Genetic characterization of AMPK-interacting genes in germline stem cell quiescence and integrity in *Caernorhabditis elegans*

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Abstract

In response to adverse conditions, *C. elegans* can enter an alternative developmental stage called dauer. In this state the larva undergoes several changes in order to conserve energy and withstand environmental stress. One such change occurs in the germline, where the germline stem cells (GSC) arrest their mitotic divisions and remain quiescent for the duration of this stage. Previous studies have identified the catalytic subunit of AMP-activated protein kinase (AMPK), *aak-2,* to be necessary in establishing this cell cycle quiescence. The *aak-2* mutants exhibit pronounced germline hyperplasia compared to the normal dauer, however the molecular mechanism through which AMPK controls the GSC proliferation remains unclear. To address this question, we identified potential AMPK targets involved in the GSC quiescence by conducting a genome-wide RNAi suppressor screen for candidates that rescue the *aak-2*-induced hyperplasia. After screening 18,432 genes, we identified 59 candidates, which were further categorized into gene ontology groups. Of particular interest are genes necessary for miRNA-dependent gene silencing and components of the extracellular matrix (ECM), important constituents of stem cell niches. In addition we also discovered that animals exhibiting the *aak-2*-dependent hyperplasia in dauer are sterile and have severe morphological germline defects following the recovery from dauer. This demonstrates the importance of AMPK-dependent dauer germline quiescence on post-dauer reproduction. Candidate suppressors of the dauer germline hyperplasia failed to rescue the sterility phenotype, suggesting that although AMPK-dependent, the processes that control the GSC quiescence in dauer and the germline recovery post-dauer are independent.

Résumé

En réponse à des conditions défavorables, les vers *C. elegans* peuvent entrer dans un état de développement alternatif appelé dauer. Dans cet état, la larve subit plusieurs changements afin d'économiser son énergie et résister aux contraintes de l'environnement. Un tel changement se produit dans la lignée germinale; les cellules souches de lignée germinale (CGC) stoppent leurs divisions mitotiques et restent au repos durant toute la durée de cette étape. Des études précédentes ont identifié la sous-unité catalytique de la protéine kinase AMP-dépendante (AMPK), *aak-2*, nécessaire à l'établissement de ce repos du cycle cellulaire. Les mutants *aak-2* présentent une hyperplasie de la lignée germinale prononcée par rapport à un dauer normal, mais le mécanisme moléculaire par lequel l'AMPK contrôle la prolifération des CGC reste incertain. Pour répondre à cette question, nous avons identifié des cibles potentielles de l'AMPK impliquées dans la quiescence des CGC, en effectuant un crible ARNi à l'échelle du genome entier pour trouver les candidats qui reduisent l'hyperplasie induite par *aak-2*. Après avoir testé 18 432 gènes, nous avons identifié 59 candidats, qui ont ensuite été classés en groupe en function de leur ontologie respective. D'un intérêt particulier sont les gènes nécessaires pour l'extinction de gènes grace au mechanisme de miARN ainsi que les composants de la matrice extracellulaire (ECM), constituants importants des niches de cellules souches. En outre, nous avons également découvert que les animaux présentant une hyperplasie durant dauer dependante de *aak-2* sont stériles et conservent de graves anomalies morphologiques de la lignée germinale après la reprise du development normal. Cela demontre l'importance de la quiescence de la lignee germinale,d'une maniere dependante de AMPK, pour la reproduction post-dauer. Nourrir les animaux en dauer avec des gènes candidats qui suppriment l'hyperplasie dauer de la lignée germinale a échoué à sauver le phénotype de stérilité, ce qui suggère que, bien que dependant de l'AMPK, les processus qui contrôlent la mise au repos de la CGC dans dauer et la récupération post-dauer de la lignée germinales.

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Preface

This is a thesis prepared and presented in accordance with the "Guidelines for thesis preparation" from the Faculty of Graduate Studies and Research. It consists of four chapters. Chapter 1 provides a literature review and research motivation; Chapter 2 and 3 are research chapters, each composed of an Introduction, Materials and Methods, Results, Discussion, Figures and a Reference list, in that respective order. Chapter 4 is a general discussion and conclusion of this thesis as a whole. My research supervisor, Dr. Richard Roy, contributed to the research presented every step of the way, guiding the research process, contributing ideas and suggesting experiments. I have written this thesis entirely, which was revised by Dr. Richard Roy. Additional contributors are recognized in the Acknowledgements section.

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Chapter 1

Literature Review

Introduction:

Stem cells play a paramount role in development, maintenance of homeostasis, tissue regeneration and cancer (Joshi *et al.*, 2010). They are mitotically dividing pluri- or multi-potent cells that give rise to various differentiated tissues. Stem cell divisions must be appropriately coordinated with the energetic status of the organism in order to avoid malignant overgrowth, and as a result, a balance between stem cell quiescence and proliferation is established (Orford & Scadden, 2008). This equilibrium is achieved through the regulation of cell cycle progression and cell division. Therefore, studying these fundamental cellular processes will provide insights into the control of stem cell dynamics.

Germline stem cells (GSCs) are a population of totipotent cells that differentiate into gametes that will eventually give rise to the entire organism. The germline is considered immortal as it passes on the genetic information to future generations (Hubbard & Greenstein, 2005). Although these stem cells are specific in their function, they possess conserved stem cell characteristics, the study of which may provide insight toward and understanding of various stem cell behaviors (Yuan & Yamashita, 2010). In the model organism *Caenorhabditis elegans,* GSCs share many similar features with the adult mammalian GSCs such as plasticity, the importance of communication between stem cells and the somatic niche, as well as the ability of these cell populations to proliferate throughout the life of the organism replenishing the differentiated germ cells (Hubbard, 2007; Johnson *et al.*, 2004). This makes the *C. elegans* germline a good model to study general stem cell dynamics.

One of the remarkable features of stem cells is their plasticity, where in response to systemic and environmental signals the cells can either proliferate or undergo quiescence (Drummond-Barbosa, 2008). In the case of epidermal disruption, the mesenchymal stem cells respond by proliferating and contributing to signalling and recruitment of factors that assist wound healing (Maxson *et al.*, 2012). During an environmental duress, animals may alter their development to conserve energy and match their physiological development to the available resources (Fielenbach & Antebi, 2008). Under unfavorable conditions stem

cells undergo mitotic quiescence and stay poised to preserve key functional features until the environment changes for the better (Cheung & Rando, 2013). This adaptive response provides an excellent context to study cell quiescence and cell division control.

