LKB1/AMPK signalling in the regulation of dauer germline stem cell quiescence and integrity in the *C. elegans* dauer larva

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Abstract

Upon encountering sub-optimal conditions, early *Caenorhabditis elegans* larvae are able to alter their developmental program and enter the dauer diapause, an alternative stage that allows larvae to endure long periods of starvation and overcrowding. During this quiescent state, the germline stem cells, which normally proliferate during reproductive development, arrest their proliferation and are consequently rendered quiescent. Previous work has implicated a role for LKB1, AMPK and PTEN orthologues in germline stem cell quiescence. AMPK is a well-characterized downstream target of LKB1, but intriguingly the hyperplasia observed in LKB1 (*par-4)* mutants is more severe than AMPK-compromised dauer larvae, suggesting that LKB1/*par-4* has alternative downstream targets in addition to AMPK that regulate germline quiescence.

We identified a subset of the genes involved in the regulation of cell polarity and cytoskeletal regulation that might act downstream of *par-4*, in an AMPK-independent manner. Moreover, we show that *par-4* temporally regulates ANI-2 and the centralspindlin complex to regulate actin cytoskeletal organization and possibly actomyosin contractility within the dauer germ line at the rachis-adjacent membrane, in an AMPK-independent manner. Additionally, we show that PAR-4-mediated AMPK-dependent pathway instructs the Notch receptor, GLP-1 to undergo subcellular re-localization, potentially in a 14-3-3/PAR-5 dependent manner. This subcellular change correlates with a reduction in Notch signalling and the establishment of germ cell quiescence upon dauer entry. Lastly, our data demonstrate that AMPK genetically interacts with a small RNA pathway to mediate appropriate chromatin modifications and gene expression, perhaps in a germline-non-autonomous manner, to regulate GSC quiescence and integrity in response to the dauer stress. In summary, this work demonstrates how nutritional stress targets several downstream regulators through the activation of the LKB1/AMPK pathway to ensure that germline

stem cells appropriately adapt and maintain their integrity until nutrient/energy contingencies are satisfied.

Résumé

Lorsqu'elles rencontrent des conditions sous-optimales, les larves de *Caenorhabditis elegans* précoces peuvent modifier leur programme de développement et entrer dans la diapause dauer, une étape alternative qui permet aux larves de supporter de longues périodes de famine et de surpopulation. Au cours de cet état de repos, les cellules souches de la lignée germinale, qui prolifèrent normalement pendant le développement de la reproduction, arrêtent leur prolifération et sont donc rendues au repos. Des travaux antérieurs ont impliqué un rôle pour les orthologues de LKB1, AMPK et PTEN dans la quiescence des cellules souches germinales. L'AMPK est une cible bien caractérisée en aval de LKB1, mais curieusement, l'hyperplasie observée chez les mutants LKB1 (*par-4*) est plus sévère que chez les larves dauer compromises par l'AMPK, ce qui suggère que LKB1/*par-4* a d'autres cibles en plus de l'AMPK qui régulent la quiescence de la lignée germinale.

Nous avons identifié un sous-ensemble des gènes impliqués dans la régulation de la polarité cellulaire et de la régulation du cytosquelette qui pourraient agir en aval de *par-4*, de manière indépendante de l'AMPK. De plus, nous montrons que *par-4* régule temporellement ANI-2 et le complexe centralspindlin pour réguler l'organisation du cytosquelette d'actine et possiblement la contractilité de l'actomyosine dans la lignée germinale dauer, au niveau de la membrane adjacente au rachis. De plus, nous montrons qu'une voie médiée par PAR-4 et dépendante de l'AMPK ordonne au récepteur Notch, GLP-1, de subir une re-localisation subcellulaire, potentiellement d'une manière dépendante de 14-3-3 / PAR-5. Ce changement subcellulaire est en corrélation avec une réduction de la signalisation Notch et l'établissement de la quiescence des cellules germinales lors de l'entrée en dauer. Enfin, nos données démontrent que l'AMPK interagit génétiquement avec une voie d'ARN petit pour assurer la médiation des modifications appropriées de la chromatine et de l'expression génique, peut-être d'une manière non autonome germinale, pour réguler la

quiescence et l'intégrité en réponse au stress dauer. En résumé, ce travail démontre comment le stress nutritionnel affecte plusieurs régulateurs en aval grâce à l'activation d'une voie LKB1/AMPK pour garantir que les cellules souches germinales s'adaptent de manière appropriée et maintiennent leur intégrité jusqu'à ce que les contingences nutriments/énergie soient satisfaites.

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Preface

The thesis is organized in accordance with the manuscript-based thesis guidelines. It consists of 5 chapters: Chapter 1 (A literature review) that is divided into 5 sub-chapters to give comprehensive review about the relevant literature and provides the rationale and the key objectives of the thesis. Chapter 2-4 (Research manuscripts) and Chapter 5 (A general discussion and a final statement). Chapter 2 has been published in *BMC Genomics*.

This thesis has been entirely written by the candidate in collaboration with the candidate's supervisor.

This thesis has been written according to the 'Guidelines for thesis preparation' from the Faculty of Graduate Studies and Research.

Contribution to original knowledge

Chapter 2 is an original research article published in *BMC Genomics.*

Chapter 3 is a manuscript in preparation and reveals new findings about how LKB1/PAR-4 acts as a tumour suppressor through acting in 2 independent pathways to block abnormal cell divisions.

Chapter 4 is an original research manuscript prepared for submission and provides novel insights about an energy sensor, AMPK, in regulating small RNA pathway and chromatin landscape under severe energetic stress.

Contribution of authors

Chapter 2 has been published in *BMC genomics* as follows,

Kadekar, P., R. Chaouni, E. Clark, A. Kazanets and R. Roy (2018). "Genome-wide surveys reveal polarity and cytoskeletal regulators mediate LKB1-associated germline stem cell quiescence." BMC Genomics 19(1): 462.

Experiments were designed by R.R and P.K. The RNAi screen to identify genes resulting in germline hyperplasia was performed by E.C. R.C, and A.K performed the *par-4* and *aak(0)* suppressor screen, respectively. P.K implemented the analysis, validation, microscopy and the suggested revisions. The manuscript was written by P.K and R.C and edited by R.R.

Chapter 3: This chapter is in preparation as follows,

Kadekar, P., Roy, R. *(in preparation)* LKB1/PAR-4 plays AMPK-dependent and -independent roles to establish germline stem cell quiescence in *C. elegans* dauer larvae

I performed all the experiments and wrote the entire manuscript in collaboration with Dr. Richard Roy.

Chapter 4: This chapter is prepared to submit for publication to *elife* as follows,

Kadekar, P., Roy, R. AMPK regulates germline stem cell quiescence and integrity through effects on a small RNA pathway.

I performed all the experiments and wrote the entire manuscript in collaboration with Dr. Richard Roy.

Chapter 1. Literature review

Chapter 1.1. General Introduction

Development of most animals is dependent on both their genetic material, or their genome, and how the genome is expressed. The regulation of this expression is often dependent on the environment, such as growth conditions. The early phase of life has a great impact on the development of most organisms. The precise interplay between the developing animal and the physical environment is critical for their survival. At several phases of development, the organism may be sensitive to unfavourable environmental cues, thus impacting normal development (Gluckman, Hanson et al. 2005). For example, in several mammals, prenatal nutrition can influence developmental and physiological processes (Vickers, Breier et al. 2000, Ozanne, Lewis et al. 2004).

Many organisms depend on several environmental factors like availability of nutrition, water and oxygen abundance, population density, temperatures, etc. for normal development. During the normal course of development, organisms can encounter sub-optimal growth conditions and extremely diverse environments. To survive these difficult periods, they have evolved several efficient strategies. For example, many organisms can enter a dormant state such as sporulation or hibernation to circumvent such hostile conditions. To prepare and execute such a dormant state, they must alter both their behavioural and physiological processes to increase available energy and reduce energy consumption to ensure long term survival (Geiser 2013). Diverse signalling pathways respond to these cues to bring about change at the genetic and molecular level to execute adaptations downstream of specific environmental challenges in order to optimize resources for long term survival.

Environmental cues also affect stem cell biology to a great extent. Stem cells are mitotically dividing cells that give rise to various differentiated tissues and thus play a crucial role in animal

development and tissue generation (Joshi, Riddle et al. 2010). One of the important characteristics of stem cells is their plasticity, as they can respond to different environmental cues and can either continue self-renewal or execute a quiescent state (Drummond-Barbosa 2008). If the animal is experiencing energetic stress, to conserve energy the stem cells take on a quiescent state and maintain their integrity until growth conditions become favourable once again. The loss of quiescence leads to the untimely exhaustion of the stem cell pool, consequently affecting tissue homeostasis later in development. This misregulation of stem cell quiescence may also contribute to various types of cancer (Orford and Scadden 2008, Rossi, Jamieson et al. 2008, Cheung and Rando 2013). This adaptive response exhibited by stem cells provides an excellent model to study how environmental cues impinge on cell cycle regulation and differentiation, and further understand the aetiology of several cancer predisposing disorders.

C. elegans as a model organism

Since 1965, *Caenorhabditis elegans* has been used as a model organism to understand several biological processes. *C. elegans* is a soil dwelling, multi-cellular eukaryotic organism which can be easily cultivated and maintained in the laboratory. Furthermore, it can be frozen for long term usage so that mutants can be stored for long durations for later analysis. *C. elegans* is a hermaphroditic animal with a short generation time and a large brood size, making it an ideal system for classic genetic analysis and forward genetic screens (Riddle, Blumenthal et al. 1997). It has been widely used for biomedical research as it shares conserved signalling pathways and possesses a significant homology at the genome level with most other animals, including humans (Kuwabara and O'Neil 2001). Numerous forward genetic screens were useful to identify a number of essential genes required for various cellular processes, including several signal transduction

pathways impinging on cell-fate specification (Sternberg and Horvitz 1986), apoptosis (Kimble and Hirsh 1979), ageing (Kimura, Tissenbaum et al. 1997), cell polarity (Kemphues, Priess et al. 1988), the RNAi and miRNA pathways (Fire, Xu et al. 1998, Reinhart, Slack et al. 2000, Simon, Madison et al. 2008), and the neurobiological basis of behaviour (Walthall and Chalfie 1988, Chan, Zheng et al. 1996). *C. elegans* have its entire genome sequenced, thus providing a great resource for several genetic/genomic studies (Wilson 1999). RNA interference (RNAi) provides an efficient reverse genetic tool to compromise gene function providing an efficient means of carrying out genome-wide surveys of gene function (Fire, Xu et al. 1998). Recently, the CRISPR/Cas9 system has been efficiently demonstrated to function in *C. elegans* allowing investigators to perform targeted, heritable genetic manipulations, thus providing an excellent approach for engineering the genome (Friedland, Tzur et al. 2013, Dickinson and Goldstein 2016).

In addition to being an excellent genetic model, there are several other advantages that favour *C. elegans* as a model organism. For example, they have a transparent cuticle which enables the visualization and *in vivo* imaging of several cell lineages, processes and structures using different staining and microscopy approaches. Also, the various fluorescent markers available to *C. elegans* investigators enable the labelling of different proteins, cellular organelles, or specific cell types, which facilitate the study of a multitude of biological processes and biochemical analyses (Chalfie, Tu et al. 1994).

C. elegans dauer larvae: A model to study how developing animals respond to environmental challenge

A special characteristic of *C. elegans* larvae is their developmental plasticity. They are sensitive to their environment and can alter their regular developmental program by sensing their growth milieu. Under replete conditions, following embryogenesis, *C. elegans* larva develops through 4 larval stages (L1-L4) to eventually molt into a reproductive adult (Cassada and Russell 1975, Riddle and Albert 1997) (Fig. 1.1). However, to adapt to adverse conditions, early larvae can enter an alternative pathway to undergo an enduring state called 'dauer', which temporarily halts their development.

The decision to execute this state takes place in the late L1 stage and the second larval stage is prolonged, as the larva undergoes a pre-dauer L2D stage before finally entering into dauer (Cassada and Russell 1975, Riddle and Albert 1997). An important feature of the dauer stage is the establishment of global cellular quiescence and altered metabolism which persist until conditions are favourable. The dauer larvae exhibit complete cell cycle arrest while rationing their energy resources for long term survival (Burnell, Houthoofd et al. 2005, Narbonne and Roy 2009). They can survive in this state for up to 6 months without feeding and once conditions are favourable, they can resume their normal development and molt into L4 larvae to become an adult with no detrimental consequence on their reproductive or somatic fitness (Riddle and Albert 1997, Burnell, Houthoofd et al. 2005, Hu 2007).

Dauer characteristics

Dauer larvae exhibit unique physical and behavioural characteristics important for their dispersal and long-term survival. Morphologically they are distinct from the other larval stages as they

appear longer and slenderer due to a radial constriction associated with a compressed hypodermis and modified cuticle. The cuticle is adapted and the openings are occluded to confer resistance to desiccation and potentially toxic environments. Their internal anatomy is regulated such that the pharynx and intestinal lumen are constricted, while the sensory neurons are rewired to affect dauer-specific behaviours. Dauer larvae are usually motionless, possibly a behaviour to conserve energy, but they are nevertheless responsive to external stimuli (Cassada and Russell 1975, Swanson and Riddle 1981, Albert and Riddle 1988, Vowels and Thomas 1992, Peixoto and De Souza 1994, Riddle and Albert 1997). They display a behaviour termed 'nictation' in which they wave their head in air, presumably as a means of dispersal (Riddle and Albert 1997).

Another important adaptation is their altered metabolism as they do not feed and therefore they must ration their lipid reserves until the environment becomes conducive to reproductive development, after which they will exit dauer (Burnell, Houthoofd et al. 2005, Narbonne and Roy 2009). Additionally, though the level of general transcription is reduced, the expression of stress-resistant genes and molecular chaperones is elevated. This indicates that stress response and mechanisms for cellular maintenance are active, thus conferring resilience to hostile environments to the dauer larva (Dalley and Golomb 1992, Larsen 1993, Riddle and Albert 1997).

Interestingly, during this diapause-like state, animals undergo global readjustments to alter genome-wide chromatin modifications that result in a significant change in gene expression when compared to the animals that never experienced dauer. The population and the level of the endogenous small RNAs regulates chromatin modifications to control genome-wide gene expression (van Wolfswinkel and Ketting 2010). Interestingly, in addition to the changes in the quantity and distribution of the specific chromatin marks, the small RNA repertoire is also altered during dauer state. The change in the levels and distribution of small endogenous RNAs is tightly

correlated with the changes in chromatin modifications. Presumably, this change in distribution of small endogenous RNAs modulates chromatin modifications and ultimately results in unique gene expression in dauer larvae. Alternatively, changes in chromatin can alter the small RNA profile (Lev, Seroussi et al. 2017). These global changes in levels and distribution of chromatin modification perhaps, acts as a molecular record of life history and may be dependent on the expression of specific endogenous small RNAs (Hall, Beverly et al. 2010, Hall, Chirn et al. 2013). Lastly, they execute a reversible global cell cycle arrest and establish a developmental quiescence, most likely to conserve energy reserves for their long term survival (Cassada and Russell 1975).

Chapter 1.2. Dauer entry and regulation

Environmental cues are often sensed and transduced via molecular signals to initiate an adaptive response. Elevated levels of a *C. elegans*-specific pheromone act as a trigger to confer dauer entry. An increased level of the pheromone is an indication of overcrowding and allows young larvae to enter dauer, a stage specialized for their dispersal and long-term survival despite a limited supply of the essential nutrients. A higher ratio of pheromone to available food favours dauer formation and halts regular reproductive development (Albert, Brown et al. 1981, Golden and Riddle 1982, Riddle and Albert 1997). Other environmental factors that influence dauer entry include abnormally high temperatures and insufficient nutrient resources (Albert, Brown et al. 1981, Golden and Riddle 1984).

Ciliated sensory neurons are essential to sense dauer pheromone and food/nutrient signals. Ablation of the amphid sensory neurons, mainly ASI, ASG, ASJ and ADF results in constitutive dauer entry, regardless of the environment. These neurons might sense dauer pheromone or food availability, or both, and then send a neuroendocrine signal to modulate downstream signalling to

prompt a required desired physiological response associated with dauer formation (Bargmann and Horvitz 1991, Hu 2007).

Several genetic analyses have revealed a network of signalling pathways that act downstream of environmental cues to induce dauer. Genetic screens of animals demonstrating abnormal dauer formation helped classify genes into at least two categories: genes which when mutated cause larvae to constitutively enter dauer, and those which when mutated prevent the larvae to enter dauer irrespective of the environmental conditions. The genes resulting in these phenotypes are termed as Daf-c (abnormal Dauer formation-constitutive) and Daf-d (abnormal Dauer formation-defective) respectively (Swanson and Riddle 1981, Riddle and Albert 1997, Hu 2007). The genetic and epistatic analysis of the Daf-c and Daf-d genes identified 3 independent parallel pathways that regulate dauer formation: insulin-like signalling, TGF-β and cGMP (Vowels and Thomas 1992, Thomas, Birnby et al. 1993, Gottlieb and Ruvkun 1994, Riddle and Albert 1997, Patterson and Padgett 2000). All the three pathways converge on a steroid-like hormone which ultimately impinges on the nuclear hormone receptor, DAF-12 to affect the decision to undergo dauer formation (Albert and Riddle 1988, Antebi, Yeh et al. 2000, Rottiers and Antebi 2006).

Insulin-like signalling

Insulin secretion is essential in many animals to regulate protein, carbohydrate and lipid metabolism during favourable growth conditions. The insulin receptor (InR) consists of 2 extracellular alpha subunits and 2 transmembrane beta subunits with tyrosine kinase activity. Insulin binds to the alpha subunits to subsequently activate trans- and autophosphorylation of the beta subunits, eliciting the catalytic activity of the receptor and leading to the activation of protein kinase cascade (Chang, Chiang et al. 2004).

In *C. elegans*, the insulin receptor/*daf-2* and *daf-23* were identified as Daf-c genes and FOXO/*daf-16* was identified as Daf-d gene (Gottlieb and Ruvkun 1994). Under optimal conditions, insulin-like peptides are secreted to stimulate InR/DAF-2 to activate the insulin-like signalling pathway (ILS) which prevents *C. elegans* larvae from entering the dauer state and favours reproductive development. The activation of DAF-2 receptor initiates a cascade of phosphorylation events to activate AKT-1/2 (Morris, Tissenbaum et al. 1996, Kimura, Tissenbaum et al. 1997, Paradis and Ruvkun 1998, Paradis, Ailion et al. 1999). The activated AKT-1 directly phosphorylates a transcription factor, DAF-16, to inhibit its entry into the nucleus to initiate transcription of the genes required for dauer entry (Lee, Hench et al. 2001, Lin, Hsin et al. 2001, Hertweck, Gobel et al. 2004). Two 14-3-3 binding proteins: PAR-5 and FTT-2 also maintain the cytoplasmic localization of DAF-16 to regulate normal growth and development, as well as life span extension (Berdichevsky, Viswanathan et al. 2006).

In contrast, when conditions are sub-optimal, the ILS pathway is not activated and DAF-16 enters the nucleus to activate genes that regulate dauer entry, stress resistance, and longevity (Lin, Hsin et al. 2001, Libina, Berman et al. 2003). In addition, DAF-18, the *C. elegans* ortholog of the phosphoinositide 3-phosphatase PTEN, antagonizes AGE-1/PI3K to regulate DAF-16, the ortholog of the FOX transcription factor protein (Ogg and Ruvkun 1998, Gil, Malone Link et al. 1999, Mihaylova, Borland et al. 1999, Rouault, Kuwabara et al. 1999).

In addition to the ILS pathway, other proteins also impinge on DAF-16 to modulate its activity. c-Jun N-terminal kinase (JNK) regulates nuclear translocation of DAF-16 (Oh, Mukhopadhyay et al. 2005), AMP-activated kinase (AMPK) directly phosphorylates DAF-16 to activate stress resistant genes (Apfeld, O'Connor et al. 2004, Greer, Dowlatshahi et al. 2007) and mitogen-

activated kinase (MAPK) indirectly regulates DAF-16-dependent transcription in parallel to ILS pathway (Troemel, Chu et al. 2006).

Global gene expression analysis of *daf-2* and *daf-2; daf-16* double mutants confirmed that DAF-16 is responsible for the expression of genes mainly responsible for metabolism, energy generation and various stress responses (McElwee, Bubb et al. 2003, Murphy, McCarroll et al. 2003). Genes responsible for growth, development and reproduction, like those regulated by the Target Of Rapamycin (TOR) pathway, are downregulated, perhaps to conserve energy (Jia, Chen et al. 2004). In *C. elegans, let-363* and *daf-15* encode TOR and its binding partner Raptor respectively. Unlike mutants of the insulin-like signalling pathway which result in complete dauer arrest, loss of *let-363* and *daf-15* result in arrest as dauer-like larvae with incomplete dauer morphogenesis (Jia, Chen et al. 2004). These phenotypes are epistatic to loss of *daf-16,* Daf-d mutations, indicating that *let-363* and *daf-15* probably act downstream, or in parallel, of *daf-16* in the dauer formation pathway.

The ILS pathway is also well characterized for its role in lifespan regulation in *C. elegans*. Compromising the insulin receptor *C. elegans* orthologue, *daf-2*, results in a significant increase in lifespan as these mutants live twice as long as the wild type adult animals (Kenyon, Chang et al. 1993). Moreover, disruption of the insulin receptor in *Drosophila* and the IGF-1 gene in mice also result in lifespan extension, suggesting that the role of ILS in lifespan regulation is evolutionarily conserved (Clancy, Gems et al. 2001, Tatar, Kopelman et al. 2001, Holzenberger, Dupont et al. 2003). In general, the role of ILS is not limited to longevity as it also confers higher resistance to several forms of stress. (Lithgow and Walker 2002, Garsin, Villanueva et al. 2003, Holzenberger, Dupont et al. 2003). In *C. elegans*, to regulate the Daf-c phenotype, DAF-16 is required in the neurons, while its expression is required in the intestine to extend life span (Apfeld

and Kenyon 1998, Libina, Berman et al. 2003, Alcedo and Kenyon 2004). In addition to its spatial regulation, ILS exhibits temporal regulation as it has been shown to regulate diapause with insignificant effect on life span during larval development and mainly impinges on longevity only during adulthood (Dillin, Crawford et al. 2002).

ILS is furthermore required to regulate germline stem cell divisions and fertility (Michaelson, Korta et al. 2010). In *C. elegans*, under normal conditions, the germline stem cells (GSC) proliferate throughout the animal's life but this can be interrupted under sub-optimal growth conditions. If there are inadequate nutrient resources during larval development, GSC arrest in the G2 phase in the L1 diapause and in G2/M phase during dauer quiescence (Fukuyama, Rougvie et al. 2006, Narbonne and Roy 2006). If *C. elegans* undergo adult reproductive diapause (ARD) in the late L4 larval stage, the germline stem cell divisions halt their proliferation and some will execute apoptosis. Genetic analysis further suggests that insulin-like signalling controls larval development and brood size in the starved adults (Tissenbaum and Ruvkun 1998, Fukuyama, Rougvie et al. 2006, Hibshman, Hung et al. 2016). Also, during adulthood, GSCs can respond to oocyte accumulation and inhibit their proliferation under the influence of ILS (Narbonne, Maddox et al. 2015). This suggests that insulin-like signalling is crucial to couple both environmental and developmental cues with germline physiology. The *C. elegans* genome is predicted to contain 40 insulin-like peptide genes (INS) (Pierce, Costa et al. 2001). Most of them are predicted to be functionally redundant but evidence suggests that some of the *ins* genes regulate dauer arrest. *Ins-1*, the orthologue of human insulin, and *ins-18* enhance dauer arrest when overexpressed in wild-type or *daf-2* animals (Pierce, Costa et al. 2001). The mechanism is still unclear but this suggests a potential antagonizing mechanism between these genes and *daf-2* signalling. Not all INS regulate dauer arrest and this could be explained on a structural basis. INS-1 and INS-18 are the only INS

molecules that contain C-peptides which are proteolytically cleaved during insulin processing. Transcription reporter assays confirm the expression of many of the *ins* genes in the amphid neurons. This suggests that the same environmental cues that affect the dauer decision may also control the expression and/or secretion of the insulin like peptides (Pierce, Costa et al. 2001). Furthermore, proteins regulating the formation of synaptic vesicles such as UNC-31, UNC-64 and ASNA-1/ATPase are essential for the secretion of these insulin-like peptides that regulate the ILS pathway, suggesting a critical role for neuronal regulation in insulin-like signalling (Ailion, Inoue et al. 1999, Kao, Nordenson et al. 2007).

TGF-β-like signalling

TGF-β is an another well characterized pathway that regulates dauer entry. Microarray based analysis of gene expression in wild type L2/L3 and *daf-7* mutant dauer larvae indicated that several genes of the ILS pathway were differentially expressed, likely highlighting a potential crosstalk and feedback mechanism between ILS and TGF-β pathway (Liu, Zimmerman et al. 2004). Moreover, transcription of DAF-16 regulated genes was also altered in *daf-7* mutant dauer larvae. A motif for DAF-16 binding activity was identified in the promotors of several genes regulated by TGF-β pathway. Similar to ILS mutants, the Daf-c mutants of the TGF-β pathway also exhibited DAF-16-dependent lifespan extension (Shaw, Luo et al. 2007). Altogether, this data set indicates a potential cross talk between ILS and TGF-β pathway which is not only limited to dauer regulation but may also be functional in other developmental processes.

The genes which well-define the TGF-β pathway include Daf-c genes: *daf-1, -4, -7, -8,* and *-14* and Daf-d genes: *daf-3* and *-5* (Patterson and Padgett 2000). Similar to the ILS pathway, binding of TGF-β family ligands activate the heteromeric receptors which further triggers a

phosphorylation cascade to eventually impinge on a SMAD4-like transcription factor. The TGF-β ligand, *daf-7*, binds to the type I receptor (*daf-1*) and the type II receptor (*daf-4*) at the cell surface (Georgi, Albert et al. 1990, Estevez, Attisano et al. 1993, Ren, Lim et al. 1996, Schackwitz, Inoue et al. 1996). This binding activity of TGF-β ligands leads to the phosphorylation of SMAD transcription factors, which translocate them to the nucleus to regulate the transcription of the target genes (Shi and Massague 2003).

daf-3, daf-8 and *daf-14* encode SMAD transcription factors and *daf-5,* the *sno/ski* homologue binds to DAF-3 (Estevez, Attisano et al. 1993, Patterson, Koweek et al. 1997, Inoue and Thomas 2000, da Graca, Zimmerman et al. 2004). Similar to mammals, SMADs positively regulate TGF-β signalling in *C. elegans* (Shi and Massague 2003). DAF-8 and DAF-14 inhibit TGF-β mediated dauer arrest but DAF-3 activity is inhibited upon the activation of the TGF-β signalling pathway (Patterson, Koweek et al. 1997, Inoue and Thomas 2000). Interestingly, recent findings reveal a temperature-dependent role of *daf-3.* At 25°C, loss of *daf-3* results in a Daf-d phenotype (Thomas, Birnby et al. 1993) while at 27°C it results in a Daf-c phenotype (Ailion and Thomas 2000), suggesting that activity of DAF-3 is certainly temperature dependent. Moreover, its activity is also dependent on genetic or environmental setting as loss of function mutation in *daf-3* suppresses Daf-c phenotype in *daf-1, -4, -7, -8* and *-14* at 25°C (Thomas, Birnby et al. 1993) and they can also enhance the weak Daf-c phenotype in some mutants (Gerisch, Weitzel et al. 2001, Ohkura, Suzuki et al. 2003). The underlying mechanism for this differential role of DAF-3 is still unknown. GFP reporter analysis using a *daf-7* promoter indicated that DAF-7 is expressed exclusively in the ASI neurons (Ren, Lim et al. 1996). The downstream receptor and the SMAD transcription factors are expressed ubiquitously in multiple tissues (Patterson, Koweek et al. 1997, Gunther, Georgi et al. 2000, Inoue and Thomas 2000). This indicates a mechanism to disseminate a signal from ASI

neurons to communicate with multiple tissues. Further studies revealed that the role of DAF-7 in regulating reproductive development is dependent on environmental cues like nutrient availability, dauer pheromone, or temperature. This is consistent with the interpretation of neurons mediating the coupling between the environmental cues and the multiple tissues (Ren, Lim et al. 1996, Schackwitz, Inoue et al. 1996). In optimal conditions, expression of *daf-7* is elevated and secreted from the ASI neurons to the surrounding tissues to activate DAF-1/4 receptor kinases to initiate phosphorylation of SMAD transcription factors DAF-8/14 and inhibit the activity of DAF-3 to activate transcription of genes regulating energy consumption and normal development. If *C. elegans* larvae encounter unfavourable conditions during development, the expression and release of DAF-7 is reduced and the activity of DAF-3 results in the transcription of genes involved in conserving energy and regulating dauer entry (Inoue and Thomas 2000, da Graca, Zimmerman et al. 2004).

cGMP signalling

The Daf-c gene, *daf-11* well defines the cGMP signalling pathway. It inhibits dauer arrest in parallel, or at least in part, to the TGF-β pathway (Thomas, Birnby et al. 1993). *daf-11* encodes a transmembrane guanylyl cyclase (GCY) that is expressed in a number of amphid sensory neurons including ASI, ASJ, ASK (Bargmann and Horvitz 1991) and in AWB, and AWC neurons which respond to various volatile odorants (Bargmann, Hartwieg et al. 1993). The role of GCY is to facilitate the conversion of GTP to cGMP in response to G-protein signalling in the chemosensory neurons (Birnby, Link et al. 2000). The Daf-c phenotype in *daf-11* is a result of a reduction in cGMP synthesis as 8-bromo-cGMP rescues the dauer arrest in *daf-11* mutants (Birnby, Link et al. 2000).

DAF-11 impinges on TAX-2 and TAX-4; two subunits of a cGMP-gated ion channel that regulates thermosensation, chemosensation and neuronal development (Coburn and Bargmann 1996, Komatsu, Mori et al. 1996, Coburn, Mori et al. 1998). They are both expressed in the sensory neurons and exhibit an expression pattern similar to *daf-11*, suggesting a functional co-operation between these neurons. Exogenous cGMP suppresses the *daf-11* dependent dauer arrest but fails to rescue the dauer arrest phenotype in the *tax-4* mutants, suggesting that TAX-4 likely acts downstream of DAF-11-dependent cGMP mediated signalling (Birnby, Link et al. 2000). DAF-11 activates TAX-2 and TAX-4 to inhibit dauer arrest through cGMP synthesis. Unlike *daf-11* mutants, *tax-2* and *tax-4* exhibit a weak Daf-c phenotype, suggesting that DAF-11 may impinge on multiple downstream targets (Coburn, Mori et al. 1998). To effect the Daf-c phenotype, other genes involved in formation of sensory cilium and the chemosensory neurons are also crucial (Vowels and Thomas 1992, Schackwitz, Inoue et al. 1996).

daf-21 is another well characterized gene involved in the cGMP pathway. It encodes a homologue of HSP-90 (Birnby, Link et al. 2000). A *daf-21* allele with recessive gain-of-function mutation exhibits the same Daf-c phenotype as *daf-11* mutants and can be rescued by an exogenous cGMP analogue. *A daf-21* null allele fails to enter the dauer state and results in larval lethality (Birnby, Link et al. 2000).

Based on epistatic analysis, the cGMP signalling pathway acts upstream of both ILS and TGF- β pathways. Compromise of DAF-16/FOXO or DAF-5/SNO-SKI can partially suppress the Daf-c phenotype in *daf-11* mutants (Vowels and Thomas 1992, Thomas, Birnby et al. 1993). Animals incapable of forming functional cilia structures suppress the Daf-c phenotype in *daf-11* mutants, but not in *daf-2* or *daf-7* mutants. Additionally, DAF-11 controls the expression of DAF-7 in the

ASI neurons and DAF-28/ILP expression in the ASI and ASJ suggesting an upstream role of the cGMP signalling pathway (Murakami, Koga et al. 2001).

Steroid hormone receptor pathway

Several genetic studies have identified a Daf-c gene, *daf-9,* and a Daf-d gene, *daf-12,* to function downstream of ILS, TGF-β and cGMP pathways to regulate dauer entry with unique pleiotropies in gonadal migration observed among all the Daf mutants (Riddle, Swanson et al. 1981, Albert and Riddle 1988, Thomas, Birnby et al. 1993, Antebi, Culotti et al. 1998, Gerisch, Weitzel et al. 2001, Jia, Albert et al. 2002). Cloning experiments identified *daf-9* as a gene encoding a cytochrome P450 and *daf-12* as a nuclear receptor homologue which acts like a transcription factor that responds to lipid hormone ligands (Antebi, Yeh et al. 2000, Gerisch, Weitzel et al. 2001, Jia, Albert et al. 2002). To execute dauer arrest, DAF-9 normally inhibits DAF-12 activity (Gerisch, Weitzel et al. 2001, Jia, Albert et al. 2002). Several DAF-9 metabolites identified as bile acid-like steroids named Δ-4 and Δ-7 dafachronic acid (DA) share a high affinity for DAF-12 (Motola, Cummins et al. 2006). Similar to more complex organisms, DAF-12 associates with coregulators in a ligand controlled fashion. Particularly, DIN-1/CoR, a homolog of mammalian SHARP, associates with DAF-12 to form a complex to allow dauer entry and exhibit associated traits (Ludewig, Kober-Eisermann et al. 2004).

Additionally, larvae lacking the *daf-12* ligand binding domain exhibit the Daf-c phenotype suggesting that the inability to bind DAF-9 is sufficient to enter dauer state (Antebi, Yeh et al. 2000). Several studies suggest that both DAF-9 and DAF-12 regulate dauer entry hormonally. Wild type, *daf-3* and *daf-16* mutants exhibit phenotypes similar to *daf-9* if grown using a cholesterol-deficient media, but *daf-12* mutants do not, despite the same growing conditions

(Gerisch, Weitzel et al. 2001). In addition, a lack of cholesterol exacerbates the Daf-c phenotypes in *daf-12* mutant larvae. This suggests that perhaps, sterols can act as a substrate and ligand for DAF-9 and DAF-12 respectively. Additionally, cholesterol sensitivity confirms the genetic position of *daf-9* in regulating dauer arrest, which is downstream of *daf-3* and *daf-16* and upstream of *daf-12*.

Identification of other components of this pathway identified *daf-36,* a gene encoding a Rieske oxygenase, which is required upstream of DAF-9 for dafachronic acid (DA) synthesis (Rottiers, Motola et al. 2006). Interestingly, DAF-36 is expressed primarily in the intestine, while DAF-9 is expressed in the hypodermis, somatic gonad and the XXX cells (Gerisch, Weitzel et al. 2001, Jia, Albert et al. 2002, Ohkura, Suzuki et al. 2003, Rottiers, Motola et al. 2006). Moreover, genetic mutants with impaired intracellular cholesterol trafficking exhibit a Daf-c phenotype. Intestinal DAF-36 dependent metabolites may possibly be transported to the neighbouring tissues with DAF-9 expression but the mechanisms are still unclear (Sym, Basson et al. 2000, Li, Brown et al. 2004, Chang, Reid et al. 2005).

miRNA pathway

The temporal regulation of developmental progression if controlled by the heterochronic genes, encoding microRNAs (miRNAs) and regulatory proteins. Pre-dauer L2d stage larvae delay the expression of L3 cell fates to eventually execute developmentally arrested dauer state (Rougvie 2001). The decision to enter dauer diapause or to develop normally depends on the activity of the DAF-12 nuclear hormone receptor (Antebi, Culotti et al. 1998). An additional role for DAF-12 has been described whereby DAF-12 directly affects the transcription of several let-7-Fam miRNAs (Bethke, Fielenbach et al. 2009). Later, a complex feedback relationship between DAF-

12 and the let-7-Fam miRNAs was established, wherein liganded DAF-12 regulates let-7-Fam miRNA expression and the let-7-Fam miRNAs temporally inhibit DAF-12. Under energetic stress, unliganded DAF-12 inhibits let-7-family-dependent cell fate transitions to favour developmental arrest (Hammell, Karp et al. 2009). For cells to progress to L3 cell fate, the transcription factor Hunchback-like-1 (HBL-1) must be downregulated. During normal development, let-7-Fam miRNA downregulates the activity of a heterochronic gene Hunchback-like-1 (HBL-1) (Karp and Ambros 2012). Thus, to promote dauer arrest, the repressive DAF-12 activity inhibits the accumulation of let-7-Fam miRNAs, resulting in persistence of HBL-1 and thus inhibiting L3 cell fates (Hammell, Karp et al. 2009).

Loss of function mutations in the components of RNA-induced silencing complex (RISC) complex can result in a complete loss of miRNA function. Mutations in *ain-1,* a component of RISC, along with mutation in *unc-3,* a neuronal transcription factor results in inappropriate dauer entry. Further genetic analysis suggests that neuronal miRNAs partly regulate endogenous cGMP signalling and potentially influences major dauer regulation pathways. Tissue-specific immunoprecipitation of miRISC, revealed several miRNA and their target mRNAs within neuronal tissue. Several of these miRNAs are likely to regulate dauer formation through multiple targets (Than, Kudlow et al. 2013). Altogether, it is evident that miRNA pathway plays a substantial role in regulating dauer entry potentially by regulating cell fate decisions and to coordinate cell fate with developmental arrest.

Conclusion

Dauer entry provides an excellent model to study how different environmental cues are sensed and transduced to alter physiology and development. Several studies and genetic screens have

identified the key pathways regulating dauer entry upon encountering unfavourable environmental conditions. During replete conditions, neuronal based signalling activates the cGMP signalling pathway resulting in release of insulin-like peptides and TGF-β ligands to trigger the ILS and TGF-β signalling pathways to stimulate synthesis of DA. Upon activation by DA and other presumptive coactivators, DAF-12 instructs the larva to undergo normal development and inhibits dauer arrest. Thus, both the pathways ultimately converge on transcriptional regulation of genes responsible for biosynthesis of steroid hormones, the ligands that activate the Nuclear Hormone Receptor/DAF-12 (Fig. 1.2).

During unfavourable conditions, the major pathways: cGMP, ILS and TGF-β are downregulated resulting in transcriptional repression of the genes required for hormone biosynthesis and thus DAF-12 remains unbound and eventually is associated with DIN-1, the co-repressor protein. This inhibition of hormone synthesis and inactivation of DAF-12 enables early larva to alter their regular development pathway and execute the dauer state, enabling their long-term survival during the sub-optimal growth conditions.

Other *C. elegans* diapauses

In response to sub-optimal growth conditions, *C. elegans* can also execute at least two types of diapause, in addition to dauer. Unlike dauer, these diapause states do not allow animals to enter an alternative development pathway, but instead delay their regular course of development. If eggs hatch in a nutrient-deprived environment, they enter a phase called the L1 diapause and arrest their development, being able to survive for 2 weeks in this quiescent state. Normal development resumes only after conditions improve and food becomes available in the surrounding environment. Ingestion of food activates ASNA-1, an ATP synthase, to stimulate insulin secretion and activate the insulin-like signalling pathway. This activated ILS pathway blocks the activity of

the transcription factor DAF-16 and instructs the animal to resume normal reproductive development (Baugh and Sternberg 2006, Kao, Nordenson et al. 2007). Under starvation conditions, the ILS pathway is downregulated and consequently, DAF-16 is translocated to the nucleus to activate the transcription of several stress response genes. During the L1 diapause, DAF-16 initiates the transcription of *cki-1*, a cyclin-dependent kinase inhibitor, to inhibit cell cycle progression in several somatic tissues (Hong, Roy et al. 1998, Baugh and Sternberg 2006). However, DAF-16 activity is not essential for establishing cell cycle arrest in the primordial germ cells (PGCs) during the L1 diapause or in the germline stem cell (GSC) population in the dauer larva, suggesting that the germ cells are regulated differently in a DAF-16-independent manner (Fukuyama, Rougvie et al. 2006, Narbonne and Roy 2006).

In addition to the L1 diapause and the dauer stage, *C. elegans* can execute the adult reproductive diapause (ARD) if the later stage L4 larva encounters limited nutritional resources. Similar to other diapauses, during ARD, both somatic and germline development is halted. To efficiently execute ARD, the apoptotic pathway is initiated in the entire germ line, except for the GSCs, and when the feeding resumes, the quiescent GSCs populate the germ line and complete the meiotic cycle to become fertile without any severe consequences on the reproductive capacity (Angelo and Van Gilst 2009). Interestingly, unlike the L1 diapause or the dauer state, ARD entry and exit is regulated by the starvation-sensing nuclear hormone receptor, NHR-49, which acts in an ILS-independent fashion (Angelo and Van Gilst 2009).

