras signaling by phosphorylating the MAPK scaffold, KSR1. *Mol. Cell* **8**, 983-993.
Narbonne, P., Hyenne, V., Li, S., Labbé, J.-C. and Roy, R. (2010). Differential requirements for STRAD in LKB1-dependent functions in C. elegans. *Development* **137**, 661-670.
Narbonne, P., Maddox, P. S. and Labbé, J.-C. (2017). DAF-18/PTEN signals through AAK-1/AMPK to inhibit MPK-1/MAPK in feedback control of germline stem cell proliferation. *PLoS Genet.* **13**, e1006738.
Nicholson, K. M. and Anderson, N. G. (2002). The protein kinase B/Akt signalling pathway in human malignancy. *Cell. Signal.* **14**, 381-395.
Ogg, S. and Ruvkun, G. (1998). The C. elegans PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway. *Mol. Cell* **2**, 887-893.
Ogg, S., Paradis, S., Gottlieb, S., Patterson, G. I., Lee, L., Tissenbaum, H. A. and Ruvkun, G. (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. *Nature* **389**, 994-999.
Padmanabhan, S., Mukhopadhyay, A., Narasimhan, S. D., Tesz, G., Czech, M. P. and Tissenbaum, H. A. (2009). A PP2A regulatory subunit regulates C. elegans insulin/IGF-1 signaling by modulating AKT-1 phosphorylation. *Cell* **136**, 939-951.
Patel, D. S., Garza-Garcia, A., Nanji, M., McElwee, J. J., Ackerman, D., Driscoll, P. C. and Gems, D. (2008). Clustering of genetically defined allele classes in the Caenorhabditis elegans DAF-2 insulin/IGF-1 receptor. *Genetics* **178**, 931-946.
Pierce, S. B., Costa, M., Wisotzkey, R., Devadhar, S., Homburger, S. A., Buchman, A. R., Ferguson, K. C., Heller, J., Platt, D. M., Pasquinelli, A. A. et al. (2001). Regulation of DAF-2 receptor signaling by human insulin and INS-1, a member of the unusually large and diverse C. elegans insulin gene family. *Genes Dev.* **15**, 672-686.
Ruvolo, P. P. (2016). The broken "Off" switch in cancer signaling: PP2A as a regulator of tumorigenesis, drug resistance, and immune surveillance. *BBA Clinical* **6**, 87-99.
Salminen, A. and Kaarniranta, K. (2012). AMP-activated protein kinase (AMPK) controls the aging process via an integrated signaling network. *Ageing Res. Rev.* **11**, 230-241.
Savage-Dunn, C. (2005). TGF-beta signaling. *WormBook : the online review of C. elegans biology*, 1-12.
Schaffer, B. E., Levin, R. S., Hertz, N. T., Maures, T. J., Schoof, M. L., Hollstein, P. E., Benayoun, B. A., Banko, M. R., Shaw, R. J., Shokat, K. M. et al. (2015). Identification of AMPK phosphorylation sites reveals a network of proteins involved in cell invasion and facilitates large-scale substrate prediction. *Cell Metab.* **22**, 907-921.
Schmidt, M., Goebeler, M., Posern, G., Feller, S. M., Seitz, C. S., Bröcker, E.-B., Rapp, U. R. and Ludwig, S. (2000). Ras-independent activation of the Raf/MEK/ERK pathway upon calcium-induced differentiation of keratinocytes. *J. Biol. Chem.* **275**, 41011-41017.
Shen, C.-H., Yuan, P., Perez-Lorenzo, R., Zhang, Y., Lee, S. X., Ou, Y., Asara, J. M., Cantley, L. C. and Zheng, B. (2013). Phosphorylation of BRAF by AMPK impairs BRAF-KSR1 association and cell proliferation. *Mol. Cell* **52**, 161-172.
Shinde, S. R. and Maddika, S. (2016). PTEN modulates EGFR late endocytic trafficking and degradation by dephosphorylating Rab7. *Nat. Commun.* **7**, 10689.
Sieburth, D. S., Sundaram, M., Howard, R. M. and Han, M. (1999). A PP2A regulatory subunit positively regulates Ras-mediated signaling during Caenorhabditis elegans vulval induction. *Genes Dev.* **13**, 2562-2569.
Solari, F., Bourbon-Piffaut, A., Masse, I., Payrastre, B., Chan, A. M.-L. and Billaud, M. (2005). The human tumour suppressor PTEN regulates longevity and dauer formation in Caenorhabditis elegans. *Oncogene* **24**, 20-27.
Stumpf, M. and den Hertog, J. (2016). Differential requirement for Pten lipid and protein phosphatase activity during zebrafish embryonic development. *PLoS ONE* **11**, e0148508.
Sundaram, M. V. (2013). Canonical RTK-Ras-ERK signaling and related alternative pathways. *WormBook : the online review of C. elegans biology*, 1-38.
Zadra, G., Batista, J. L. and Loda, M. (2015). Dissecting the dual role of ampk in cancer: from experimental to human studies. *Mol. Cancer Res.* **13**, 1059-1072.
Zheng, S. and Chin-Sang, I. D. (2016). C. elegans methods to study PTEN. *Methods Mol. Biol.* **1388**, 307-321.