Caenorhabditis elegans **as a model organism**

For almost half a century, *C. elegans* has been used as a model to study fundamental biological processes. The transparent soil dwelling nematode, 1.5 mm in length, was established as a model organism in 1965 by Sydney Brenner due to its rapid life cycle and relatively simple physiology (Riddle *et al.*, 1997). The invariance of the cell lineage from animal to animal, as well as the constant cell number and their fixed position, indicates tight developmental control, making the worm an amenable model for developmental biological research (Kipreos, 2005). In the field of genetics, the worm is appreciated for its amenability to laboratory cultivation and short life cycle, which allows execution of high-throughput forward and reverse genetic screens (Jorgensen & Mango, 2002). *C. elegans* exist as self-fertilizing hermaphrodites but under certain conditions may develop into males, which enables genetic crossing and further enhances their capacity as a good model for genetic analysis (Brenner, 1974).

C. elegans were first chosen as a model to study genetic basis of behavior, but it has proven to be extremely useful in studying many cellular and developmental processes including cell proliferation, cell signalling, apoptosis, metabolism and aging (Kaletta & Hengartner, 2006). *C. elegans* has also been at the forefront of biomedical research as it shares conserved signalling pathways, as well as considerable homology at the genome level with most higher order organisms (Kuwabara & O'Neil, 2001). The highly regulated cell divisions provide insights into mechanisms of cell cycle regulation and cell polarity (Gonczy, 2008). Ultimately, *C. elegans* is a system in which numerous fundamental processes can be studied in a context of the entire multicellular organism.

An important advantage of the *C. elegans* is their transparency, making it an excellent model to study cell biology. This property allowed scientists to monitor the highly stereotypical cell divisions in the early embryo *in vivo*, and tracing the entire embryonic cell lineage of the organism using light microscopy (Oegema & Hyman, 2006; Sulston *et al.*, 1983) Differential interference microscopy (DIC) allows observation of tissues and cellular organelles (Shaham, 2006). Transgenenic approaches using the GFP-fusion transgenes allow the monitoring of intracellular processes *in vivo*, while functional GFP-fusion proteins can provide positional and temporal information about virtually any proteins of interest (Muller-Reichert *et al.*, 2008; Shaham, 2006).

For geneticists, one major advantage of using *C. elegans* is the ability to perform large-scale genetic screens. Large-scale forward and reverse screens allow scientists to identify components of important biological pathways while further epistatic analysis can yield hierarchical information about gene function. A particularly useful reverse genetic tool is RNA interference (RNAi). RNAi technology, combined with the progress made in whole-genome sequencing, became a standard for large-scale reverse genetic screens (Perrimon *et al.*, 2010). RNAi is a post-transcriptional gene silencing mechanism that occurs through a conserved pathway that is triggered by dsRNA (Bass, 2000; Fire *et al.*, 1998; Grishok, 2005). Whether it is introduced exogenously or produced endogenously within a cell, the dsRNA is first processed by Dicer, which cleaves the dsRNA into 21-23 nucleotide-long fragments of small interfering RNAs (siRNAs). These siRNAs consequently bind to the RISC complex, which guide them to a target mRNA, which is subsequently cleaved by an endonuclease in the RISC complex (Bass, 2000; Hammond, 2005; Mello & Conte, 2004; Perrimon *et al.*, 2010). In *C. elegans* RNAi is a particularly potent molecular tool because it can be easily administered using different techniques and has few off-target effects (Kamath & Ahringer, 2003; Tabara *et al.*, 1998; Timmons & Fire, 1998)

Caenorhabditis elegans dauer stage

In order to thrive in their environment, organisms elicit a coordinated physiological response to environmental fluctuations (Baugh, 2013). When they experience environmental stress, they may enter a hypometabolic state (e.g. hibernation, quiescence or diapause) where their metabolism and cell divisions slow down (Padilla & Ladage, 2012). The nematode *C. elegans* is well adapted to sensing its milieu and altering its course of development accordingly. Under plentiful conditions, following embryogenesis, the worm transitions through four larval stages and eventually molts into an adult. This process takes approximately three days (Figure 1.1) (Cassada & Russell, 1975; Riddle & Albert, 1997). At the optimal temperature range of 15-20°C, *C. elegans* has a lifespan of two to three weeks (Kenyon *et al.*, 1993). This normal course is interrupted when the worm is subjected to an environmental duress, namely when the population density in a given area increases and food supply becomes scarce. In response to these conditions young larvae can enter an alternative enduring dauer larva state. During this stage, the animals undergo morphological and physiological changes, which allow them to survive the harsh conditions. When the stress is alleviated and conditions become suitable for further development and reproduction, the animal can exit dauer directly into the L4 stage and resume normal development, producing a healthy, reproductively competent adult (Cassada & Russell, 1975; Riddle & Albert, 1997).

Entry into dauer: environmental cues

The complex changes that the worm undergoes prior to entering the dauer state are triggered by changes in its surroundings, the assessment which begins at the L1 stage (Riddle & Albert, 1997). Environmental cues are sensed and transduced via molecular signalling, prompting a systemic response. The primary sensory cue is the dauer pheromone which compose a family of fatty acid-like ascaroside molecules (Butcher *et al.*, 2007; Ludewig & Schroeder, 2013). Increased levels of the pheromone indicate overcrowding and prompt the young larva to go into dauer, all the while preventing dauer exit (Golden & Riddle, 1982; J. Golden & D. Riddle, 1984).

Acting antagonistically to the levels of pheromone is the food signal present in bacterial cultures and yeast extracts (J. W. Golden & D. L. Riddle, 1984a; Riddle & Albert, 1997) This hydrophilic signal has chromatographic properties similar to carbohydrates, however its exact identity is unknown (Golden & Riddle, 1982; J. W. Golden & D. L. Riddle, 1984a; Riddle & Albert, 1997). It is the high ratio of pheromone to food that favors dauer induction and maintenance, and prevents the shift back to reproductive development (Golden & Riddle, 1982). Finally, temperature modulates the response to the pheromone/food balance, and it plays a role in conditions of intermediate pheromone levels, where higher temperatures potentiate dauer entry (J. W. Golden & D. L. Riddle, 1984b; Riddle & Albert, 1997).

Entry into dauer: molecular cues

Sensing the pheromone and the food signals depends on the function of ciliated sensory neurons (Albert *et al.*, 1981). The ablation of amphid sensory cells, specifically ADF, ASG and ASI, leads to constitutive dauer entry, suggesting that it is these neurons that interpret the environmental signal, prompting dauer formation (Bargmann & Horvitz, 1991). These cells therefore sense the dauer pheromone, the food signal or both, and then convey a neuroendocrine signal to the downstream pathways to elicit a physiological response.