Chapter 1.3. *C. elegans* gonad

The *C. elegans* adult gonad comprises the germline stem cells (GSCs), the meiocytes and the somatic gonad. Considering that the GSCs carry all the required genetic information for the next

generation, their response to dauer formation is of great biological interest, raising questions regarding stem cell dynamics and quiescence. In *C. elegans* hermaphrodites, the gonad consists of 2 identical U-shaped gonadal arms with one on the anterior side of the midline (vulva) and the other at the posterior. They are connected proximally, near the ventral midline by a shared uterus. In the L4/adult animal, each gonadal arm is divided into a distal or dorsal gonad (distal to the uterus and vulva) and a proximal or ventral gonad. The distal gonad consists of the distal tip cell (DTC), which forms a niche for the proliferating germ cells, while the proximal end of each gonadal arm includes differentiated gametes: oocytes and sperm. During development, the proliferating germ cells are situated at the distal end of the gonad and form a syncytium. Each germ cell possesses a stable intercellular bridge that connects it to a central core of common cytoplasm, known as the rachis (Hirsh, Oppenheim et al. 1976, McCarter, Bartlett et al. 1997, Hubbard and Greenstein 2005). The gonad is thus spatially organized, with the mitotic germ cells remaining distinct in the distal end from the differentiated cells at the proximal end. This enables easy identification of the proliferating GSCs and thus facilitates the study of their cell cycle dynamics.

Development of the somatic gonad

The somatic gonad is comprised of the non-germ cell elements and consists of five tissues with specialized functions and characteristic features: the DTCs, gonadal sheath, spermathecal-uterine valve, and the uterus. In particular the DTCs and the sheath cells are of prime importance as they are intimately associated with several aspects of germline development (Kimble and White 1981, McCarter, Bartlett et al. 1997, Hall, Winfrey et al. 1999). At hatching, the L1 larva comprises 4 gonadal primordial cells: Z1 and Z4 (somatic gonad precursor cells), and Z2 and Z3 (primordial

germ cells) which they flank. Under normal conditions, Z1 and Z4 divide to generate 12 descendants: two DTCs, nine blast cells and one anchor cell. The DTCs are situated at the anterior and the posterior gonad and the remaining cells migrate to the center of the gonad to form the somatic gonad primordium, and also divide the germ cells into anterior and posterior population (Kimble and Hirsh 1979).

The DTC is a large, single somatic cell situated at the distal most extreme of both dorsal gonadal arms. In adults, numerous thin cytoplasmic extensions (cytonemes) arise from the DTC and extend up to 8-12 germ cell rows. The DTCs are required to: 1) Promote mitosis and inhibit meiosis in the germ cells and 2) to promote the elongation of the gonadal arms (Kimble and White 1981, Austin and Kimble 1987, Blelloch and Kimble 1999).

Gonadal sheath cells develop to form a layer to cover the germ line. The sheath cells are closely associated with the germ line and are absolutely essential to promote germ cell proliferation, pachytene exit, gametogenesis, and to specify male gamete fate during sex determination (Seydoux, Schedl et al. 1990, McCarter, Bartlett et al. 1997, Rose, Winfrey et al. 1997, Killian and Hubbard 2004).

C. elegans germline development

During the early stages of embryogenesis, germline determinants are isolated to the P blastomeres, terminating in P4, the germline precursor cell that divides symmetrically to generate the 2 PGCs, Z2 and Z3. P granules, the specialized perinuclear particles specific to the germ line, are maternally contributed to the P blastomeres. Normal germline development is altered in the absence of their defining proteins (Hubbard and Greenstein 2005). The PIE-1 protein was identified as one of the proteins required to specify germline fate. PIE-1 is a CCCH-type zinc finger protein and exhibits

a dynamic localization pattern during early embryogenesis (Mello, Schubert et al. 1996). Several studies demonstrate that to specify germline fate, PIE-1 represses transcriptional elongation and blocks the expression of genes required for somatic differentiation (Seydoux and Dunn 1997, Batchelder, Dunn et al. 1999). Interestingly, PIE-1 also directly regulates MEP-1, a component of the nucleosome remodeling and deacetylase (NuRD) complex, which functions in the somatic cells to prevent them from acquiring germline fate (Belfiore, Mathies et al. 2002, Unhavaithaya, Shin et al. 2002).

At hatching, the L1 larval gonad is comprised of 2 primordial germ cells (PGCs), Z2 and Z3, flanked by the somatic precursor cells (SPCs), Z1 and Z4, enclosed in a basement membrane. If conditions are favourable, all 4 precursor cells proliferate, and the somatic cells rearrange to position DTCs at the cap of the gonadal arms, creating a niche for the GSCs to proliferate (Kimble and Hirsh 1979, Hubbard 2011).

Under normal conditions, these cell divisions continue uninterrupted and the germ cells remain undifferentiated until the L3 stage. During the L4 stage when the gonadal arms have developed substantially, they begin to show distinct distal and proximal polarity, whereby germ cells at the proximal end are now too far away from the niche to receive the signal that drives mitosis from the DTCs. As a result, these germ cells lose their proliferative fate and initiate the meiotic program to eventually produce haploid gametes (Hubbard and Greenstein 2005) (Fig. 1.3 A).

Maintenance of the proliferative germ line

The *C. elegans* germ line is spatially and temporally organized with the proliferative cells positioned at the distal end and the differentiated haploid gametes at the proximal end. Unremitting mitotic divisions are essential to allow the production of oocytes to replace those that are fertilized

in a continuous assembly line-like arrangement. The niche-like relationship between the germ cells and the DTCs is crucial for maintaining the proliferative fate and preventing germ cell differentiation. Laser ablation of the DTC results in a loss of GSC proliferation and initiates their meiotic program (Kimble and White 1981). One of the best-studied pathways to date that directly regulates GSC proliferation is the Notch signalling pathway. Loss-of-function mutations in the genes positively regulating this pathway result in the loss of mitotic fate in the germ cells (Kimble and White 1981, Austin and Kimble 1987, Pepper, Lo et al. 2003). Dissection of this niche and GSCs interaction has identified various genetic and molecular interactions that result in germ cell proliferation.

Notch signalling maintains proliferation while blocking differentiation in the GSCs. The Notch receptor, GLP-1, is present at the surface of the germ cells, while the Notch ligand, LAG-2/Delta, is expressed on the surface of the DTCs (Crittenden, Troemel et al. 1994, Henderson, Gao et al. 1994). Within the niche, LAG-2 activates the Notch receptor/GLP-1 and leading to the cleavage of the intracellular domain of activated GLP-1. Upon cleavage, the intracellular domain enters the nucleus and forms a complex with a CSL family protein, LAG-1, and LAG-3 to regulate transcription (Hansen and Schedl 2006, Kimble and Crittenden 2007). Activation of the Notch pathway blocks the activity of the genetic pathways that regulate meiotic entry. GLD-1, a KH-domain containing RNA-binding protein, and GLD-2, a cytoplasmic poly-A polymerase protein, interact and form a complex regulatory network to initiate meiotic entry (Francis, Barton et al. 1995, Eckmann, Kraemer et al. 2002, Eckmann, Crittenden et al. 2004). The activation of Notch/GLP-1 interferes with the accumulation of GLD-1, thereby blocking meiotic entry. FBF-1 and FBF-2, two Pumilio-related RNA binding proteins, are required to mediate the activity of the Notch signalling pathway. These PUF family (Pumilio and FBF) at least in part promote mitosis

by repressing the levels of *gld-1* mRNA (Crittenden, Bernstein et al. 2002, Lamont, Crittenden et al. 2004) (Fig. 1.3 B). Recently, it has been shown that the two Notch targets SYGL-1 and LST-1, link niche signalling to FBF. Both the proteins physically interact with FBF to form a repressive complex that is crucial for stem cell proliferation (Shin, Haupt et al. 2017). As the dividing GSCs presumably push the other mitotic cells towards the proximal end, GLP-1 is out of proximity to receive an activation signal from the DTCs. This diminishes the Notch signal, which in turn relieves FBF target RNAs from repression and allows the germ cells to enter meiotic prophase 1. The Notch signalling is further reduced as GLD-1 negatively regulates GLP-1 translation (Marin and Evans 2003). The reduced GLP-1 expression allows for the controlled and restricted proliferation of the germ cells at the distal end of the gonad.

Chapter 1.4. Dauer germ line

Most organisms delay their GSC divisions if they encounter nutritional stress. For example, *C. elegans* larvae require cholesterol to maintain their reproductive fitness. If the larvae are grown without cholesterol, their brood size is significantly compromised due to a reduction in germline proliferation and differentiation (Shim, Chun et al. 2002). Similarly, in female *Drosophila,* the germline stem cells and their differentiated progeny cells alter their divisions in response to nutrient availability (Drummond-Barbosa and Spradling 2001). Probably, to protect germline stem cells from accumulating defects while undergoing proliferation during periods of starvation, they possess an ability to respond to the external environment and adjust their proliferation rate accordingly (Hand, Denlinger et al. 2016).

Cell division is an energy-consuming process and thus as a strategy to conserve energy, upon dauer entry, *C. elegans* larvae exhibit global cell cycle quiescence (Cassada and Russell 1975, Hand,

Denlinger et al. 2016). In larvae with reduced levels of insulin-like signalling, similar to somatic tissues, GSC proliferation gradually slows down during the preparation for dauer entry and eventually exhibits complete arrest such that no germ cell division occurs during dauer state (Narbonne and Roy 2006). Similarly, inhibiting insulin-like signalling in the late larval stages also results in significant reduction of germ cell proliferation (Gems, Sutton et al. 1998). Thus, upon starvation, insulin-like signalling directly or indirectly impinges on GSC divisions and reduces their proliferation rate.

Quiescent GSCs exhibit G2/M phase arrest

In mammalian cells, insulin signalling regulates Akt/PKB activity to modulate the cell cycle machinery to progress through G1/S and G2/M checkpoints. Akt/PKB-dependent G1/S regulation mainly depends upon the activity of the two CDK inhibitors: p21 and p27 (Brazil, Yang et al. 2004). Thus, it seems reasonable to hypothesize that the GSC quiescence in *C. elegans* dauer larvae occurs at the G1/S checkpoint through blocking the inhibition of p21/p27. Consistent with this hypothesis, RNAi mediated knockdown of *cki-1* (*C. elegans* homolog of p21/p27) results in abnormal GSC divisions in dauer larvae (Hong, Roy et al. 1998). Although G1 specific CDK inhibitors are required for dauer GSC quiescence, GSCs fail to arrest in G1 phase and exhibit G2/M phase arrest in *daf-2* compromised dauer larvae. The quiescent GSCs contain twice the amount of DNA of G1 arrested somatic cells (Narbonne and Roy 2006). This suggests that in *C. elegans* dauer larvae, GSC quiescence results in G2/M phase arrest and G1 specific CDK inhibitors contribute to the inhibition of the GSC divisions. The surprising differences in the cell cycle regulation are possibly at the organismal level and the significance of the G2/M phase arrest of GSCs in response to environmental stress remains unclear.

Moreover, reduced insulin-like signalling does not affect the proliferative signal within a niche. The Delta/Serrate-like ligand, LAG-2, is still expressed in the DTCs and the Notch receptor GLP-1 is present in the quiescent dauer germ line. Presumably, Notch signalling is still active as both the ligand and the receptor are expressed, but germ cells still exhibit G2/M phase arrest and fail to either proliferate or differentiate (Narbonne and Roy 2006). This suggests that perhaps, there are other regulators which may block germ cell proliferation in parallel or downstream of presumably active Notch signalling to establish a quiescent state in the dauer germ line (Narbonne and Roy 2006).

A forward genetic screen was performed to identify genes required to establish quiescence in nutritionally-challenged larvae. Using this strategy, a dominant negative mutation in the *C. elegans* orthologue of the catalytic subunit of AMP-activated kinase (AMPK) was isolated, and further genetic analysis indicated that *par-4*/LKB1 and *daf-18/*PTEN impairment also resulted in aberrant, untimely germ cell proliferation in dauer larvae (Narbonne and Roy 2006). *daf-18/*PTEN acts downstream of the *C. elegans* insulin receptor orthologue DAF-2, and counteracts the activity of PI3 kinase. Not surprisingly, PTEN loss of function mutations commonly result in several types of human cancer (Di Cristofano and Pandolfi 2000). Identification of the other downstream targets of insulin-like signalling that link GSC proliferation with nutrient availability, perhaps novel regulators of PTEN signalling will help to understand its tumour inhibiting properties.

Chapter 1.5. LKB1/AMPK signalling

Liver Kinase B1 (LKB1) is a tumour suppressor, and if misregulated in humans, it gives rise to Peutz Jeghers syndrome (PJS), an autosomal dominant disease that predisposes patients to various cancers (Hemminki, Markie et al. 1998). The protein kinase activity of LKB1 is important for its tumour suppression function as many of the mutations that have been identified in PJS lie within its highly conserved kinase domain (Jenne, Reimann et al. 1998). Two accessory proteins: Ste20-related adaptor protein-α (STRADα) and mouse protein 25-α (MO25α), are essential to activate the kinase activity of LKB1 and also to regulate its cellular localization in mammals (Baas, Boudeau et al. 2003, Boudeau, Baas et al. 2003). This complex likely impinges on downstream targets to block tumour growth. However, several of its substrates are also well defined protein kinases that fall into a class called Microtubule Affinity Related Kinases, or MARK kinases (Lizcano, Goransson et al. 2004). LKB1-mediated phosphorylation of these targets leads to their activation and consequently their downstream pathways (Kullmann and Krahn 2018).

The essential role of LKB1 was initially identified for the *C. elegans* orthologue, PAR-4, during early embryogenesis as the mutations in *par-4* lead to defects in partitioning of cellular components during the first zygotic division (Kemphues, Priess et al. 1988, Rose and Kemphues 1998). One of the downstream MARK kinases is another *par* gene product called PAR-1 that is essential in regulating polarity in the early embryo. In the absence of PAR-4, posterior localization of PAR-1/PAR-2 and anterior localization of PAR-6 fails to set up correctly and results in a symmetrical division (Watts, Morton et al. 2000). LKB1 was similarly implicated in polarity regulation in *Drosophila* and mammalian cells in culture suggesting that its role in regulating polarity is highly conserved (Martin and St Johnston 2003, Nakano and Takashima 2012).

However, LKB1 has other downstream targets, many of which affect cellular processes other than cell polarity (Shackelford and Shaw 2009). The well-characterized downstream target of LKB1 is the major metabolic regulatory kinase; AMP-activated protein kinase (AMPK), which is crucial for the appropriate energy fine-tuning in most organisms (Hardie and Lin 2017).

In *C. elegans* dauer larvae, similar to *daf-18/*PTEN, loss of *par-4*, and *aak-2,* which encodes a homolog of the α2 catalytic subunit of AMPK results in dauer germline hyperplasia. RNAi mediated depletion of the other catalytic subunit of AMPK, *aak-1*, also results in supernumerary germ cells in dauer larvae. Moreover, in the animals lacking both the catalytic subunits (henceforth referred as *aak(0*) or AMPK null) the phenotype is additive, with a significantly greater number of germ cells as compared to the individual mutants.

Interestingly, the loss of LKB1/PAR-4 in *aak(0)* dauer larvae resulted in an additive germline hyperplasia, suggesting that LKB1/PAR-4 must impinge on additional targets, independently of AMPK to establish quiescence in the dauer germline (Narbonne and Roy 2006, Kadekar, Chaouni et al. 2018). These results are consistent with previous findings where LKB1 plays both AMPK-dependent and AMPK-independent roles to regulate cell cycle dynamics in haematopoietic stem cells (Gurumurthy, Xie et al. 2010, Nakada, Saunders et al. 2010)

Structure and regulation of AMPK

AMPK was identified as a regulator of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and acetyl coenzyme A (Acetyl Co-A) carboxylase in two independent findings (Beg, Allmann et al. 1973, Carlson and Kim 1973). Further studies of these enzymes revealed that the kinase involved in their phosphorylation and inhibition required AMP as a cofactor to activate itself and hence was named AMPK (Ferrer, Caelles et al. 1985, Carling, Zammit et al. 1987).

AMPK is a heterotrimeric complex comprised of a catalytic subunit (α) and 2 regulatory subunits (β and γ). There exist at least 2 isoforms for each subunit and can form different combinations resulting in differential tissue expression and subcellular localization (Salt, Celler et al. 1998, Cheung, Salt et al. 2000). The catalytic subunit consists of a Ser/Thr kinase domain at the N-terminus and a β regulatory binding domain is present at the C-terminus (Hardie, Ross et al. 2012). The γ subunit contains four cystathionine-β-synthase (CBS) domains in two tandem repeats referred to as a Bateman domain (Carling, Thornton et al. 2012). Crystal structure of the AMPK core identified that 3 of these domains bind to adenine bases. One of the domains binds strongly to AMP while the other two domains bind to AMP, ADP or ATP in a competitive manner (Xiao, Heath et al. 2007). In all organisms, AMP and ADP act as activators of the AMPK complex. Addition of AMP to recombinant AMPK *in vitro* results in a ten-fold increase in AMPK phosphorylation activity (Suter, Riek et al. 2006). AMP or ADP binding to the Bateman domains result in a conformational change in the γ subunit to expose the activation loop centered by a conserved Thr residue named T172 based on its position in the original sequence (Hawley, Davison et al. 1996). This conformational change and the exposure of the T172 residue allows an upstream activating kinase to phosphorylate and activate the complex. Binding of ATP instead blocks the exposure of T172 and thus inhibits the allosteric activation of AMPK complex (Carling, Thornton et al. 2012). Thus, AMPK activity is dependent on AMP/ADP:ATP ratios and adjusts metabolism by impinging on key downstream targets.

Another regulator of AMPK has been identified as another kinase, Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ). It responds to increased levels of intracellular Ca^{2+} and is independent of AMP/ADP:ATP ratio. Increasing intracellular Ca^{2+} levels can help activate AMPK

in tumour cells that exhibit an increase in AMP and/or ADP and lack LKB1 activity (Hawley, Boudeau et al. 2003, Fogarty, Ross et al. 2016).

AMP mediated activation of AMPK is also dependent on myristoylation of the N-terminal domain of the β subunit. In the absence of myristoyl group, AMPK is unable to bind AMP and the phosphorylation of the T172 residue is insufficient for AMPK activity (Oakhill, Chen et al. 2010). Additionally, hydrogen peroxide (H_2O_2) also activates AMPK in mammalian cells by oxidizing the cysteine residues of the α subunit, suggesting that the AMPK is also responsive to the cellular redox status (Zmijewski, Banerjee et al. 2010).

Physiological implications of AMPK signalling

Upon activation, AMPK plays important roles in regulating several processes which are depicted here in an overview of the current mechanistic understanding of AMPK function in regulating growth, metabolism, cell polarity, epigenetics etc. (Fig 1.4).

1) AMPK modulates growth and autophagy

Under energetic stress, AMPK acts as a molecular switch to inhibit cellular growth to conserve energy resources. In mammals under nutritional stress, the LKB1/AMPK pathway modulates cell growth by impinging on the mammalian target of rapamycin complex 1 (mTORC1). AMPK directly phosphorylates the TSC1/TSC2 complex, which acts as a GAP and inhibits the active Rheb-GTP into the inactive form of Rheb-GDP. Active Rheb-GTP triggers the mTOR pathway to upregulate protein synthesis and cellular growth in general. Thus, its inactivation in AMPK-dependent manner attenuates mTOR signalling and inhibits cellular growth (Schmelzle and Hall 2000, Inoki, Zhu et al. 2003, Shaw, Bardeesy et al. 2004). In *C. elegans,* no TSC1/TSC2 homologues have been identified yet, but a recent study has implicated a novel role of Ral-GAP

in the *C. elegans* mTOR signalling pathway (Martin, Chen et al. 2014). Moreover, AMPK also directly phosphorylates Raptor, a scaffold protein required to assemble the mTOR complex, to inhibit its activity (Gwinn, Shackelford et al. 2008). It is still unclear whether AMPK modulates the mTOR pathway through any of the above-mentioned effectors to inhibit germ cell proliferation to establish dauer quiescence and this finding further suggests that alternative pathways must exist to establish GSC quiescence downstream of LKB1/AMPK signalling, which are independent of TSC1/2.

In addition to regulating growth through mTOR, AMPK also modulates autophagy; an intracellular degradation system that provides energy in response to nutrient or other stress. The most upstream components of this pathway include a serine/threonine kinase Atg1 and its regulatory proteins Atg13 and Atg17 (Mizushima 2010, Yang and Klionsky 2010). The activity of Atg1 complex is suppressed by mTORC1, presumably through phosphorylation of Atg1 orthologs of ULK1 and ULK2 and their regulatory subunits (Mizushima 2010). Unlike mTORC1, AMPK directly phosphorylates ULK1 to activate the complex to regulate autophagy (Egan, Shackelford et al. 2011, Hardie 2011). Genetic studies in yeast and *C. elegans* also suggest that Atg1 orthologs function downstream of AMPK to regulate autophagy (Wang, Wilson et al. 2001, Egan, Shackelford et al. 2011). Altogether, these studies demonstrate that AMPK regulates autophagy through direct activation of ULK1 as well as by inhibiting the negative regulator of ULK1, mTORC1.

2) Role of AMPK in regulating metabolic pathways

AMPK is generally activated under nutrient-deficient conditions. Several physiological stimuli like glucose depletion, ischaemia, extreme muscular exercise, hypoxia and heat shock are known

to activate AMPK (Zhang, Zhou et al. 2009, Carling, Thornton et al. 2012, Hardie, Ross et al. 2012). AMPK activation results in phosphorylation of several key metabolic proteins to alter several metabolic pathways. Upon activation, AMPK is known to alter the activities of acetyl-CoA carboxylase 1 (ACC1), HMG-CoA reductase, and glycogen synthase kinase 3β (GSK-3β) to modulate carbohydrate and lipid metabolism, resulting in a suppression of the energy consuming anabolic events like fatty acid synthesis, gluconeogenesis and cholesterol synthesis. It also promotes energy producing processes such as fatty acid oxidation and glucose transport into the muscle tissues (Schimmack, Defronzo et al. 2006). However, AMPK can directly phosphorylate and block the activity of a catabolic enzyme adipose triglyceride lipase (ATGL), the enzyme required to catalyze the rate-limiting step of lipolysis in *C. elegans* dauer larva, suggesting a catabolism suppressing role of AMPK under energetic stress (Narbonne and Roy 2009).

Upon dauer entry, AMPK null mutants deplete their fat reserves at a faster rate due to the increased activity of the lipase ATGL-1. Moreover, these animals become osmosensitive, resulting in a severe decline in their viability. AMPK directly phosphorylates ATGL-1 at S303 to block lipolysis for their long-term survival. This ATGL-1 phosphorylation generates 14-3-3 binding sites on ATGL-1 which is further recognized by the 14-3-3 binding protein, PAR-5, allowing for the sequestration of ATGL-1 away from the lipid droplets and targeting it for proteasome-mediated degradation (Xie and Roy 2015). Altogether, AMPK ensures long-term dauer survival by downregulating the activity of ATGL-1 to ration lipid reserves along with appropriate osmoregulation (Narbonne and Roy 2009).

The long-term survival of AMPK mutants is vastly dependent on generating new energy sources. Recently, it has been shown that the viability of *aak(0)* mutant dauer larvae can be significantly improved by elevating HIF-1-dependent expression of the genes involved in fatty acid

biosynthesis. This mechanism is downstream of suppressed catalase activity which allows an increase in the cellular levels of hydrogen peroxide during dauer. Reactive Oxygen Species (ROS) is often regarded as a toxic by-product, although at low levels, ROS has a beneficial role and promotes longevity and as such hydrogen peroxide plays a crucial role in the induction of hormesis (Schulz, Zarse et al. 2007, Ludovico and Burhans 2014). Similarly, at low levels, ROS modulates lipid biosynthesis downstream of active HIF-1 to provide cellular energy, which enhances the survival of the *aak(0)* dauer larvae (Xie and Roy 2012). Several genes involved in enhancing survival of *aak(0)* dauer larvae were identified from the genome-wide screens and further characterization will reveal the regulatory pathways involved in modulating lipid metabolism (Xie and Roy 2012).

3) Role of AMPK in regulating behavioural response.

Several studies have implicated AMPK in the hypothalamic regulation of food intake behaviour (Kola 2008, Yang, Atasoy et al. 2011). However, understanding how starvation impacts behaviour in an AMPK-dependent manner is elusive due to complexity of the nervous system in the higher animals. Under energetic stress, AMPK induces adaptive behaviours in *C. elegans* and understanding and dissecting the mechanisms influencing foraging behaviours is relatively simpler than other complex organisms (Lee, Cho et al. 2008, Cunningham, Bouagnon et al. 2014). Food abundance affects several aspects of *C. elegans* locomotive behaviour (Sawin, Ranganathan et al. 2000, Gray, Hill et al. 2005, Chalasani, Chronis et al. 2007). In the absence of food, well-fed animals exhibit frequent reversals and thus limited dispersal, presumably a memory of food availability in the locomotory circuit results in efficient limited exploration. Starvation suppresses reversal behaviour and triggers forward movements, allowing animals to explore and disperse

(Gray, Hill et al. 2005). During periods of starvation, AMPK acts as a molecular trigger in two interneurons; AIB and AIY to suppress reversals and induce dispersal behaviour by regulating glutamatergic inputs (Ahmadi and Roy 2016). Furthermore, under oxidative stress, AAK-2 is expressed in the ventral cord and several neurons to protect the animal from oxidative stress and modulate its motility and behavioural responses (Lee, Cho et al. 2008). Mutations in either PAR-4 or AMPK reduce body bending during locomotion and fail to reduce head oscillation in response to anterior touch (Lee, Cho et al. 2008). Thus, the activity of AMPK is crucial to integrate sensory cues with the neuronal system to accordingly induce adaptive behavioural changes.

4)AMPK modulates cell polarity, migration, and cytoskeletal dynamics.

LKB1 is a well-studied cell polarity regulator during critical asymmetric cell divisions in the early *C. elegans* embryo and several other organisms (Kullmann and Krahn 2018). AMPK-related MARKs (Microtubule Affinity Regulating Kinases), mainly act downstream of LKB1 to establish cell polarity (Jansen, Ten Klooster et al. 2009). However, in mammalian cells, AMPK activity is also essential for polarization of proteins involved in the formation of both tight and adherens junctions (Zhang, Li et al. 2006, Zhang, Jouret et al. 2011). AMPK is further implicated in microtubule assembly by phosphorylating a microtubule plus end protein CLIP-170, as mutations in its AMPK phosphorylation sites result in abnormal microtubule assembly. Interestingly, mTORC1 was reported to be a regulator of CLIP-70 (Choi, Bertram et al. 2002), suggesting that mTOR and AMPK signalling perhaps converge on CLIP-70. Furthermore, LKB1/AMPK-dependent regulation of the mTOR pathway has been linked with regulation of ciliogenesis and neuronal polarization under metabolic stress (Boehlke, Kotsis et al. 2010, Williams, Courchet et al. 2011).

5) Role of AMPK in chromatin modification and epigenetics.

Recently, in human umbilical vein cells, AMPK has been implicated in the regulation of gene expression through chromatin remodeling. DNA methyltransferase 1 (DNMT1), retinoblastoma binding protein 7 (RBBP7), and histone acetyltransferase 1 (HAT1) carry AMPK consensus phosphorylation sequences. DNMT1 controls DNA methylation to limit access of transcription factors to promoters and is inhibited by RBBP7 (Rhee, Bachman et al. 2002, Marin, Gongol et al. 2017). HAT1 mediates the acetylation of histones to relax chromatin-DNA structure to promote transcription (Sterner and Berger 2000). AMPK-dependent phosphorylation results in activation of HAT1 and inhibition of DNMT1. In general, AMPK-mediated phosphorylation enhances DNMT1-RBBP7 and HAT1-RBBP7 interactions to increase the expression of nuclear genes required for mitochondrial biogenesis (Marin, Gongol et al. 2017). In mammals, AMPK phosphorylates histone H2B to activate stress-promoted transcription leading to cellular adjustment to stress (Bungard, Fuerth et al. 2010).

In *C. elegans,* during L1 starvation*,* AMPK plays a critical role in blocking abnormal chromatin modifications to ensure that gene expression is tightly coordinated with available energy resources. In the absence of AMPK, inappropriate chromatin marks are established, which are correlated with compromised germline integrity because of abnormal gene expression. These abnormalities persist for several generations resulting in a mortal germline phenotype. AMPK regulates the chromatin modifying COMPASS complex to ensure that abnormal marks are not established under starved conditions (Demoinet, Li et al. 2017). Furthermore, these findings also suggest that the activity of AMPK might be important to also resolve the abnormal epigenetic modifications in the following generations. Altogether AMPK is an important link between chromatin regulation and the metabolic status of the animal to ensure appropriate gene expression during periods of starvation.

Figures

Figure 1.1. *C. elegans* can alter their developmental pathway in response to unfavourable environment.

In replete conditions at 20^{O}C, after hatching, *C. elegans* develops through four larval stages to become a reproductive adult. However, if the conditions are sub-optimal during their early development, L1 larvae can enter a developmentally quiescent dauer state instead of L3 and can remain in this state for up to four months, or until conditions become normal. When the conditions improve, dauer larvae can directly molt into L4 and resume their normal development. Figure used with permission from WormAtlas. Altun, Z.F., Herndon, L.A., Wolkow, C.A., Crocker, C., Lints, R. and Hall, D.H. (ed.s) 2002-2018. http://www.wormatlas.org

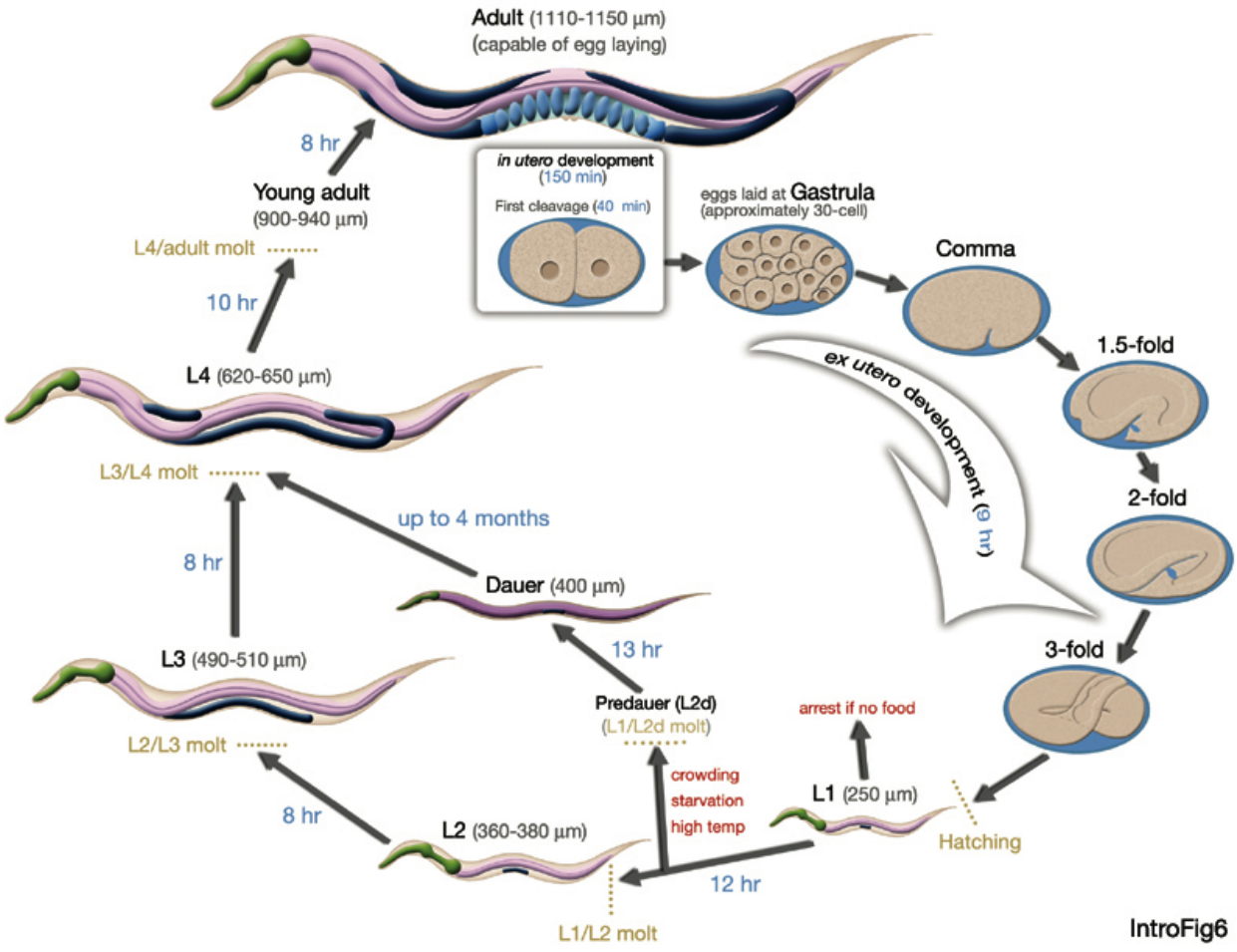

Adult (1110-1150 µm)
(capable of egg laying)
8 hr
Young adult
(900-940 µm)
L4/adult molt
10 hr
L4 (620-650 µm)
L3/L4 molt
8 hr
up to 4 months
Dauer (400 µm)
13 hr
L3 (490-510 µm)
L2/L3 molt
8 hr
L2 (360-380 µm)
L1/L2 molt
Predauer (L2d)
(L1/L2d molt)
crowding
starvation
high temp
12 hr
L1 (250 µm)
arrest if no food
Hatching
in utero development
(150 min)
First cleavage (40 min)
eggs laid at Gastrula
(approximately 30-cell)
Comma
1.5-fold
2-fold
3-fold
ex utero development (9 hr)
IntroFig6

Figure 1.2. Regulatory Pathways for Dauer Arrest in *C. elegans.*

Under favourable condition, cGMP pathway stimulates both insulin-like and TGF-β pathway to promote transcription of DAF-9 to produce dafachronic acids (DA) that can bind DAF-12 to initiate normal development. Arrows and bars represent positive and negative interaction respectively. Based on (Rottiers and Antebi 2006).

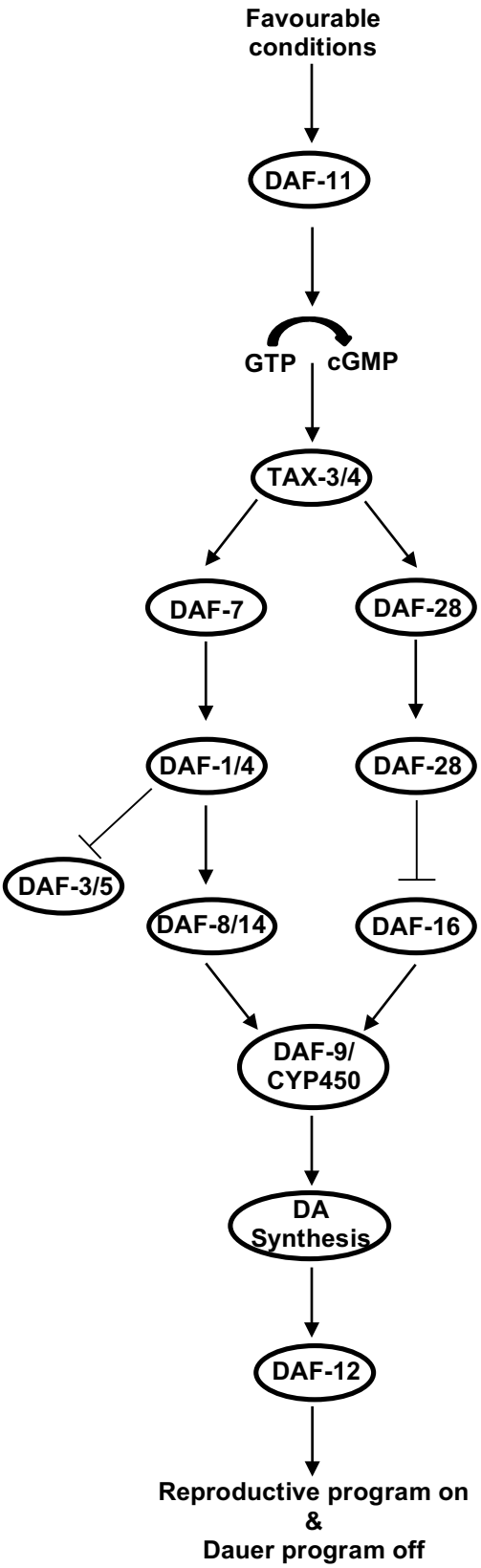
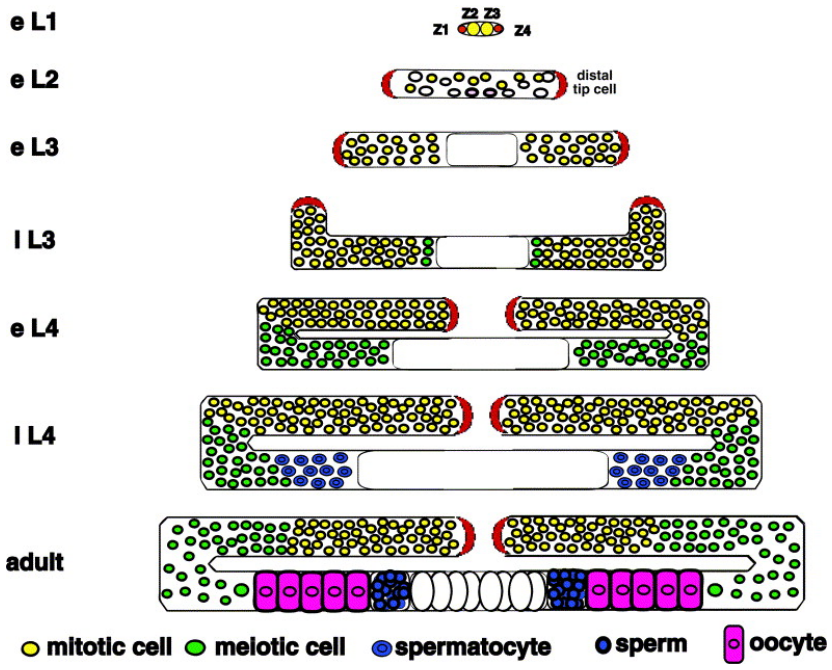

Favourable conditions
DAF-11
GTP
cGMP
TAX-3/4
DAF-7
DAF-28
DAF-1/4
DAF-28
DAF-3/5
DAF-8/14
DAF-16
DAF-9/ CYP450
DA Synthesis
DAF-12
Reproductive program on & Dauer program off

Figure 1.3. Normal *C. elegans* germline development and mechanisms to regulate proliferation vs. differentiation decision in the germ line.

A) *C. elegans* hatch with a gonad primordium consisting of two germline precursor cells (Z2 and Z3) flanked by two somatic precursor cells (Z1 and Z4). Z2 and Z3 proliferate throughout gonadal development to populate the distal end of the gonad. Uninterrupted germ cell divisions are maintained through their interaction with the distal tip cell (DTC) via Notch signalling. As the germ cells are pushed proximally, they fail to receive a proliferative signal from the DTCs and are able to differentiate into sperm during the L4 stage and also into oocytes after reaching adulthood. The figure is adapted from (Pepper, Lo et al. 2003). B) A simplified genetic pathway of the proteins involved in regulating the mitosis vs meiotic entry decisions. LAG-2 is expressed in the DTCs and interacts with the Notch receptor GLP-1, present on the germ cells to activate genes required for germ cell proliferation. Active Notch signalling inhibits the activity of GLD-1 and GLD-2 in FBF-1/2-dependent manner to block meiotic entry and maintain proliferative fate. Arrows and bars represent positive and negative interaction respectively. Based on (Kimble and Crittenden 2007).

A

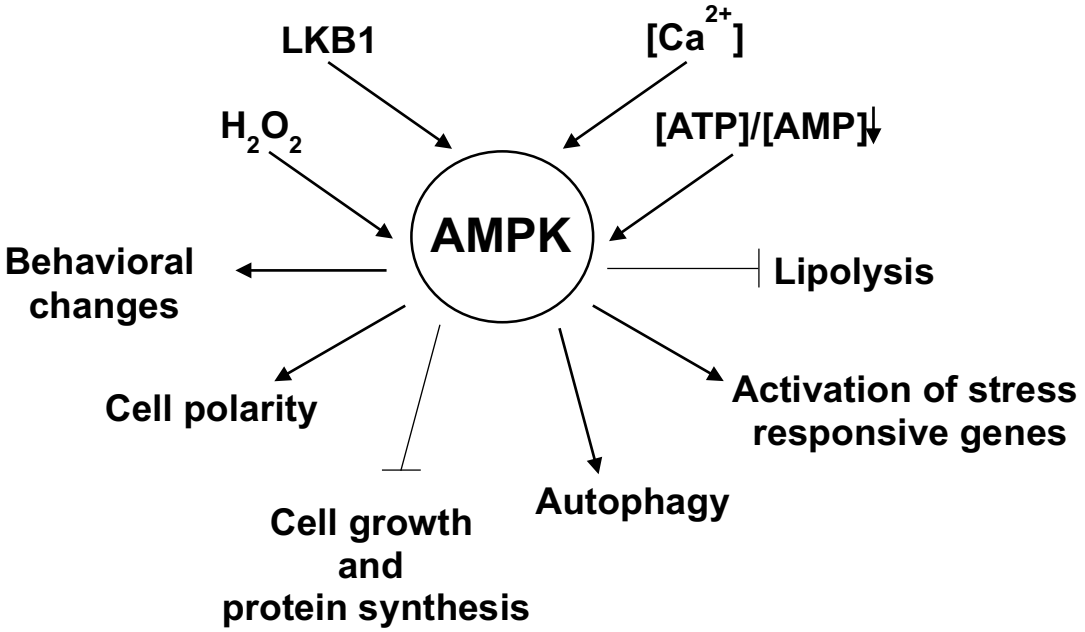

e L1
Z2 Z3
Z1
Z4
e L2
distal
tip cell
e L3
l L3
e L4
l L4
adult
mitotic cell
meiotic cell
spermatocyte
sperm
oocyte

B


LAG-2
GLP-1
LAG-1
FBF-1/2
GLD-1/2
Meiotic
entry
Proliferative
fate

Figure 1.4. Physiological implications of AMPK.

Upon activation by several upstream signals such as low ATP/AMP ratio, intracellular Ca^{2+}, LKB1 kinase activity and H_2O_2 level, AMPK can impinge on several processes to manage energy resources.


LKB1
[Ca2+]
H2O2
[ATP]/[AMP]
AMPK
Behavioral changes
Lipolysis
Cell polarity
Activation of stress responsive genes
Cell growth and protein synthesis
Autophagy

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Chapter 1.6. Research statement

During optimal conditions, most animals will develop without interruption. However, if the conditions become unfavourable, many animals have evolved strategies to endure these periods of stress. The main objective of this research is to comprehend how environmental cues impinge on the various signalling pathways that affect various physiological processes to ensure adaptation and survival, while minimizing potential negative impacts on their reproductive fitness.

In *C. elegans*, if larvae are subjected to nutrient scarcity during early development, they change their regular developmental program to execute a quiescent dauer stage. In dauer larvae that are impaired for LKB1 or AMPK signalling, the germ cells undergo hyperproliferation, giving rise to a pronounced germline hyperplasia. Although it is clear that this signalling cascade is essential to mediate germ cell quiescence during this diapause stage, the downstream mechanisms are still unclear. LKB1 acts upstream of AMPK in most complex organisms, but based on previous studies we know that it may work through additional targets to establish quiescence independently of AMPK (Narbonne and Roy 2006). We reasoned that any one of these targets, or a combination thereof, could confer the tumour suppressor activity of LKB1. To obtain more comprehensive understanding and further characterize the role of LKB1/AMPK signalling in regulating dauer germline quiescence, we first performed a series of non-biased, genome-wide genetic screens aimed to identify potential genes involved in the regulation of dauer germ cell quiescence. Combining the results of these screens will help us to establish a genetic framework and categorize potential genes acting downstream of LKB1/PAR-4 in AMPK-dependent or -interdependent fashion. Further characterization of these genes will help us to understand the mechanisms by which PAR-4 and AMPK regulate germline stem cell quiescence in response to energetic stress. Understanding how LKB1 signalling blocks abnormal cell proliferation is also of particular

biomedical interest, as LKB1 is the causative gene in Peutz Jeghers Syndrome (PJS), an autosomal dominant disease with a surprisingly high predisposition to cancer (Hemminki, Markie et al. 1998). We hope that these findings will eventually also shed light on developing novel therapies to modulate cancer stem cell divisions that often underlie tumour growth.

Chapter 2: Genome-wide surveys reveal polarity and cytoskeletal regulators mediate LKB1-associated germline stem cell quiescence

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Abstract

Caenorhabditis elegans can endure long periods of environmental stress by altering their development to execute a quiescent state called "dauer". Previous work has implicated LKB1 - the causative gene in the autosomal dominant, cancer pre-disposing disease called Peutz-Jeghers Syndrome (PJS), and its downstream target AMPK, in the establishment of germline stem cell (GSC) quiescence during the dauer stage. Loss of function mutations in both LKB1/*par-4* and AMPK/*aak(0)* result in untimely GSC proliferation during the onset of the dauer stage, although the molecular mechanism through which these factors regulate quiescence remains unclear. Curiously, the hyperplasia observed in *par-4* mutants is more severe than AMPK-compromised dauer larvae, suggesting that *par-4* has alternative downstream targets in addition to AMPK to regulate germline quiescence.

We conducted three genome-wide RNAi screens to identify potential downstream targets of the protein kinases PAR-4 and AMPK that mediate dauer-dependent GSC quiescence. First, we screened to identify genes that phenocopy the par-4-dependent hyperplasia when compromised by RNAi. Two additional RNAi screens were performed to identify genes that suppressed the germline hyperplasia in *par-4* and *aak(0)* dauer larvae, respectively. Interestingly, a subset of the candidates we identified are involved in the regulation of cell polarity and cytoskeletal function downstream of *par-4*, in an AMPK-independent manner. Moreover, we show that *par-4* temporally regulates actin cytoskeletal organization within the dauer germ line at the rachis-adjacent membrane, in an AMPK-independent manner. Our data suggest that the regulation of the cytoskeleton and cell polarity may contribute significantly to the tumour suppressor function of LKB1/*par-4*.

Introduction

One of the defining features of any multicellular organism is their capacity to organize essentially identical cellular units into multiple individual cell types with distinct functional properties. These cells will then sort themselves to later give rise to the diverse tissues and organs that will function in a coordinated manner to support growth and reproduction. To achieve this degree of functional complexity, the contributing cell units must respond to regulatory cues that convey information that dictates such functional distinctions. Spatial/positional and temporal cues are critical throughout development as cells must know precisely when they may need to respond to a given signal and also where they are oriented among their cellular neighbours with respect to the overall body plan.

Tissues themselves must also be polarized to provide structural integrity that is unique to their function, but also for the correct partitioning of cellular constituents including intracellular proteins, organelles and cytoskeletal components (Bryant and Mostov 2008). This permits cells to sense and respond to spatiotemporal signals from adjacent cells and/or the surrounding microenvironment. Consistent with this important role, loss of cell polarity often causes cells to become unresponsive to growth inhibitory signals, allowing cells to circumvent differentiation, senescence, and/or apoptosis (St Johnston and Ahringer 2010).

Recently, regulators of cell polarity have also been found to converge on signalling pathways that control cell growth and proliferation and energy metabolism (Banks and Humbert 2008, Martin-Belmonte and Perez-Moreno 2011). It is therefore not surprising that many tumours possess abnormalities in tissue architecture that are often associated with, or caused by, the inappropriate expression of key cell polarity regulators. Consistent with this, mutations in the polarity-regulating protein kinase LKB1/STK11 have been identified in individuals with Peutz-Jeghers Syndrome

(PJS), an autosomal dominant disease that predisposes patients to various types of cancer (Hemminki, Markie et al. 1998, Jenne, Reimann et al. 1998).

LKB1 is a serine/threonine kinase that phosphorylates 13 other protein kinase substrates in the cell, enabling them to subsequently phosphorylate their respective downstream targets more efficiently (Alessi, Sakamoto et al. 2006). The misregulation of these LKB1 targets may explain how loss of this kinase results in aberrant tissue growth and tumour formation, as they are involved in diverse cellular processes ranging from metabolic control to regulation of epithelial and neuronal polarity.

Among the known LKB1 targets, the activation of AMP-activated protein kinase (AMPK) is perhaps the best characterized, where LKB1 activation of AMPK was found to modulate the TOR pathway to coordinate growth with the metabolic status of the cell (Hardie 2005, Alexander and Walker 2011). Curiously, PJS shares many clinical features with other hamartomatous syndromes that are presumed to be associated with abnormally increased TOR signalling. These include Tuberous Sclerosis, Cowden's Disease, and Juvenile Polyposis; all of which share a significantly increased frequency of rare benign hamartomas. AMPK has been shown to phosphorylate Tuberous Sclerosis 2 (TSC2), a GAP protein that blocks TOR activation, and Raptor, an activator of the mTOR pathway that is blocked during periods of starvation (Inoki, Zhu et al. 2003, Gwinn, Shackelford et al. 2008). It is therefore compelling to conclude that mutations in LKB1 disrupt this coordination of nutrient/energy availability and growth by compromising AMPK activation, thus leaving TOR signalling unchecked.

However, PJS is an autosomal dominant disease and many patients with PJS retain one wild type copy of LKB1, which is sufficient to activate AMPK (Sakamoto, McCarthy et al. 2005). Moreover, no mutation has yet been identified in AMPK in any PJS patient to date, suggesting that LKB1

may act independently of AMPK, and potentially TOR signalling, to suppress tumour growth (de Leng, Jansen et al. 2007). Furthermore, data obtained from studies in mice suggest that the tumour suppressor function of LKB1 is not solely TOR-dependent (Wei, Amos et al. 2008). Moreover, although hyperactivation of TOR signalling has been associated with PJS, inhibition of TOR using rapamycin in LKB1 heterozygous mice indeed reduces polyp numbers in the gut, but does not prevent polyp formation altogether (Wei, Amos et al. 2008). Therefore, the hyperactivation of TOR that occurs in LKB1+/- individuals may exacerbate the defects in PJS, but it is unlikely to be the unique underlying cause of the disease. This suggests that LKB1 must work through additional TOR-independent targets that contribute to its tumour suppressor function.

We previously showed that both LKB1 and AMPK regulate germline stem cell quiescence during periods of energy stress. When either of these genes, or the tumour suppressor PTEN, is impaired the germline stem cells (GSCs) proliferate when they should normally arrest (Narbonne and Roy 2006). Although mutations in either LKB1 or AMPK cause hyperplasia, LKB1 mutations always result in a greater degree of hyperplasia than null mutations that disrupt all AMPK signalling, suggesting that other genes that act downstream of LKB1, and independent of AMPK, must be phosphorylated in order to elicit both cell cycle and developmental quiescence in the GSCs during the dauer stage (Narbonne and Roy 2006).

In *C. elegans,* the defects associated with LKB1 or AMPK disruption are most obvious in the gonads of animals subjected to energy stress. The *C. elegans* germ line develops from two cells that are born during embryogenesis and remain quiescent until the L1 stage. The two cells are referred to as the primordial germ cells Z2 and Z3, which will divide continuously during development in replete conditions to generate all the germ cells that will constitute the adult germ line. The continuous division of these cells is dependent on signalling between the distal-most

germ cells and two somatic gonadal cells called distal tip cells (DTCs) that are located at the distal end of each identical growing gonad arm (Hubbard and Greenstein 2005).

The DTCs form a niche for the GSCs and their mitotic divisions are maintained through Notch signalling. The Delta-like ligand LAG-2 is expressed in the DTCs, while the GSCs express the Notch-like receptor, GLP-1 (Henderson, Gao et al. 1994). Active Notch signalling instructs these GSCs to proliferate, while blocking them from executing their alternative meiotic pathway. The ongoing divisions driven by Notch signalling physically displace these dividing cells proximally until they no longer receive the Notch signal from the DTCs, allowing them to execute their alternate meiotic pathway (Crittenden, Leonhard et al. 2006).

Under optimal environmental conditions germline proliferation continues uninterrupted. However, if environmental conditions deteriorate, three independent signalling pathways: insulin-like signalling, TGF- β or cGMP signalling can regulate the decision to execute the alternative development pathway and enter dauer state (Riddle and Albert 1997, Patterson and Padgett 2000). All three signals are required to block a nuclear hormone receptor from activating the dauer gene expression program. However, loss of any one of the three signals is sufficient to induce *C. elegans* larvae to execute dauer development (Antebi 2006). These pathways can be manipulated at the molecular or genetic level to specifically induce or suppress dauer formation.

Upon executing dauer development, GSCs undergo a G2/M cell cycle arrest despite the presence of active Notch ligand in the DTCs and GLP-1 expression in the GSCs (Narbonne and Roy 2006), suggesting that the germ cell proliferation is blocked either downstream of, or in parallel to, Notch signalling. The orthologues of LKB1 (*par-4)* and AMPK (the two catalytic subunits *aak-1* and *aak-2*) and PTEN (*daf-18*) co-operate during this stage to trigger the cell cycle quiescence typical

of the GSCs during the dauer stage (Narbonne and Roy 2006). However, the underlying mechanisms involved in establishing this quiescence are unknown.

To determine what pathways might act downstream of LKB1/AMPK in the establishment and/or maintenance of GSC quiescence, we used dauer-induced germline quiescence as a read out to find genes that modulate these germ cell divisions. Using an unbiased reverse genetic approach, we identified genes that could act as potential targets of LKB1/AMPK signalling that are critical for its role in dauer-associated GSC quiescence and potentially for the tumour suppressor function of LKB1. Since genetic analysis suggests that LKB1/*par-4* must impinge on targets other than AMPK to induce cell cycle arrest, we designed unbiased RNAi-based screens that would favour the identification of genes that act downstream of LKB1/*par-4*, but in a manner that is independent of AMPK. Our data indicate that *par-4* can indeed act independently of AMPK to regulate germline quiescence in the dauer stage. Moreover, most of the genes that act with *par-4,* but do not rely on AMPK to regulate germline quiescence, have documented roles in cell polarity and cytoskeletal regulation. These genes may therefore act downstream of LKB1 such that when LKB1 function is compromised in PJS patients, their misregulation may contribute to the aetiology of the disease.

Results

To better understand how germline stem cell cycle quiescence is regulated during periods of reduced insulin-like signalling, we performed three independent genome-wide RNAi screens based on feeding dsRNA corresponding to every predicted gene in *C. elegans* (Kamath and Ahringer 2003). One analysis was designed to isolate genes that result in germline hyperplasia typical of LKB1(*daf-2; par-4)* or AMPK compromise (*daf-2; aak(0))* during the dauer stage. Two additional genome-wide RNAi surveys were performed to identify genes that, when blocked, would suppress the germline hyperplasia phenotype typical of both LKB1*/par-4* or AMPK mutant dauer larvae. The activity of the identified genes would presumably be under LKB1/PAR-4 and/or AMPK-mediated regulation, and in the absence of either of these genes the activity of these targets would go unchecked during the dauer stage. The results from these two screens could contribute to the identification of novel genes that suppress hyperplasia exclusively in (i) the LKB1/*par-4* mutant background, (ii) the AMPK mutant background, and (iii) in both the *par-4* and AMPK mutant backgrounds. All the strains used for the above mentioned screens express a LAG-2::GFP transgene allowing us to visually assess the distance between the DTCs; a proxy for the degree of germline hyperplasia (Narbonne and Roy 2006) (Fig. 2.1).

To eliminate as many false positives as possible from the primary screens, secondary and tertiary screens were performed. Thus, only candidates that demonstrated a reproducible response to the RNAi treatment in each round were considered as the potential candidates. For each RNAi treatment, at least 100 dauer larvae were screened to analyze the distance between the DTCs. As RNAi can exhibit differential phenotypic penetrance, expressivity, and variance, we applied a threshold such that a gene candidate would only qualify for the next round of analysis if greater than 5% of the larvae (as 1-5% animals treated with an empty RNAi vector displayed DTC displacement) showed detectable DTC displacement. In the event that an RNAi only partially

affects the DTC displacement (expressivity), but in more than 5% dauer larvae (penetrance), then it would be retained, as this would still indicate that this gene must somehow affect dauer germline quiescence.

From our genome-wide analysis, we identified a total of 39 genes that resulted in dauer germline hyperplasia when they were subjected to feeding RNAi (Table 2.1). The suppressor screens that were carried out for *par-4* and *aak(0)* allowed us to identify 49 and 55 candidate genes, respectively; all of which resulted in a pronounced and reproducible reduction in dauer germline hyperplasia when mutant animals were fed the corresponding dsRNA (Table 2.2 and Table 2.3). The candidate genes were classified according to their predicted roles in diverse biological processes using Gene Ontology (GO) annotation (Huang da, Sherman et al. 2009, Huang da, Sherman et al. 2009). The most prevalent of these-included regulation of cell polarity and/or the cytoskeleton, nutrient signalling and metabolism, tumour suppressor genes, intracellular trafficking regulation, the extracellular matrix (ECM) and cell adhesion, protein processing, gene expression regulation and lastly, a significant number of genes with unknown function (Fig. 2.2A-C).

To further validate our candidates, dauer germ cell quantification was performed upon RNAi of each individual candidate gene identified in all the screens to confirm their role in maintaining dauer germline quiescence (Fig 2.2E-G, subset of genes shown). To verify that the identified candidate genes from the screens were not specific to the disruption of insulin-like signalling, but rather, their activity is required in other signalling pathways that control dauer formation, we quantified the number of germ cell nuclei and validated the function of each candidate gene identified in mutants with compromised TGF-β signalling (*daf-7)* (Tables 2.1-3). We observed no differences in RNAi-mediated phenotypes suggesting that each of the identified genes was

required for germline quiescence downstream of at least two different triggers for dauer formation. Genetic evidence suggests that *par-4* acts to suppress the germline proliferation in a manner that is, at least in part, independent of AMPK (Narbonne and Roy 2006). We therefore compared the candidate genes that suppressed *par-4*-dependent dauer germline hyperplasia and those that suppressed the hyperplasia in the *aak(0)* background. If the two genes function in a linear pathway where PAR-4 activates AMPK to phosphorylate critical targets involved in cell cycle quiescence, the genes that were identified in the two independent suppressor screens should be identical. This was however not what we observed. Our analysis revealed that only 17 candidates were common to both data sets, implying that PAR-4 mediates its control over cell proliferation via AMPK through these targets (Fig. 2.2D). More importantly, the remaining candidates must act downstream of, or in parallel to, PAR-4. These genes may also act independently of AMPK, since they do not affect the hyperplasia caused by the loss of AMPK signalling.

The GO term analysis suggests that the majority of the genes that act exclusively downstream of LKB1/*par-4* have documented roles in cytoskeletal regulation and cell polarity (Table 2.4). This is somewhat counterintuitive given that the cytoskeletal profile of mitotic germ cells in *C. elegans* remains elusive, while germ cell polarity, particularly with respect to the apical/basal axis, must be quite unique given the syncytial nature of germ cells within the gonad.

Genes involved in cytoskeleton and polarity

The tumour-like effects of disrupting apico-basal cell polarity has been well documented in *Drosophila melanogaster* where mutations that affect cell polarity result in severe hyperplasia of the affected somatic tissues (Martin and St Johnston 2003). 18% of the genes that caused the germline hyperplasia and 14% of the candidates from our *par-4* suppressor screen belonged to the

subset of genes that affect these processes, respectively. This number is surprisingly high considering that no apical/basal polarity has been previously ascribed to the *C. elegans* germ cells. Interestingly, compromising the function of the Partitioning defective gene *par-3* resulted in the germline hyperplasia typical of *par-4* and *aak(0)* mutants. *par-3* has a well characterized role in the polarization of the early *C. elegans* embryo where it is required for the initial establishment of anterior-posterior polarity and the segregation of the germline determinants in the one cell zygote (Etemad-Moghadam, Guo et al. 1995, Bowerman, Ingram et al. 1997, Nance and Priess 2002). In mammalian cells PAR-3 acts at the apical region of epithelial cells to distinguish this region from the basolateral cortex (Afonso and Henrique 2006).

In *C. elegans* PAR-3 forms a complex at the anterior cortex of the zygote with PAR-6 and PKC-3, which excludes PAR-1 and PAR-2 to the posterior cortex (Nance, Munro et al. 2003). Formation of the anterior and posterior PAR complexes is essential to specify the germline blastomere (Mello, Schubert et al. 1996, Schubert, Lin et al. 2000, Cuenca, Schetter et al. 2003). Later in development, the PAR-3/PAR-6/aPKC-3 complex localizes to the apical cortex of epithelial cells and is required for the maintenance of adherens junctions (known as apical junctions in *C. elegans*) (Bossinger, Klebes et al. 2001, McMahon, Legouis et al. 2001).

To determine if *par-3* acts with *par-4* in an AMPK-dependent or -independent manner, we compromised *par-3* in both *par-4* and the *aak(0)* mutant animals. Loss of *par-3* was not additive to the hyperplasia caused by loss of *par-4* based on the germ cell numbers that were present in the compound mutants. On the other hand, loss of *par-3* was indeed additive in the *aak(0)* background (Fig. 2.3B, C). This suggests that *par-3* and *par-4* function together in a linear genetic pathway, yet it acts independently of *aak(0)*/AMPK to affect germ cell quiescence in the *C. elegans* dauer larva.

Another set of genes required for GSC quiescence include *mig-5* and *let-413*, which are homologues of the *Drosophila* tumour suppressor genes Dishevelled and Scribble, respectively. Dishevelled is a key regulator of planar cell polarity (PCP) in imaginal disc epithelia (Adler 1992, Gubb 1993, Eaton 1997), while Scribble is part of a protein complex, along with Lethal giant larvae (Lgl) and Discs-large (Dlg), that defines the basolateral domain of epithelial cells (Bilder and Perrimon 2000, Peng, Manning et al. 2000). Our genetic analyses suggest that these genes, which we identified in our genetic screen, act independently of *par-4* or *aak(0),* since they both enhance germline hyperplasia when compromised in either the *par-4* or the *aak(0)* background (Fig. 2.3B, C).

Two other candidates in this category include *cdc-42* and *rho-1*, small Rho-like GTPases with many well characterized functions, including polarization of the early embryo, tight junction assembly, acto-myosin contractility, and endocytosis (Gotta, Abraham et al. 2001, Hurd, Gao et al. 2003, Schonegg and Hyman 2006, Balklava, Pant et al. 2007). Specifically, together with *rho-1*, *cdc-42* is required for the initial actinomyosin contractions of the *C. elegans* one-cell embryo to generate a cortical flow of proteins, which is the first asymmetry-generating event in development (Schonegg and Hyman 2006). CDC-42-dependent activation of WASP (Wiskott-Aldrich Syndrome Protein) is essential to activate the Arp2/3 complex and consequently stimulate actin assembly (Millard, Sharp et al. 2004).

To determine if *cdc-42* impinges on this pathway and whether actin cytoskeletal assembly is vital to establish GSC quiescence in dauer larvae, we performed RNAi experiments against *wsp-1* (*C. elegans* WASP orthologue) and *arx-2* and *arx-3;* the *C. elegans* orthologues of the Arp2/3 complex subunits followed by quantification of the resulting germ cell numbers. Disruption of either the WASP or the Arp2/3 complex resulted in significant increase in the germ cell counts (Fig. 2.3A),

suggesting that *cdc-42* activity is essential for the maintenance of germline stem cell quiescence during the dauer stage, presumably through its role in regulating actin cytoskeletal organization. Furthermore, when *cdc-42* or *rho-1* are compromised in either a *par-4* or an *aak(0)* mutant, we observed an additive increase in germ cell counts (Fig. 2.3B, C), typical of these two gene products acting, most likely together, in an independent pathway that regulates germline quiescence in concert with AMPK and LKB1 during the dauer stage.

Among the collection of genes that suppress the *par-4*-dependent hyperplasia in dauer larvae, 7 identified genes are involved in regulating the cytoskeleton and cell polarity. Of particular interest are *cyk-4* and *pac-1*; two Rho-GAPs that regulate cytoskeletal dynamics by deactivating Rho-GTPases (Lundquist 2006). Since at least two small GTPases were identified in our genetic screen for genes that resulted in dauer germline hyperplasia, the discovery that their inactivation rescues hyperplasia in a *par-4* background strongly suggests that a mechanism involving the Rho-family GTPases is required to establish or maintain quiescence in the germ line during the dauer stage.

The RhoGAP-encoding *cyk-4* is a conserved component of Centralspindlin, a complex that lies at the heart of central spindle assembly and cytokinesis in metazoans (Mishima, Kaitna et al. 2002, Glotzer 2009, White and Glotzer 2012). Recently, a novel role of *cyk-4* that is independent of its function in cytokinesis was demonstrated through its ability to regulate germline architecture by maintaining intracellular bridges between the germ cells in *C. elegans* (Zhou, Rolls et al. 2013). Intercellular bridges (or rachis bridges) are formed and stabilized along the distal gonad arm between the individual germ cells and the rachis. Rachis bridges therefore resemble the mitotic cleavage furrow and actinomyosin ring, retaining many of the factors found in these structures (Hime, Brill et al. 1996, Zhou, Rolls et al. 2013).

To determine if other components of the Centralspindlin complex cooperate with CYK-4 to regulate germline quiescence during the dauer stage we used RNAi to disable a second Centralspindlin component, *zen-4,* which belongs to the kinesin-6 subfamily of plus end directed microtubule motor proteins (Siddiqui 2002). *zen-4*(RNAi) significantly suppressed *par-4*-dependent hyperplasia (Fig. 2.2F), suggesting that *par-4* may impinge upon the Centralspindlin complex to either affect its assembly, stability, and/or function, either directly or indirectly, to mediate dauer GSC quiescence. The pseudokinase adaptor protein STRAD is differentially required to enhance the activity of LKB1/PAR-4 and is essential to establish dauer germline quiescence (Narbonne, Hyenne et al. 2010). To test if *strd-1* is required for the AMPK-independent function of *par-4* to establish dauer germline quiescence, we compromised its function in both *par-4* and *aak(0)* mutants and quantified the number of germ cells in the resulting dauer gonads. Based on our results, *strd-1* acts in a *par-4*-dependent pathway that functions in parallel to AMPK (Fig 2.3B, C).

Interestingly, the candidate genes that we isolated through the *par-4* suppressor screen that are involved in cell polarity and cytoskeletal regulation did not suppress the germline hyperplasia typical of *aak(0)* mutant dauer larvae (Table 2.4). This is consistent with our genetic analysis that indicated that the germ cell numbers observed in *par-4; aak(0)* mutant dauer are significantly greater than in *aak(0)* mutant animals alone (Narbonne and Roy 2006). Therefore, PAR-4 likely regulates germline quiescence through its ability to control cellular mechanisms that are both dependent and independent of AMPK kinase targets during the dauer stage in *C. elegans*. These AMPK-independent targets suggest that PAR-4 must interact with key regulators of cell polarity and cytoskeletal dynamics.

***par-4* mutants show temporal defects in actin organization at the rachis-adjacent membrane independently of AMPK**

Because we identified a surprisingly high number of genes that act in cytoskeletal and cell polarity organization, we wondered whether the loss of *par-4* function might affect the actin cytoskeletal network and consequently permit the germ cells to undergo supernumerary cell divisions during the dauer stage. To examine the changes in cytoskeletal profiles, we monitored actin cytoskeletal organization in the dauer quiescent germ line and how it is impacted by the absence of PAR-4 and/or AMPK.

Using a *daf-2(e1370)* strain that harbours a transgenic reporter *Ppie-1::*GFP::MOE; a fusion protein that decorates actin filaments with GFP and recapitulates the distribution of F-actin *in vivo* specifically in the germ line (Velarde, Gunsalus et al. 2007), we studied the changes in the actin cytoskeletal re-arrangements within the larval germ line at varying time intervals following the initiation of dauer development. In the quiescent germ line of most dauer larvae the actin filaments are localized at the membrane adjacent to the rachis, but in the *par-4* mutant dauer germline actin organization at the membrane adjacent to the rachis is temporally perturbed. At 48h after switching to their restrictive temperature of 25^0C, actin filaments are completely disorganized, but at 72h the disorganization of the network is resolved and appears organized, similar to *daf-2* dauer larvae (Fig. 2.4). These observations suggest that the early re-organization of the actin cytoskeleton that occurs in the dauer germ line is controlled by *par-4*, but later during the dauer stage other regulators that control the cytoskeletal arrangement become active and can compensate in its absence to re-organize the actin network.

No obvious temporal or spatial defects in cytoskeletal arrangement were observed in *daf-2; aak-1* dauer larvae treated with *aak-2*(RNAi). Actin filaments localized at the membrane adjacent to the

rachis in a configuration very similar to *daf-2* mutants (Fig. 2.4). Therefore, *par-4* regulates actin organization at the rachis bridge during the early phase of the dauer diapause, which may contribute to the establishment of quiescence in the dauer germ line. Most importantly however, it does this independently of AMPK.

Based on our genetic analysis, we propose that *par-4* may require *par-3* in an AMPK-independent manner to establish quiescence in the dauer germ line. To confirm if PAR-3 is essential to regulate temporal actin cytoskeletal organization, we treated *daf-2* animals with *par-3*(RNAi) and monitored actin cytoskeletal organization in the dauer germ line. Similar to *par-4* mutants, compromise of *par-3* results in temporal defects in the arrangement of actin at the rachis-adjacent membrane within the dauer germ line (Fig. 2.4). When examined in light of previous data obtained in the one cell zygote, these data suggest that PAR-3 may be one of the downstream targets of PAR-4 required to mediate temporal cytoskeletal changes to ensure that germ cells undergo quiescence in response to dauer cues. However, our data cannot rule out that PAR-3 could formally function upstream of PAR-4.

Compromise of *cdc-42* also resulted in perturbed actin cytoskeletal arrangement within the dauer germ line (Supplemental fig. 2.1) thus confirming that appropriate cytoskeletal re-organization is crucial to maintain the quiescent state of the germ line typical of the dauer larva and mis-regulation of such cytoskeletal rearrangements at the onset of dauer formation results in aberrant germ cell proliferation.

Germline autonomous vs non-autonomous functions

The germ line is highly responsive to environmental conditions and the germ cells will alter their cell divisions to meet contingencies that reflect the growth status of the organism (Baugh 2013).

The signals that impinge on the germ cells to regulate these decisions can be transduced either autonomously by sensing energy restriction, or alternatively, signals from the soma could be eventually transmitted to the germ line in a non-autonomous manner to control processes involved in their proliferation or apoptosis (Ito, Greiss et al. 2010, Korta and Hubbard 2010).

To determine whether the candidate genes we identified in our screens exert their function in a germline autonomous fashion, we tested them in an *rrf-1 (pk1417)* background. *rrf-1* encodes one of the RNA-dependent RNA polymerases in *C. elegans* that acts in a tissue-specific manner where it is required for the amplification of dsRNA in somatic tissues. Mutations in *rrf-1* result in a significant reduction in somatic RNAi, leaving germline-specific RNAi intact (Sijen, Fleenor et al. 2001, Grishok 2005). Although some somatic tissues do remain somewhat RNAi proficient, despite these limitations, this method provides a reasonable approach to test large gene sets to understand whether gene function is required in the soma or the germ line.

By performing feeding RNAi directed to all our candidate genes that resulted in dauer germline hyperplasia, we found that 20 of the 39 candidates resulted in germline hyperplasia in *daf-2; rrf-1* dauer larvae (Table 2.1); 31 out of 49 candidates identified in *par-4* suppressor screen exhibited reduced germline hyperplasia in *rrf-1* compromised *par-4* mutant dauer larvae (Table 2.2); and 35 out of 55 candidates isolated in *aak(0)* suppressor screen had a germline non-autonomous role to regulate quiescence (Table 2.3). Cytoskeletal and polarity regulators identified in the genomic screens acted predominantly in a germline autonomous fashion. This suggests that the activity of these genes is important within the germ line to regulate their polarity; a feature that is critical for the establishment or maintenance of quiescence. Other gene categories identified in all the 3 genomic screens which acted in a germline autonomous manner include genes that belong to the categories of intracellular trafficking, cell cycle regulation, ECM regulation, intracellular

signalling and gene expression (Table 2.1-3). Therefore, a considerable number of the candidates we identified in our screens act in a germline non-autonomous manner, suggesting that PAR-4 and AMPK activity might be sufficient in the soma to instruct the germ cells to arrest proliferation and enter a quiescent state in response to energy stress.

Discussion

In order to withstand long periods of environmental stress, *C. elegans* can execute an alternative developmental stage called "dauer" (Golden and Riddle 1982). The dauer stage is associated with a global cell cycle arrest presumably as a consequence of diverting the available energy resources that might normally fuel the energetically taxing process of cell proliferation to more essential processes required for long term survival (Kipreos 2005, Tobin and Saito 2012). Previous work revealed that AMPK and LKB1/*par-4* mediate this cycle arrest in the dauer germ line (Narbonne and Roy 2006). The disruption of either of these genes causes dauer-specific germline hyperplasia, while depletion of both enhances the hyperplasia significantly, suggesting that the genes do not work in a simple linear pathway to establish or maintain germline quiescence (Narbonne and Roy 2006).

Because of this inconsistency we carried out a series of genome-wide RNAi surveys to obtain a more comprehensive view of the various genes involved in the regulation of germline quiescence during the dauer stage. The analysis would provide a more detailed understanding of how gene products work downstream or in parallel with these two protein kinases to ensure germline cell cycle arrest during this environmentally challenging period. Furthermore, because of the role of these protein kinases in Peutz-Jeghers Syndrome these genes could correspond to potential kinase targets of these enzymes that become misregulated in this disorder.

From our RNAi analysis, we identified a total of 39 genes that resulted in dauer germline hyperplasia and identified 49 and 55 candidate genes that suppressed *par-4* and *aak(0)* dependent germline hyperplasia, respectively. Further analysis of the suppressor screens revealed the subsets of genes that exclusively suppressed the germline hyperplasia in (i) the LKB1/*par-4* mutant background and (ii) the AMPK mutant background. LKB1 dependent activation of AMPK has

been confirmed in several different models, but it appears that genes that suppress *aak(0)* dependent hyperplasia fail to suppress the germline hyperplasia in *par-4* mutants. These data are consistent with previous findings indicating that AMPK could be activated by alternative LKB1/PAR-4-independent pathways. Indeed, CaMKK2 is a significant contributor to AMPK activation in specific contexts, particularly in neurons, but also in LKB1-deficient tumour cells (Mairet-Coello, Courchet et al. 2013, Fogarty, Ross et al. 2016).

Though RNAi is a powerful genetic tool, it is important to accept the caveats and limitations associated with the method, namely the associated variation in penetrance and expressivity, in addition to the refractory nature of stable protein to the RNAi procedure. Based on this caveat the number of genes we identified is almost certainly an underestimate. Many essential genes that may be involved in maintaining germline quiescence could not be identified due to their essential role during early stages of embryogenesis (Anderson and Kimble 1997). RNAi at the L1 stage may result in the depletion of the newly transcribed mRNA, but it will not affect proteins that have been translated earlier. This may explain why we detected so few genes involved in general cell cycle progression, as many cell cycle regulators are maternally contributed (Bao, Zhao et al. 2008). Based on the systematic RNAi analysis in wild-type animals, 1170 genes demonstrate lethal and sterile RNAi phenotypes (Kamath, Fraser et al. 2003). It is likely that many of these could potentially play a role in the maintenance of dauer germline quiescence, but it would require a more laborious screen design to identify them.

While the germ cells of *C. elegans* show no apparent apical/basal polarity, the screen performed to identify genes that could phenocopy that loss of LKB1/AMPK signalling in the germ line, uncovered numerous genes that have well-defined roles in the regulation of apicobasal polarity and/or in regulating the cytoskeleton. This includes the Par gene, *par-3*, which has roles in both

embryonic and epithelial polarity (Etemad-Moghadam, Guo et al. 1995, Nance, Munro et al. 2003); *cdc-42*, which is a master regulator of cell division and polarity, along with regulating diverse cytoskeletal changes (Etienne-Manneville 2004), as well as the *C. elegans* orthologues of the Scribble and Dishevelled tumour suppressor genes in *Drosophila*, known as *let-413* and *mig-5* in *C. elegans*, respectively (Legouis, Gansmuller et al. 2000, Walston, Guo et al. 2006). Moreover, combining the results of the *par-4* and AMPK suppressor screens, we found that candidate genes encoding cytoskeletal and polarity regulators exclusively suppressed the *par-4*-dependent proliferation, with no effect on the hyperplasia observed in the AMPK mutant animals. Given the role of PAR-4 in establishing early embryonic asymmetry and the identification of polarity regulators from the screen for factors required to maintain the germline cell cycle arrest in dauer, we propose a model where PAR-4 establishes or maintains germline quiescence by at least two mechanisms: one that includes its canonical activation/regulation of AMPK, while another that involves the regulation of some aspect of germline cell polarity and cytoskeleton that is independent of AMPK.

It is puzzling that genes with such well-characterized roles in the establishment and maintenance of apical/basal cell or anterior/posterior polarity impinge on *C. elegans* germ cells, which are essentially symmetrical, and show no clear polarization, while also developing in the shared cytoplasm typical of the gonadal syncytium. Interestingly, we show that actin organization at the rachis-adjacent membrane might be the first indication of germ cell-associated polarization. Furthermore, we show that this regulation of the actin cytoskeleton is downstream of PAR-4 but is independent of AMPK.

How might the actin cytoskeletal regulation at the rachis bridge inform germ cell function and affect proliferation? In general, the physical properties of the cytoskeleton can have a profound

influence on cell function and therefore contribute to proliferative behaviour (Guilak, Cohen et al. 2009). One possibility is that localized actinomyosin contraction near the rachis-adjacent membrane could set up a polarized state, perhaps by altering the cellular milieu by modifying actin-dependent cytoplasmic movements of key determinants that are necessary for the proper establishment of cell quiescence.

Other interesting genes required for the dauer germline quiescence include those that are involved with various aspects of the extracellular matrix (ECM)—either encoding factors that constitute the ECM, or factors that couple the cytoskeletal network to the overlying matrix. Genes identified in this subset were identified as suppressors of both *par-4*- and AMPK-dependent dauer germline hyperplasia. The germ line is enclosed within a basement membrane, with which both the GSCs and DTCs are in direct contact (Kramer 2005). Dauer-dependent remodeling of the extracellular matrix may convey important cues necessary for stem cell cycle arrest. The ECM may signal to the germline stem cells directly, where the EGF-like repeats on the extracellular domain of GLP-1 could interact with components of the basement membrane (Maine and Kimble 1989) affecting its activity. Alternatively, changes in ECM composition may affect the migration of DTCs, which could in turn affect germline stem cell proliferation. In *C. elegans* the hemidesmosomes anchor cells to the matrix and have recently been shown to be involved in mechanotransduction (Borradori and Sonnenberg 1999, Labouesse 2012). The identification as *vab-10* as a germline autonomous gene may suggest that a process involving mechanosensing between the DTCs and the germ cells is important for germ cell cycle regulation; germline stem cell proliferation may be coordinated with DTC migration, or lack thereof.

A number of genes found to be required for germline stem cell quiescence during dauer have demonstrated roles in nutrient signalling and metabolism. These genes were of interest because of

the implications of the TOR-mediated growth signalling pathway in the maintenance of germline quiescence during dauer. The genes which resulted in germline hyperplasia upon their RNAi-mediated knockdown are *aap-1* (AGE-1 adaptor protein), *gpi-1* (glucose phosphate isomerase), *sptl-1*(serine palmitoyltransferase) and *sams-3* (S-adenosylmethionine synthetase). Mutations in either of *aap-1* or *gpi-1* result in extended lifespan (Wolkow, Munoz et al. 2002, Schulz, Zarse et al. 2007). GPI-1 acts during an early event in glycolysis, upstream of GPD-2 and GPD-3, which are both specifically required for a response to anoxic conditions (Mendenhall, LaRue et al. 2006). While it is intuitive that *gpi-1* would be required for the normal dauer response to anoxia, it is puzzling as to how *gpi-1* may have an effect on the germ line. There were no other members of the glycolytic pathway identified in the RNAi screen, so it seems unlikely that defects in glycolysis are directly responsible for the germline hyperplasia phenotype or that the RNAi effects of these genes are highly penetrant embryonic lethal.