Genetic studies have elucidated a network of molecular pathways that act downstream of the environmental signals to induce dauer. Screens for animals that were abnormal in dauer formation revealed two classes of mutants: those that constitutively entered dauer and those that did not enter dauer at all, irrespective of the conditions. The genes responsible for causing the two phenotypes were termed Daf-c and Daf-d genes, respectively (Riddle & Albert, 1997; Swanson & Riddle, 1981). Genetic and epistatic analysis allowed these genes to be ordered in a hierarchical manner, determining that parallel insulin-like, TGF-beta and cGMP pathways comprised the intricate signalling of dauer formation, and converge on the DAF-12 nuclear hormone receptor, the activity of

which is required for appropriate dauer formation (Antebi *et al.*, 2000; Riddle *et al.*, 1981; Thomas *et al.*, 1993; Vowels & Thomas, 1992)

Insulin-Like Signalling

Much of the information about nutritional availability is conveyed via insulin-like signaling (Tobin & Saito, 2012). In *C. elegans,* the upstream component of the insulin-like signalling (ILS) pathway is the DAF-2 receptor, which belongs to the insulin/insulin-like growth factor family of receptors, and is activated by agonistic and antagonistic binding of insulin-like peptides (Kimura *et al.*, 1997). During the early experiments on genes involved in dauer formation, deletion of *daf-2* resulted in a dauer-constitutive, Daf-c*,* phenotype (Riddle *et al.*, 1981). In addition, when adult animals with a temperature sensitive *daf-2* mutation are shifted to a restricted temperature, they exhibit life-span extension (Kenyon *et al.*, 1993).

Ligand binding to the DAF-2 receptor on the plasma membrane initiates a signalling cascade that is required for reproductive growth and metabolism (Ogg & Ruvkun, 1998). Downstream, DAF-2 recruits a phosphoinositide-3-kinase (PI3K) AGE-1, to convert phosphoinositide-3, 4-P2 (PIP2) to phosphoinositide-3, 4, 5-P3 (PIP3) (Dorman *et al.*, 1995). This conversion increases the concentration of PIP3, which then recruits Akt/PKB to the plasma membrane. The two members of the Akt/PKB family in *C. elegans* are AKT-1 and AKT-2. Inactivation of *C. elegans* Akt/PKB signalling results in a dauer constitutive phenotype. An activating mutation in *akt-1* suppresses the dauer arrest phenotype of *age-1* mutants, indicating that it acts downstream of *age-1* (Morris *et al.*, 1996; Murphy & Hu, 2013; Paradis & Ruvkun, 1998). The DAF-2 and AGE-1 signals are modulated by the PTEN phosphatase homologue DAF-18 as it dephosphorylates PIP3 through its phosphatase activity (Ogg & Ruvkun, 1998).

The downstream effector of the ILS pathway is DAF-16, a forkhead transcription factor, similar to FoxO, which regulates various cellular processes such as metabolism, ageing and a number of stress-responses (Murphy *et al.*, 2003). *daf-16* was identified

during the initial screens for dauer formation, where mutation in this gene caused a dauer-defective phenotype (Riddle *et al.*, 1981). Later, it was shown that active DAF-2 signaling results in DAF-16 being sequestered in the cytosol, preventing nuclear translocation and subsequent transcriptional activation of its target genes (Lin *et al.*, 2001; Mukhopadhyay *et al.*, 2006). When DAF-2 signal is reduced, whether due to a mutation of one of its pathway components or due to the low endocrine signal in response to low food availability, the nuclear DAF-16 concentration increases, leading to transcription of DAF-16 target genes that ultimately affect dauer formation and life-span extension genes (Mukhopadhyay *et al.*, 2006).

The ILS pathway has been implicated in control of cell division in *C. elegans*. The *C. elegans* germline stem cells that normally proliferate throughout the life of the organism stop dividing at various stages of development under conditions of nutrient stress. If the animal encounters inadequate food supply upon hatching, it undergoes an L1 diapause during which the two germline precursor cells arrest at the G2 stage of the cell cycle (Fukuyama *et al.*, 2006). In response to nutrient stress later in development, the animal goes into dauer. The dauer germline consists of approximately 32 cells and the cells are kept quiescent at the G2/M phase (Narbonne & Roy, 2006c). Finally, the *C. elegans* can undergo a starvation-induced adult reproductive diapause (ADR) at L4, during which the germline arrests its cell divisions and undergoes apoptosis to reduce the germ cell number in each of the gonadal arms (Angelo & Van Gilst, 2009). These findings highlight the role of ILS in coordinating an appropriate stem cell division response to nutritional deprivation.

TGF-Beta signaling

TGF-beta is another signal required for dauer decision (Patterson & Padgett, 2000; Zimmerman & Padgett, 2000). The TGF-beta related ligand is encoded by *daf-7* gene, loss of which causes a Daf-c phenotype, suggesting that active DAF-7 signalling is required for normal development (T. L. Gumienny & Savage-Dunn, 2013; Swanson & Riddle, 1981). The *daf-7* expression is restricted to the ASI chemosensory neurons, suggesting that it negatively regulates dauer development by processing sensory cues (Ren *et al.*, 1996). In

addition to *daf-7, daf-1, daf-4, daf-8* and *daf-14* were identified as components of the TGF-beta pathway. Loss of any of these genes also resulted in a dauer constitutive phenotype. Additionally, *daf-3, daf-5* and *daf-12* genes were demonstrated to act downstream of the aforementioned pathway, since their compromise suppresses the Daf-c phenotype (Thomas *et al.*, 1993).

The DAF-7 ligand acts through binding to DAF-1 and DAF-4; the Class I and II TGF-beta receptors, respectively (Estevez *et al.*, 1993; Ren *et al.*, 1996). The serine/threonine receptors form a complex, and phosphorylate and activate the downstream SMAD DAF-8 and an atypical SMAD DAF-14 (Patterson & Padgett, 2000; Riddle & Albert, 1997). These SMADs, in turn, inhibit DAF-3/DAF-5, and acts as a negative transcriptional regulatory complex that resembles the canonical SMAD that antagonizes normal reproductive development. In essence, active DAF-7 signalling leads to repression of DAF-3/DAF-5, and in the absence of DAF-7, this repression is alleviated, and dauer program ensues (T. L. Gumienny & Savage-Dunn, 2013; Hanna-Rose & Han, 2000; Inoue & Thomas, 2000; Y. Wang & Levy, 2006).

cGMP signaling

cGMP pathway is the third signalling cascade implicated in dauer formation. *daf-11* encodes the membrane bound guanyl cyclase receptor which catalyzes a secondary messenger molecule cGMP from GTP (Barna *et al.*, 2012; Birnby *et al.*, 2000), while genetic evidence also implied activity of *daf-21* gene, a member of the Hsp90 family of molecular chaperones. Mutations in both *daf-11* and *daf-21* showed pleiotropic phenotypes, distinct from other dauer constitutive mutations (Vowels & Thomas, 1992). It is suggested that DAF-21 is needed to stabilize DAF-11 in order for it to carry out its proper function and inhibit dauer formation (Birnby *et al.*, 2000). DAF-11 is expressed in several chemosensory neurons, where it affects cGMP-gated channels and facilitates sensory transduction.