Few genes identified in the *par-4* and *aak(0)* suppressor screen encode proteins that are involved in regulating growth and metabolism. 3 out of 4 genes identified in our *par-4* suppressor screen also suppressed AMPK-dependent germline hyperplasia. This suggests that *par-4*/LKB1 regulates growth and metabolism to establish dauer germline quiescence predominantly in an AMPK-dependent manner. This subset includes *cyc-1* (Cytochrome C), *cco-1* (Cytochrome C Oxidase) and *vha-2* (Vacuolar H ATPase). Both *cyc-1* and *cco-1* regulate lifespan and *vha-2(RNAi)* results in sterile animals with polyploidy and premature oocytes (Marcotte, Xenarios et al. 2000, Oka and Futai 2000, Dillin, Hsu et al. 2002, Durieux, Wolff et al. 2011). All these genes act in a germline non-autonomous fashion making it even more intriguing to characterize how these genes communicate with the germ line during energy stress.

Although one might intuitively assume that the genes identified in our screens function in a germline autonomous fashion, our analysis with *rrf-1* suggested that several genes (Table 2.1-3) regulate dauer germline quiescence in a germline non-autonomous manner. This represents a novel implication of the somatic tissues in directing mitotic proliferation of GSCs. Further investigation of the subcellular and intercellular structure within the distal germ line or between the germ cells and gonadal sheath cells will enhance our understanding of how these genes influence the regulation of the germ cell population. Though this is a useful approach there are indeed some caveats to consider. The subset of genes that we identified as germline autonomous should not be over-interpreted, as it has been demonstrated that *rrf-1(pk1417)* mutants are still capable of executing RNAi at least in some somatic cells, although this may be at a reduced level (Kumsta and Hansen 2012). This suggests that some of the genes that are documented as acting in a germline autonomous manner may have also been reduced in somatic tissue, where their function might also contribute to the regulation of germline quiescence.

Conclusion

Although our analyses are revealing, they are not comprehensive, mainly due to limitations in our RNAi strategy and the essential nature of the process at hand; namely cell cycle dynamics. Nevertheless, our work has revealed the role of several gene families that could act downstream of LKB1/PAR-4 in an AMPK-dependent or -independent fashion to promote germline stem cell cycle arrest under reduced insulin signalling. The data presented here suggest that the tumour suppressor function of LKB1 that is disrupted in Peutz-Jeghers Syndrome patients may be related to its roles in the regulation of cell polarity, and not uniquely due to its ability to activate AMPK. While AMPK has been shown to be required for the dauer germline quiescence, a direct role for AMPK in PJS has not been established. This suggests that the maintenance of cell polarity by LKB1 that may be disrupted in PJS patients might not involve AMPK, but rather, it may be more due to its disruption in an entirely independent pathway. Alternatively, it could be the combined effect of disrupting both pathways that contribute to the aetiology of the disease. This would be consistent with the poor results obtained using inhibitors of the TOR pathway in the clinic (Korsse, Peppelenbosch et al. 2013). In this scenario only the downstream effectors of TOR are attenuated, while the polarity of the cells remains compromised, potentially sensitizing PJS cells to continue proliferating through TOR-independent cues.

To our knowledge LKB1 is the sole causative gene in PJS, although others may contribute epistatically. The genes we identified in our unbiased screening approach indicate that the tumour suppressor function of LKB1 may lie in its ability to control cell polarity, and not exclusively in its ability to modulate TOR signalling. This is largely because our *C. elegans*-based strategy allows us to identify genes that affect this LKB1-mediated process that would otherwise be difficult or impossible to characterize in other models, mainly due to their essential nature. It is

possible that any functional compromise of these genes in humans would result in lethality, confounding any such functional characterization and thus the absence of additional genes that contribute to the disease.

Further study of the genes we have identified in these screens will provide additional insight as to how PAR-4/LKB1 signalling blocks tumour growth by regulating cell cycle arrest under energetic stress, while providing additional LKB1 or AMPK downstream kinase substrates that could be useful for the development of new therapies to benefit PJS patients or other cancers that arise due to the loss of LKB1 function.

Materials and Methods

C. elegans genetics

All *C. elegans* strains were maintained at 15°C and according to standard protocols (Brenner 1974). The strains used for the screen include MR155 [*daf-2 (e1370) III; qIs56(lag-2::GFP) V*], MR0671 [*rrf-1(pk1417) I; daf-2(e1370) III*], CB1372 [*daf-7(e1372)III*], MR0674 [*daf-2 (e1370) III; par-4(it57) qIs56(lag-2::GFP) V*], MR0672 [*daf-7(e1372) III; par-4(it57) V*], MR0863 [*rrf-1(pk1417) I; daf-2(e1370) III; par-4(it57) V*], MR0998 [*daf-2(e1370) aak-1(tm1944) III; aak-2(ok523) X; qIs56(lag-2::GFP) V*], MR0480[*daf-7(e1372) III; aak-2(ok523) X*], MR0868 [*rrf-1(pk1417) I; daf-2(e1370) III; aak-2(ok523 X)*], MR1842 [*daf-2(e1370) III; orIs20[unc-119(+)Ppie-1::gfp::moesin*], MR2036 [*daf-2(e1370) III; par-4(it57) V; orIs20[unc-119(+)Ppie1::gfp::moesin*], MR2037 [*daf-2 (e1370) aak-1(tm1944) III; orIs20[unc-119(+)Ppie1::gfp::moesin*]. All the strains that possess single gene mutations were obtained from the *Caenorhabditis* Genetic Centre (CGC) unless mentioned otherwise. Transgenic lines and compound mutants were created in the laboratory using standard molecular genetic approaches.

RNAi Screening

Bacterial clones expressing dsRNA from the RNAi library were grown in LB medium with ampicillin at 37°C overnight. The bacterial culture was seeded onto 12-well NGM plates containing ampicillin and IPTG. Seeded plates were incubated at room temperature for 24 hours to induce dsRNA expression. Meanwhile, a population of the animals was synchronized and resulting L1s were incubated on the dsRNA containing plates at the restrictive temperature (25°C) to compromise the function of *daf-2* (to induce dauer) and *par-4*. All the worms carrying a *lag-*

2::*gfp* transgene were examined for DTC displacement, as a proxy for degree of germline hyperplasia (Narbonne and Roy 2006).

DAPI staining and germ cell nuclei count

For whole animal DAPI (4′,6-diamidino-2-phenylindole) staining, *C. elegans* dauer larvae were washed off plates and soaked in Carnoy's solution (60% ethanol, 30% acetic acid, 10% chloroform) for overnight. Animals were then washed twice in PBST (1×PBS + 0.1% Tween 20), and stained in 0.1 mg/ml DAPI solution for 30 minutes. Finally, larvae were washed four times (20 minutes each) in PBST, and mounted in Vectashield (Vector Laboratories) medium. The germ cell nuclei count was determined per dauer larve based on their position and nuclear morphology. The germ cell nuclei count was performed to validate the identified genes from the RNAi screens. The strains (MR0671, CB1372, MR0672, MR0863, MR0480, MR0868) used for the further characterization of the identified genes, didn't harbour the *lag-2::GFP* transgene and thus the germ cell quantification was performed to study the RNAi effect on the dauer germline quiescence. Two-tailed *t-test* was performed to calculate the P value to compare the germ cell count between different genotypes.

Immunostaining and Microscopy

For extruded dauer gonad staining, gonads were dissected, fixed and stained as described elsewhere (Arduengo, Appleberry et al. 1998). Images were captured using the Leica DMR compound microscope equipped with a Hamamatsu C4742-95 digital camera. Image analysis, computational deconvolution and pseudocolouring were performed using Openlab 3.01 software from Improvision. Images were merged and stacked using Image J.

Abbreviations

Peutz-Jeghers Syndrome (PJS)

Germline stem cell (GSC)

AMP-activated protein kinase (AMPK)

Tuberous Sclerosis 2 (TSC2)

Distal tip cells (DTCs)

Gene Ontology (GO)

Extracellular matrix (ECM)

Wiskott-Aldrich Syndrome Protein (WASP)

Declarations

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Author contributions

Experiments were designed by R.R and P.K. The RNAi screen to identify genes resulting in germline hyperplasia was performed by E.C. R.C, and A.K performed the *par-4* and *aak(0)* suppressor screen, respectively. P.K implemented the analysis, validation, microscopy and the suggested revisions. The manuscript was written by P.K and R.C and edited by R.R. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interest.

Figures and Tables

Figure 2.1. Experimental design for genome-wide RNAi screens to identify genes that interact with LKB1/*par-4* or AMPK/*aak(0)*.

All the strains used for the three genome-wide screens contain a temperature sensitive *daf-2(e1370)* mutation that allowed us to induce dauer by shifting synchronized L1 larvae to 25˚C. Importantly, all the strains carry a *lag-2*::GFP transgene that is expressed in the DTCs, marking the distal extremities of the germ line. This expression allowed us to indirectly evaluate the degree of germline stem cell hyperplasia (Narbonne and Roy 2006). **A)** The screen was designed to identify genes that result in dauer germline hyperplasia when their function is compromised by RNAi. *daf-2* mutants carrying the *lag*-2::GFP transgene were synchronized and L1 larvae were then put on plates containing IPTG to induce dsRNA expressed from each bacterial clone and were shifted to 25°C to induce dauer formation. The dauer larvae were subsequently screened for increased DTC displacement; a proxy for germline hyperplasia (Narbonne and Roy 2006). The diagrams in **B** and **C** describe screens designed to identify suppressors of *par-4*- and *aak(0)*-induced dauer germline hyperplasia, respectively. For **B)**, the screen was performed with a strain that contains a temperature sensitive *par-4* mutation, while in **C)** an AMPK null mutant (*aak(0)*) that harboured deletions in both catalytic subunits was used. Upon dauer induction, animals were monitored for reduced displacement between the DTCs indicating suppression of the germline hyperplasia typical of both *par-4* and *aak(0)* mutants. See Materials and Methods for more details.

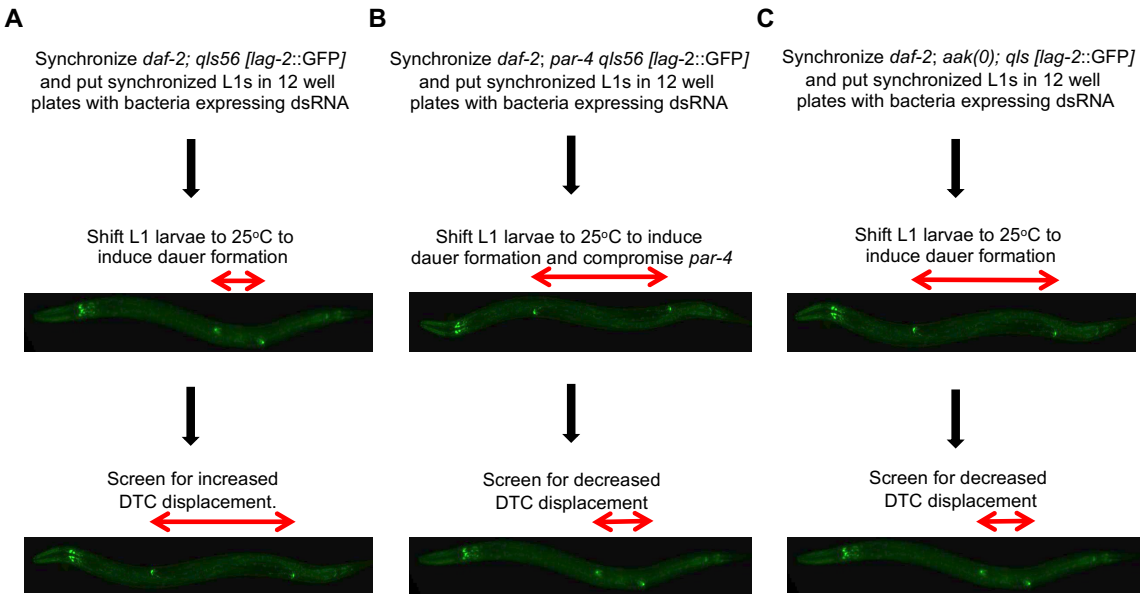

A
Synchronize daf-2; qls56 [lag-2::GFP] and put synchronized L1s in 12 well plates with bacteria expressing dsRNA
Shift L1 larvae to 25°C to induce dauer formation
Screen for increased DTC displacement.
B
Synchronize daf-2; par-4 qls56 [lag-2::GFP] and put synchronized L1s in 12 well plates with bacteria expressing dsRNA
Shift L1 larvae to 25°C to induce dauer formation and compromise par-4
Screen for decreased DTC displacement
C
Synchronize daf-2; aak(0); qls [lag-2::GFP] and put synchronized L1s in 12 well plates with bacteria expressing dsRNA
Shift L1 larvae to 25°C to induce dauer formation
Screen for decreased DTC displacement

Figure 2.2. Identification of genes that interact with AMPK/*aak(0)* and LKB1/*par-4* to regulate germline quiescence during the dauer stage.

Candidate genes identified in the genome-wide RNAi screens were categorized based on their functional description on WormBase. **A)** Gene ontology (GO) terms of genes that phenocopied *par-4* and *aak(0)* mutations and caused germline hyperplasia when subjected to RNAi. **B)** and **C)** Genes that suppressed *par-4* and *aak(0)* dependent dauer germline hyperplasia respectively were categorized by GO terms and shown in a pie chart. The most common GO terms include genes with known functions in the regulation of gene expression, DNA and RNA metabolism; in cytoskeletal regulation and cell polarity; in the regulation of cell-ECM adhesion and ECM integrity; cell signal transduction; cell cycle progression; cell growth signalling and metabolism; intracellular trafficking and protein processing. A significant portion of candidate genes remain uncharacterized and fall into the Unknown functional group. **D)** A Venn diagram that illustrates the total number of RNAi candidate genes that suppress the hyperplasia in the *par-4* and *aak(0)* backgrounds. The overlap (green) represents the number of candidates that suppressed the hyperplasia in both genotypes, implying that they act downstream of PAR-4 in an AMPK-dependent manner. **E)** To validate the candidates identified that phenocopy the hyperplasia observed in *par-4* and AMPK mutants, *daf-2(e1370)* dauer larvae were subjected to RNAi against genes representing different functional classes. The germ cell nuclei counts were performed following DAPI staining. RNAi against the LKB1 pseudokinase component *strd-1* was used as a positive control. **F, G)** To validate the identified gene candidates and confirm the suppression of *par-4* and *aak(0)* dependent dauer germline hyperplasia, genes from different functional categories were depleted using RNAi and germ cell nuclei counts were performed on extruded DAPI-stained gonads. RNAi of these genes resulted in a significant decrease in the germline hyperplasia typical

of *par-4* and *aak(0*) mutant dauer larvae. ***P<0.0001, **P<0.001, *P<0.05 when compared to L4440 using two-tailed *t-test*, n=50.

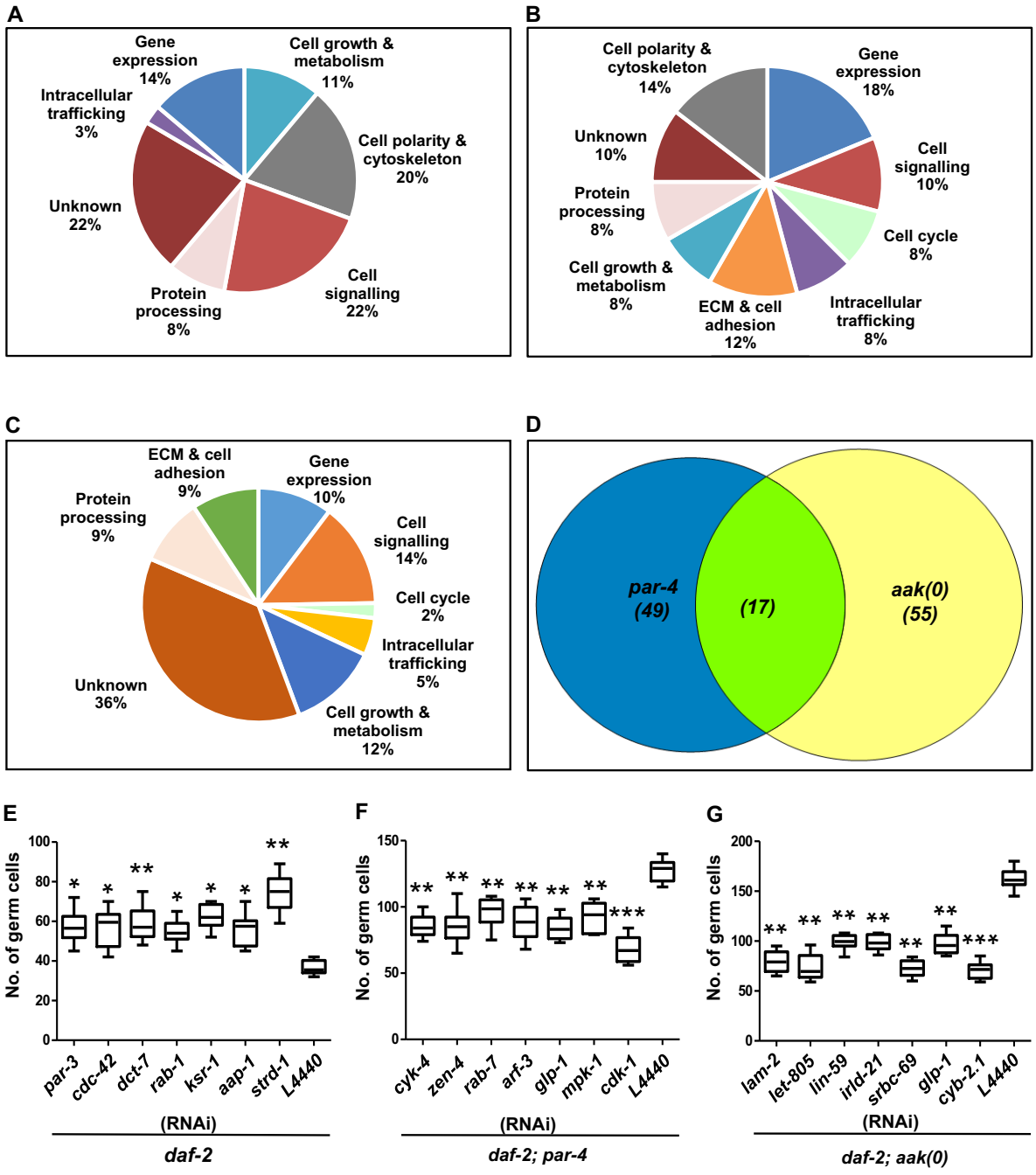

A
Gene expression 14%
Cell growth & metabolism 11%
Intracellular trafficking 3%
Cell polarity & cytoskeleton 20%
Unknown 22%
Cell signalling 22%
Protein processing 8%
B
Cell polarity & cytoskeleton 14%
Gene expression 18%
Unknown 10%
Cell signalling 10%
Protein processing 8%
Cell cycle 8%
Cell growth & metabolism 8%
ECM & cell adhesion 12%
Intracellular trafficking 8%
C
ECM & cell adhesion 9%
Gene expression 10%
Protein processing 9%
Cell signalling 14%
Cell cycle 2%
Intracellular trafficking 5%
Unknown 36%
Cell growth & metabolism 12%
D
par-4 (49)
(17)
aak(0) (55)
E
No. of germ cells
par-3
cdc-42
dct-7
rab-1
ksr-1
aap-1
strd-1
L4440
(RNAi)
daf-2
F
No. of germ cells
cyk-4
zen-4
rab-7
arf-3
glp-1
mpk-1
cdk-1
L4440
(RNAi)
daf-2; par-4
G
No. of germ cells
lam-2
let-805
lin-59
irld-21
srbc-69
glp-1
cyb-2.1
L4440
(RNAi)
daf-2; aak(0)

Figure 2.3. Compromise of both cell polarity and actin cytoskeletal regulators results in dauer germline hyperplasia.

A) Canonical actin cytoskeleton regulators which were not identified in the screen were depleted using RNAi and germ cell nuclei counts were performed. Depletion of *wsp-1, arx-2, arx-3* resulted in significant increase in the number of germ cells. **B)** and **C)** To test if *par-4* mediates cell polarity independently of AMPK to establish dauer GSC quiescence, genes belonging to the cell polarity and cytoskeleton category were depleted in either a *par-4* or an *aak(0)* background and germ cell nuclei counts were performed. **P<0.001, *P<0.05 when compared to L4440 using two-tailed *t-test*, n=50

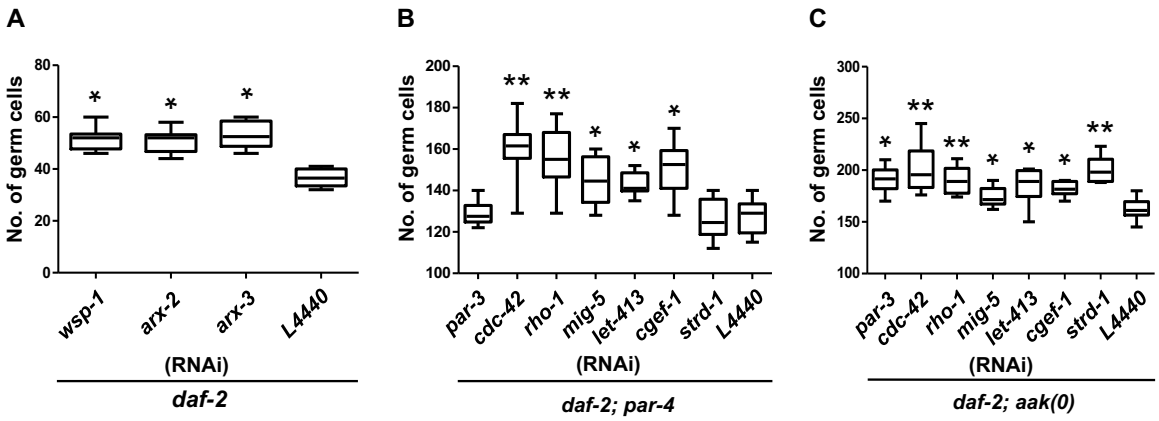
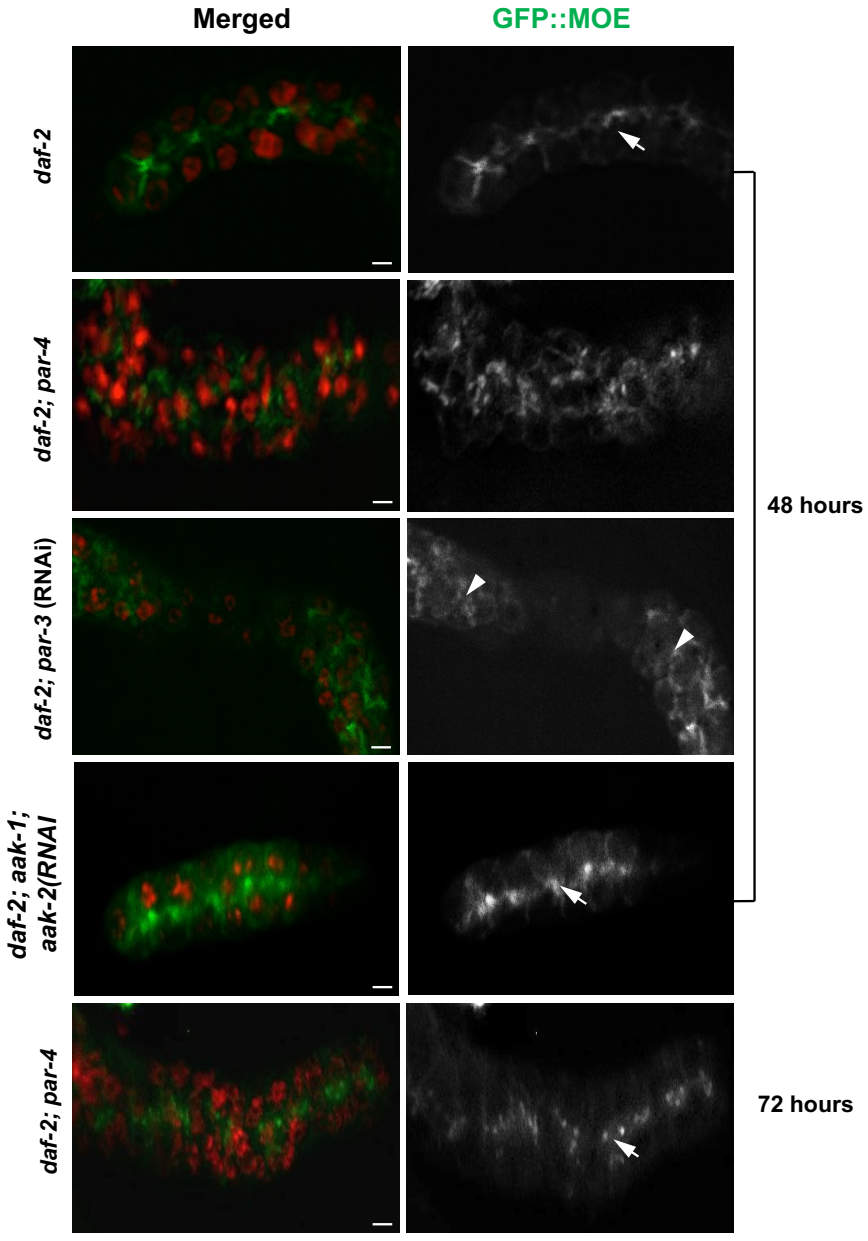

A
No. of germ cells
80
60
40
20
0
wsp-1
arx-2
arx-3
L4440
(RNAi)
daf-2
B
No. of germ cells
200
180
160
140
120
100
par-3
cdc-42
rho-1
mig-5
let-413
cgef-1
strd-1
L4440
(RNAi)
daf-2; par-4
C
No. of germ cells
300
250
200
150
100
par-3
cdc-42
rho-1
mig-5
let-413
cgef-1
strd-1
L4440
(RNAi)
daf-2; aak(0)

Figure 2.4. The organization of the actin cytoskeleton is initially perturbed but is resolved later in the *par-4* mutant dauer germline.

daf-2(e1370) dauer larvae that express a GFP reporter that marks the actin cytoskeleton were examined to monitor any change to the actin cytoskeletal network in the germ line. All images in the left panel are merged, condensed Z stacks. Actin filaments are organized at the membrane adjacent to the rachis in the *daf-2* dauer germ line (arrow) at 48 hours after shifting to the restrictive temperature of 25^0C. Actin organization is perturbed (arrowhead) in the germ line of *par-4* dauer larvae at 48 hours, but is resolved at 72 hours (arrow). Similarly, *par-3* compromised dauer larvae exhibit defects in actin organization at the rachis-adjacent membrane. No defects were observed in the actin organization at the rachis-adjacent membrane in the *daf-2; aak-1; aak-2*(RNAi) treated dauer germ line at 48 hours (arrow). n=15, scale bar: 4μm


Merged
GFP::MOE
daf-2
daf-2; par-4
daf-2; par-3 (RNAi)
daf-2; aak-1; aak-2(RNAi
daf-2; par-4
48 hours
72 hours

Supplemental figure 2.1. The organization of the actin cytoskeleton is perturbed in the *cdc-42* compromised dauer germline.

daf-2(e1370) dauer larvae that express the actin cytoskeleton marker were subjected to *cdc-42*(RNAi) and monitored for change in the actin cytoskeletal network in the germline. The image in the left panel consists of merged, condensed Z stacks. Actin filament organization at the membrane adjacent to the rachis is disrupted at 48 hours after shifting to their restrictive temperature (arrowhead). n=10 Scale bar: 4μm

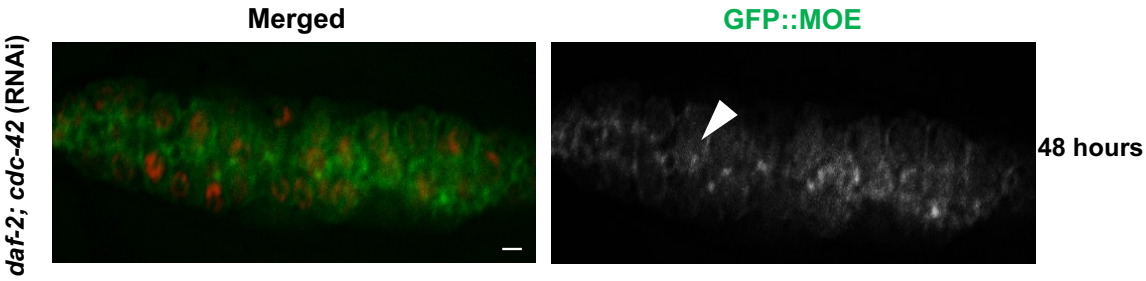

Merged
GFP::MOE
daf-2; cdc-42 (RNAi)
48 hours

Table 2.1. **Candidate genes that resulted in germline hyperplasia during the dauer stage.**

Chromo-some	Cosmid Identifier	Gene	Brief description
I	ZC123.3		Homeobox protein; homologue to human ATBF1 transcription factor and tumour suppressor
I	Y110A7A.10[$]	*aap-1*	PI3K p50/55 adaptor/regulatory subunit orthologue
I	T08B2.10	*rps-17*	40S ribosomal protein S17
I	F57B10.8		Activator of basal transcription
I	F26E4.4[$]		Activator of basal transcription
I	T06G6.9[$]	*pfd-3*	Molecular chaperon Prefoldin, subunit 3; orthologue to human VHL-binding protein tumour suppressor
I	Y87G2A.h[$]	*gpi-1*	Putative glucose 6-phosphate isomerase; Required for normally short lifespan
II	C23H3.4[$]	*sptl-1*	Putative serine palmitoyltransferase
II	F53A10.2		Rap-1 GTPase-activating protein; orthologue to a human tumour suppressor in squamous cell carcinoma
II	T05C1.6[$]		Calmodulin-binding transcriptional activator
II	R07G3.1	*cdc-42*	RHO small GTPase; regulator of polarity
II	T05C12.6	*mig-5*	One of three homologues to Dishevelled in *Drosophila*
II	T22C8.2	*chhy-1*	Chondroitin hydrolase; similar to human HYAL1, a potential tumour suppressor in lung cancer cell lines
II	F40F8.9[$]	*lsm-1*	Small nuclear ribonucleoprotein splicing factor
II	T06D8.2[$]		No description available
II	C50E10.3[$]	*sre-53*	G protein-coupled chemoreceptor
II	W01D2.2	*nhr-61*	Nuclear hormone receptor
III	R74.2[$]		No description available
III	Y44F5A.1		Protein containing WD40 repeats; required for protein-protein interactions
III	C27F2.4[$]		Predicted protein carboxyl methylase
III	R02F2.7[$]		No description available
III	F54E7.3	*par-3*	PDZ-domain containing protein; required for polarization of the early embryo
III	B0336.3[$]		Protein containing and RNA recognition motif
III	C07H6.5	*cgh-1*	Putative RNA helicase; inhibits apoptosis in oocytes
III	C50C3.9[$]	*unc-36*	Voltage-dependent calcium ion channe
III	Y52D3.1	*strd-1*	Activator of LKB1
IV	F15E6.8	*dct-7*	RNA binding protein controlled by DAF-16/FOXO; affects germline tumours
IV	H35B03.2[$]		Subunit of nuclear ribonuclease P
IV	C06E7.1[$]	*sams-3*	S-adenosylmethionine synthetase
IV	F12F6.5	*sgrp-1*	Homologue to Cdc42-interacting protein CIP4
IV	Y51H4A.c	*rho-1*	Rho GTPase; required for actin filament-based processes including embryonic polarity
V	F48G7.9		Serine/threonine protein kinase

V	C39F7.4	*rab-1*	Ras-like GTPase, orthologue to Rab1
V	T03D3.11[$]	*srj-44*	7-transmembrane olfactory receptor
V	F26D11.11[$]	*let-413*	Localizes to basolateral region of epithelial cells and required for adherens junction formation; strong homology to *Drosophila* Scribble
X	C14A11.3	*cgef-1*	Guanosine nucleotide exchange factor for Rho and Rac GTPases
X	C38C5.1[$]		No description available
X	F13B9.5	*ksr-1*	Kinase suppressor of Ras
X	T09B9.3[$]		Glycerophosphoryl diester phosphodiesterase

Table 2.2. Candidate genes that suppress *par-4*-dependent hyperplasia during the dauer stage.

Chromo-some	Cosmid Identifier	Gene	Brief Description
I	H26D21.1[$]	*hus-1*	DNA damage checkpoint protein required for DNA damage-induced cell cycle arrest in *C. elegans*
I	C43E11.6	*nab-1*	Neurabin orthologue that regulate interactions between actin and microtubules during cell division, migration and growth cone guidance
I	F27C1.7[$]	*atp-3*	Subunit of mitochondrial ATP synthase, which regulates growth rate, body size, and ageing.
I	C34G6.6[$]	*noah-1*	ECM component required for molting, development, vulval development, and normal body morphology
I	H15N14.1	*adr-1*	Adenosine deaminase that acts on RNA by deaminating adenosines and generating inosines in dsRNA; protects transgenic RNA from RNAi silencing
I	C54G4.8[$]	*cyc-1*	Subunit of complex III cytochrome c reductase required for normal ATP production
I	F26E4.9[$]	*cco-1*	Subunit of cytochrome c oxidase-1, a component the electron transport chain in mitochondria
I	E03H4.8[$]		Uncharacterized
I	H28O16.1[$]		Uncharacterized
I	M01D7.1[$]		Uncharacterized
I	ZK1151.1	*vab-10*	A spectraplakin, component of the hemidesmosome in *C. elegans;* required for transducing mechanical signals from muscle cells to epidermis, and DTC migration
I	B0414.2	*rnt-1*	Transcription factor required for seam cell proliferation; interacts with SMA-4 and regulates expression of CDK-inhibitor *cki-1*
II	T15H9.3[$]	*hlh-6*	Helix loop helix transcription factor
II	C47D12.6[$]	*tars-1*	Threonyl amino-acyl tRNA synthetase
II	ZK930.3[$]	*vab-23*	Transcription factor involved in ventral closure, elongation
II	W08F4.6[$]	*mlt-8*	Putative signalling peptide secreted from cells involved in the L2/L3 molting process
II	W03C9.3	*rab-7*	Rab-GTPase required for endosome to lysosome trafficking
II	F59G1.3[$]	*vps-35*	Vacuolar protein sorting factor
II	R09D1.7[$]	*chil-20*	Chitinase-like protein
II	C41C4.4[$]	*ire-1*	Transmembrane serine/threonine kinase and endoribonuclease necessary for unfolded protein response (UPR)
II	F10B5.1[$]	*rpl-10*	Ribosomal subunit L10
III	T05G5.3	*cdk-1*	Cyclin-dependent kinase required for cell cycle progression through the G2/M checkpoint

III	R01H10.1[$]	*div-1*	DNA polymerase subunit required for normal interphase timing and asymmetric distribution of PIE-1 and P granules
III	K12H4.4		Uncharacterized
III	C04D8.1	*pac-1*	Rho-GAP involved in establishing radial asymmetry during *C. elegans* development by regulating the spatial localization of CDC-42
III	F43C1.2[$]	*mpk-1*	Mitogen-activated-protein-kinase
III	F58A4.8	*tbg-1*	γ-tubulin
III	K08E3.6	*cyk-4*	Rho-GAP and member of the centralspindlin complex required for cytokinesis; also factor localized to intercellular bridge in the rachis required for gonad structural integrity
III	R10E11.2[$]	*vha-2*	V-ATPase subunit involved in protein sorting and receptor mediated endocytosis
III	F26A1.14[$]		Uncharacterized
III	H19M22.2[$]	*let-805*	Myotactin; subunit of the hemidesmosome complex
III	F26F4.10	*rars-1*	Arginyl-tRNA synthetase
III	F02A9.6	*glp-1*	Notch receptor homologue and required for germline proliferation
III	M88.6	*pan-1*	Transmembrane protein required for completion of larval molts; expression enriched in the germ line
III	B0336.2	*arf-1.2*	ADP-ribosylation factor homolog, a GTPase that regulates intracellular trafficking and the actin cytoskeleton
III	T12A2.2[$]	*stt-3*	Yeast oligosaccharyltransferase subunit homologue
III	F37C12.4	*rpl-36*	Large ribosomal subunit L36
III	F58A4.11[$]	*gei-13*	Predicted DNA binding protein involved in body shape regulation, cuticle synthesis and locomotion
IV	C46A5.2	*del-7*	Degenerin-like protein; degenerin family of proteins are sodium ion channels essential for homeostasis and involved in mechanotransduction
IV	F56H11.1[$]	*fbl-1*	Fibulin, a component of the extracellular matrix required for DTC migration initiation
IV	F56H9.5[$]	*lin-25*	Subunit of the Mediator complex, which functions downstream of LET-60 to regulate differentiation of a number of cell types
IV	F57H12.1	*arf-3*	ADP-ribosylation factor homolog, a GTPase that regulates intracellular trafficking and the actin cytoskeleton
IV	R13H7.1[$]	*srx-20*	Serpentine receptor, class X
V	K06A4.3	*gsnl-1*	Gelsolin-related protein predicted to function as an actin regulatory protein, capping barbed ends of actin filaments
V	Y49A3A.2[$]		Uncharacterized
V	F33E11.1[$]	*nhr-15*	A nuclear hormone receptor
V	E02C12.3[$]	*srx-47*	Serpentine receptor, class X
V	F53A9.10[$]	*tnt-2*	Troponin, a tropomyosin binding protein
V	C54D1.5[$]	*lam-2*	Subunit of laminin required for basement membrane integrity and gonad morphology

Table 2.3. Candidate genes that suppress AMPK-dependent hyperplasia during the dauer stage.

Chromo-some	Cosmid identifier	Gene	Brief description
I	T12F5.4	*lin-59*	SET domain-containing protein, closely related to *Drosophila* tri-thorax ASH1 protein implicated in chromatin remodeling.
I	H15N14.1[$]	*adr-1*	Adenosine deaminase that acts on RNA by deaminating adenosines and generating inosines in dsRNA; protects transgenic RNA from RNAi silencing
I	C54G4.8[$]	*cyc-1*	Subunit of complex III cytochrome c reductase required for normal ATP production.
I	F26E4.9[$]	*cco-1*	Subunit of cytochrome c oxidase-1, a component of the electron transport chain in mitochondria
I	F35C12.1[$]		Uncharacterized
I	I-5 Q4 G9*		A *vab-10* isoform. A spectraplakin, component of the hemidesmosome in *C. elegans;* required for transducing mechanical signals from the muscle cells to epidermis
I	E03H4.8		Uncharacterized
I	H28O16.1[$]		Uncharacterized
I	Y18D10A.13	*pad-1*	Unfamiliar conserved protein required for embryonic development
II	T08E11.5[$]	*fbxc-19*	F-box C protein
II	B0281.6		Uncharacterized
II	F42G2.4[$]	*fbxa-182*	F-box C protein
II	K02F6.1		Uncharacterized
II	T24E12.9[$]		Uncharacterized
II	F29G1.3[$]	*vps-35*	Vacuolar protein sorting factor
II	T02G5.9[$]	*kars-1*	Lysyl(K) Amino-acyl tRNA Synthetase
II	F22B5.2	*eif-3.G*	Encodes a homologue of eukaryotic translation initiation factor 3, subunit 4. Affects embryonic viability, fertility and growth
II	C50E10.2[$]		Uncharacterized
II	C50E10.3[$]	*sre-53*	Serpentine receptor, Class E
II	Y53F4B.g		Uncharacterized
III	H19M22.2[$]	*let-805*	Myoactin; subunit of the hemidesmosome complex
III	T20B6.3		Uncharacterized
III	F26A1.13[$]		Uncharacterized
III	B0336.2	*arf-1.2*	ADP-ribosylation factor homologue, a GTPase that regulates intracellular trafficking and the actin cytoskeleton
III	C28H8.11[$]	*tdo-2*	Tryptophan 2,3-DiOxygenase
III	T12A2.2[$]	*stt-3*	Yeast oligosaccharyltransferase subunit homologue
III	C18F10.4[$]	*srg-1*	Serpentine Receptor, Class G

III	F23F12.6	*rpt-3*	A triple A ATPase subunit of the 26S proetosome's 19S regulatory particle base subcomplex; functions as a reverse chaperone by unfolding substrates and translocating them into the core proteolytic particle (CP) of proteasome
III	R13A5.7[$]		Uncharacterized
III	K12H4.4[$]		Uncharacterized
III	F02A9.6	*glp-1*	Notch receptor homologue required for germline stem cell mitotic proliferation
III	F54G8.1[$]	*irld-34*	Insulin/EGF-receptor L Domain protein
III	R10E11.2[$]	*vha-2*	V-ATPase subunit involved in protein sorting and receptor-mediated endocytosis
IV	F47C12.6		Uncharacterized
IV	W03B1.6[$]	*oac-51*	O-ACyltransferase homologue
IV	F57H12.1	*arf-3*	ADP-ribosylation factor homologue, a GTPase that regulates intracellular trafficking and the actin cytoskeleton
IV	Y43E12A.1	*cyb-2.1*	A cyclin B isoform
IV	C28C12.11[$]		Uncharacterized
IV	K07F5.7		Uncharacterized
IV	C04G2.11[$]	*irld-21*	Insulin/EGF-receptor L Domain protein
IV	T17B5.1[$]		Uncharacterized
IV	F25H8.3	*gon-1*	A metalloprotease involved in ECM degradation; controls gonadal morphogenesis
V	F33E11.1[$]	*nhr-15*	A nuclear hormone receptor
V	F48G7.11[$]	*nhr-190*	A nuclear hormone receptor
V	C24B9.7[$]	*srg-59*	Serpentine Receptor, Class G
V	F35F10.3		Uncharacterized
V	F32D1.2	*hpo-18*	Hypersensitive to POre-forming toxin
V	T24A6.12[$]	*srbc-69*	Serpentine Receptor, Class BC
V	Y47D7A_1[$]43.d		Uncharacterized
V	C10G8.8[$]		Uncharacterized
V	F07G11.4		Uncharacterized
V	C54F6.10[$]	*str-31*	Seven-transmembrane G-protein coupled receptor
V	C12D5.7[$]	*cyp-33A1*	Cytochrome P450 family
V	C37C6.6[$]	*mig-6*	Similar to extracellular matrix proteins papilin and lacunin; required for DTC migration at all phases
X	C54D1.5[$]	*lam-2*	Subunit of laminin required for basement membrane integrity and gonad morphology

Table 2.1-3. The gene identity of each RNAi clone was determined by the database available with the commercially available Ahringer RNAi library. The brief descriptions for each gene locus are derived from WormBase or determined according to their functional homology. [$] RNA candidates

that potentially function germline non-autonomously. * These wells did not appear to be in the Ahringer library database. The RNAi clone from this well was sequenced and the sequence was compared to other species to identify potential orthologues.

Table 2.4. Candidate genes that exclusively suppress *par-4*-dependent hyperplasia during the dauer stage.

Chromo-some	Cosmid Identifier	Gene	Brief Description
I	H26D21.1	*hus-1*	DNA damage checkpoint protein required for DNA damage-induced cell cycle arrest in *C. elegans*
I	C43E11.6	*nab-1*	Neurabin orthologue that regulate interactions between actin and microtubules during cell division, migration and growth cone guidance
I	F27C1.7	*atp-3*	Subunit of mitochondrial ATP synthase, which regulates growth rate, body size, and ageing
I	C34G6.6	*noah-1*	ECM component required for molting, development, vulval development, and normal body morphology
I	M01D7.1		Uncharacterized
I	B0414.2	*rnt-1*	Transcription factor required for seam cell proliferation; interacts with SMA-4 and regulates expression of CDK- inhibitor *cki-1*
I	T05F1.6	*hsr-9*	Cell cycle checkpoint protein in response to DNA damage
II	T15H9.3	*hlh-6*	Helix loop helix transcription factor
II	R09D1.7	*chil-20*	Chitinase-like protein
II	C47D12.6	*tars-1*	Threonyl amino-acyl tRNA synthetase
II	ZK930.3	*vab-23*	Transcription factor involved in ventral closure, elongation
II	W03C9.3	*rab-7*	Rab-GTPase required for endosome to lysosome trafficking
II	F10B5.1	*rpl-10*	Ribosomal subunit L10
II	W08F4.6	*mlt-8*	Putative signalling peptide secreted from cells involved in the L2/L3 molting process
II	C41C4.4	*ire-1*	Transmembrane serine/threonine kinase and endoribonuclease necessary for unfolded protein response (UPR).
III	T05G5.3	*cdk-1*	Cyclin-dependent kinase required for cell cycle progression through the G2/M checkpoint
III	R01H10.1	*div-1*	DNA polymerase subunit required for normal interphase timing and asymmetric distribution of PIE-1 and P granules
III	K12H4.4		Uncharacterized
III	C04D8.1	*pac-1*	Rho-GAP involved in establishing radial asymmetry during *C. elegans* development by regulating the spatial localization of CDC-42
III	F43C1.2	*mpk-1*	Mitogen-activated-protein-kinase
III	F58A4.8	*tbg-1*	γ-tubulin
III	K08E3.6	*cyk-4*	Rho-GAP and member of the centralspindlin complex required for cytokinesis; also factor localized to

			intercellular bridge in the rachis required for gonad structural integrity
III	F26F4.10	*rars-1*	Arginyl-tRNA synthetase
III	M88.6	*pan-1*	Transmembrane protein required for completion of larval molts; expression enriched in the germ line
III	T12A2.2	*stt-3*	Yeast oligosaccharyltransferase subunit homologue
III	F37C12.4	*rpl-36*	Large ribosomal subunit L36
III	F58A4.11	*gei-13*	Predicted DNA binding protein involved in body shape regulation, cuticle synthesis, and locomotion
IV	C46A5.2	*del-7*	Degenerin-like protein; degenerin family of proteins are sodium ion channels essential for homeostasis and involved in mechanotransduction
IV	F56H11.1	*fbl-1*	Fibulin, a component of the extracellular matrix required for DTC migration initiation
IV	F56H9.5	*lin-25*	Subunit of the Mediator complex, which functions downstream of LET-60 to regulate differentiation of a number of cell types
IV	R13H7.1	*srx-20*	Serpentine receptor, class X
V	K06A4.3	*gsnl-1*	Gelsolin-related protein predicted to function as an actin regulatory protein, capping barbed ends of actin filaments

Table 2.4. The gene identity of each RNAi clone was determined by the database available with the commercially available Ahringer RNAi library. The brief descriptions for each gene locus are derived from WormBase or determined according to their functional homology.

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Connecting statement: Bridging chapter 2 to chapter 3

Having performed the whole genome-wide screens and identified the potential targets acting downstream of LKB1/PAR-4 in AMPK-dependent and -independent manner to regulate dauer germlin quiescence, to further extend our understanding I decided to investigate how PAR-4 /AMPK might impinge on Notch signalling to inhibit germ cell proliferation. Furthermore, I also characterized the AMPK-independent function of LKB1/PAR-4 to regulate centralspindlin complex to inhibit abnormal germ cell divisions.

Chapter 3: LKB1/PAR-4 plays AMPK-dependent and -independent roles to establish germline stem cell quiescence in *C. elegans* dauer larvae

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Manuscript in preparation

Abstract

C. elegans larvae undergo a global developmental arrest in response to harsh growth conditions by executing an alternative diapause-like stage called 'dauer'. It was previously shown that LKB1, AMPK and PTEN are all required to establish quiescence in the germ line of *C. elegans* dauer larvae. We noted that in the quiescent germ line of insulin receptor mutants (*daf-2*), GLP-1(Notch receptor) undergoes subcellular re-localization from the cell membrane of the germ cells to the rachis-adjacent membrane. This re-localization is associated with the germ line stem cell (GSC) cycle quiescence typical of the dauer.

In the germ line of LKB1 (*par-4)* mutants and AMPK (*aak-0)* mutant dauer larvae, GLP-1 is still present around the GSCs membrane and fails to re-localize and correlates with the observed germline hyperplasia typical of these mutants. We show that AMPK phosphorylates GLP-1 at S1199 to possibly generate a 14-3-3 binding site that is recognized by PAR-5, the germline 14-3-3 protein, and that PAR-5 is required to mediate dauer-dependent GLP-1 re-localization. Though LKB1/PAR-4 acts through AMPK in order to regulate the cell cycle arrest, curiously, LKB1 knockdown in AMPK null mutants shows an additive effect suggesting an AMPK-independent role for LKB1 in the regulation of this quiescence. We show PAR-4 plays an AMPK-independent role to regulate ANI-2 localization, perhaps to stabilize the centralspindlin complex to regulate actin organization and NMY-2-dependent actomyosin contractility at the rachis bridges to block germ cell divisions. Altogether, in this study we demonstrate that PAR-4 impinges on at least 2 independent pathways where it regulates Notch signalling in an AMPK-dependent manner and the centralspindlin complex in an AMPK-independent manner to establish quiescence in the germline stem cells during energetic stress.

Introduction

During unfavourable growth conditions, many organisms can delay their developmental program by slowing or arresting their cell divisions and/or by altering their rate of metabolism. This is often achieved by triggering signal transduction pathways that impinge on key regulators that appropriately block energy consuming pathways presumably as a means of conserving energy for their long-term survival (Hardie, Carling et al. 1998, Hand, Denlinger et al. 2016). When *C. elegans* larvae are confronted with sub-optimal growth conditions and/or overcrowding during the early stages of development, three parallel signalling pathways (Insulin-like signalling, TGF-β and cGMP) can independently affect a neuroendocrine signal that is responsible for a developmental switch, instructing the larva to execute an alternative diapause-like stage called `dauer' instead of the third larval (L3) stage typical of reproductive development. Upon entering this stage, the dauer larva undergoes a phase of global quiescence that likely occurs as a means of conserving energy, but also to ensure that cell divisions do not occur during a paucity of appropriate molecular building blocks. In this morphologically and metabolically adaptive state, dauer larvae can survive up to six months, and when conditions become favourable for growth, they exit dauer and resume their regular reproductive development with little to no negative consequence on their fitness (Albert, Brown et al. 1981, Golden and Riddle 1982, Riddle and Albert 1997).

The *C. elegans* germ line is sensitive to fluctuations in the environment, particularly to nutrient availability, as demonstrated by the germline stem cell quiescence during dauer, the L1 diapause and in the nutrient-deprived L4 (Fukuyama, Rougvie et al. 2006, Narbonne and Roy 2006, Angelo and Van Gilst 2009, Fukuyama, Sakuma et al. 2012). During the dauer stage, in addition to the general cell cycle arrest that occurs during this period, the germline stem cell population remains

quiescent until reproductive development is resumed, when the germline stem cells will re-enter the cell cycle to eventually generate germ cells that will differentiate into gametes. This ability to exit the cell cycle and re-enter provides the germline stem cell (GSC) with a plasticity, providing a means to interrogate how environmental cues impinge upon the dynamic relationship between cell cycle progression and differentiation. Previous studies have demonstrated that LKB1, AMP-activated protein kinase (AMPK) and PTEN co-operate during the dauer stage to establish cell cycle quiescence in the GSCs (Narbonne and Roy 2006), however, the underlying mechanism(s) affected downstream of these genes that impact quiescence is presently unclear.

The gonad of L1 larva contains 4 progenitor cells Z1, Z2, Z3 and Z4. Among these, the somatic gonad precursors Z1 and Z4 will undergo multiple cell divisions to eventually give rise to the somatic gonad which comprises two identical reflexed arms capped at each extremity by the distal tip cells (DTCs). The germline precursor cells, Z2 and Z3 will give rise to a population of germline stem cells (GSCs) that divide continuously throughout larval development (Hubbard and Greenstein 2005). The distal gonad arms capped by the DTCs act as a niche for the GSCs, wherein the mitotic population of the GSCs is maintained through Notch signalling between the somatic DTCs and the distal germ cells. The Delta-like ligand, LAG-2 is expressed in the somatic DTCs, while the GSCs express the Notch-like receptor, GLP-1(Henderson, Gao et al. 1994). Active Notch signalling maintains the GSCs in a mitotically-competent state while blocking them from executing their alternative meiotic pathway. These dividing cells physically push their daughters proximally, where cells no longer receive the LAG-2 signal from the DTCs, leaving the Notch receptor in its unliganded inactive form, thereby instructing the cells to differentiate and execute the meiotic program (Kimble and Crittenden 2007).

Moreover, during metazoan development, Notch signalling is implicated in diverse functions required during embryogenesis and tissue development. Previous studies have demonstrated the role of Notch signalling in *Drosophila* wing and eye development, mammalian angiogenesis and neuronal fate decisions, and stem cell renewal (Fanto and Mlodzik 1999, Baron, Aslam et al. 2002, Hitoshi, Alexson et al. 2002, Liu, Shirakawa et al. 2003, Aguirre, Rubio et al. 2010). The interaction between a Notch ligand and the Notch receptor activates two proteolytic cleavages that result in the release of the Notch intracellular domain (NICD) from the cellular membrane (Baron 2003). Post-cleavage, NICD enters the nucleus and interacts with other transcription factors to activate the expression of target genes. However, Notch signalling needs to be regulated at certain time points as aberrant Notch signalling is associated with several types of human cancer (Stylianou, Clarke et al. 2006, Sharma, Draheim et al. 2007). Multiple mutations resulting in active Notch signalling were identified in most of the patients suffering from T-cell acute lymphoblastic leukemia. Recent studies have implicated the role of LKB1/AMPK in regulating Notch signalling and in the absence of LKB1/AMPK, Notch signalling is abnormally activated resulting in severe pathological disorders but the mechanism(s) is still unclear (Li, Lee et al. 2014, Shan, Zhang et al. 2016).

In *C. elegans,* germline proliferation continues uninterrupted throughout reproductive development, but as they prepare for dauer, the GSCs decrease their division rate in L2d to eventually arrest in G2/M phase during the dauer stage (Narbonne and Roy 2006). Curiously, in the quiescent *C. elegans* dauer germ line, LAG-2 is still present and active in the DTCs (Narbonne and Roy 2006) suggesting that the Notch receptor is regulated downstream of its activation. Our genome-wide RNAi screens indicated that eliminating GLP-1/Notch function could suppress the dauer germline hyperplasia in LKB1/AMPK mutants, suggesting that misregulation of Notch in

this mutant background may be partially responsible for the decision to exit quiescence. Thus to further investigate how Notch signalling is regulated to maintain dauer germline quiescence will help us to understand how LKB1/AMPK pathway integrates with Notch signalling under starvation to block abnormal cell divisions.

In *C. elegans*, loss *of par-4* in mutants that lack all AMPK signalling due to mutations in both catalytic subunits (henceforth referred as *aak(0)*) results in animals with enhanced germline hyperplasia compared to AMPK mutants. Since these phenotypes are additive*, par-4* likely functions through other alternative downstream targets, independently of AMPK to establish quiescence in the dauer germ line (Narbonne and Roy 2006, Kadekar, Chaouni et al. 2018). In addition to regulating dauer germline quiescence, *par-4* is also involved in modulating actin contractility to regulate early *C. elegans* embryonic polarization and cell division (Chartier, Salazar Ospina et al. 2011). Recently, in *C. elegans* dauer larvae, genome-wide RNAi screens identified a subset of cytoskeleton and polarity regulating genes that suppressed *par-4*-dependent hyperplasia but did not affect the hyperplasia caused by the loss of AMPK signalling (Kadekar, Chaouni et al. 2018). Taken together these observations suggest that LKB1/PAR-4 affect different targets/pathways that block cell growth in an AMPK/TOR-independent manner.

This work provides a mechanistic insight about how under nutrient stress, LKB1/PAR-4 can act in a canonical AMPK-dependent pathway to downregulate Notch signalling by modulating asymmetric localization of the Notch receptor in apparently unpolarized *C. elegans* germ line. Furthermore, we also show how LKB1/PAR-4 might act in an AMPK-independent manner to regulate centralspindlin complex at the rachis bridge and modulate actin contractility to establish dauer germline quiescence. This enables us to better understand the role of LKB1/PAR-4 as a tumour suppressor and how its loss of function might result in Peutz Jeghers Syndrome.

Results

PAR-4/AMPK signalling regulates GLP-1 re-localization to the rachis-adjacent membrane in the dauer germ line

In *C. elegans* the loss of AMPK and/or LKB1 signalling results in hyperplasia in the dauer germ line, suggesting that these protein kinases are important for the establishment of quiescence during the onset of dauer formation through phosphorylation of targets that inhibit cell proliferation (Narbonne and Roy 2006). Germ cell proliferation occurs at the distal end of the gonad in *C. elegans,* where Notch receptor activation occurs through its interaction with a Delta-like ligand produced in a somatic distal tip cell (DTC). Activation of Notch through this physical interaction signals germ cells to block them from executing their meiotic program, while alternatively instructing them to undergo mitotic proliferation (Henderson, Gao et al. 1994, Kimble and Crittenden 2007).

Curiously, all cell proliferation is arrested during the dauer stage, yet both the Delta-like ligand LAG-2 that is expressed in the DTC, and the Notch receptor GLP-1 are still expressed (Narbonne and Roy 2006)). Notch signalling should be active and, consistent with this, the germline stem cells (GSCs) do not execute their meiotic program.

However, if Notch is indeed activated, the germ cells should respond by proliferating. This is not what is observed during the dauer diapause; in the dauer germ line the GSCs reduce their rate of division prior to dauer entry and arrest in the G2 phase of the cell cycle while they remain quiescent throughout the duration of the dauer stage. Interestingly, even when dauer larvae harbour a constitutively active form of GLP-1 that normally generates a tumourous germ line in adult hermaphrodites, the GSCs still maintain quiescence (data not shown). This suggests that despite activation of the Notch receptor in the dauer germ line, the germ line stem cell divisions must be

modulated downstream or in parallel to GLP-1 activation, while the meiotic program still remains blocked since none of the dauer germ cells enter meiosis (Narbonne and Roy 2006).

The elimination of *glp-1* partially corrects the supernumerary cell divisions typical of the AMPK and LKB1 mutant dauer germ lines indicating that the proliferating cells require activated GLP-1 and that Notch signalling pathway must act downstream or in parallel with LKB1 and AMPK to establish GSC quiescence in the dauer larvae (Kadekar, Chaouni et al. 2018). It is therefore plausible that one or more components in the Notch signalling pathway may act as substrates of either LKB1 or AMPK to regulate quiescence during the dauer stage.

Similar to the proliferating adult germ line, GLP-1 is present in the quiescent dauer germ line, and therefore is probably not regulated through its abundance (Narbonne and Roy 2006). However, when we examined the germ cells present in *daf-2* dauer larvae, we noted that GLP-1 undergoes a subcellular re-localization from its distribution around the cell membrane to the membrane most adjacent to the rachis in the germ cells (Fig. 3.1A, B). To quantify this we examined the ratio of GLP-1 to PLC::mCherry (germ cell membrane marker) in the germ line in *daf-2* adult hermaphrodites and compared it to that of the *daf-2* dauer germ line. Our data indicate that the ratios were significantly lower in the dauer germ line compared that of the *daf-2* adults (Fig. 3.1E). This confirms that in the quiescent dauer germ line, GLP-1 is no longer randomly distributed around the germ cell membrane. Instead it undergoes a dauer-dependent subcellular re-distribution to the rachis-adjacent membrane that coincides with the block in GSC divisions typical of the dauer germ line, while still delaying execution of the meiotic program.

Because the GSCs undergo Notch-dependent proliferation prior to dauer entry in both AMPK and LKB1 mutants (Narbonne and Roy 2006)), we tested whether the subcellular re-distribution of the GLP-1/Notch receptor might be perturbed in the absence of these protein kinases. We stained *daf-*

2; aak(0) and *daf-2; par-4* gonads extruded from dauer larvae and noted that GLP-1/Notch fails to re-localize to the rachis-adjacent membranes in these mutant dauer larvae and, alternatively, it is present around the cell membranes of all the germ cells like in non-dauer animals (Fig. 3.1C, D). This was further quantified by comparing the ratios of GLP-1 to PLC::mCherry, which were significantly higher when compared to *daf-2* mutant dauer germ lines (Fig. 3.1E). This suggests that LKB1 and AMPK regulate dauer GSC quiescence by triggering GLP-1 re-localization to the rachis-adjacent membranes of the GSCs. More importantly, this modification of the GLP-1 distribution within the GSCs correlates with the ability of GLP-1 to maintain a meiotic block while modulating cell proliferation of the GSCs in the dauer germ line.

If the re-localization of GLP-1 has any effect on Notch signalling in the dauer germ line, then one would expect that Notch targets that are involved in promoting proliferation should reflect this. To determine if Notch signalling is indeed active in the mutants that affect GLP-1 re-localization we performed semi-quantitative RT-PCR on *fbf-2* (Lamont, Crittenden et al. 2004), an RNA binding protein that binds to and blocks the translation of germline mRNAs and is one of the best characterized downstream targets of GLP-1 in the germ line. Consistent with the observed GSC cell cycle arrest during the dauer stage, the levels of *fbf-2* mRNA are lower in the quiescent dauer germ line compared to the proliferating germ line (Fig. 3.2A). However, the *fbf-2* levels in the LKB1/*par-4* and AMPK/*aak(0)* dauer larvae were significantly higher than control *daf-2* dauer larvae, and were comparable to those observed in the proliferating germ lines *of daf-2* adult hermaphrodites (Fig. 3.2A). Our data are consistent with a role of the observed re-localization of the Notch receptor in affecting its ability to activate mitotic divisions of the GSCs during dauer such that when GLP-1 is present around the germ cell membranes, the GSCs are proliferative, while meiosis remains blocked. However, when GLP-1 undergoes subcellular re-localization to

the rachis-adjacent membrane, only the meiotic function remains active, resulting in cells that arrest divisions but that do not differentiate to meiocytes.

GLP-1 is not required for meiotic arrest in the dauer germ line

That GLP-1 actively blocks meiosis during reproductive development has been convincingly shown, while we have shown that loss of *glp-1* during the dauer stage allows cells to enter meiosis where they arrest in pachytene of prophase I (Narbonne and Roy 2006). However, although our data suggest that, in addition to AMPK, *glp-1* is required to fully arrest meiotic progress during the dauer stage, our experiments do not address whether GLP-1 may be required at an earlier step prior to dauer entry, as animals were shifted to restrictive temperature early during the L1 stage. To address the role of GLP-1 in regulating the meiotic arrest typical of the dauer germ line, we disabled GLP-1 specifically during the dauer stage and monitored meiotic entry and the number of cells arrested in G2 phase at various points during dauer formation and during the duration of the dauer stage.

We therefore induced *daf-2, glp-1* to form dauer larvae using pheromone at permissive temperature then upshifted them to restrictive temperature for 48 hours to compromise *glp-1* function. We used HTP-3 as a marker to confirm if the GSCs enter meiosis. HTP-3 expression is diffuse in premeiotic nuclei and discernably enriched at the axes of meiotic chromosomes (Goodyer, Kaitna et al. 2008). Following the temperature shift during the L1 stage, germline proliferation is arrested and HTP-3 localizes along the chromosomal axes in most of the germ cells present in the dauer gonad, consistent with our previous observations that *glp-1* compromise will allow dauer germ cells to enter meiosis where they will arrest at pachytene (Fig. 3.2B). However, when *glp-1* is compromised specifically when the animals enter the dauer stage, HTP-3 retains its diffuse

expression pattern most likely because the GSCs do not form an axial element typical of meiotic cells (Fig. 3.2C). Although, we cannot rule out that some stable non-detectable level of GLP-1 persists following the temperature shift that may be sufficient to maintain the meiotic block, our data suggests that other regulators likely contribute to the meiotic arrest typical of the dauer germ line. Based on these observations and in light of our subcellular re-localization data, GLP-1 re-distribution correlates with a complete loss of GLP-1/Notch signalling in the dauer germ line.

AMPK phosphorylates GLP-1 to possibly recruit the *C. elegans* 14-3-3 protein, PAR-5, to target GLP-1 for re-localization

We and others have demonstrated that AMPK-mediated phosphorylation can generate 14-3-3 binding sites on its target proteins. Recognition of the phosphorylated target protein by cellular 14-3-3 proteins can affect numerous aspects of protein function including protein stability and/or its subcellular localization (Gwinn, Shackelford et al. 2008, Xie and Roy 2015). Since GLP-1 fails to re-localize in the LKB1/*par-4* and AMPK mutant dauer germ lines, we questioned whether AMPK could phosphorylate GLP-1 directly to signal its re-localization by recruiting a 14-3-3 binding protein. Consistent with this possibility we identified 3 potential AMPK phosphorylation sites that are conserved in *C. briggsae* and also in *Drosophila* Notch using a Group-based Prediction System (GPS) (Xue, Zhou et al. 2005) (Fig. 3.3A). Peptides containing these 3 sites were expressed as GST fusion proteins and used as substrates in an *in vitro* protein kinase assay to evaluate whether AMPK could phosphorylate these substrates directly. Only the peptide containing the conserved S1199 site was phosphorylated *in vitro* (Fig. 3.3B).

To confirm if AMPK-mediated phosphorylation at S1199 contributes to dauer germline quiescence, we mutated S1199 to a non-phosphorylable alanine and quantified the germ cell

numbers in transgenic mutant dauer germ lines. If this site is physiologically important to ensure germline quiescence during dauer following AMPK phosphorylation, then its elimination should phenocopy a compromise in AMPK. Interestingly, *daf-2* animals bearing this mutation (S1199A) exhibited mild dauer germline hyperplasia (Fig. 3.3C), consistent with this site contributing to the germ cell quiescence conferred by AMPK during the dauer stage. This finding suggests that AMPK regulates dauer germline quiescence, at least in part, by phosphorylating GLP-1 at S1199. There are 7 identified 14-3-3 binding proteins in humans but only 2 in *C. elegans,* namely PAR-5 and FTT-2. FTT-2 is specific to the soma and plays an important role in controlling FoxO function, while PAR-5 is the only 14-3-3 binding protein expressed in the germ line. (Aristizabal-Corrales, Fontrodona et al. 2012). If AMPK phosphorylation recruits a 14-3-3 binding protein to mediate the re-localization of GLP-1 in the dauer germ line, compromising the function of PAR-5 in the dauer larvae should phenocopy the hyperplasia observed in the germ line of *aak(0)* mutant dauer larvae. To confirm this, *daf-2* animal were treated with *par*-5(RNAi) prior to dauer formation. We observed a significant increase in the number of germ cells in *daf-2*; *par-5*(RNAi) dauer larvae consistent with it acting in the regulation of GSC quiescence during the dauer stage (Fig 3.4A). Also, compromise of PAR-5 in the AMPK mutants failed to enhance the number of germ cells, suggesting that PAR-5 acts with AMPK in a linear pathway (Fig 3.4A). To confirm if PAR-5 is crucial for the re-localization of GLP-1 during the dauer stage GLP-1 localization was monitored in *par-5*(RNAi) treated dauer germ line. Similar to the AMPK and PAR-4 mutants, GLP-1 failed to re-localize to the rachis-adjacent membrane of the dauer GSCs upon compromising the function of PAR-5 (Fig, 3.4B, C). Overall, these data suggest that AMPK phosphorylates GLP-1 directly and the phosphorylated version of GLP-1 is further possibly recognized by PAR-5 to ultimately

change GLP-1 localization, thereby temporarily blocking GSC proliferation during the dauer diapause.

ani-2 suppresses the dauer germline hyperplasia and the temporal defect in actin organization in the *par-4* mutants

The hyperplasia that occurs in the dauer germ line is more severe in *par-4* mutants than in *aak(0)* animals suggesting that PAR-4 may have alternative targets independent of AMPK that may contribute to the supernumerary GSC divisions when misregulated (Narbonne and Roy 2006, Kadekar, Chaouni et al. 2018).

The germ cells share a common cytoplasm and are connected to each other via intercellular bridges. ANI-2 is a scaffold protein that localizes to these bridges within the germ line where it remains throughout the duration of gonad development, as it is required for the stability of these structures (Maddox, Habermann et al. 2005). In the early embryo ANI-2 has been demonstrated to regulate the organization of the actin cytoskeleton and is dependent on the activity of PAR-4 (Chartier, Salazar Ospina et al. 2011, Amini, Goupil et al. 2014). ANI-2, a homologue of the actomyosin scaffold protein Anillin, is required for appropriate localization of centralspindlin, composed of the subunits ZEN-4 and CYK-4, which recruits and regulates proteins that modulate the actin cytoskeleton to promote the formation and the progression of the cleavage furrow during cytokinesis (Zhou, Rolls et al. 2013). Recently, genome-wide screens identified *zen-4* and *cyk-4,* to significantly reduce *par-4*-dependent hyperplasia, but failed to suppress the defect in *aak(0)* dauer larvae (Kadekar, Chaouni et al. 2018). To determine if *ani-2* might also suppress the *par-4*-dependent dauer germline hyperplasia in a manner that is independent of AMPK, we performed *ani-2*(RNAi) in both *par-4* and *aak(0)* L4 larvae and monitored germ cell counts in their progeny

following dauer induction. We found that *ani-2* suppressed the dauer germline hyperplasia exclusively in *par-4* mutant dauer larvae (Fig 3.5A). This data further supports an AMPK-independent role for PAR-4 through its direct or indirect regulation of *ani-2* and centralspindlin complex to establish dauer GSC quiescence.

PAR-4 is required for temporal regulation of actin cytoskeleton at the rachis-adjacent membrane in the dauer germ line (Kadekar, Chaouni et al. 2018) and in the early embryo PAR-4 impinges on ANI-2 to regulate actin cytoskeleton (Chartier, Salazar Ospina et al. 2011). We therefore questioned whether PAR-4 could block ANI-2 to modify the actin cytoskeletal network of the GSCs so that it is incompatible with cell proliferation during the dauer stage. Curiously, *ani-2* was also found to suppress the observed temporal defect in the actin organization in the *par-4* mutants, as actin filaments are organized at the rachis-adjacent membrane (Fig. 3.5B). These data suggest that *par-4* negatively regulates *ani-2* to maintain actin organization at the rachis bridge, which may contribute to the establishment of quiescence in the dauer germ line, independently of AMPK.

PAR-4 is required to maintain ANI-2 localization and actomyosin contractility at the rachis bridge

Since *par-4* regulates the cytoplasmic localization of the scaffold protein ANI-2 in the early *C. elegans* embryo to maintain actomyosin contractility (Chartier, Salazar Ospina et al. 2011), we questioned whether *par-4* may also affect the localization of ANI-2 at the rachis-adjacent membrane to ensure correct actin organization during the onset of dauer. To test this, we monitored ANI-2 expression in the germ cells of control *daf-2* and *daf-2; par-4* dauer larvae at different time points following the upshift to induce dauer formation. If *par-4* affects ANI-2 localization during the onset of dauer then following the shift to restrictive temperature, the misregulated ANI-2

should remain evenly distributed on the cell membranes rather than accumulating preferentially at the rachis-adjacent boundary. Our data are consistent with this regulatory relationship, whereby the normal localization of ANI-2 at the rachis-adjacent membrane typical of *daf-2* dauer larvae is perturbed, as its localization is not restricted to the rachis-adjacent membrane in the *par-4* dauer germ line (Fig. 3.5C). This result suggests that *par-4* is required to maintain the appropriate localization of ANI-2 at the intercellular bridges in the dauer germ line to regulate actin organization. In the absence of *par-4*, ANI-2 becomes mislocalized and might contribute to the compromise of the establishment and/or the maintenance of GSC quiescence during dauer development.

Our results suggest that PAR-4 activity is required to regulate ANI-2 localization and its loss results in abnormal actin cytoskeletal organization and possibly actomyosin contractility within the dauer germ line since the loss of PAR-4 function compromises actomyosin contractility in the early embryos (Chartier, Salazar Ospina et al. 2011). To test if actomyosin contractility is required to block unscheduled cell divisions, we compromised the function of *nmy-2* by introducing a temperature sensitive allele of *nmy-2* in the *daf-2* background and quantified the number of germ cells in the dauer germ line. Curiously, by the disrupting the activity of *nmy-2,* and presumably actin contractility, the germ cells undergo extra cell divisions and result in mild germline hyperplasia (Fig. 3.5D). This preliminary data suggests that PAR-4 may regulate actomyosin contractility at the rachis bridge in *ani-2* and *nmy-2* dependent manner to block abnormal cell divisions.

Discussion

C. elegans can adapt to environmental stress during their early development by making a short term fitness trade off by opting out of its reproductive life cycle to enter a highly stress-resistant, quiescent dauer stage (Cassada and Russell 1975). During this diapause stage, all somatic and germline cell divisions are arrested, as is the progression through larval development. Previous studies have demonstrated the role of LKB1 and AMPK signalling in mediating dauer germline quiescence (Narbonne and Roy 2006). Although AMPK is a well-characterized downstream target of the LKB1/PAR-4 kinase, genetic analyses suggest that PAR-4 likely regulates AMPK-independent pathway/s to arrest germ cell divisions upon dauer entry (Narbonne and Roy 2006, Kadekar, Chaouni et al. 2018). These AMPK-independent pathways/s have not been characterized beyond genetic analysis, but it is likely that the polarity regulatory properties of LKB1/PAR-4 may contribute significantly to the establishment or maintenance of the germline quiescence that occurs during the dauer stage.

In the developing germ line, active Notch signalling plays a crucial role to maintain germ cell proliferation, while simultaneously blocking them from executing their meiotic program (Kimble and Crittenden 2007). We show that in the quiescent dauer germ line, GLP-1/Notch undergoes re-localization from its position around the germ cell membranes to the rachis-adjacent membrane (Fig. 3.1A, B). The significance of this subcellular re-localization is unclear but it correlates with the modulation of Notch signalling, as the expression of *fbf-2,* one of the downstream Notch targets associated with germ cell proliferation, is significantly reduced in *daf-2* dauer larvae. Although the reduction in germ cell divisions typical of *daf-2* dauer larvae correlates with the down-regulation of the Notch signalling, unlike *glp-1(lf)* mutants, the germ cells do not initiate their meiotic program. Therefore, the ability of Notch to enhance

proliferation is inhibited while in this new cellular location, but the germ cells do not execute meiosis, suggesting that Notch function may still be active. This separation of Notch function is unlikely, however. By compromising Notch function specifically during the dauer stage, germ cells still retain the G2/M phase arrest and fail to enter meiosis, contrary to what is observed when GLP-1 is compromised in early L1 larvae. These data support a role for additional regulators that, during the conditions of energetic stress typical of dauer, are sufficient to block meiotic differentiation in a manner that is independent of Notch/GLP-1 (Fig. 3.2B, C).

Recently, genome-wide RNAi screens indicated that compromising GLP-1/Notch function could restore quiescence in the dauer GSCs in LKB1/AMPK mutants, suggesting that misgregulation of Notch in this mutant background may be partially responsible for the unscheduled cell divisions. When we examined GLP-1/Notch localization in both PAR-4 or AMPK mutant dauer germ lines we noted that GLP-1/Notch does not re-localize and is still present around all the cell membranes (Fig. 3.1A, B). Furthermore, the levels of *fbf-2* expression in these mutants are comparable to those observed in the adult proliferating germ line (Fig.3.2A)

Because GLP-1/Notch possesses consensus AMPK phosphorylation sites we wondered if it might be a direct kinase target downstream of AMPK activation during the onset of dauer formation. We showed that AMPK phosphorylates a GLP-1 peptide containing S1199 *in vitro* and mutating S1199 to a non-phosphorylable alanine, partially recapitulates the dauer germline hyperplasia typical of *par-4* and AMPK mutants. This suggest that under energetic stress AMPK presumably phosphorylates GLP-1 at S1199 to inhibit the Notch signalling cascade and establish germline quiescence during the dauer stage.

Phosphorylation can act as a functional trigger that can be read by effectors to initiate a multitude of downstream cellular responses. AMPK phosphorylation often generates 14-3-3 binding sites on its substrates followed by recruitment of a 14-3-3 binding protein (Gwinn, Shackelford et al. 2008, Ahmadian, Abbott et al. 2011, Xie and Roy 2015). The recruitment of a 14-3-3 binding protein can result in changes in subcellular localization, structure and protein abundance, enzymatic activity…etc (Obsilova, Kopecka et al. 2014). Our data suggests that AMPK phosphorylation of GLP-1 on S1199 possibly recruits the *C. elegans* 14-3-3 binding protein PAR-5, to modulate GLP-1 re-localization. In its absence, GLP-1 fails to re-localize to the rachis-adjacent membrane which correlates with supernumerary germ cells in the dauer germ line (Fig. 3.4A-C). Our recent genome-wide screens identified a subset of genes involved in endocytic sorting to regulate dauer germline quiescence (Kadekar, Chaouni et al. 2018). This raises the possibility that Notch re-localization might require the endocytic machinery. Although we are currently testing this possibility, it is consistent with the studies in mammals, where depletion of LKB1 activates Notch signalling in AMPK-dependent manner (Shan, Zhang et al. 2016).

Although the S1199A modification and the *par-5*(RNAi) experiments both indicate that the subcellular GLP-1 localization is important for germline quiescence during dauer, they cannot fully account for the extensive germline hyperplasia we observe in AMPK null mutants or in *par-4* mutants. This is most likely because other regulators that are targeted by these protein kinases act collectively to mediate dauer germline quiescence.

Depletion of both PAR-4 and AMPK enhances dauer germline hyperplasia significantly, suggesting that the genes do not work in a simple linear pathway to establish or maintain germline quiescence. Our genome-wide screens also identified a subset of genes involved in

the regulation of the cytoskeleton and cell polarity that exclusively suppress the *par-4*-dependent proliferation, with no effect on the germline hyperplasia in the *aak(0)* dauer larvae (Kadekar, Chaouni et al. 2018). This subset includes conserved components of centralspindlin (CYK-4, ZEN-4), a complex that regulates central spindle assembly and cytokinesis in metazoans and is also required to maintain germ line architecture in *C. elegans* (Glotzer 2009, Zhou, Rolls et al. 2013). ANI-2 is a scaffold protein that localizes to the rachis and is required to dock CYK-4 and maintain the integrity of the rachis bridge. (Maddox, Habermann et al. 2005, Zhou, Rolls et al. 2013, Amini, Goupil et al. 2014). Moreover, it is regulated in a PAR-4-dependent manner to regulate actomyosin contraction in at the cell cortex in early *C. elegans* embryo (Chartier, Salazar Ospina et al. 2011).

Due to its essential role during embryogenesis, ANI-2 was not isolated in our screens, but we explored its role in a dauer context since it not only regulates CYK-4 localization, but it is also subject to *par-4*-dependent regulation. Consistent with our results from our genome-wide suppressor screens (Kadekar, Chaouni et al. 2018), loss of *ani-2* suppressed *par-4*-dependent germline hyperplasia, while it also restored the temporal organization of the actin filaments at the rachis-adjacent membrane in *par-4* mutants (Kadekar, Chaouni et al. 2018). Furthermore, we show that PAR-4 is required to specify the localization of ANI-2 at the rachis bridge as in *par-4* mutants, ANI-2 is aberrantly localized with the dauer germ line (Fig 3.5A-C).

Based on its antagonistic association with NMY-2 in the early embryo (Chartier, Salazar Ospina et al. 2011), it is plausible that PAR-4 regulates ANI-2 to promote actin contractility upon dauer formation. Consistent with this hypothesis we show that the compromise of *nmy-2,* and presumably actomyosin contractility in *daf-2* animals, also results in supernumerary germ cells in the dauer germ line (Fig. 3.5D).

How might the actomyosin contraction present at the rachis bridge impinge on germ cell proliferation? In general, the physical properties of the cytoskeleton can affect the cellular functions and therefore can contribute to establish quiescence (Guilak, Cohen et al. 2009). As we show that upon dauer entry, GLP-1 gets disappeared from around the germ cell membranes and is re-localized to the rachis, one possibility is that 'basally' localized actomyosin contraction could set up a polarity or a cellular orientation that might be important in guiding this process of asymmetric distribution. Understanding how GLP-1 re-localization is affected in the mutants with compromised actomyosin contractility, can provide us further insights about Notch regulation. Considering the antagonistic relationship between ANI-2 and NMY-2, it is imaginable that the contractile ring at the rachis is in relaxed and stable state in the presence of ANI-2. This relaxed state is essential for normal germline development, and facilitates the maturation of oocytes (Zhou, Rolls et al. 2013). The other possibility is that upon dauer entry, PAR-4 impinges on ANI-2 to inhibit its activity and promote the contractility at the rachis bridge to inhibit the germ cell proliferation

Overall, our findings provide evidence that PAR-4 regulates at least two independent pathways to deter germ cell divisions when the energy resources are limited (Fig. 3.6). This may be significant in the aetiology of PJS, where the protein kinase function of LKB1 is critical for its tumour suppression function. The inappropriate regulation of several kinase substrates, that include AMPK, probably function to maintain tissue homeostasis collectively, and loss of any single branch of these kinase networks could contribute to, but cannot fully account for the tumourigenic phenotype associated with LKB1 mutations. Using *C. elegans* we have shown that the role of LKB1 in the regulation of cell polarity and its effects on cytoskeletal regulators and actomyosin contractility may be an equally important contributor to stem cell divisions.

While AMPK may also impinge on other targets, like Notch, that act independent of the TOR signalling pathway, all of which together constitute the tumour suppressive function of LKB1.