Ultimately, the three pathways converge on the DAF-12 nuclear hormone receptor (Riddle & Albert, 1997). Daf-d mutations in *daf-12* are epistatic to most Daf-c genes (Antebi *et al.*, 2000; Riddle *et al.*, 1981). Nuclear hormone receptors are a class of transcription factors that are activated in response to binding of a lipophilic hormone (Rottiers & Antebi, 2006). They initiate broad changes in gene expression, thus regulating developmental transitions (Antebi, 2013). Dafachronic Acid (DA) is a class of acid-like steroids that bind to DAF-12 to prevent dauer formation and promote reproductive development (Motola *et al.*, 2006). The production of these ligands requires DAF-9, a cytochrome p450 enzyme, to generate the DA molecules from sterol precursors (Gerisch & Antebi, 2004; Motola *et al.*, 2006). *daf-9* loss of function is a Daf-c mutation, which acts downstream or in parallel to *daf-16, daf-3* and *daf-5* but upstream of *daf-12* with respect to dauer formation (Gerisch *et al.*, 2001). The Insulin-Like and TGF-beta pathways converge within the steroidogenic tissues to influence the DA hormone production. The hormones are then distributed throughout the various tissues of the organism, and eventually bind to the DAF-12 receptor to activate target genes, allowing the animal to bypass the dauer arrest (Antebi, 2013). Thus, the transcriptional output of active TGF-beta and Insulin -signaling stimulates the DA hormone production, which activates the DAF-12 transcription factor. This activation guides the decision whether to execute reproductive development. In the absence of either of the signals, the hormones are no longer produced and DAF-12 remains associated with its co-repressor DIN-1, thereby specifying the dauer stage (Antebi, 2013; Riddle & Albert, 1997; Rottiers & Antebi, 2006).

Physiological changes during the *C. elegans* dauer stage

During the dauer stage, *C. elegans* dramatically changes its morphology and behavior to facilitate dispersal and long-term survival (Riddle & Albert, 1997).The body becomes radially constricted, and impermeable to chemicals and resistant to desiccation due to the thickening of the cuticle and sealing off its orifices (Cassada & Russell, 1975). The dauer larvae are motionless, but in order to facilitate dispersion, they are capable of rapid movement and a specialized behavior called nictation, where the larvae erect

themselves and wave in the air, presumably in order to attach themselves to other organisms and be transferred to a different location (Riddle & Albert, 1997).

When in dauer, the animals suspend pharyngeal pumping and do not feed. (Cassada & Russell, 1975). Consistent with the need to adapt to an environment with poor food availability, dauer metabolism is altered to utilize internal energy resources. Dauer larvae show a decreased rate of oxidative metabolism while it relies on the breakdown of lipid and glycogen reserves (O'Riordan & Burnell, 1989; Riddle & Albert, 1997). Additionally, dauer larvae display increased transcriptional activity of anti-ROS proteins, molecular chaperones and components involved in cellular detoxification (Burnell *et al.*, 2005; Dalley & Golomb, 1992). This indicates that stress resistance and cellular maintenance processes are upregulated, presumably, to render dauer larvae resilient to the taxing environment.

Caenorhabditis elegans **dauer as a context to study cell cycle quiescence**

One of the advantages of the *C. elegans* as a model is the invariance and tight control of cell division (van den Heuvel & Kipreos, 2012). Completion of the cell cycle is an energetically costly event, and it is conceivable why under environmental stress this process is arrested. To conserve energy in the dauer larvae, somatic cells arrest at the G1 stage of the cell cycle, but can be induced to execute the S-phase under specific conditions, such as depletion of *cki-1*, a cyclin-dependent kinase inhibitor (Hong *et al.*, 1998; Kipreos, 2005). An alternative developmental state such as dauer, where an organism undergoes changes to conserve energy to endure the environmental strain, provides a scenario in which we can study how cell cycle quiescence is established and maintained, and uncover regulatory networks that are involved in this process.

The *C. elegans* germline

The germline is a unique tissue that establishes a link between generations, passing on genetic material to the progeny. Germline stem cells are totipotent cells that have the capacity to differentiate into gametes, which contain the genetic information that will be

passed on to the next generation. The *C. elegans* germline shares common features with germlines of other organisms. It contains the only stem cell population that proliferates throughout the life of the organism, making it a good model to study the dynamics of stem cell division (Hubbard, 2007).

The germline of the *C. elegans* hermaphrodite animals resides within the two U-shaped tubes of the gonadal arms joined by the common uterus (Pazdernik & Schedl, 2013). Each tube contains germ cells that are organized in a spatio-temporal manner with respect to their proliferation, meiotic progression and gametogenesis, progressing from the distal to the proximal ends of the gonad (Schedl, 1997). The germline nuclei reside in a syncytium, however they are often referred to as individual "cells". They are separated by an incomplete plasma membrane and each cell has an opening into the shared cytoplasmic lumen or rachis (Hirsh *et al.*, 1976).

Interactions between the somatic gonad and the germline are critical for stem cell proliferation and proper gametogenesis. The germline is enveloped by a basement membrane, which helps to maintain structural integrity and guides cell migration (J. M. Kramer, 2005). Somatic epithelial sheath cells play critical roles throughout germline development. During early larval development they are required for larval germline amplification and determination of the size of the proliferative stem cell region. Later they become necessary for proper gonad elongation during the final stages of development (Killian & Hubbard, 2005). Finally, flanking the hermaphrodite germline are the two distal tip cells (DTCs) derived from the somatic gonadal precursors Z1 and Z4 (Hubbard & Greenstein, 2005) Each constitutes a distal niche to maintain the proliferative zone of mitotically dividing cells in the adult worm (Hall *et al.*, 1999). The proximity of the germline to its niche will control the decision to exit mitotic proliferation and initiate meiosis.