Materials and Methods

C. elegans genetics

All nematode strains were maintained at 15°C and grown on standard NGM plates seeded with E. coli (OP50) (Brenner 1974), unless otherwise stated. Following strains, alleles and transgenes were used, CB1370 [*daf-2 (e1370) III*], MR1000 [*daf-2(e1370) aak-1(tm1944) III; aak-2(ok524) X*], MR593 [*daf-2(e1370); par-4(it57)*], MR548 [*daf-2(e1370) glp-1(e2141) III*]. MR2137 [*daf-2(e1370) aak-1(tm1944) III; aak-2(ok523) X; ltIs4 [unc-119(+)Ppie1::plc::mCherry]*], MR2138 [*daf-2 (e1370) III; ltIs44[unc-119(+)Ppie1::plc::mCherry]*], MR2139 [*daf-2 (e1370) III; par-4(it57); ltIs44[unc-119(+)Ppie1::plc::mCherry]*], MR2036 [*daf-2(e1370) III; par-4(it57) V; orIs20[unc-119(+)Ppie1::gfp::moesin]*]

RNAi Feeding

Bacterial clones expressing dsRNA from the RNAi library were grown in LB medium with ampicillin at 37°C overnight. The bacterial culture was seeded onto regular NGM plates containing ampilicilin and IPTG. Seeded plates were incubated at room temperature for 24 hours to induce dsRNA expression. L4 larvae were fed on the RNAi plates and were allowed to lay eggs at 15°C and then the eggs were switched to 25°C to induce dauer.

DAPI staining and germ cell nuclei count

For whole worm DAPI (4',6-diamidino-2-phenlindole) staining, dauer larvae were washed off plates and soaked in Carnoy's solution (60% ethanol, 30% acetic acid, 10% chloroform) on a shaker overnight. Animals were washed twice in PBST (1XPBS + 0.1% Tween 20), and stained in 0.1 mg/ml DAPi solution for 30 minutes. Finally, larvae were washed four times (20 minutes

each) in PBST, and mounted in Vectashield medium. The total number of germ cell nuclei per dauer gonad was then determined based on their position and nuclear morphology.

Immunostaining

For extruded dauer gonad staining, gonads were dissected, fixed and stained as described elsewhere (Arduengo, Appleberry et al. 1998). Following primary anti-bodies were used, rabbit polyclonal anti-GLP-1 (1:50), rabbit anti-ANI-2(1:1000), guniea pig anti-HTP-3(1:600), mouse anti-mCherry (1:200). Secondary antibodies were Alexa Fluor 488-coupled goat anti-rabbit, Alexa Fluor 555-coupled donkey anti-mouse, Alexa Fluor 555-coupled donkey anti-guniea pig (1:500 ; Molecular probes). Microscopy was performed as described in (Kostic, Li et al. 2003)

Semi quantitative RT PCR

Expression analyses of *fbf-2* in different genotypes was conducted by semi-quantitative RT PCR. Total RNA was isolated from ~300 dauer larvae for each of the tested genotypes using Trizol. RT reactions were performed with 200 ng RNA and MLV reverse transcriptase (NEB) in 20 µl total volume and the resulting cDNA was used to perform PCR using *fbf-2* specific primers. 35 PCR cycles were performed and amplified products were resolved on 2% agarose gels and visualized by ethidium bromide staining. Relative levels were quantified using ImageJ and were normalized to *tba-1* as internal loading control.

GST fusion protein production

To perform *in vitro* kinase assay, we generated three bacterially expressed fragments of GLP-1, each containing a predicted AMPK phosphorylation site. The different GLP-1 fragments were amplified by PCR from cDNA with the following primers:

S185 F: GATC CCCGGG GAAGGAATCGATCATTGTGCTC

R: GATC GCGGCCGC GTATCCCGAGTCGCATACAC

S488 F: GATC CCCGGG TCCAGACATCCATGCAAGAACG

R: GATC GCGGCCGC TTCCAAATTGCATTCCGGGTTG

S1199 F: GATC CCCGGG CTTCGCCAAGTGGCAAAC

R: GATC GCGGCCGC TGAGAACGATCCATCAGACG

PCR fragments were then cloned into pGEX 6P-3 (Promega) between Xma1 and Not1. BL21 bacteria were transformed and cultured overnight. IPTG at 0.1mM was added to induce peptide production at 37^0C for 3 hours. Then proteins were purified by standard protocol for GST fusion protein purification.

AMPK *in vitro* kinase assay

Kinase assay was performed by using 0.06 U (20ng) per reaction of purified human active AMPK (Millipore cat# 14-840) and ~1.5µg of purified GST peptides at 30^0C for 15 minutes. Then the reaction was subjected to SDS-PAGE and autoradiography. The phosphorylation sites were further assessed by immunoblotting with rabbit anti-phospho-Ser binding motif antibody (NEB).

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Figures

Figure 3.1. *par-4* and *aak(0)* regulate GLP-1 re-localization to the rachis-adjacent membrane in the *daf-2* dauer germ line.

All animals analyzed harbour a *Ppie-1::*PLC::mCherry transgene as a germ cell membrane marker. The images in the left panel are merged, condensed Z stacks. A) In a *daf-2* adult proliferating germ line, GLP-1 is present around the membrane of the mitotic germ cells (white arrow head). B) In the *daf-2* dauer germ line, GLP-1 re-localizes to the rachis-adjacent membrane of the germ line (white arrow). C, D) In the dauer germ line of *par-4* and *aak(0)* larvae, GLP-1 fails to re-localize and remains equally distributed around the membrane of the germ cells (white arrowhead). E) Quantification of GLP-1 levels normalized to the PLC::mCherry using Image J. n=20 different animals. ***$p<0.0001$ when compared to *daf-2* adult, *par-4* and *aak(0)* dauer larvae using one way ANOVA. Scale bar for A: 10 μm and for others scale bar: 4μm.

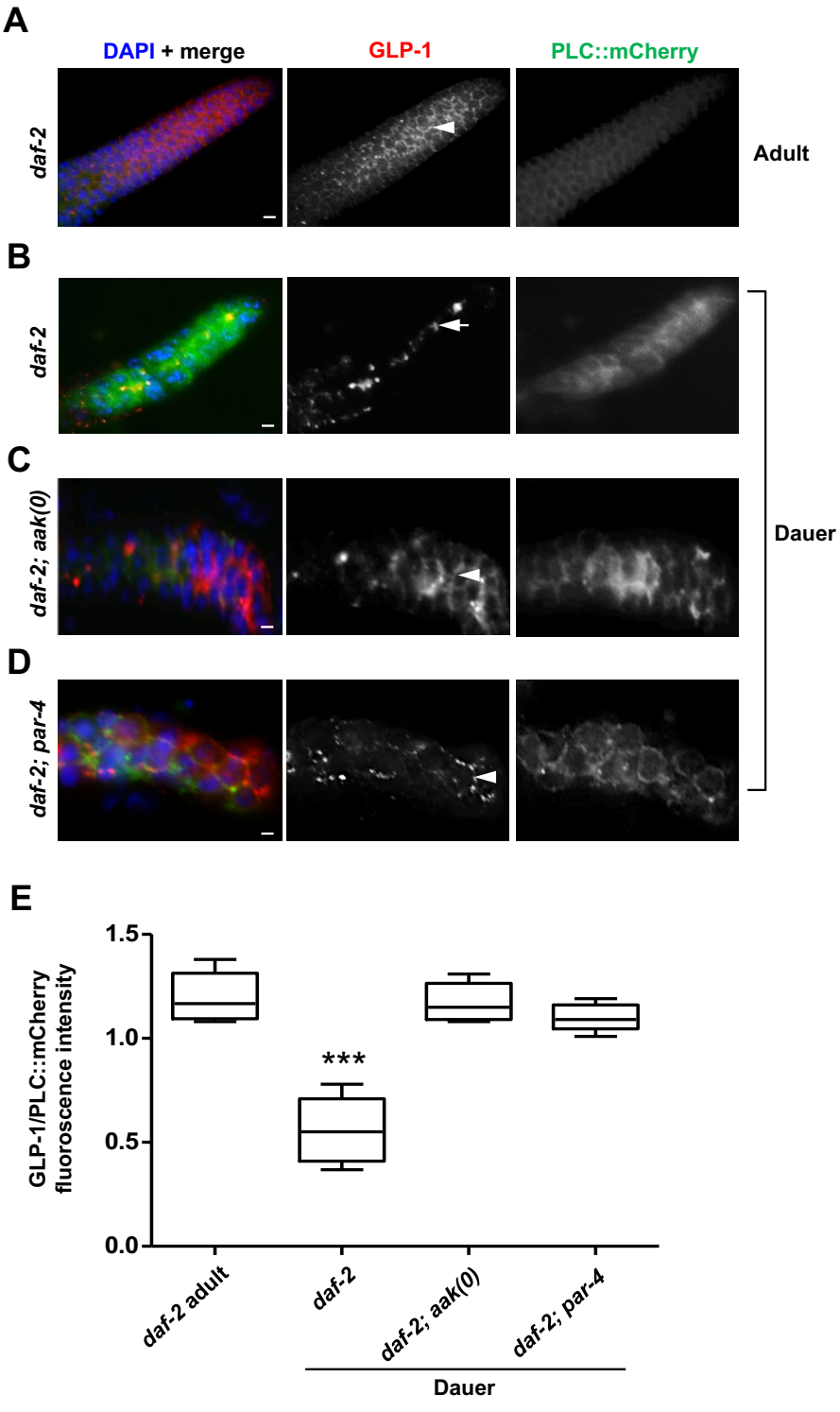

A
DAPI + merge
GLP-1
PLC::mCherry
daf-2
Adult
B
daf-2
C
daf-2; aak(0)
D
daf-2; par-4
Dauer
E
GLP-1/PLC::mCherry fluoroscence intensity
1.5
1.0
0.5
0.0
***
daf-2 adult
daf-2
daf-2; aak(0)
daf-2; par-4
Dauer

Figure 3.2. Notch signalling is reduced in the dauer germ line, while additional GLP-1 - independent mechanisms block meiotic progression in dauer larvae.

A) Semi-quantitative RT-PCR was performed and levels of *fbf-2* were normalized to tubulin and quantified using Image J software. RNA was isolated from *daf-2* adults, 1-day old *daf-2*, *par-4* and *aak(0)* dauer larvae. *daf-2; glp*-1dauer larvae were used as a control. B) In *daf-2; glp-1* dauer germ line, where GLP-1 is compromised following upshift to restrictive temperature during the L1 stage, HTP-3 is localized along the chromosomal axes in the germ cells (white arrowhead) as in the meiotic cells and thus in the L1 larva, GLP-1 function is required to promote mitosis and to establish meiotic arrest. C) In the *daf-2; glp-1* germ line, GLP-1 is compromised only after the larvae enter the dauer stage. HTP-3 is localized in a diffused pattern within the nuclei of the germ cells (white arrow) as in the mitotic cells and thus in the dauer state. n=15; Scale bar: 4μm.

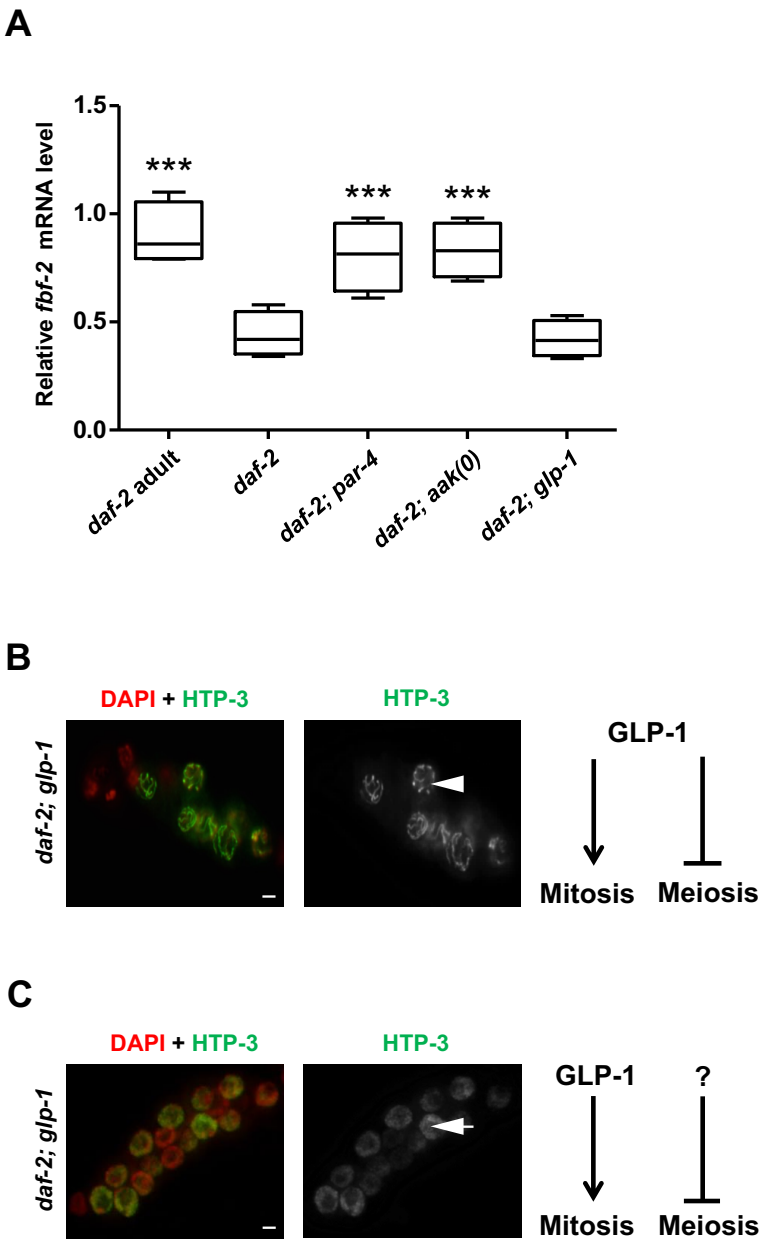

A
Relative fbf-2 mRNA level
1.5
1.0
0.5
0.0
***
***
***
daf-2 adult
daf-2
daf-2; par-4
daf-2; aak(0)
daf-2; glp-1
B
DAPI + HTP-3
HTP-3
daf-2; glp-1
GLP-1
Mitosis
Meiosis
C
DAPI + HTP-3
HTP-3
daf-2; glp-1
GLP-1
?
Mitosis
Meiosis

Figure 3.3. AMPK phosphorylates GLP-1 at S1199 which contributes to dauer germline quiescence.

A) A consensus AMPK phosphorylation sequence was identified at residues S185, S488 , and S1199 (red). B) 3 GLP-1 peptides containing conserved AMPK phosphorylation consensus sites were used in an *in vitro* kinase assay. ATGL was used a positive control (See materials and methods for details. C) Whole animal DAPI staining was performed to quantify the number of dauer germ cells in *daf-2* animals expressing a non-phosphorylable S1199A variant. Statistical analysis was performed using the two-tailed t-test when compared to *daf-2* animals where ** $P<0.001$; $n=100$.

A

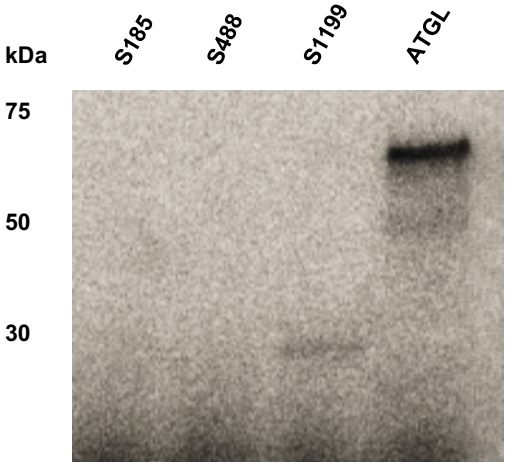
Optimal AMPK motif L R R V x S x x N L
Secondary Selection M K K S x S x P D E

```
C. elegans          VYNYYCDCPIGKSGRYCER--TECALM  197
C. briggsae         VNGYICACPPGRGGAFCEI--TNCTLM  254
D. melanogaster     ISDYTCRCPPNFTGRFCQDDVDECAQR  338
                    : .* * ** .  * :*:     *:

C. elegans          YGYTGPTCEEVLVI--------------EKSKETVIRDLC  497
C. briggsae         YGYTGTRCQEK-VI--------------DKSKEIMFRELC  560
D. melanogaster     SKWKGKRCDIYDANYPGWNGGSGSGNDRYAADLEQQRAMC  1482
                      :.*  *:   .                :.    * :*

C. elegans          GRQTVKNIK-------------------------------RAGSRKTPTSAASSRETNHL  1215
C. briggsae         GRQTMKKVK-------------------------------RNGSKKTP----MIQETNHL  1250
D. melanogaster     GNASGKQSNQTAKQKAAKKAKLIEGSPDNGLDATGSLRRKASSKKTSAASKKAANLNGL  2245
                    *. : *: :                                : .*:**.       : *
```

B



^{32}P autoradiogram

C

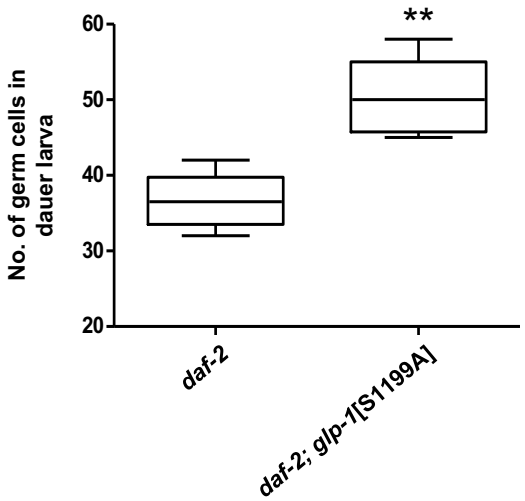
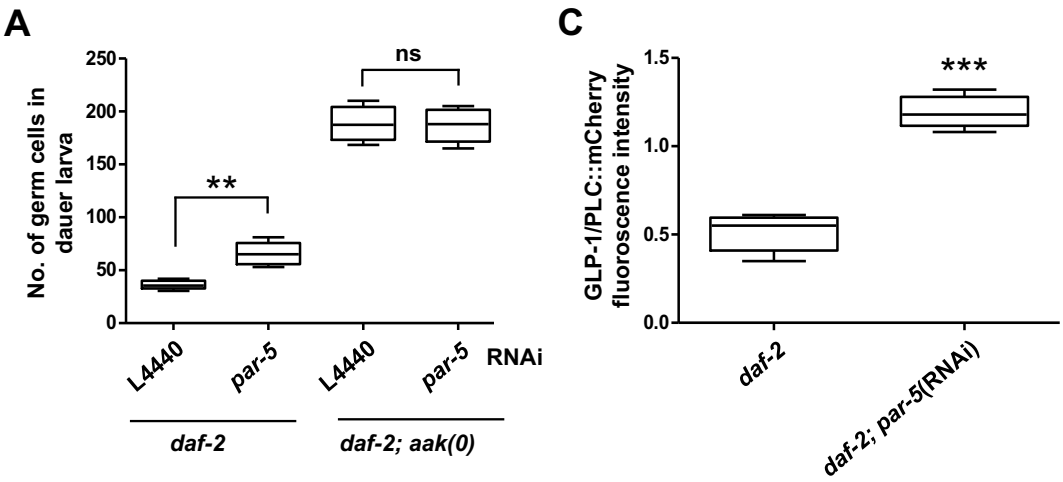


Figure 3.4. Compromise of the 14-3-3 binding protein PAR-5 exhibits germline hyperplasia and GLP-1 mislocalization in *daf-2* dauer larvae.

A) Whole animal DAPI staining was performed to quantify the number of dauer germ cells in the *daf-2* and *daf-2; aak(0)* animals following *par-5*(RNAi) treatment. Statistical analysis was performed using the two-tailed t-test. ** P<0.001 and ns= non-significant n=100. B) All the animals analyzed carry *Ppie-1::*PLC::mCherry transgene as a germ cell membrane marker. All the images in the left panel are merged, condensed Z stacks. GLP-1 fails to re-localize to the rachis-adjacent membrane in *par-5* compromised *daf-2* dauer larvae (white arrowhead). C) Quantification of GLP-1 levels normalized to the PLC::mCherry using Image J software. ***P<0.0001 when compared to *daf-2* via one-way ANOVA; n=25. Scale bar: 4μm.


A
No. of germ cells in dauer larva
250
200
150
100
50
0
**
ns
L4440
par-5
L4440
par-5
RNAi
daf-2
daf-2; aak(0)
C
GLP-1/PLC::mCherry fluoroscence intensity
1.5
1.0
0.5
0.0
***
daf-2
daf-2; par-5(RNAi)

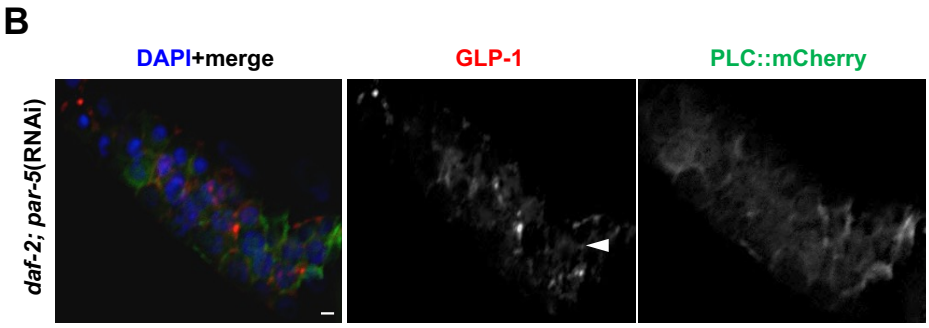

B
DAPI+merge
GLP-1
PLC::mCherry
daf-2; par-5(RNAi)

Figure 3.5. *ani-2* suppresses *par-4*-dependent germline hyperplasia and temporal actin organization defects.

A) Whole animal DAPI staining was performed to quantify the number of dauer germ cells in the *daf-2; par-4* and *daf-2; aak(0)* animals following *ani-2*(RNAi) treatment. Statistical analysis was performed using the two-tailed t-test. ** P<0.001 and ns= non-significant n=100. B) All the animals tested carry *Ppie1*::MOE::GFP transgene to visualize actin filaments. All the images in the left panel are merged, condensed Z stacks acquired following control or *ani-2*(RNAi) in the *par-4* dauer germ line at 48 hours after shifting to the restrictive temperature (25^0C) (white arrow). C) All the images in the left panel are merged, condensed Z stacks. In *daf-2* dauer germ line at 48 hours after shifting to the restrictive temperature (25^0C), ANI-2 is present at the rachis-adjacent membrane (white arrow). ANI-2 localization is perturbed in the *par-4* dauer germ line at 48 hours after shifting to the restrictive temperature of 25^0C (white arrowhead). Scale bar: 4μm. D) Whole animal DAPI staining was performed to quantify the number of dauer germ cells in the *daf-2; nmy-2* dauer larvae 48 hours after shifting to the restrictive temperature (25^0C). Statistical analysis was performed using the two-tailed t-test. ** P<0.001 n=100.

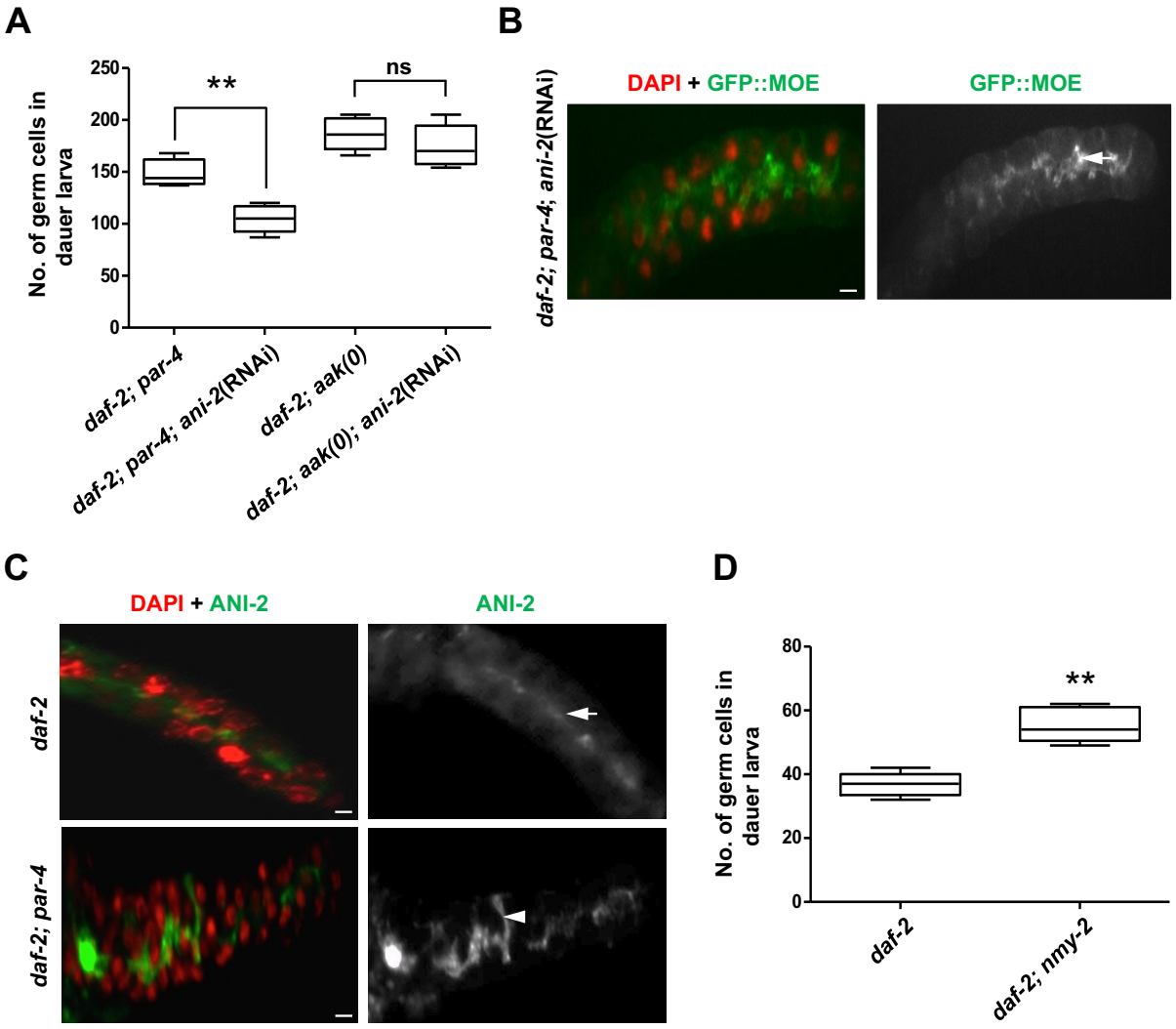
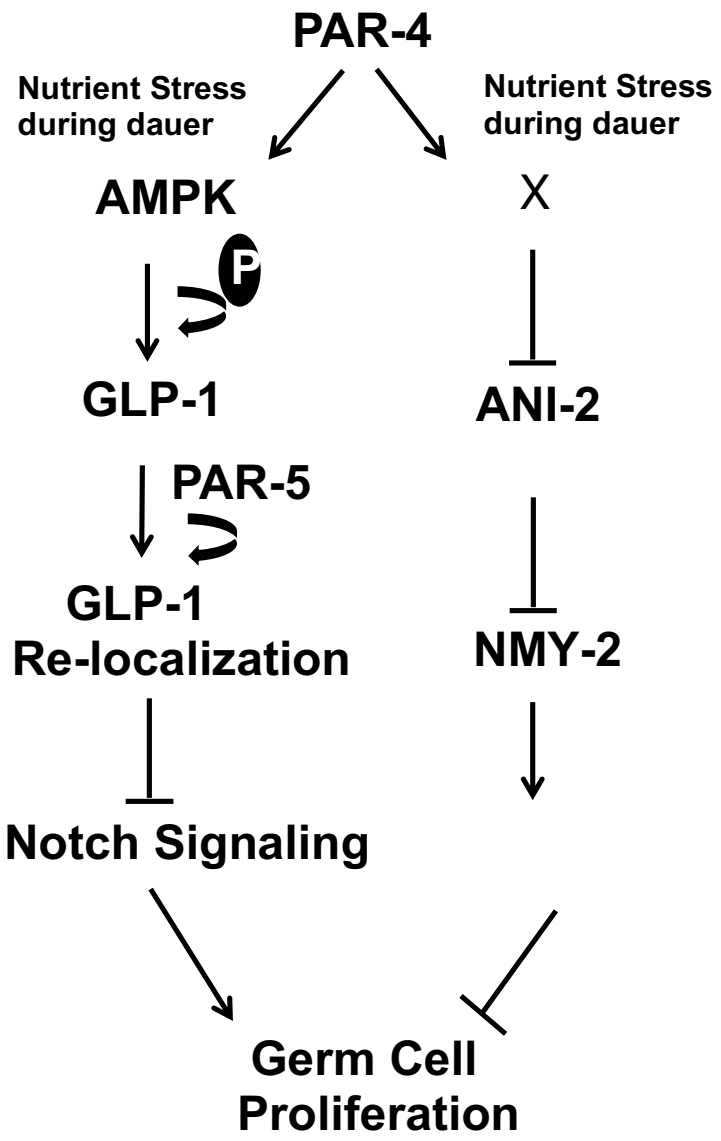

A
No. of germ cells in dauer larva
250
200
150
100
50
0
**
ns
daf-2; par-4
daf-2; par-4; ani-2(RNAi)
daf-2; aak(0)
daf-2; aak(0); ani-2(RNAi)
B
DAPI + GFP::MOE
GFP::MOE
daf-2; par-4; ani-2(RNAi)
C
DAPI + ANI-2
ANI-2
daf-2
daf-2; par-4
D
No. of germ cells in dauer larva
80
60
40
20
0
**
daf-2
daf-2; nmy-2

Figure 3.6. Model depicting PAR-4 mediated AMPK-dependent and -independent mechanisms to establish dauer germline quiescence.

Under energetic stress, PAR-4 acts as a major regulator to mediate dauer germline quiescence and acts in AMPK-dependent and -independent manner. Arrows and bars represent positive and negative genetic interactions, respectively.


PAR-4
Nutrient Stress during dauer
Nutrient Stress during dauer
AMPK
X
P
GLP-1
ANI-2
PAR-5
GLP-1 Re-localization
NMY-2
Notch Signaling
Germ Cell Proliferation

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Connecting statement: Bridging chapter 3 to chapter 4

After elucidating the potential downstream mechanism of LKB1 and AMPK signalling cascade to establish dauer germ cell quiescence, I was perplexed with new questions and wanted to study the consequence of these untimely germ cell divisions. In the following chapter, I performed experiments to understand the role of AMPK signalling in maintaining the germ cell integrity under energetic stress. This analysis provides novel insights about the role of AMPK in regulating the chromatin landscape and the small RNA pathway to ensure appropriate gene expression in response to the environmental conditions that induce dauer development.

Chapter 4: AMPK regulates germline stem cell quiescence and integrity through its effects on a small RNA pathway

Pratik Kadekar and Richard Roy

Manuscript prepared for submission to elife

Abstract

C. elegans larvae can undergo a global developmental arrest following the execution of a diapause-like state called 'dauer' in response to unfavourable growth conditions. Germline stem cell (GSC) quiescence in the dauer state requires the activity of AMPK and its upstream activator LKB1 (*par-4*). Survival in this quiescent state surpasses the normal lifespan of reproductive animals quite dramatically, and without any apparent negative impact on their reproductive fitness. Conversely, AMPK mutant animals exhibit complete sterility after dauer exit, suggesting that germ cells lose their integrity during dauer in the absence of AMPK. The germ lines of these animals are disorganized and they fail to make functional gametes. Partial rescue of the post-dauer sterility could be achieved by compromising the function of two major upstream effectors of the small interfering RNA pathway (*dcr-1* and *rde-4*), as well as the primary Argonaute protein *ergo-1*. Disruption of these gene products also partially suppressed the germline hyperplasia in AMPK mutant dauer larvae. Small RNAs are well known to regulate gene expression by affecting histone mark deposition at the chromatin level, and in AMPK mutant dauer larvae, the H3K4me3 and H3K9me3 chromatin marks are both increased and aberrantly distributed in the germline, consistent with the misrelated RNA. The higher levels of H3K4me3 and H3K9me3 persist and fail to get resolved in the PD adult germ line resulting in abnormal germline gene expression. These changes may require a cell non-autonomous effect of small RNAs since the PD sterility is partially restored in animals that lack the dsRNA importer SID-1 while AMPK expression in the neurons and the excretory system is sufficient to restore fertility in the AMPK mutant PD adults. Collectively, our data suggest that AMPK regulates a small RNA pathway, perhaps in a germline non-autonomous manner, to establish and/or maintain GSC quiescence and integrity in response to the energy stress associated with the dauer stage. Our findings provide a model to understand

how the soma communicates with the germ line to initiate the appropriate epigenetic modifications required to adapt to acute environmental challenges

Introduction

It is becoming more widely accepted that life history can potentially affect developmental and behavioural outcomes, either in a temporary, or often in a more permanent manner. These modifications can occur downstream of a broad spectrum of environmental factors, including temperature, light, resource availability, population density, and even the presence of predators; all of which can influence gene-expression, often with phenotypic consequences (Gilbert 2001, Lee, Bussiere et al. 2013). Furthermore, these consequences are not restricted to the generation that experienced the event, but rather, they can be transmitted to subsequent generations.

Studies have shown that the molecular record of these events is encoded in the form of epigenetic changes associated with histone modifications, DNA methylation and/or base modification, or alterations in the small RNA repertoire (Bonasio, Tu et al. 2010). Because the transmission of these molecular memories can span one or several generations, these modifications must impinge in some way upon the germline, thus providing some adaptive phenotypic change in the unexposed future generations (Sharma 2013, Rechavi, Houri-Ze'evi et al. 2014, Demoinet, Li et al. 2017, Klosin, Casas et al. 2017) . These epigenetic modifications of the germ cells can have a significant impact on successive generations, yet the molecular mechanisms through which “experience" is transduced to the genome across several generations remain elusive.

C. elegans has been used successfully to demonstrate how environmental cues can modulate epigenetic change and behaviour (Kelly 2014). Furthermore, a subset of these modifications and associated traits can be transmitted to subsequent generations in a manner dependent on small heritable RNAs (Remy 2010). Recently, it was shown that acute starvation at the L1 larval stage leads to the generation of small RNA species that are inherited for at least three generations. This

heritable pool of RNAs could reflect the adaptive change in expression of genes involved in nutrition and metabolism (Rechavi, Houri-Ze'evi et al. 2014, Houri-Ze'evi, Korem et al. 2016).

In addition to the L1 stage, later in development, larvae can execute an alternative developmental program to enhance survival and fitness in response to overcrowding or sub-optimal survival conditions. During this diapause-like state called dauer, they undergo a global, genome-wide, adjustment of chromatin modifications that is accompanied by a significant change in gene expression when compared to the animals that never transited through this stage. These changes in the abundance and distribution of chromatin marks likely contribute to the molecular record of life history and the adaptive adjustment of these chromatin modifications is most probably dependent on the expression of specific endogenous small RNAs (Hall, Beverly et al. 2010, Hall, Chirn et al. 2013). Currently, it is still unclear how the physiological stress associated with the dauer stage might impact the population of small RNAs and, in transgenerational contexts, how these changes are transmitted across generations, in light of the erasure of histone marks that normally takes place during each cycle of embryogenesis.

Global cell cycle arrest is one of several distinctive features of *C. elegans* dauer larvae. Upon entry into the dauer stage the germline stem cell divisions begin to slow to finally establish a state of quiescence, which they maintain until they recover from dauer and resume normal development. Despite potentially long periods in this diapause stage, this cell cycle/developmental quiescence has no impact on their reproductive fitness (Hall, Beverly et al. 2010). The activity of the cellular energy sensor AMPK and its upstream kinase LKB1/PAR-4, as well as the activity of tumour suppressor PTEN, are all independently required for the quiescent state of the germline stem cells (GSC) in response to dauer signalling (Narbonne and Roy 2006). Maintaining a quiescent state in response to energetic stress may be favourable for survival, presumably because it reduces energy

consumption during a period when energy is limited (Rossi, Jamieson et al. 2008, Cheung and Rando 2013) . Moreover, when quiescence fails to establish and/or maintained during periods of energy stress, germ cell integrity is compromised, resulting in reduced brood sizes (Demoinet, Li et al. 2017).

We show here that during the dauer stage, AMPK activity is not only required to block GSC proliferation, but also to maintain GSC integrity to ensure reproductive success following recovery to replete conditions. In the absence of AMPK, several chromatin modifications become misregulated, resulting in inappropriate gene expression that has a detrimental effect on reproductive fitness following exit from the dauer stage. Using genetic analysis, we reveal the importance of the endogenous small RNA pathway and its regulation by AMPK. Moreover, this pathway acts at least partially in a cell non-autonomous manner, to adjust the GSC-specific chromatin landscape in favour of an adaptive gene expression program fine-tuned toward maintaining germ cell integrity during the long-term energy stress typical of the dauer stage.

Results

Compound reproductive defects in the dauer germ line result in post-dauer sterility in AMPK mutants

In *C. elegans,* the decision to execute dauer development is regulated by three independent signalling pathways that converge on a nuclear hormone receptor to ultimately affect multiple developmental and physiological processes (Hu 2007). Many of these processes involve measures to conserve energy for the duration of the diapause, which are mediated through a significant metabolic adjustment that occurs downstream of all these signalling pathways (Penkov, Erkut et al. 2018).

To conserve the energy associated with cell divisions, while also ensuring germ cell integrity throughout the dauer stage, the *C. elegans* orthologues of LKB1 (*par-4*) and the regulatory and catalytic components of AMPK cooperate to establish cell cycle and developmental quiescence in the germline stem cells (GSC). Animals that have reduced, or lack all AMPK activity (*aak(0)*) undergo pronounced germline hyperplasia due to supernumerary cell divisions upon dauer entry, however it is unclear if these extra cells retain their germ cell integrity and are competent to yield functional gametes (Narbonne and Roy 2006).

The dauer larva is remarkable in that it can remain in a quiescent state for months longer than it would normally survive while in its reproductive mode. Nevertheless, it can exit this quiescence upon improvement in growth conditions, to resume reproductive development with no compromise of their reproductive fitness, regardless of the duration of the developmental arrest *per se* (Ouellet, Li et al. 2008). The germ cells must therefore retain the appropriate information to maintain their totipotency over lengthy periods so that upon recovery from the diapause the animal can still reproduce without any loss in fitness. Since AMPK and LKB1 are critical to block

germ cell divisions during the dauer stage we were curious whether the supernumerary germ cells that are produced in *aak(0)* mutants are indeed competent to generate functional gametes and/or embryos. We therefore quantified the brood size of *daf-2* (control) and *daf-2; aak(0)* animals after allowing both of these mutants to recover after remaining at least 24h in the dauer stage. In contrast to control *daf-2* animals that recover from dauer with very little to no negative reproductive consequence, AMPK mutant animals that transit through dauer for 24 hours or more exhibit highly penetrant post-dauer (PD) sterility upon recovery (Fig. 4.1A, Fig. S4.2). The brood size of *daf-2* PD adults was not significantly different from *daf-2* animals that never transit through dauer (data not shown) suggesting that passage through the dauer stage has no impact on reproductive fitness provided that AMPK signalling is active.

To confirm that the AMPK activity is not exclusive to the insulin-like signalling branch involved in dauer formation, but rather, it is required downstream of the other signalling pathways that control dauer formation, we determined if AMPK may play a more general role in PD fertility by testing if it is also required in *daf-7* mutants (TGF-β pathway), or in the *aak(0)* mutants treated with dauer pheromone (Hu 2007). Similar to downregulation of insulin-like signalling, compromise of both the TGF-β or the pheromone pathway results in high sterility in the absence of AMPK (Fig. S4.1). This suggests that activity of AMPK is critical for fertility, and hence the maintenance of germ cell integrity, downstream of the major pathways required for dauer entry.

The observed sterility in *aak(0)* PD animals is also accompanied by severe defects in vulva development. These animals exhibited protruding vulva (Pvl), multi vulva (Muv) and burst vulva (Rup) phenotypes, while PD lethality (prematurely expire before reaching adulthood) was also observed at a comparable frequency (Fig. 4.1B, Table S4.1). Thus, it is not only the germ line

which is compromised in the absence of AMPK, but at least some of the somatic tissues are also sensitive to AMPK function.

AMPK post-dauer animals have abnormal germline morphology and the germ cells fail to exit pachytene

To determine the physiological basis of the observed sterility in the *aak(0)* PD animals, we examined their germline morphology and organization using a germ cell membrane marker (Amini, Goupil et al. 2015). We noted that in 95% of the animals, the oocyte morphology appeared abnormal and they also lacked the typical, single-file organization seen in the control *daf-2* PD animals (Fig. 4.2A, B, Table S4.2). Also, in 60% of the *aak(0)* PD animals, the general gonad symmetry was abnormal in terms of size and shape of the gonadal arms. (Table S4.2).

We stained the germ lines of PD control and AMPK mutant animals and examined chromosome morphology within the germ cells as they progress through the distinct regions of meiotic prophase to generate fully differentiated oocytes (Kimble and Crittenden 2007). There were no obvious defects in the size of the mitotic zone or the spatio-temporal arrangement of the transition zone. For further characterization we binned the post-transition zone germ cells into 3 different zones: in Zone 1, germ cells enter the pachytene stage; in Zone 2 the cells exit pachytene and initiate the separation of the paired homologous chromosomes (diplotene); in Zone 3 separation of homologues is complete, forming 6 tightly condensed DAPI stained bodies representing 6 pairs of homologous chromosomes (diakinesis).

In the *daf-2* PD germline, there were no observed abnormalities as the germ cells complete meiotic progression to eventually give rise to oocytes with 6 condensed DAPI-stained bodies (Fig. 4.2B). However, in the PD germ lines of *aak(0)* mutants, the germ cells do enter pachytene in Zone 1, but

then fail to exit in Zone 2. The pachytene arrest persists into Zone 3, as the chromosomes fail to separate and condense (Fig. 4.2C). This suggests that in *aak(0)* PD animals, the germ cells fail to exit pachytene and thus never undergo diakinesis to produce mature oocytes.

To further determine, if the sterility was a result of abnormal sperm formation or function, or whether the defect was associated with the oocytes, we performed reciprocal crosses and monitored the brood size of the resulting cross progeny (Fig. 4.2E, F). Using an anti-MSP antibody we noted that while sperm was present in the *aak(0)* PD hermaphrodites, it may have been produced during dauer stage (Narbonne and Roy 2006), persisting into the adult stage. To determine if the sperm present in the *aak(0)* PD adults is functional, we mated *aak(0)* hermaphrodites that never transited through dauer, with *aak(0)* PD males (a ratio of 20 males per hermaphrodite was maintained). If the mating was successful and the PD males produced functional sperm, we would expect ~50% of the progeny to be male. However, no significant F1 male progeny were observed suggesting that the sperm is defective (Fig. 4.2E). We cannot rule out however that, despite our monitoring, these mutants could be mating incompetent.

Similarly, when we mated PD *aak(0)* hermaphrodites and *aak(0)* males that never transited through dauer, *aak(0)* PD hermaphrodites still exhibited high sterility (Fig. 4.2F) suggesting that integrity of the oocytes is also compromised.

These results collectively suggest that germline development is sensitive to periods of energetic stress, and in the absence of AMPK, the germ line becomes severely perturbed; the integrity of both the oocytes and the sperm is affected ultimately rendering the PD animals sterile.

Post-dauer sterility and germline hyperplasia are not necessarily directly linked in AMPK mutants

As *aak(0)* dauer larvae fail to maintain the GSC arrest and exhibit sterility upon exiting the dauer stage, we questioned whether the observed sterility might result from the inappropriate germ cell divisions that occur during the dauer stage. To test this, we performed RNAi on genes that were shown to suppress the supernumerary germ cell divisions in the *aak(0)* dauer larvae (Kadekar, Chaouni et al. 2018). We subsequently allowed these animals to recover to form adults, after which we assessed their fertility and brood size. None of the suppressors of dauer germline hyperplasia that we tested were capable of restoring the PD fertility in the *aak(0)* mutants, although the germline hyperplasia typical of *aak(0)* mutant dauer larvae was visibly ameliorated (Fig. 4.3A, B). Albeit, RNAi of these genes does not completely suppress the germline hyperplasia, leaving the possibility that the extra number of germ cells could be responsible for the PD sterility. Nevertheless, our data suggest that the PD sterility of *aak(0)* animals is not necessarily a direct consequence of the dauer germline hyperplasia, but may involve additional processes that could act in concert with, but possibly independent of, the regulation of cell cycle quiescence during the dauer stage.

Many chromatin marks are misregulated both globally and in the germ line in *aak(0)* dauer larvae

In *C. elegans,* dauer larvae exhibit a significantly different gene expression profile when compared to animals that never transit through the dauer stage. Furthermore, these changes in the gene expression persist after the animals exit dauer and become reproductive adults. Thus, a molecular memory of the passage through dauer is recorded, and has been shown to influence fertility in the

PD animals (Hall, Beverly et al. 2010). The observed changes in gene expression are highly correlated with the changes that occur in the various chromatin marks detected in dauer and in PD larvae compared to controls that never transited through dauer (Hall, Beverly et al. 2010).

AMPK has been implicated in the regulation of gene expression through its ability to modify chromatin by directly phosphorylating histone H2B to activate stress-promoted transcription (Bungard, Fuerth et al. 2010). Furthermore, recently we showed that AMPK modulates the chromatin landscape to ensure that transcriptional activity is blocked in the primordial germ cells until animals have sufficient cellular energy levels (Demoinet, Li et al. 2017). Since AMPK may directly regulate histone modifying enzymes to bring about changes in gene expression we wondered whether chromatin modification may be perturbed in the dauer germ cells resulting in changes in the adaptive gene expression program that would normally occur in dauer. This inability to appropriately adjust to the energy stress associated with dauer development might explain the loss of integrity in the PD germ cells.

We therefore examined the global levels of diverse chromatin marks that were previously found to change following transit through the dauer program. We first performed western blot analysis on whole extracts from *daf-2* and *daf-2; aak(0)* mutant dauer larvae using antibodies specific to specific for histone modification associated both with transcription activation (H3K4me3 and H3K9ac) and repression (H3K9me3 and H3K27me3). Interestingly, all the marks we tested were abnormally high in the absence of AMPK (Fig. 4.4A). To confirm if the increased level of these chromatin marks was associated with the hyperplasia associated with AMPK mutant dauer larvae, we performed the same experiments in animals that lack *glp-1* (Fig. S4.3) (Kimble and Crittenden 2007) and quantified the levels of the chromatin marks. The reduction of germ cells significantly decreased the levels of the chromatin marks, suggesting that the germ cells are the major

contributors to the global increase in the levels of the chromatin marks in AMPK mutant dauer larvae, although the levels also increase in the soma (Fig. 4.4B).

The distribution of chromatin marks is dramatically altered in *aak(0)* dauer germ cells and higher levels of these marks persist in the post-dauer germ line

From our western analysis we could not discern if the levels in the chromatin marks were abundant simply because of the supernumerary germ cells in the AMPK mutant dauer larvae or whether the levels were higher in each individual nucleus *per se.* We therefore dissected gonads from both *daf-2* and *daf-2; aak(0)* dauer larvae and quantified the levels of H3K4me3 and H3K9me3 to better evaluate their levels per nucleus and to determine if there were any changes in their proximal-distal distribution through the gonad. In the control *daf-2* dauer germ line, the levels of both H3K4me3 and H3K9me3 are consistent across all nuclei throughout the dauer germline, but in the AMPK mutant dauer larvae, the pattern of H3K4me3 and H3K9me3 expression in the gonad is altered, while their levels are highly variable in individual nuclei (Fig. 4.5A, B). Of particular interest, we noted that the expression of H3K4me3 in individual nuclei is comparatively weak in the distal gonad, but gradually increases toward the proximal goal where it is much higher.

To test whether the abnormal distribution and abundance of the H3K4me3 and H3K9me3 marks are resolved after the *aak(0)* larvae exit dauer, we the stained PD adult gonads. Interestingly, we noted that higher levels of both H3K4me3 and H3K9me3 persist in the *aak(0)* PD adult germ line when compared to the control *daf-2* PD animals (Fig. 4.5C, D).

Altogether, these data confirm the role of AMPK in the appropriate regulation of both transcriptionally activating and repressive chromatin marks in the germ line under energetic stress.

In its absence the levels of each mark we tested increased, while the distribution of these marks was dramatically disrupted.

Gene expression is altered in both the *aak(0)* dauer and post-dauer germ line

To examine if aberrant chromatin modifications result in abnormal gene expression in animals that lack AMPK signalling, we performed qRT-PCR to quantify the transcript levels of selected germline-specific genes which were found to be significantly altered in dauer and in the PD adults (Hall, Beverly et al. 2010). The abundance of these transcripts was considerably different in *aak(0)* dauer larvae; some of the genes (*ppk-2, pmk-1, mek-2)* were present at lower levels, while others (*spe-26, pro-2)* were detected at significantly higher levels when compared to control *daf-2* animals (Fig. 4.6A). Furthermore, some of these differences in transcript abundances are not resolved in the PD adult germ line of AMPK mutants (Fig. 4.6B). These data suggest that the modifications that we observed in both the dauer and PD animals that lack all AMPK signalling result in dramatic changes in the gene expression program that would normally occur as a result of transit through the dauer stage.

Compromise of small RNA pathway components partially suppresses *aak(0)* post-dauer sterility and dauer germline hyperplasia

Many endogenous small RNAs are critical in distinguishing loci to be targeted by chromatin modifying enzymes, in addition to specifying whether the modification will be active or repressive. They act as mediators of gene expression in order to adapt to cell type information and to varying environmental situations (van Wolfswinkel and Ketting 2010). It is therefore not surprising that the small RNA repertoire is dramatically altered in both dauer and PD adults compared to animals

that develop in a replete environment (Hall, Chirn et al. 2013). We were therefore curious to know whether AMPK might regulate the changes that occur in the suite of small RNA species during dauer owing to its role in regulating chromatin marks in response to energetic stress. To test this possibility, we compromised critical components of the miRNA (*ain-1*), and germ line/nuclear RNAi (*hrde-1, csr-1*) pathways, and some common upstream effectors that impinge on all the small RNA pathways (*dcr-1, rde-4*), to determine if disabling any of these pathways might affect the sterility of the PD AMPK mutant adults. The miRNA pathway is essential for executing dauer entry making it difficult to interpret their potential role in this process (Than, Kudlow et al. 2013). On the other hand, we found that the individual disruption of the RNAse III-like Dicer (*dcr-1*), its accessory factor RDE-4, or the primary Argonaute protein ERGO, could partially rescue the sterility of PD AMPK mutants, the uncontrolled proliferation in the germ line of AMPK mutant dauer larvae, in addition to some of the somatic defects (Fig. 4.7A, B, Fig. S4.2, Table S4.1). In contrast, the compromise of the nuclear Argonaute proteins HRDE-1, or the germline licensing Argonaute CSR-1, had little effect on the hyperplasia or the PD sterility of the AMPK mutants. These data indicate that AMPK must directly or indirectly regulate a small RNA pathway that affects both GSC proliferation and integrity, but does not include the canonical nuclear Argonaute proteins that have been characterized to regulate germline gene expression.

Since both the dauer-associated hyperplasia and the PD sterility of the AMPK mutants correlated with the misregulation of chromatin modifications, we wanted to confirm if the compromise of these various RNAi pathway components might also restore the inappropriate levels and distribution of the chromatin marks in the AMPK mutant germ line. We therefore quantified the global levels of both H3K4me3 and H3K9me3 in *dcr-1*(RNAi) and *rde-4*(RNAi) treated dauer larvae. The levels of both of these marks were significantly reduced in the *dcr-1*(RNAi) AMPK

mutant dauer animals, with no effect in control *daf-2* dauer larvae. Surprisingly, only the level of H3K4me3 was significantly reduced in the *rde-4*(RNAi) AMPK mutants (Fig. 4.7C, D, E), although this may reflect the weak and variable RNAi penetrance typical of *rde-4*. These data confirm that AMPK impinges on the endogenous small RNA pathway to modulate chromatin modifications that affect both GSC proliferation and integrity, although it is currently unclear how AMPK might control these processes, and whether phospho-regulation of key targets might be involved.

Somatic AMPK activity is sufficient to regulate dauer germ cell quiescence and integrity

Recent studies have shown that AMPK can act cell non-autonomously to regulate lifespan, and the L1 survival of AMPK mutants is greatly improved when AMPK is restored in neurons (Fukuyama, Sakuma et al. 2012, Burkewitz, Morantte et al. 2015). To determine if AMPK plays a non-autonomous role in maintaining GSC quiescence and integrity, we expressed the catalytic subunit of AMPK (*aak-2*) ubiquitously in the soma (*sur-5p)* of *aak-2* mutants. Interestingly, ubiquitous somatic expression of *aak-2* restores the fertility in the PD of *aak-2* mutants and also rescues the dauer germline hyperplasia (Fig. 4.8A, B and Fig. S4.2). This suggests that AMPK activity in the soma is sufficient to maintain the integrity and quiescence in the germ cells during the dauer stage. To determine in which somatic tissue AMPK function can restore quiescence and integrity to the GSCs during the dauer stage, we used tissue-specific promoters to express *aak-2* and quantified PD sterility and the degree of germline hyperplasia in the AMPK mutants. Using a transgenic strain collection, we generated AMPK null mutants that express *aak-2* exclusively in the neurons (*unc-119)*, the excretory system (*sulp-5)*, the skin (*dpy-7*), the gut (*elt-2*), and the muscles (*unc-54*) (Narbonne and Roy 2009). Restoration of AMPK function in the neurons and

the excretory system partially rescued the fertility and GSC quiescence. This suggests that AMPK is required in at least 2 tissues to non-autonomously regulate germ cells under the energetic stress. Ubiquitous somatic expression of *aak-2* also restores the normal levels and distribution of H3K4me3 and H3K9me3 marks in the dauer germline (Fig. 4.8C, D). These data confirm the non-germline role of AMPK is sufficient to establish quiescence and maintain germ cell integrity during sub-optimal growth conditions.

To further investigate if AMPK modulates some aspect of soma to germline communication we investigated the role of the dsRNA importer, *sid-1,* as it plays an important role in the transfer of small endogenous RNAs from the soma to the germ cells (Devanapally, Ravikumar et al. 2015). To investigate if an aberrant transfer of small RNAs to the germline could mediate the defects in *aak(0)* PD animals, we compromised the function of *sid-1* in AMPK mutants and assessed PD fertility. Interestingly, loss of *sid-1* partially restored fertility in AMPK PD animals (Fig. 4.8E), suggesting that an abnormal transfer of small endogenous RNAs to the germ line occurs in the AMPK mutants and is responsible, at least in part, for the observed PD sterility.

Discussion

During periods of energetic stress, *C. elegans* larvae can alter their normal reproductive development and enter a quiescent diapause-like state called dauer. The dauer stage is often an obligate step in the normal development of several parasitic nematodes as it is typically associated with exceptional stress resistance that is accompanied by a global developmental arrest, including a temporary attenuation of germ cell divisions. One of the predominant cellular energy sensors, AMPK, and its activating kinase PAR-4, become highly active during this state and regulate the germline quiescence that occurs during the dauer stage. In the absence of either of these kinases, the dauer germ cells proliferate abnormally resulting in a dramatic over-proliferation of the germline (Narbonne and Roy 2006).

The consequences of these unscheduled germ cell divisions have never been interrogated. If these cells are competent, the excessive proliferation could result in a significant increase in reproductive fitness. Alternatively, if these supernumerary cells are abnormal it could have detrimental effects on subsequent generations.

In this study, we show that the extra cells that arise during dauer in AMPK mutants do not provide any reproductive advantage and that AMPK signalling is critical to coordinate germ cell quiescence with the perceived organismal energy stress associated with dauer. Although both the dauer germline hyperplasia and the PD sterility are AMPK dependent, our data suggest that the observed sterility may not necessarily be a direct consequence of abnormal cell divisions within the germline stem cells, since the aberrant cell divisions can be suppressed without ameliorating the sterility of the AMPK mutant dauer larvae. The AMPK-dependent processes that are required for PD fertility may be independent of its role in modulating germ cell proliferation (Fig. 4.3).

We show that upon dauer exit, *daf-2* animals develop normally with no consequence in their germline development or their reproductive fitness. However, AMPK PD animals show striking defects in germline development and organization (Fig. 4.2B, Table S4.2). While at the cellular level, the germ cells progress into meiotic prophase, only to subsequently arrest in a pachytene-like state. Although, morphologically many of the proximal germ cells appear to undergo cellularization and oogenesis, at the nuclear level they fail to complete diakinesis to form 6 condensed nuclear bodies; a hallmark of a mature oocyte and thus fails to produce a functional matured oocyte (McCarter, Bartlett et al. 1999). Collectively, these results suggest the role of AMPK activity in protecting the gametes by establishing and maintaining GSC cell cycle quiescence and germ cell integrity during periods of extreme energetic stress.

But what might constitute germ cell integrity and how might it be maintained by AMPK over the duration of the dauer stage? One possibility might include changes in the gene expression program to mitigate the adaptive cellular and metabolic adjustments necessary to endure the energetic stress of the dauer stage, whether it last 24h or 6 months. As *C. elegans* larvae transit through dauer state, the chromatin is concomitantly remodelled, altering gene expression significantly (Hall, Beverly et al. 2010). In additions, these modifications are tightly correlated with changes in the small RNA repertoire such that the expression of most endo-siRNAs are affected in both dauer and PD larvae when compared with animals that never transit through the dauer stage. Based on the mechanism of small RNA-mediated changes to the chromatin, these changes in small RNA population likely presage chromatin remodelling, which together provide a molecular memory of this life history event and possibly provides a template for the consequent establishment of distinct adaptive cellular responses or behaviour(s) (Hall, Beverly et al. 2010).

We show that AMPK is critical to ensure that these global chromatin modifications take place in a regulated manner. In its absence, the abundance of both the transcriptional activating (H3K4me3 and H3K9ac) and repressive (H3K9me3 and H3K27me3) marks increase aberrantly in the dauer larvae. In addition, the H3K4me3 and H3K9me3 chromatin marks become abnormally distributed within the dauer germline and fail to resolve upon dauer exit, persisting in to the adult PD germline, consistent with AMPK executing an important role in resolving these modifications upon dauer exit. Although we have not identified the penultimate AMPK target(s) that mediate these changes in chromatin regulation, but the relationship between AMPK and chromatin regulators is akin to its role during the L1 diapause, where germ cell quiescence and integrity are compromised due to irregular chromatin modifications in the absence of AMPK (Demoinet, Li et al. 2017).

The observed anomalies in both the abundance and the distribution of the activating and repressive marks likely perturbs the coordination of germline gene expression with the energy stress of dauer, which would normally be mediated through a specific chromatin syntax in both dauer and PD AMPK animals. In the absence of AMPK, gene expression may no longer correspond to that of a germ cell, or at least a germ cell that has been subjected to the challenge of surviving the dauer stage, and this abnormal gene expression program may drive the abnormal germline development in addition to the somatic defects observed in the AMPK PD adults.

Furthermore, our findings suggest that these abnormalities in AMPK mutants are dependent on various components of a small RNA pathway. These results suggest that a small RNA based process that is regulated by Dicer and RDE-4 to modulate chromatin modifications is under direct or indirect control of AMPK. Although we have not yet identified the key AMPK targets that might be critical for mediating this change in small RNA function, we did note that Dicer contains multiple consensus AMPK phosphorylation sites, while RDE-4 also could be a potential substrate.

Alternatively, in addition to the primary ARGONAUTE protein ERGO, a number of ARGONAUTE orthologues remain to be characterized. We cannot rule out that one of these ARGONAUTE family members may somehow respond to AMPK signalling to affect this small RNA-mediated change in the chromatin landscape that occurs during dauer and PD recovery in *C. elegans*.

Using *rrf-1* to address in what cells AMPK is required, we concluded that AMPK acted in a germline-autonomous manner to maintain GSC quiescence in the dauer larvae (Narbonne and Roy 2006). The technical flaws of this strategy have since been well documented (Kumsta and Hansen 2012) and our recent transgenic experiments confirm that AMPK activity is sufficient in the neurons, or the excretory system, to regulate germ cell quiescence and integrity. Moreover, the somatic expression of AAK-2 also restored the normal levels of chromatin modifications in the dauer germline (Fig. 4.8). However, this neuron-specific function of AMPK is unlikely to be a general feature of AMPK signalling, since its neuronal expression failed to suppress the supernumerary germ cell divisions in AMPK mutants during L1 diapause (Fukuyama, Sakuma et al. 2012). Recently it was shown that AMPK expression in the neurons can extend lifespan in *C. elegans* under energetic stress (Fukuyama, Sakuma et al. 2012, Burkewitz, Morantte et al. 2015). The neurons may therefore sense the environment and accordingly signal, in a neuroendocrine manner, to other tissues to adapt. AMPK could be one of the intermediaries in transducing the signals from the neurons, potentially regulating some diffusible molecule, to enhance their survival without any compromise on their fitness. In some cases, these other tissues may be the germ cells. This would place AMPK at a critical position in sensing environmental challenges to ultimately impinge on the germ line, potentially to mediate some chromatin-mediated adaptation that could benefit subsequent generations.

But what neuron-derived diffusible signal could affect the chromatin in the germ cells? Curiously, like in plants, RNAi is systemic in *C. elegans*. Injection of dsRNA into the somatic tissue can result in RNA-mediated gene silencing in the germline (Fire, Xu et al. 1998, Winston, Molodowitch et al. 2002, Devanapally, Ravikumar et al. 2015). Furthermore, the endo-siRNA pathway that is active in the somatic tissues can contribute, at least in part, to the changes in the germline gene expression and the brood size upon passage through dauer (Hall, Chirn et al. 2013). Our results support and extend these findings, as the compromise of the dsRNA importer, *sid-1* partially rescues the AMPK-dependent sterility of PD AMPK mutants, suggesting that the abnormal transfer of small RNAs to the germline that occurs in the absence of AMPK can culminate in sterility. At present we cannot confirm if AMPK might regulate the systemic transfer of small RNAs directly or whether it is a problem with the composition of the small RNAs that are mobilized.

Overall our data suggest that AMPK activity is critical to regulate both the abundance and the distribution of various chromatin modifications in order to preserve the integrity of the germ cells under energetic stress. This is presumably achieved by directly or indirectly modulating a small RNA pathway in a germline non-autonomous manner. Perhaps most importantly, our findings provide a novel model to reveal the mechanisms through which the soma communicates with the germ line to adapt to acute environmental challenges and provide some selective epigenetic advantage to future generations.

Materials and Methods

C. elegans genetics

All *C. elegans* strains were maintained at 15°C and according to standard protocols (Brenner 1974). The strains used for the study include CB1370 [*daf-2(e1370 III*], MR1000 [*daf-2(e1370) aak-1(tm1944) III; aak-2(ok523) X*], MR0480 [*daf-7(e1372) III; aak-2(ok523) X*], MR1175 [*aak-1(tm1944) III; aak-2(ok523) X*], MR2137 [*daf-2(e1370) aak-1(tm1944) III; aak-2(ok523) X; ltIs4[unc-119(+)Ppie1::plc::mCherry]*], MR2156 [*daf-2; ltIs44[unc-119(+)Ppie1::plc::mCherry]*], MR1973 [*daf-2(e1370) aak-1(tm1944) III; aak-2(ok523) X; sid-1(rr167) V*]. Transgenic lines and compound mutants were created in the laboratory using standard molecular genetic approaches. To create transgenic lines to express tissue-specific *aak-2,* MR1000 animals were injected with different constructs as per (Narbonne and Roy 2009).

RNAi Feeding

Bacterial clones expressing dsRNA from the RNAi library were grown in LB medium with ampicillin at 37°C overnight. The bacterial culture was seeded onto regular NGM plates containing ampicillin and IPTG. Seeded plates were incubated at room temperature for 24 hours to induce dsRNA expression. L4 larvae were fed on the RNAi plates and were allowed to lay eggs at 15°C and then the eggs were switched to 25°C to induce dauer.

DAPI staining and germ cell nuclei count

For whole worm DAPI (4' ,6-diamidino-2-phenlindole) staining, dauer larvae were washed off plates and soaked in Carnoy's solution (60% ethanol, 30% acetic acid, 10% chloroform) on a shaker overnight. Animals were washed twice in PBST (1XPBS + 0.1% Tween 20), and stained

in 0.1 mg/ml DAPi solution for 30 minutes. Finally, larvae were washed four times (20 minutes each) in PBST, and mounted in Vectashield medium. The total number of germ cell nuclei per dauer gonad was then determined based on their position and nuclear morphology.

Dauer recovery assay

A population of the studied strains were synchronized, and the resulting embryos were added to normal NGM plates seeded with *E. coli*, and incubated at 25°C for 72 hours in order to induce dauer formation and allow animals to spend at least 24 hours in dauer state. Following this window, dauer larvae were shifted to the permissive temperature of 15°C to allow dauer exit and initiate regular development. Upon dauer exit, the L4 larvae were individually isolated onto separate plates and were transferred to new plates every 24 hour intervals to quantify their brood size. The brood size of each animal was the sum of non-hatched and hatched progeny.

Immunostainings and quantification

For extruded dauer gonad staining, gonads were dissected, fixed and stained as described elsewhere (Arduengo, Appleberry et al. 1998). Following primary anti-bodies were used: rabbit polyclonal anti-H3K4me3 (1:500), anti-H3K9me3 (1:500), rabbit anti-HIM-3 (1:200). Secondary antibodies were Alexa Fluor 488-coupled goat anti-rabbit (1:500)Microscopy was performed as described in (Kostic, Li et al. 2003). Ratios for the fluorescence intensity across the germ line were determined using Image J.

Western blot

C. elegans dauer larvae and post-dauer adults were lysed by sonication in lysis buffer (50mM Hepes pH7.5, 150mM NaCl, 10% glycerol, 1% Triton X-100, 1.5mM $MgCl_2$, 1mM EDTA and protease inhibitors. Protein concentrations were determined using nanodrop 2000c spectrophotometer (Thermo Scientific). Nitrocellulose membranes were incubated with primary antibodies: rabbit anti-H3K4me3, anti-H3K9me3, anti-H3K27me3, anti-H3K9ac (1:1,000; Diagenode); mouse anti–α-tubulin (1:3,000; Sigma). Proteins were visualized using horseradish peroxidase conjugated anti-rabbit or anti-mouse secondary antibody (Bio-Rad).

RNA Isolation and Real Time PCR

Total RNA was extracted with Trizol (Invitrogen). RNA concentration and purity were determined with a NanoDrop 2000c spectrophotometer. Purified RNA (400 ng) was used to synthesize complementary DNA. Gene expression levels were determined by real time PCR with the SYBR Green Supermix and BioRad iCycler Real Time PCRSystem (BioRad). Relative gene expression was normalized to *tba-1* as loading control.

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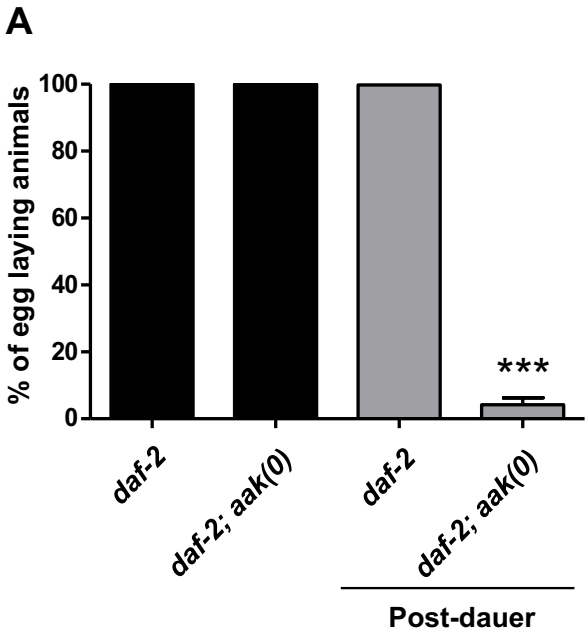
Figures and Tables

Figure 4.1. Post-dauer *aak(0)* adults exhibit severe sterility and somatic defects.

A) All adult animals that laid eggs (dead or hatched) were considered as fertile. Both *daf-2* and *daf-2; aak(0)* animals cultivated under permissive conditions showed no fertility defect compared to wild type. To assess the fertility of the PD adults, animals were maintained in the dauer stage for 24 hours after which they were switched to permissive temperature to resume their normal development (See materials and methods). Egg laying animals were counted, the means calculated, and the values are shown with SD. Upon recovery, *daf-2* PD adults were fertile, but *daf-2; aak(0)* PD adults were almost entirely sterile; ***P<0.0001 using Marascuilo procedure. Assays were performed three times and the data represent the mean ±SD; n=50. **B)** In *daf-2; aak(0)* PD animals, the high degree of sterility is also accompanied with severe somatic defects that affect vulva development. Values represent means ±SD; n=50. A proportion of 16.5±3.5% of these animals prematurely expired during their recovery phase and failed to reach adulthood.


A
% of egg laying animals
100
80
60
40
20
0
***
daf-2
daf-2; aak(0)
daf-2
daf-2; aak(0)
Post-dauer

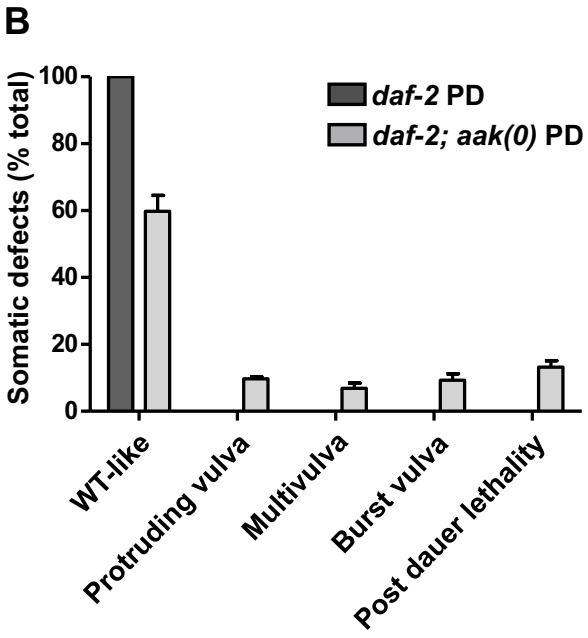

B
Somatic defects (% total)
100
80
60
40
20
0
daf-2 PD
daf-2; aak(0) PD
WT-like
Protruding vulva
Multivulva
Burst vulva
Post dauer lethality

Figure 4.2. AMPK post-dauer animals show abnormal germline morphology and the germ cells fail to exit pachytene.

All animals analyzed express a *Ppie-1::*PLC::mCherry transgene to monitor germ cell membranes/organization. **A, B**) In *daf-2* PD adults, the germ line develops normally and no obvious defects were observed, but *daf-2; aak(0)* PD adults exhibit various defects in germline development and organization. Oocyte morphology is abnormal (white arrowhead) and they lacked the typical, file-like organization observed in the control *daf-2* PD animals. **C, D)** For further characterization, the post-transition zone germ cells were divided into 3 different subregions. In the first subregion after the transition zone (Zone 1), germ cells enter pachytene stage; in Zone 2, the cells exit pachytene and initiate the separation of the paired chromosomes (diplotene); in Zone 3 separation of the paired chromosomes is complete, forming 6 tightly condensed DAPI-stained bodies representing 6 pairs of homologous chromosomes (diakinesis). In *daf*-2 PD, the germ cells go through all these processes to eventually give rise to 6 condensed DAPI-stained bodies (white arrowhead), but in *daf-2; aak(0)* PD adults, the germ cells enter pachtytene in Zone 1, but fail to completely exit the pachytene stage based on the continued presence of long chromosome tracks (white arrow). A-D) n= 20. Scale bar: 10um in A and B, 4 um in C and D. **E,F)** Reciprocal crosses were performed and a ratio of 20 males per hermaphrodite was maintained for all the crosses. *daf-2; aak(0)* PD males were mated with normal *daf-2; aak(0)* 15 hermaphrodites and a number of males/hermaphrodite in F1 were counted. Few to no male progeny were identified in the F1 generation of this mating. The mean is shown ± SD. **F)** *daf-2; aak(0)* PD hermaphrodites were crossed with normal *daf-2; aak(0)* males. 15 animals were quantified and PD *aak(0)* hermaphrodites exhibited a high frequency of sterility. The mean is represented ± SD. ***P<0.0001 using Marascuilo procedure.

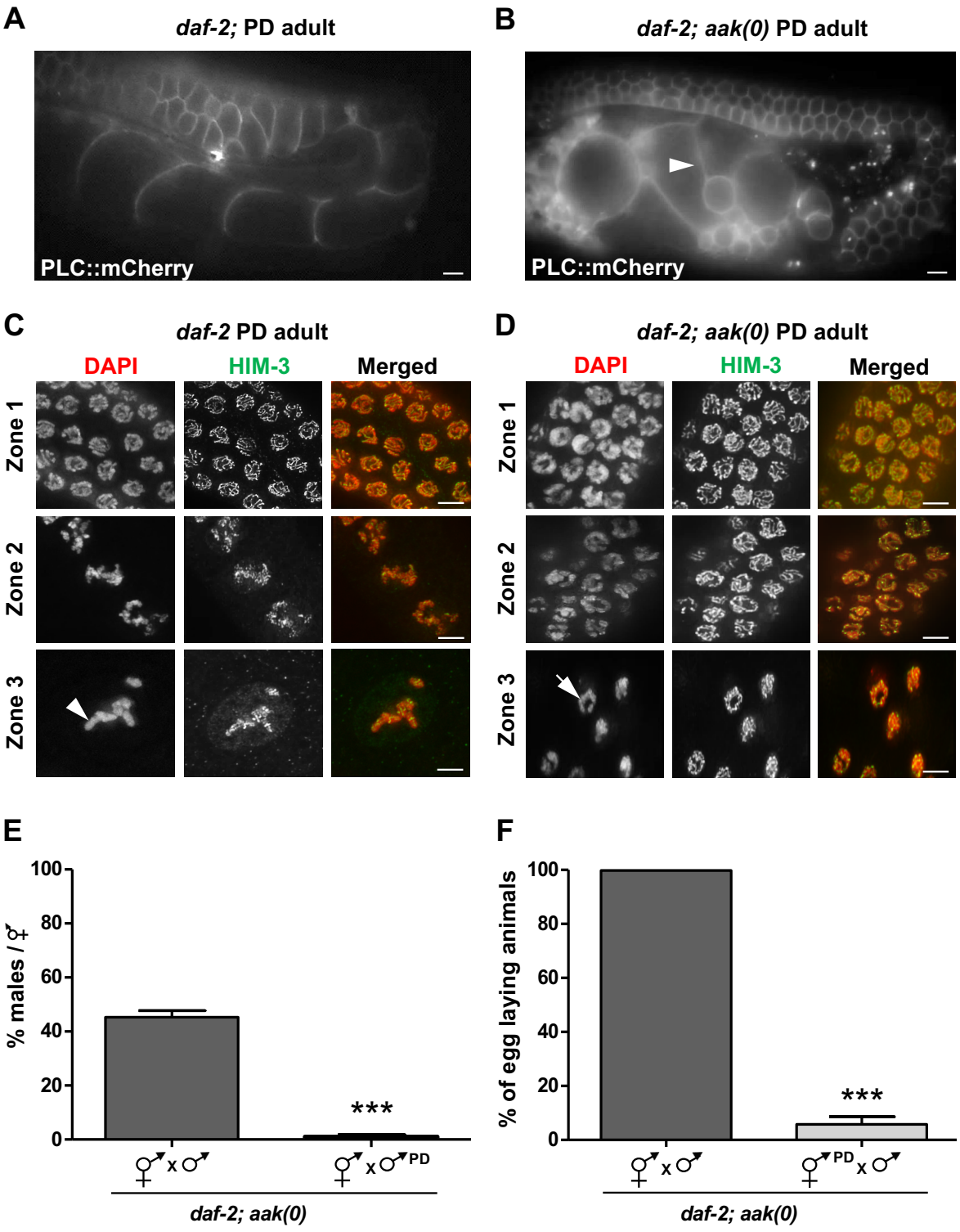

A
daf-2; PD adult
PLC::mCherry
B
daf-2; aak(0) PD adult
PLC::mCherry
C
daf-2 PD adult
DAPI
HIM-3
Merged
Zone 1
Zone 2
Zone 3
D
daf-2; aak(0) PD adult
DAPI
HIM-3
Merged
Zone 1
Zone 2
Zone 3
E
% males / ♂
100
80
60
40
20
0
***
♂ x ♂
♂ x ♂PD
daf-2; aak(0)
F
% of egg laying animals
100
80
60
40
20
0
***
♂ x ♂
♂PD x ♂
daf-2; aak(0)

Figure 4.3. Post-dauer sterility and germline hyperplasia are not necessarily interdependent in AMPK mutants.

A, B) Whole animal DAPI staining was performed to quantify the number of dauer germ cells. The number of egg-laying animals was quantified and the mean is represented ± SD. To test if the germ cell integrity defect results from the dater-dependent germline hyperplasia genes that were previously found to suppress germline hyperplasia in dauer larvae (Kadekar, Chaouni et al. 2018) were subjected to RNAi. Larvae were switched to permissive temperature to exit dauer and resume reproductive development. Fertility was assessed 48h after the temperature shift by counting egg-laying adults. L4440 is an empty RNAi vector and is used as a control. ***P<0.0001 when compared with L4440 using the two-tailed t-test. n=50.

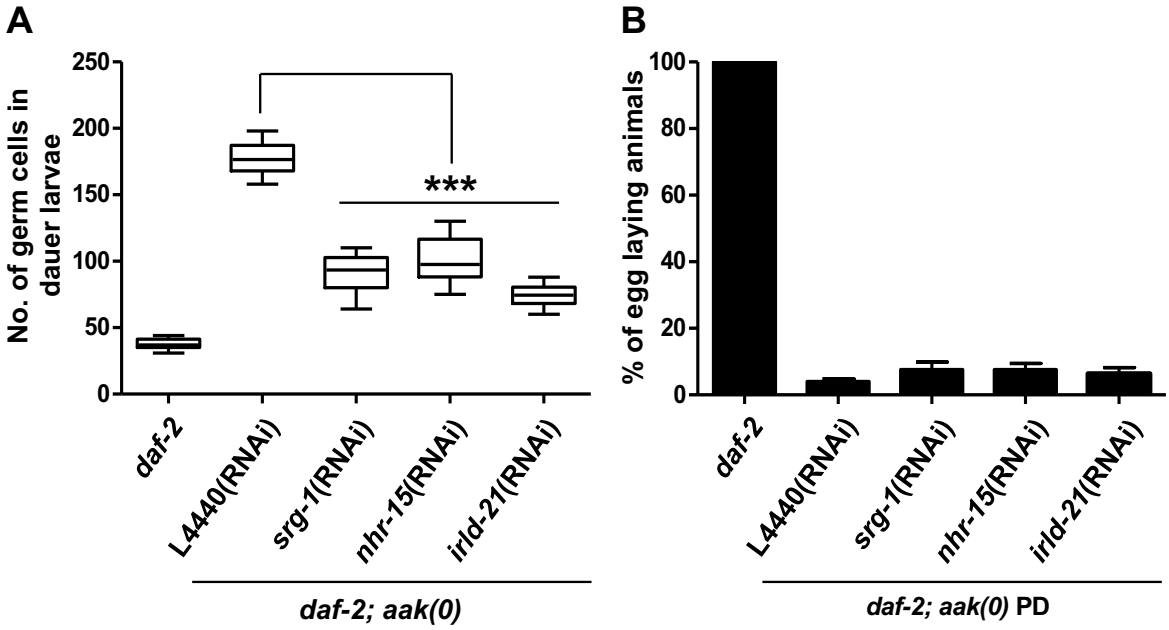

A
No. of germ cells in dauer larvae
250
200
150
100
50
0
***
daf-2
L4440(RNAi)
srg-1(RNAi)
nhr-15(RNAi)
irld-21(RNAi)
daf-2; aak(0)
B
% of egg laying animals
100
80
60
40
20
0
daf-2
L4440(RNAi)
srg-1(RNAi)
nhr-15(RNAi)
irld-21(RNAi)
daf-2; aak(0) PD

Figure 4.4. Many chromatin marks are misregulated both globally and in the germ line in *aak(0)* dauer larvae.

A, B) Global levels of H3K4me3, H3K9me3, H3K9ac, and H3K27me3 were quantified by performing whole animal western blot analysis of *daf-2* and *daf-2; aak(0)* dauer larvae. *glp-1*(RNAi) was performed post-embryonically using dsRNA feeding in order to compromise germline development without affecting early embryogenesis.