Normal germline development

When the larva hatches after embryogenesis, it contains 4 cells that compose the gonadal primordium: the primordial germ cells, Z2 and Z3, and somatic gonad precursors,

Z1 and Z4, enclosed in a basement membrane (Figure 1.2) (Hubbard & Greenstein, 2005). In order to form a functional gonad, somatic and germline development must be carefully coordinated. Under favorable conditions both somatic and germline cells proliferate, and the somatic cells rearrange to produce the characteristic gonadal "arms" capped by the DTCs on each end, providing a niche for the germline stem cells to mitotically divide (Hubbard, 2011; J. Kimble & Hirsh, 1979). Until the L3, the germline proliferates but remains undifferentiated. During the L3, the germline proliferates at a much greater rate to reach a size where the proximal-most areas do not receive the instructing signal responsible for maintenance of mitotic divisions from the DTCs, and this results in entry into meiosis, a specialized cell cycle that results in production of haploid gametes.

Mitotic proliferation of the germline during normal development

The self-fertilizing hermaphrodite *C. elegans* must produces gametes throughout its life cycle. The gonad can be considered as an assembly line of continuous gamete production. Persistent mitotic divisions of germline stem cells of the proliferative zone in the distal part of each gonadal arm allow for constant replenishment of oocytes to replace those that were fertilized. This self-renewal and amplification of germline stem cells is essential to produce 200-300 progeny, typical of the adult hermaphrodite (Hodgkin & Barnes, 1991).

The *C. elegans* hermaphrodite germline is spatially and temporally organized from undifferentiated stem cells at the distal end, to fully differentiated gametes at the proximal end. In adult hermaphrodites, a pool of approximately 200 stem cells is maintained at the distal end (Cinquin *et al.*, 2010). These cells maintain their identity via the interaction with the DTCs (J. E. Kimble & White, 1981). As the cells proliferate they move further from this niche, they no longer receive the signals emanating from the niche, and progress into meiosis - a specialized cell cycle that results in production of haploid gametes (J. Kimble, 2011). Already, at the most proximal region of the proliferative zone, some cells are in pre-

meiotic S-phase, and as they move further away into the transition zone, the cells enter early meiotic prophase.

DTCs instruct mitotic division of the germline stem cells via Notch/GLP-1 signaling at all stages of development (Austin & Kimble, 1987; S. Crittenden *et al.*, 1994). The distal tip cells express the ligand LAG-2, which binds to the GLP-1 receptor on the surface of the germ cells (S. Crittenden *et al.*, 1994; Henderson *et al.*, 1994). Studies of the *glp-1* gene conclude that it is both necessary and sufficient for maintenance of the mitotic fate in the germline (Berry *et al.*, 1997; S. Crittenden *et al.*, 1994). GLP-1 dictates mitotic proliferation by inhibiting the GLD-1 and GLD-2 pathways that down-regulate mitosis-promoting genes at the mRNA level (Kadyk & Kimble, 1998; J. Kimble & Crittenden, 2007). GLP-1 achieves this via an RNA-binding protein called FBF-2, which, when activated, post-transcriptionally represses GLD-1 and GLD-2 (J. Kimble & Crittenden, 2007; Lamont *et al.*, 2004). As the mitotic cells leave the DTC/LAG-2 ligand expression region, GLP-1 signalling is no longer activated, resulting in cells entering meiotic prophase I, and committing to differentiation. The GLP-1 signalling is further diminished due to the negative feedback of GLD-1 on GLP-1, where GLD-1 acts to repress the *glp-1* translation (Marin & Evans, 2003). This results in the restricted localization of the germline stem cells to the distal area of the gonadal arm.

Dauer germline development

As *C. elegans* larva prepares for dauer entry during the L2 stage, the germline is at its proliferative expanding state, where germ cells have not yet left the niche, so the germline stem cell fate is maintained. The dauer state is accompanied by global somatic cell cycle arrest (Kipreos, 2005). Concomitantly, the germline stem cells also arrest cell divisions and remain quiescent until normal development is resumed (Narbonne & Roy, 2006a, 2006b). The dauer germline contains approximately 32 cells, paused at the G2/M stage of mitosis. Curiously, the DTCs continue to produce the LAG-2 signal, and the GLP-1 expression remains present in dauer, yet the cell divisions remain blocked (Narbonne & Roy, 2006a).

Understanding the AMPK-dependent regulation of dauer germline quiescence

Maintaining cells in a quiescent state during environmentally unfavorable conditions is advantageous for the animal's survival, presumably because it lowers the energetic cost of completing the cell cycle in times when energy is limited (Tobin & Saito, 2012). This cellular safeguarding occurs in both the soma and the germline. Previously our laboratory performed a large-scale genetic screen to identify genes required to maintain the germline cell cycle arrest (Narbonne & Roy, 2006a). The screen revealed that AMP-activated kinase (AMPK), PAR-4/LKB1 and DAF-18/PTEN are required for quiescence (Narbonne & Roy, 2006a). AMPK is a cellular energy sensor required to modulate an array of cellular processes in response to a reduction in cellular energy. PAR-4/LKB1 and DAF-18/PTEN are tumor suppressors, misregulation of which has been associated with the cancer predisposing Peutz-Jeghers Syndrome and Cowden's Syndrome, respectively (Hemminki *et al.*, 1998; Liaw *et al.*, 1997).

ATP hydrolysis generates the energy used to fuel most cellular processes. In a well-functioning system, the ATP supply must be matched to the energetic needs of the cell (Carling *et al.*, 2012). AMP-activated kinase (AMPK) is a universal energy sensor, with orthologs present in all eukaryotes (Hardie, 2011). It senses the energy status of the cell by detecting the ratio of AMP and ADP (products of ATP hydrolysis) to ATP, a higher ratio indicating a reduced energy stockpile. AMPK down-regulates anabolic and up-regulates catabolic pathways in response to the elevated levels of AMP (Carling *et al.*, 1987; Hardie, 2011). AMPK exists as a heterotrimeric complex, consisting of two regulatory subunits ϒ and β, and a catalytic subunit α (Hardie *et al.*, 2012). AMP binds directly to the ϒ subunit of AMPK and allosterically activates it, enhancing the interaction with the upstream LKB1/MO25/STRAD complex, which results in optimal AMPK activity (Hardie *et al.*, 2012; Woods *et al.*, 2003). The heterotrimeric LKB1/MO25/STRAD complex provides a basal level of phosphorylation at Thr172 of the AMPK catalytic subunit, making it a worse substrate for the inactivating phosphatases (Stein *et al.*, 2000).