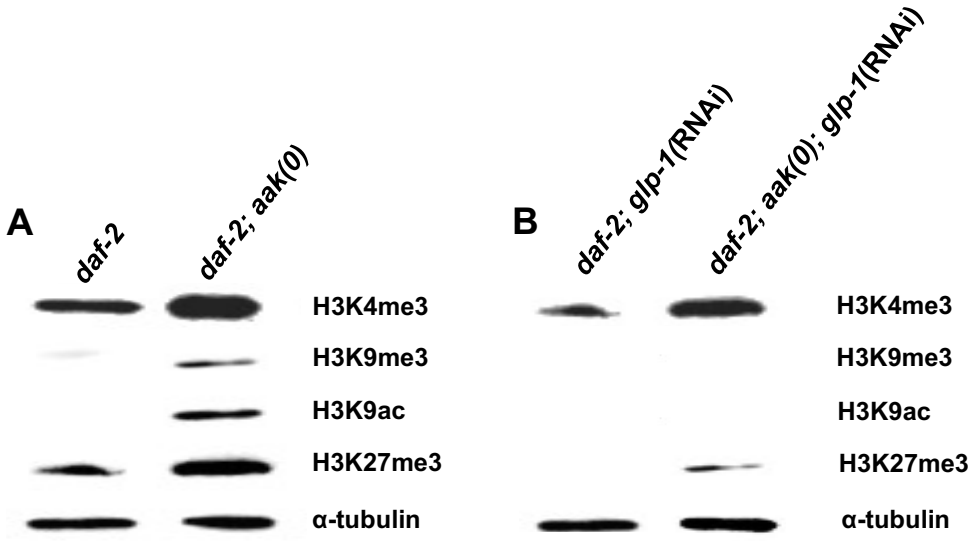

A
daf-2
daf-2; aak(0)
H3K4me3
H3K9me3
H3K9ac
H3K27me3
α-tubulin
B
daf-2; glp-1(RNAi)
daf-2; aak(0); glp-1(RNAi)
H3K4me3
H3K9me3
H3K9ac
H3K27me3
α-tubulin

Figure 4.5. The distribution and abundance of both activating and repressive chromatin marks are dramatically altered in the *aak(0)* dauer and post-dauer germ cells

All images are merged, condensed Z stacks. The graphs represent the average immuno-fluorescence signal of anti-H3K4me3 and anti-H3K9me3 normalized to DAPI across the dissected germ line. For the micrographs of *daf-2* dauer germ lines, the entire dauer germ line was analyzed (distal, proximal, distal). Due to technical difficulties only a single gonadal arm of the *daf-2; aak(0)* germ line was analyzed (distal, proximal). Images in A', B', C, C', D and D' are aligned such that distal is left side and the proximal is right. **A-A')** The left panel (*daf-2*) and right panel (*daf-2; aak(0)*) show H3K4me3 (green), and in **B, B')** H3K9me3 (green) staining merged with DAPI (red). **C-C', D-D')** PD *daf-2* and *daf-2; aak(0)* adult gonads were extruded and stained with anti-H3K4me3 and H3K9me3 (green) and signal intensity was quantified across the germ line using Image J software **P<0.001 using the F-test for variance when compared to *daf-2; aak(0)*. Scale bar: 4um n=15 for all the experiments.

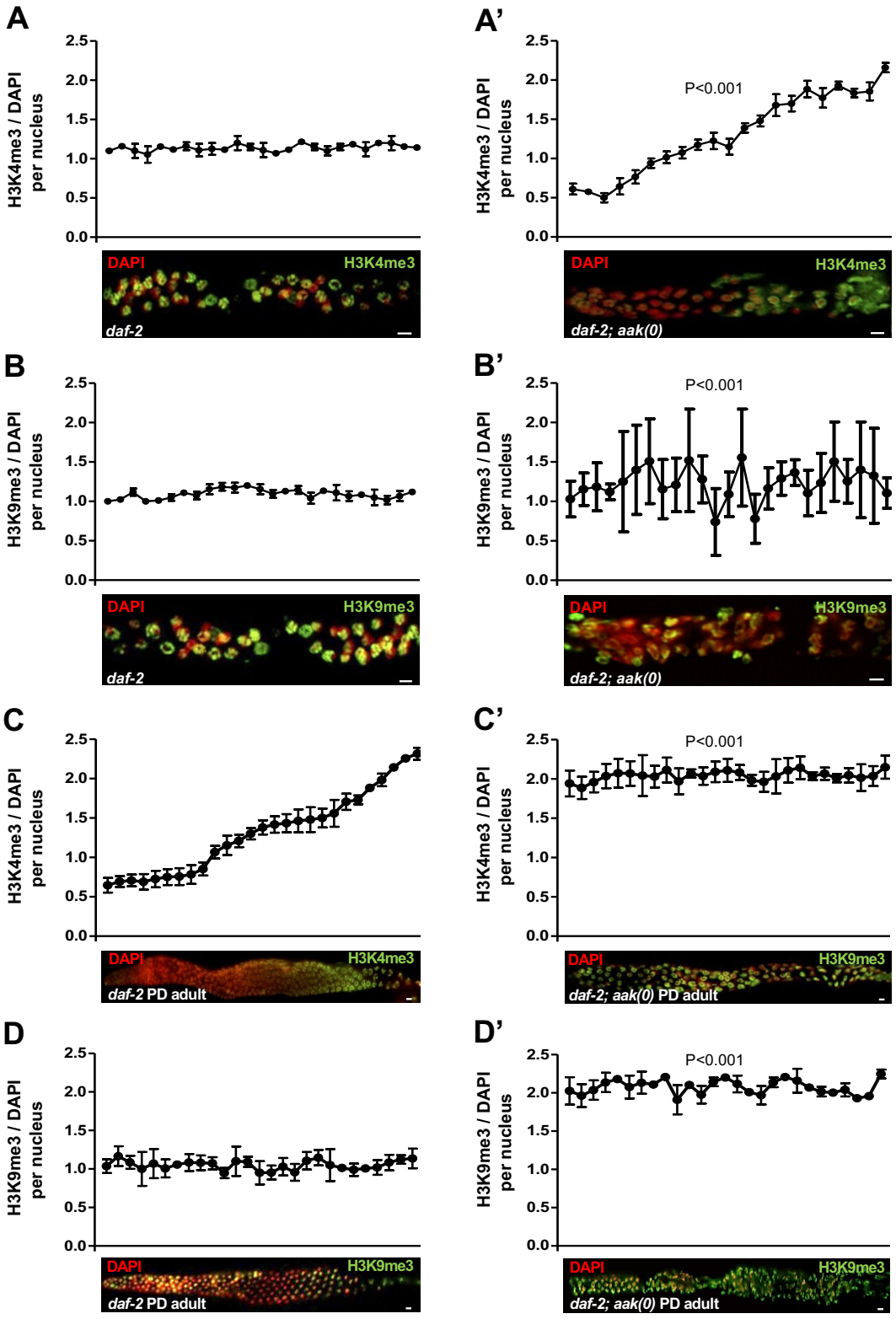

A
H3K4me3 / DAPI per nucleus
2.5
2.0
1.5
1.0
0.5
0.0
DAPI
H3K4me3
daf-2
A'
P<0.001
daf-2; aak(0)
B
H3K9me3 / DAPI per nucleus
daf-2
B'
daf-2; aak(0)
C
daf-2 PD adult
C'
daf-2; aak(0) PD adult
D
D'

Figure 4.6. Gene expression is altered in both the *aak(0)* dauer and post-dauer germ line.

A, B) Germline genes which were previously shown to be differentially expressed during and after transit through the dauer stage (Hall, Beverly et al. 2010), were quantified in *daf-2* and *daf-2; aak(0)* dauer and PD animals. The relative mRNA levels were analyzed using quantitative real-time PCR in both *daf-2* and *daf-2; aak(0)* dauer and PD adults. The expression of these germline genes was significantly altered in *daf-2; aak(0)* dauer and PD animals, when compared to *daf-2*. Error bars, indicate SD from 3 independent experiments. **P<0.001 using one-way ANOVA when compared to *daf-2*.

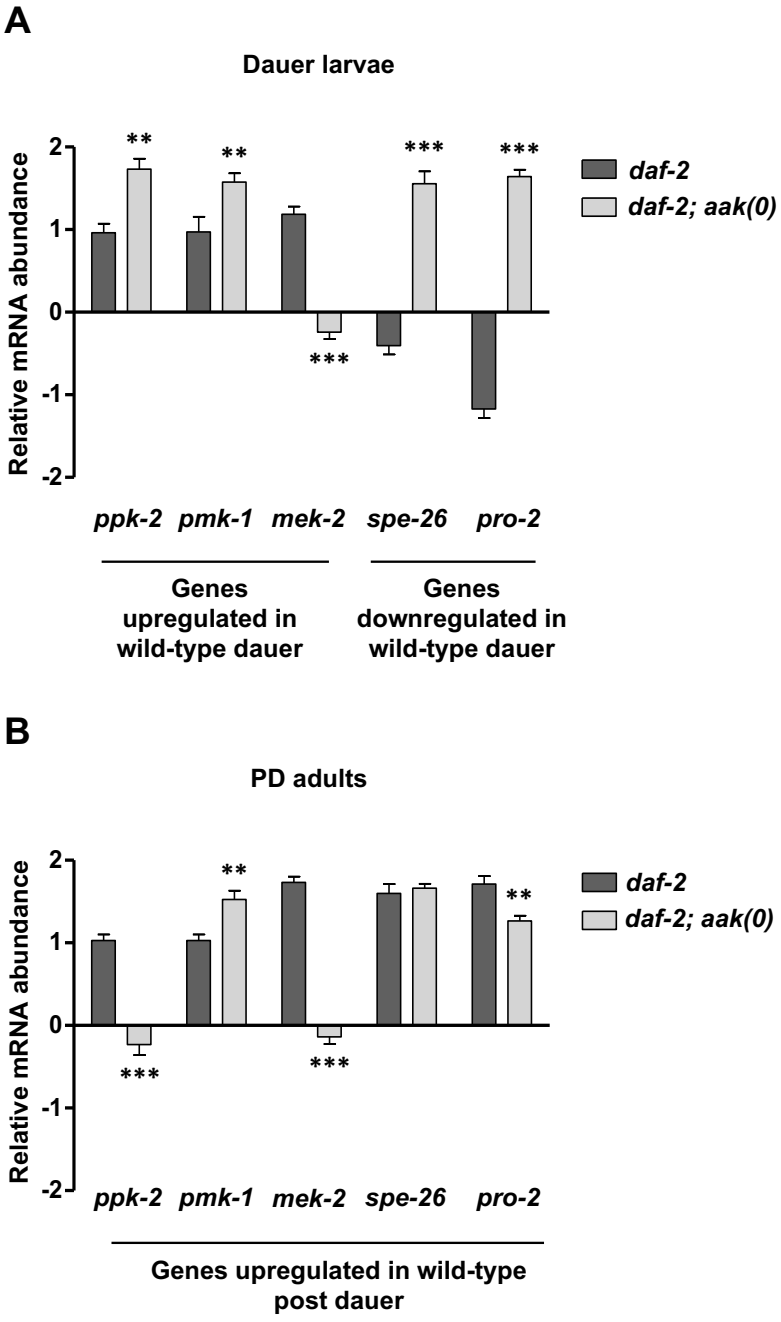

A
Dauer larvae
Relative mRNA abundance
2
1
0
-1
-2
**
**
***
***
***
***
daf-2
daf-2; aak(0)
ppk-2
pmk-1
mek-2
spe-26
pro-2
Genes upregulated in wild-type dauer
Genes downregulated in wild-type dauer
B
PD adults
Relative mRNA abundance
2
1
0
-1
-2
***
**
***
**
daf-2
daf-2; aak(0)
ppk-2
pmk-1
mek-2
spe-26
pro-2
Genes upregulated in wild-type post dauer

Figure 4.7. Compromise of small RNA pathway components partially suppresses *aak(0)* post-dauer sterility and dauer germline hyperplasia.

To compromise the function of small RNAi pathway, *daf-2; aak(0)* animals were subjected to RNAi by dsRNA feeding against multiple components of the small RNAi pathway. The L4440 empty RNAi vector was used as a control. **A)** The PD sterility observed in the *daf-2; aak(0)* animals was partially rescued by *dcr-1, rde-4* and *ergo-1* RNAi, while RNAi for the germline Argonautes, *csr-1* and *hrde-1* failed to suppress the observed sterility. **P<0.001 and *P<0.05 using Marascuilo procedure and n=100 **B)** Whole animal DAPI staining was performed to quantify the number of germ cells and the germline hyperplasia in the *daf-2; aak(0)* dauer larvae. Statistical analysis was performed using the two-tailed t-test when compared to L4440 treated animals where **P0.001 and *P<0.05; n=100. **C, D, E)** Followed by the RNAi treatment, global levels of H3K4me3 and H3K9me3 were quantified using whole animal western analysis. Global levels of H3K4me3 were significantly decreased in both *dcr-1* and *rde-4* compromised dauer animals, while

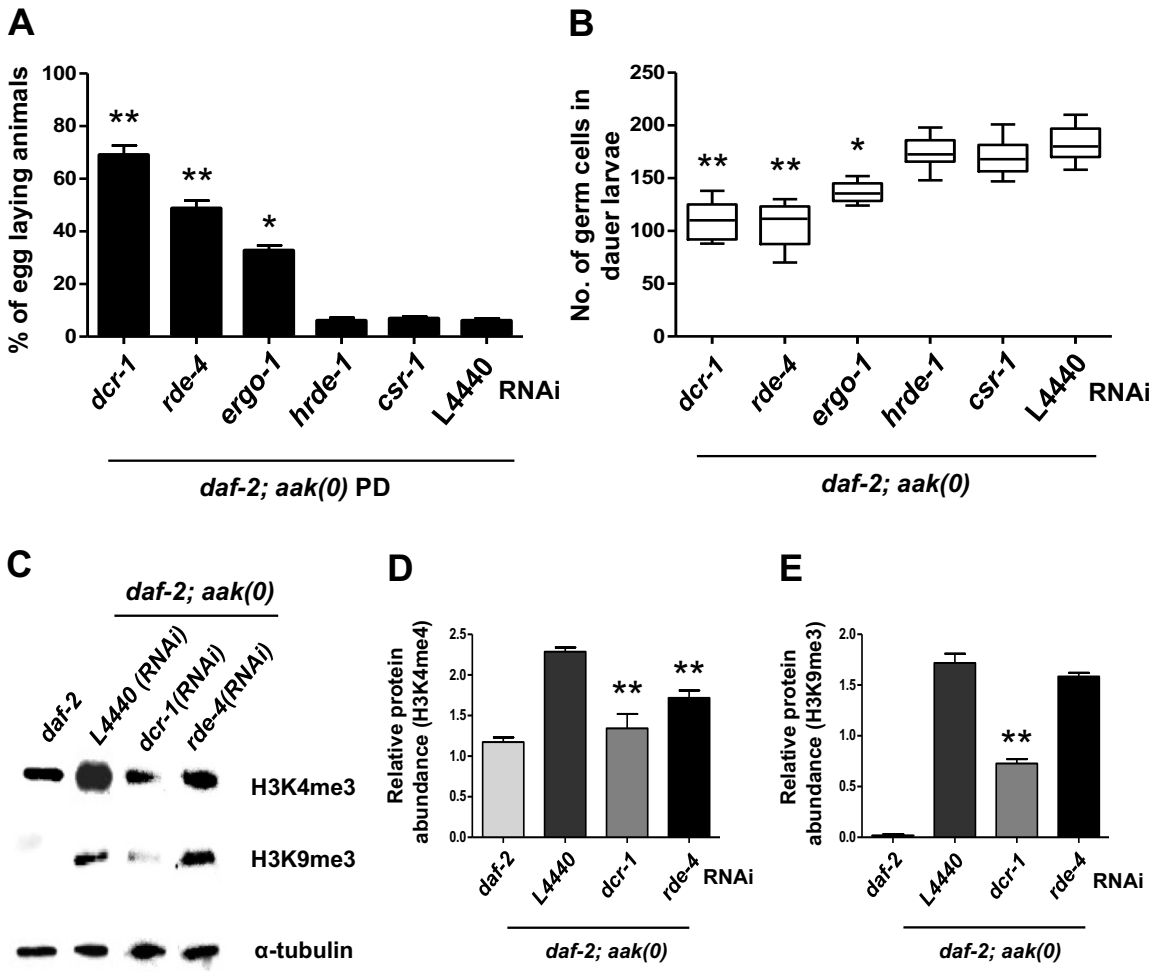
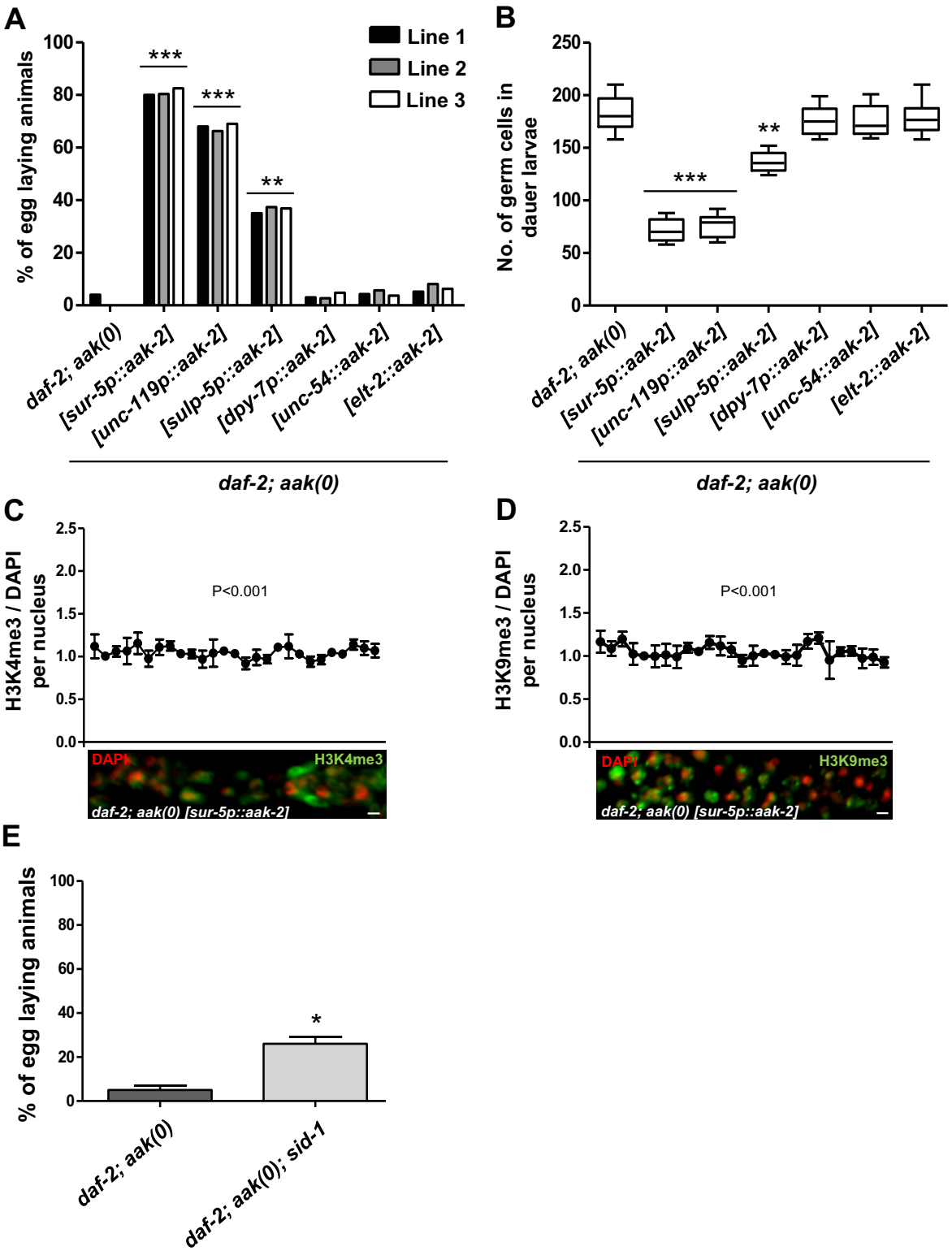
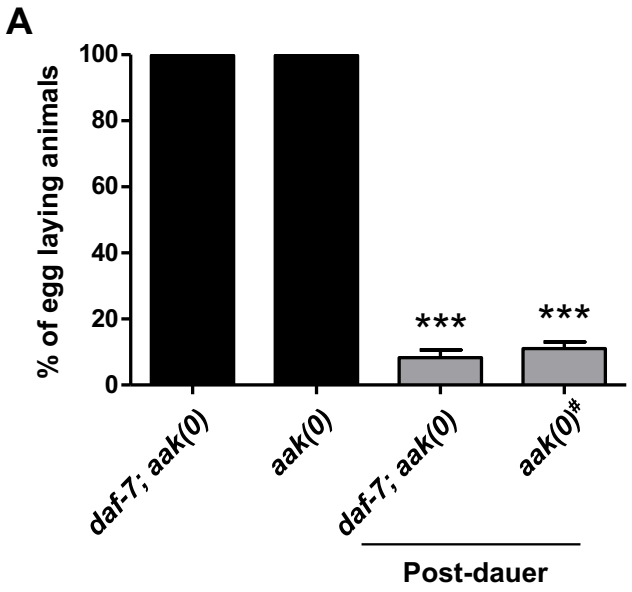

A
% of egg laying animals
100
80
60
40
20
0
**
**
*
dcr-1
rde-4
ergo-1
hrde-1
csr-1
L4440
RNAi
daf-2; aak(0) PD
B
No. of germ cells in dauer larvae
250
200
150
100
50
0
**
**
*
dcr-1
rde-4
ergo-1
hrde-1
csr-1
L4440
RNAi
daf-2; aak(0)
C
daf-2; aak(0)
daf-2
L4440 (RNAi)
dcr-1(RNAi)
rde-4(RNAi)
H3K4me3
H3K9me3
α-tubulin
D
Relative protein abundance (H3K4me4)
2.5
2.0
1.5
1.0
0.5
0.0
**
**
daf-2
L4440
dcr-1
rde-4
RNAi
daf-2; aak(0)
E
Relative protein abundance (H3K9me3)
2.0
1.5
1.0
0.5
0.0
**
daf-2
L4440
dcr-1
rde-4
RNAi
daf-2; aak(0)

Figure 4.8. Somatic AMPK activity is sufficient to restore germ cell quiescence and integrity in *aak(0)* mutants.

A) Plasmid constructs that contain *aak-2* cDNA driven by tissue-specific promoters was injected into *daf-2; aak(0)* mutants and both the dauer dependent germline hyperplasia and the PD sterility were evaluated for each transgenic strain. All transgenic lines are extrachromosomal and are represented by square brackets, and 3 independently generated lines were used for quantification. PD fertility was assessed 24h following the temperature shift after animals were maintained minimally 24 hour in dauer. ***P<0.0001 and **P<0.001 using Marascuilo procedure when compared to *daf-2; aak(0).* n=50 **B)** Whole animal DAPI staining was performed to quantify the number of germ cells present in the dauer gonad in the transgenic lines and compared to controls. ***P0.0001 and **P<0.001 using the two-tailed t-test when compared to *daf-2; aak(0).* n=50. **C, D)** All the analyzed images are merged, condensed Z stacks. The graphs represent the average immunofluorescence for H3K4me3 and H3K9me3 normalized to DAPI across the dissected gonad. **P 0.001 using F-test of variance when compared to *daf-2; aak(0)* and n=10. **E)** Disrupting soma to germline transmission of double-stranded RNA by compromising the function of *sid-1* partially restores fertility in the *daf-2; aak(0)* PD animals. A number of animals laying eggs (dead or hatched) were counted and the mean is shown ± SD. *P<0.05 using Marascuilo procedure when compared to *daf-2; aak(0)* and n=100.

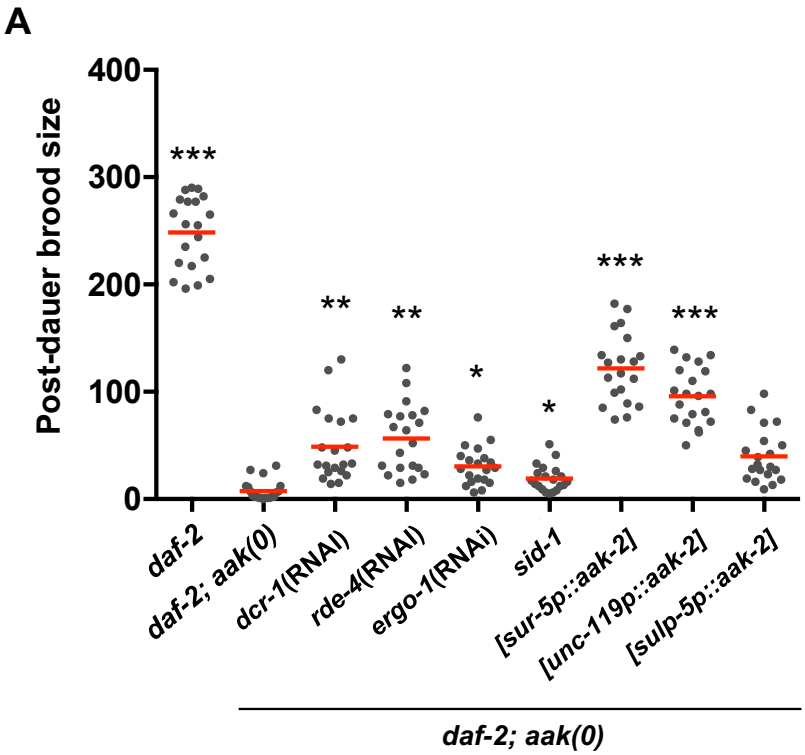

A
% of egg laying animals
Line 1
Line 2
Line 3
***
***
**
daf-2; aak(0)
[sur-5p::aak-2]
[unc-119p::aak-2]
[sulp-5p::aak-2]
[dpy-7p::aak-2]
[unc-54::aak-2]
[elt-2::aak-2]
daf-2; aak(0)
B
No. of germ cells in dauer larvae
***
**
daf-2; aak(0)
[sur-5p::aak-2]
[unc-119p::aak-2]
[sulp-5p::aak-2]
[dpy-7p::aak-2]
[unc-54::aak-2]
[elt-2::aak-2]
daf-2; aak(0)
C
H3K4me3 / DAPI per nucleus
P<0.001
DAPI
H3K4me3
daf-2; aak(0) [sur-5p::aak-2]
D
H3K9me3 / DAPI per nucleus
P<0.001
DAPI
H3K9me3
daf-2; aak(0) [sur-5p::aak-2]
E
% of egg laying animals
*
daf-2; aak(0)
daf-2; aak(0); sid-1

Supplemental figure 4.1. AMPK acts downstream of major pathways regulating dauer entry. **A)** If AMPK mutants along with the mutations in *daf-7*/TGF-b are grown under normal conditions, they are absolutely fertile. But upon dauer passage, *daf-7; aak(0)* animals display severe sterility. Similarly, *aak(0)* animals treated with dauer pheromone [*aak(0)*$^{\#}$], exhibit high sterility upon dauer recovery. ***P<0.0001 using Marascuilo procedure. Assays were performed three times and the data represent the mean ± SD for n=50.


A
% of egg laying animals
100
80
60
40
20
0
***
***
daf-7; aak(0)
aak(0)
daf-7; aak(0)
aak(0)#
Post-dauer

Supplemental figure 4.2. Compromise of the small RNA pathway and the somatic expression of AAK-2 partially rescues low brood size in AMPK post-dauer animals.

A) To assess general reproductive capability, F_1 progeny number were counted in the fertile animals following dauer recovery and the total distribution is plotted. The mean brood size for each group is depicted by the horizontal red line. ***P<0.0001, **P 0.001 and *P<0.05 using one way ANOVA when compared to *daf-2; aak(0.*


A
Post-dauer brood size
400
300
200
100
0
***
**
**
*
*
***
***
daf-2
daf-2; aak(0)
dcr-1(RNAi)
rde-4(RNAi)
ergo-1(RNAi)
sid-1
[sur-5p::aak-2]
[unc-119p::aak-2]
[sulp-5p::aak-2]
daf-2; aak(0)

Supplemental figure 4.3. Loss of *glp-1* reduces the number of germ cells in the dauer germ line.

A) *glp-1*(RNAi) was used to reduce the number of germ cells in the dauer larvae. Whole worm DAPI staining was performed to quantify the number of germ cells and *glp-1*(RNAi) results in significant reduction in the number of germ cells. ***P<0.0001 using the two-tailed t-test. n=25.

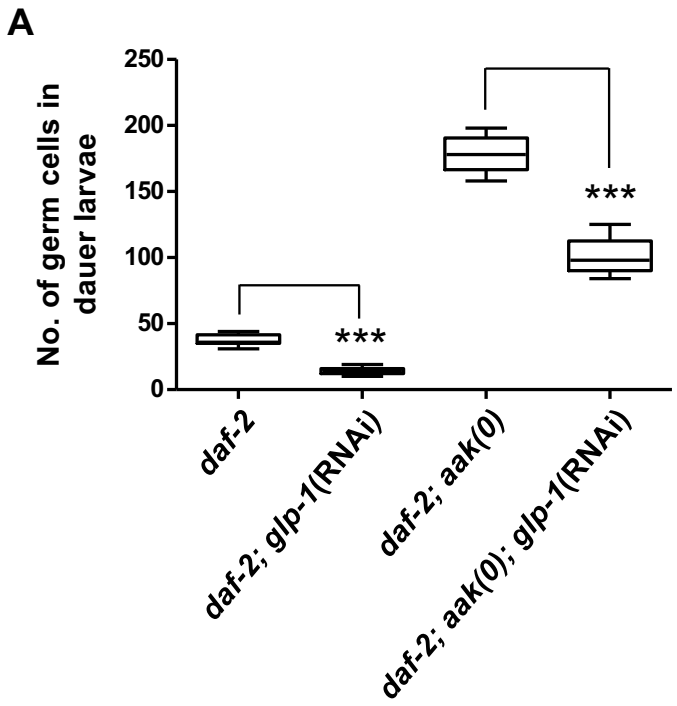

A
No. of germ cells in dauer larvae
250
200
150
100
50
0
***
***
daf-2
daf-2; glp-1(RNAi)
daf-2; aak(0)
daf-2; aak(0); glp-1(RNAi)

Table S4.1

Somatic defects in mutants that suppress sterility in post-dauer AMPK mutant adults.

Genotype	Post-dauer somatic phenotypes (%)					
	WT-like	**Protruding Vulva**	**Multivulva**	**Burst Vulva**	**Post-dauer Lethality**	**n**
daf-2; aak(0)	66.3 ± 6.5	10.12 ± 2.5	5.16 ± 1.8	7.32 ± 1.3	11.11 ± 3.3	50
***daf-2; aak(0) rde-4(*RNAi)**	84.2 ± 3.6 **	7.44 ± 2.5	0	2 ± 1.3	2.86 ± 1.22	50
***daf-2; aak(0) dcr-1*(RNAi)**	82.3 ± 4.5**	9.53 ± 2.32	0	3.1 ± 3.43	3.04 ± 1.1	50
***daf-2; aak(0) ergo-1*(RNAi)**	74.64 ± 2.1*	14.2 ± 4.2	2.05 ± 4.2	5.1 ± 4.4	5.15 ± 3.1	50
daf-2; aak(0); sid-1	75.76 ± 3.5*	8 ± 4.3	2.53 ± 3.73	4 ± 1.2	10.23 ± 2.7	50
daf-2; aak(0) [sur5p:aak-2]	93 ± 5.2***	2.5 ± 1.2	0	1.03 ± 0.8	3 ± 2.1	50
daf-2; aak(0) [unc-119p::aak-2]	87.3 ± 4.6**	1.2 ± 0.3	0	6.22 ± 2.8	6.05 ± 3.2	50
daf-2; aak(0) [sulp-5p::aak-2]	82 ± 4.1*	7.44 ± 3.3	2.1 ± 1.6	3 ± 1.3	6.22 ± 2.4	50

Somatic defects were quantified in various mutant backgrounds that suppress the sterility typical of AMPK mutant PD adults. Mutation that suppress the PD sterility also partially suppress the somatic defects in the *daf-2; aak(0)* PD animals. ***P<0.0001, **P 0.001 and *P<0.05 using the chi-square test.

Table S4.2

The PD AMPK germ line exhibits severe architectural defects in oocyte organization and gonadal symmetry.

Germline phenotype	Percentage of *daf-2;aak(0)* PD adults exhibiting germline defects (n=50)
Disorganized oocytes*	95 $\pm$ 2.8
Asymmetrical gonad**	60 $\pm$ 5.9

Germline morphology was monitored in AMPK mutant PD animals using a germ cell membrane marker. *Germlines that lacked the typical single-file organization. ** An asymmetric gonad refers to a gonad with irregular gonadal symmetry in terms of size and shape of the gonadal arms.

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Chapter 5: General discussion

C. elegans larvae can alter their normal development and enter the dauer diapause that enables them to thrive in the unfavourable conditions. As a part of this quiescent stage, the germline stem cells halt their proliferation and exhibit quiescence (Cassada and Russell 1975, Narbonne and Roy 2006). Previous work has demonstrated that LKB1/PAR-4 and AMPK cooperate to establish germ cell arrest under nutrient-deficient conditions (Narbonne and Roy 2006). Loss of either of these proteins causes severe dauer germline hyperplasia, while compromise of *par-4* in *aak(0)* mutants causes enhanced hyperplasia. Although AMPK is a well-characterized downstream target of LKB1/PAR-4, genetic analyses suggest that AMPK is unlikely to be the sole mediator of germ cell quiescence (Narbonne and Roy 2006). Based on this, we conducted three independent genome-wide RNAi screens with an aim to identify genes i) that result in germline hyperplasia typical of LKB1(*daf-2; par-4)* or AMPK compromise (*daf-2; aak(0)*) during the dauer stage, ii, iii) and genes that suppressed the germline hyperplasia phenotype typical of both LKB1*/par-4 or* AMPK mutant dauer larvae, respectively. This work has revealed the role of several gene families that potentially act downstream of LKB1/PAR-4 in an AMPK-dependent or -independent fashion, or in a parallel pathway, to establish germline stem cell cycle arrest during conditions of reduced insulin signalling.

In *C. elegans,* though germ cells show some asymmetry, and are clearly polarized in the gonad, no obvious conventional apical/basal polarity has been assigned to these cells. Our screens revealed several genes that have well-characterized roles in the regulation of apical/basal cell polarity and/or in mediating cytoskeleton regulation. Interestingly, our genetic analysis revealed that the genes encoding the cytoskeletal and polarity regulators act in a linear fashion, or in parallel to LKB1/PAR-4, but independent of AMPK (Kadekar, Chaouni et al. 2018). Based on this study, and the well-characterized role of PAR-4 in establishing early embryonic polarity and regulating actin

cytoskeleton (Kemphues, Priess et al. 1988, Chartier, Salazar Ospina et al. 2011), we propose a model where LKB1/PAR-4 plays an AMPK-independent role in regulating polarity and cytoskeleton within the dauer germline to establish or maintain dauer germ cell quiescence. This suggests that the LKB1-dependent regulation of cell polarity may be perturbed in PJS patients and might not necessarily be uniquely due to a reduction in AMPK function. It could be the additive effect of disrupting both pathways that contribute to the aetiology of the disease. Further characterization of these genes in *C. elegans* and the PJS mouse model (Rossi, Ylikorkala et al. 2002) will provide novel insights as to how LKB1/PAR-4 acts as a tumour suppressor to block abnormal cell divisions.

Our genetic suppressor screens also indicated that the elimination of the Notch receptor, *glp-1*, partially suppresses the abnormal cell divisions typical of both the AMPK and LKB1 mutant dauer germ lines, suggesting that the proliferating cells require active GLP-1 and that Notch signalling pathway must act downstream, or in parallel, with LKB1 and AMPK to establish GSC quiescence in the dauer larvae (Kadekar, Chaouni et al. 2018). We show that LKB1/AMPK modulates GLP-1 localization to downregulate the proliferative function of Notch signalling. We noted that upon dauer entry, GLP-1 undergoes re-localization from its position around the cell membranes of the GSCs to the membrane immediately adjacent to the rachis. Our data suggest that AMPK might directly phosphorylate the Notch receptor to target in for re-localization in a 14-3-3 binding protein-dependent manner. It has been shown that Notch signalling is the major oncogenic trigger in T cell acute lymphoblastic leukemia (T-ALL) and is abnormally regulated in many human malignancies (Liu, Zhang et al. 2013). AMPK has been implicated in regulating Notch-dependent cell acute lymphoblastic leukemia, suggesting that the function of Notch in these cells might be subject to AMPK-mediated regulation (Kishton, Barnes et al. 2016). We have identified genes

involved in endocytosis in our genomic screens (Kadekar, Chaouni et al. 2018) and understanding how these genes affect Notch localization or abundance upon their impairment will give us further insights as of how AMPK might regulate Notch signalling in general to inhibit aberrant cell proliferation.

Our further studies show that AMPK activity is not only important to maintain quiescence, but also to maintain the integrity of the GSCs. We noted that upon exiting the dauer stage, post-dauer AMPK animals exhibit severe sterility as they fail to produce functional gametes. During dauer entry and dauer exit, animals alter their global chromatin landscape and gene expression profile (Hall, Beverly et al. 2010). Our findings suggest that AMPK activity is essential to regulate appropriate global chromatin modifications in the dauer larvae to ensure appropriate gene expression. As the abnormal levels persist in the post-dauer AMPK mutants, we propose that AMPK might be important to resolve the aberrant chromatin modification upon dauer exit. Our genetic data suggest that AMPK directly or indirectly impacts on a small RNA pathway to regulate the chromatin modifications required for germline integrity both during dauer and following dauer recovery. To verify if AMPK impacts on the types and abundance of small RNAs generated in response to starvation, high throughput sequencing will be performed to identify and quantify both the small RNAs and mRNAs (the genes of which are potential targets of the small RNAs) in AMPK mutants compared with *daf-2* control dauer animals. In mammals, AMPK directly phosphorylates histone proteins to activate stress-promoted gene transcription (Bungard, Fuerth et al. 2010). Abnormal changes to the chromatin landscape and associated aberrant gene expression and altered epigenomic patterns are hallmarks of cancer (Ellis, Atadja et al. 2009, Audia and Campbell 2016). Thus our research provides an excellent framework to study how LKB1/AMPK

signalling pathway mediates chromatin modifications, which may contribute to their roles in tumour suppression.

Lastly, we identified that AMPK activity in the soma, especially in the neurons and the excretory system is sufficient to partially restore dauer germline quiescence and fertility in the post-dauer AMPK animals. This suggests that AMPK might also impinge on the mechanisms that help to transmit the signals from the neurons, or the excretory system, to other tissues; most importantly to the germ cells to mediate the appropriate adaptation in response to conditions in the surrounding environment. Further, we show that the abnormal small RNAs transduced from the somatic tissues to the germ cells may result in the abnormal chromatin modifications and consequent gene expression, compromising germ cell integrity. Further reverse or forward genetic screens along with biochemical analyses will help to identify the potential targets of AMPK that might be critical for regulating the endogenous levels and/or the systemic transport of the small RNAs to ensure the quiescence and integrity of the germ cells under energetic stress.

Final statement

Using *C. elegans* as a model system we have identified several genes that potentially act downstream of, or in parallel to, LKB1/AMPK signalling to regulate germ cell divisions during periods of nutrient stress. We demonstrate that AMPK is not the sole target of LKB1/PAR-4 signalling and a collection of cell polarity and cytoskeletal regulators act downstream of LKB1/PAR-4, but are independent of AMPK. Further characterization of these genes and how they interact with LKB1/PAR-4 in *C. elegans* will further inform us as to how LKB1/PAR-4 acts as a tumour suppressor. LKB1 is the causative gene in cancer-predisposing disease Peutz Jeghers

Syndrome and thus implications of these findings can be also applied to design novel drug therapies to circumvent untimely cell divisions.

Our work also delineates how AMPK signalling impinges on critical aspects of germline stem cell biology to maintain its quiescence and integrity through appropriate chromatin modifications. This work provides an excellent model to comprehend how disruption of these chromatin-based mechanisms contribute to several abnormalities. These findings further extend our understanding of how organisms adapt at the molecular level to ensure their survival, and potentially the survival of successive generations, during exposure to extreme growth conditions.

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