Active AMPK plays a role in controlling various processes in order to maintain cellular homeostasis. It up-regulates glucose uptake and catabolism in the muscle, as well as systemic mitochondrial biogenesis (Grahame Hardie, 2014). AMPK inhibits fatty acid and cholesterol synthesis, as these are highly energy consuming anabolic processes(Habegger *et al.*, 2012; Krishan *et al.*, 2014; Li *et al.*, 2011). AMPK does not only control the cellular metabolic processes, but also seems to play a role in cell polarity. AMPK phosphorylates the microtubule plus end protein CLIP-170, suggesting that it may play a role in polarity by controlling the microtubule regulation (Nakano *et al.*, 2010). The cells depleted of AMPK, demonstrated loss of polarity and inappropriate cell migration in mouse cell lines (Nakano *et al.*, 2010). Finally, AMPK inhibits protein synthesis and promotes cell cycle arrest (Hardie *et al.*, 2012).

Given the diversity of the AMPK target processes, it is not surprising that its misregulation is implicated in the pathophysiology of many disorders, including cancer. Although AMPK complex components are rarely mutated in cancers, its cooperation with other factors has been shown to promote tumor progression (Faubert *et al.*, 2014). The link between AMPK and cancer, was first recognized due to the discovery that LKB1 was the upstream activator of AMPK (Woods *et al.*, 2003). Mutations in the gene encoding LKB1 are associated with Peutz-Jehger cancer-predisposing syndrome in humans. The tumor suppressing properties of LKB1 may be mediated by AMPK, potentially through regulation of the core cell growth and proliferation pathway, mTOR (Shaw *et al.*, 2004). AMPK phosphorylates and activates TSC2, part of the TSC1/TSC2 heterodimer, which acts as a GTPase-activating protein (GAP), converting Rheb-GTP into its inactive, GDP-bound form. Active Rheb-GTP phosphorylates and activates the mTOR protein and therefore its inactivation results in attenuated mTOR signalling (Inoki *et al.*, 2003; Laplante & Sabatini, 2012; Schmelzle & Hall, 2000). In addition, AMPK phosphorylates and inhibits Raptor, a scaffolding protein on which the mTOR complex assembles (Gwinn *et al.*, 2008). It is also plausible that LKB1 acts independently of AMPK as the mutation in the Peutz-Jehger syndrome is autosomal dominant and dosage-dependent, where one copy of the wild type allele is most likely enough to phosphorylate AMPK, suggesting that in PJS patients, the compromised LKB1 may be involved in a different process.

Inappropriate oncogenic activation of mTOR has been implicated in aberrant cell growth and cell cycle, partially due to the inhibition of mRNA translation repressor 4E-BP1 (Dowling *et al.*, 2010; Laplante & Sabatini, 2012). Therefore AMPK-mediated regulation of mTOR, presents another link between AMPK and cancer. Loss of AMPK leads to cellular changes that are characteristic of the metabolic changes that occur in tumor cells, specifically to inappropriate switch to glycolysis under aerobic conditions (Faubert *et al.*, 2013). Furthermore, loss of cell polarity has been shown to assist tumor invasion (Williams & Brenman, 2008). Thus the role of AMPK in regulation of cell polarity suggests another avenue through which AMPK can impact tumor formation and development (Nakano *et al.*, 2010). Given this combined evidence, it is clear that in different organisms functional AMPK is necessary to control cell division and proliferation in many contexts.

The *C. elegans* dauer state is an alternative developmental stage that occurs in response to unfavorable environmental conditions, and it is accompanied by a global cell cycle arrest. Convincing genetic evidence has demonstrated that loss of *aak-2* results in dauer animals possessing a much greater number of germ cell nuclei as compared to the normal dauers (Narbonne & Roy, 2006a)(Figure 1.3). *aak-2* encodes an isoform of the *C. elegans* α catalytic subunit homolog of AMPK (Apfeld *et al.*, 2004). Loss of function of *aak-2* together with *daf-18*, a gene that encodes a homolog of PTEN lipid phosphatase, a tumor suppressor that acts as a negative regulator of insulin-like signalling and the mTOR pathway, resulted in an even greater number of nuclei leading to an additive phenotype (Figure 1.3) (Narbonne & Roy, 2006a; Ogg & Ruvkun, 1998). This suggests that AMPK and PTEN act in parallel and may have different target mechanisms that suppress the hyperplasia. Another intriguing finding revealed by the work of (Narbonne & Roy, 2006a) is that PAR-4/LKB1-dependent regulation of germline quiescence is likely to impinge on downstream targets other than AMPK, as double mutant worms displayed an enhanced hyperplasia as compared to either of the single mutants (Figure 1.4) (Narbonne & Roy, 2006a).

Research motivation

Among many developmental changes that occur during dauer, germline proliferation during this stage arrests at the G2/M stage of the cell cycle. (Narbonne & Roy, 2006). Despite showing clear evidence that *aak-2* is sufficient to mediate GSC quiescence, it remains unclear how this is achieved. AMPK is a protein kinase, suggesting that phosphorylation of specific targets is responsible for the establishment and maintenance of germline stem cell quiescence during the dauer stage. The goal of my research was to better characterize the role of AMPK in keeping germline stem cells in a quiescent state and the impact of the AMPK-dependent hyperplasia on post-dauer reproductive development. It was carried out in parallel with the work that aimed to uncover the mechanisms of PAR-4/LKB1-mediated stem cell quiescence, independent of AMPK. Combining the evidence from these two projects will help to better understand and characterize this germline arrest, and it will be a stepping-stone to understanding the network responsible for the germ stem cell proliferation control during an environmental, mainly nutritional challenge. We conducted a genome-wide RNAi screen to identify all genes that suppress the *aak-2*-dependent germline hyperplasia. This reverse genetic approach allowed us to identify and further characterize potential AMPK targets in a non-biased manner, providing information on which gene families may be involved in mediating AMPK-dependent quiescence. To identify genes that could be direct targets of AMPK, the candidates were inspected for the AMPK phosphorylation sites. Further characterization and analysis of direct or indirect targets will help to uncover the mechanisms by which AMPK mediates the dauer germ stem cell quiescence.

Figures

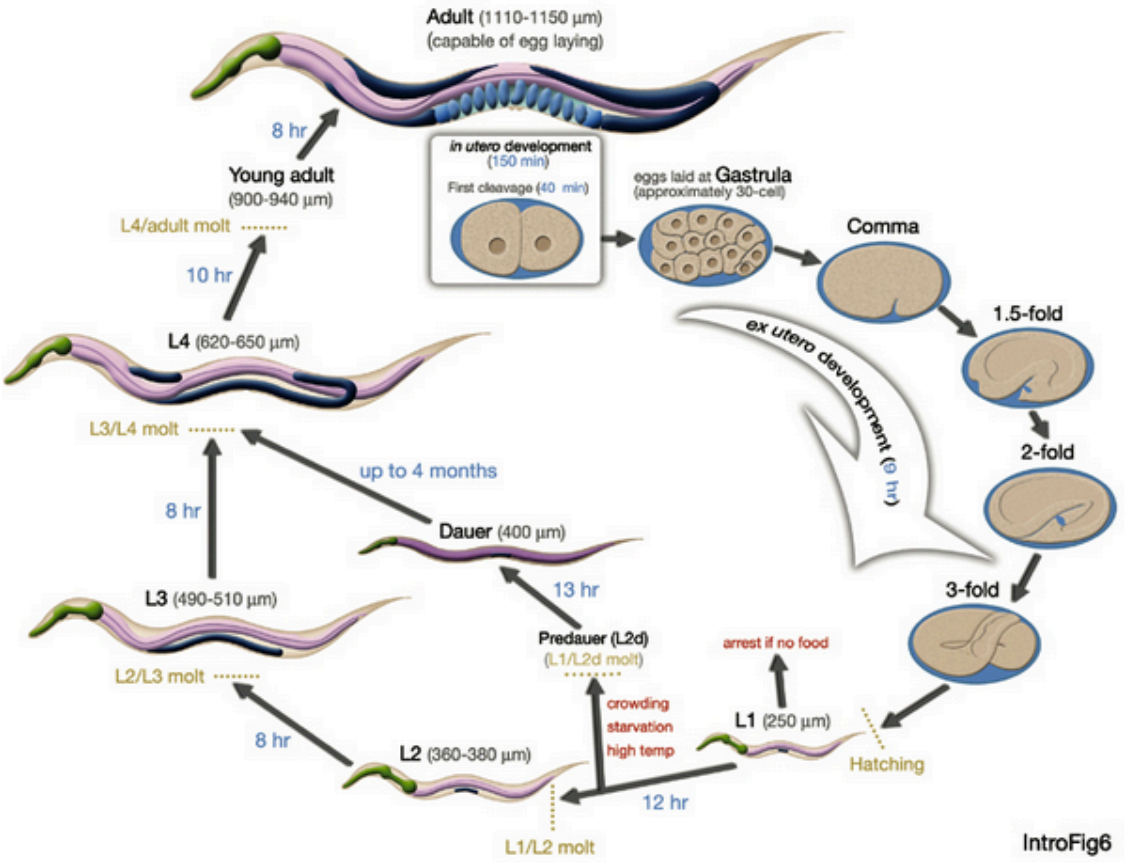

Figure 1.1 – *C. elegans* development may be altered in response to the environmental conditions

C. elegans development at 20°C in replete growth conditions follows the reproductive life cycle trajectory: after hatching the *C. elegans* undergo four larval molts until it reaches adulthood. Upon hatching, if conditions are suboptimal, the hatchling enters the L1 diapause, enabling it to survive for up to 2 weeks if it does not find a food source. If the environmental duress is encountered during L1, the larva will enter a pre-dauer L2d stage. During this time the L2d larva stockpiles lipid reserves and slows down its metabolism and cell divisions in anticipation of the subsequent dormant dauer state. Once the conditions improve, the worms molts directly into the L4 state (Altun, 2009). Figure used with permission from WormAtlas, Altun, Z. F., Herndon, L. A., Crocker, C., Lints, R. and Hall D. H. (ed.s) 2002-2012. http://www.wormatlas.org

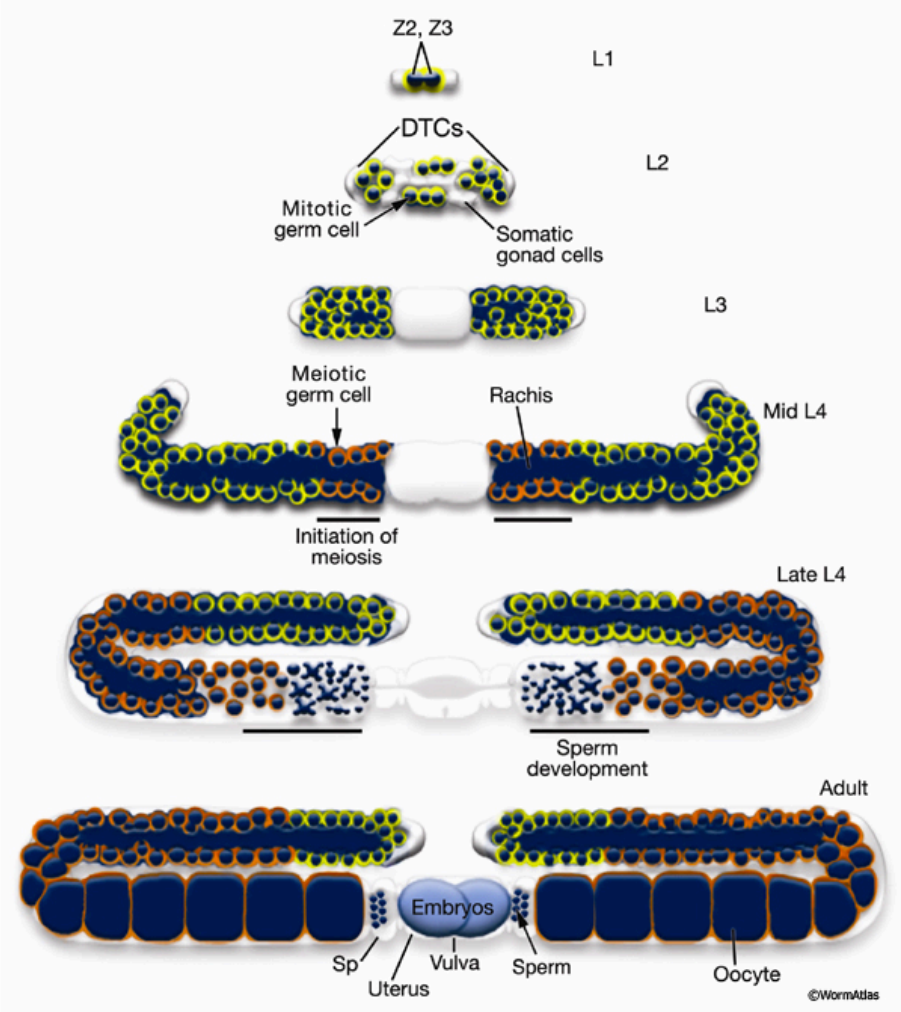

Figure 1.2 - *C. elegans* germline development under normal conditions

The germline is specified early in development from Z2 and Z3 precursors, while Z1 and Z4 give rise to the somatic gonad encompassing the germ cells. Z2 and Z3 proliferate to create a pool of mitotically dividing stem cells during early larval development. These divisions are maintained through the interactions with Distal Tip Cells (DTCs) that express the LAG-2/Delta signal and trigger the GLP-1/Notch signaling, which inhibits the meiotic program and promotes mitosis. As the germline expands, the proximal-most cells do not receive the signal from the niche and the cells begin to undergo meiosis, differentiating into gametes. During the dauer stage, the germline resembles that of L2 larvae, containing approximately 30 germ cell nuclei. Figure used with permission from WormAtlas, Altun, Z. F., Herndon, L. A., Crocker, C., Lints, R. and Hall, D. H. (ed.s) 2002-2012. http://www.wormatlas.org

Genotype	% Dauer arrest (n)	Number of germ cell nuclei in dauer *(n)
daf-2(e1370)	0 (148)	35.44±3.85 (25)
daf-2(e1370); aak-2(ok524)	100 (290)	69.96±9.08[1] (25)
daf-2(e1370); daf-18(e1375)	99.48 (743)	98.17±13.61[1] (30)
daf-2(e1370); daf-18(e1375);aak-2(ok524); aak-1(RNAi)	85.13 (316)	189.53±23.71[1] (15)

*Mean values ± s.d; all strains were 100% dauer constitutive at 25 °C
[1] Statistical significance using the one-tailed-*t*—test with unequal variance ($P \leq 0.0005$)

Figure 1.3 - Depletion of AMPK causes germline hyperplasia in dauer

Germ cell nuclei count was performed in dauer larvae that possessed mutations in AMPK catalytic subunit homologue *aak-2*, in C-terminal regulatory domain of *daf-18*, RNAi for *aak-1* – the second isoform of the AMPK catalytic subunit and *daf-2* mutation, which allows constitutive dauer entry at restrictive temperature of 25°C. Compared to normal dauers, *daf-2(e1370); aak-2(ok524)* and *daf-2(e1370); daf-18(e1375)* animals had a much greater number of germ cell nuclei, as compared to *daf-2(e1370)* mutants. The loss of both *daf-18* and *aak-2* led to an enhanced hyperplasia when compared to either of the mutations alone. This table adapted from (Narbonne & Roy, 2006a) demonstrates that the tumor suppressor PTEN and AMPK act in parallel and possibly through different mechanisms to induce dauer germline stem cell quiescence (Narbonne & Roy, 2006a).

Genotype	Number of germ cell nuclei in dauer* (n)	Dauer lifespan (days)* (n)
daf-2(e1370)	35.44±3.85 (25)	>15 (100)
daf-2(e1370); aak-2(ok524)	69.96±9.08 (25)	8.18±2.41[1] (60)
daf-2(e1370); par-4(it57)	148.28±30.90 (25)	>15[1] (60)
daf-2(e1370); par-4(it57);aak-2(ok524)	202.65±24.14 (17)	6.85±1.83[1] (59)

*Mean values ± s.d; all strains were 100% dauer constitutive at 25 °C
[1] Statistical significance using the one-tailed-*t*—test with unequal variance ($P \leq 0.0005$)

Figure 1.4 - AMPK does not solely account for PAR-4/LKB1-mediated germ cell quiescence in *C. elegans* dauer

Due to the known role of AMPK in mediating PAR-4/LKB1 function, it is plausible that PAR-4/LKB1 is acting through AMPK to suppress the mTOR pathway and thus control cell growth and induce germline quiescence. The dauer germ cell nuclei were counted in both AMPK and PAR-4/LKB1 deficient strains. The mutations in *daf-2* and *aak-2* have been previously described in Figure 3. *par-4(it57)* is a temperature sensitive allele in which the kinase function of PAR-4 is impaired when shifted to the restrictive temperature. Mutations in *daf-2(e1370); par-4(it57)* resulted in a greatly enhanced number of germ cells as compared to the *daf-2(e1370)*. In a triple mutant *daf-2(e1370); par-4(it57);aak-2(ok524)*, the animals display a much greater number of germ cells than in either *daf-2(e1370); aak-2(ok524)* or *daf-2(e1370); par-4(it57).* This additive phenotype suggests that PAR-4/LKB1 maybe playing a role independent of AMPK in mediating the stem cell quiescence in dauer (Narbonne & Roy, 2006a).

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Chapter 2

A global genomic survey to investigate the role of AMPK in germline stem cell quiescence in the *C. elegans* dauer larvae

Introduction

When a young *C. elegans* larva senses suboptimal growth conditions, it arrests reproductive development at the L3 stage and enters the dauer diapause (Fielenbach & Antebi, 2008; J. W. Golden & D. L. Riddle, 1984b; Riddle & Albert, 1997). This alternative state allows the animal to endure harsh conditions and survive for long periods of time. As a result, to conserve energy, cells undergo a general somatic cell cycle arrest, where the expanding stem cell population in the germline arrests and remains quiescent until conditions improve, triggering the animals to exit the dauer stage and enter the reproductive L4 stage (Hubbard, 2011; Narbonne & Roy, 2006a; Padilla & Ladage, 2012). How this dauer-dependent regulation of dividing stem cell proliferation is exerted is however not understood.

In order to better understand the mechanism of germline stem cell quiescence, an EMS screen was performed to identify factors that cause inappropriate growth of the germline in dauer animals. We identified a single mutant *rr48* that possessed a dominant negative mutation in *aak-2*, the alpha catalytic subunit homologue of mammalian AMP-activated kinase (AMPK) (Narbonne & Roy, 2006a). AMPK is a metabolic energy sensor that modulates a variety of cellular processes in response to fluctuations in cellular energy levels, and it has a known role in regulating cell growth (Imamura *et al.*, 2001). In addition to AMPK, *daf-18* and *par-4* are also necessary to maintain the germline arrest (Narbonne & Roy, 2006a). Despite identifying these factors, the underlying mechanism of how they establish the dauer-dependent germ cell quiescence remains unclear.

AMPK is a protein kinase that acts as a master regulator of cellular homeostasis as it matches energy production to the energetic needs of the cell (Carling *et al.*, 2012). This is achieved by responding to changes in the AMP:ATP ratio followed by adaptive metabolic adjustment leading to down-regulation of energetically costly cellular processes (Carling *et al.*, 1987). In addition to being allosterically activated by AMP, AMPK is phosphorylated by a heterotrimeric serine/threonine kinase complex, LKB1, which plays a role in numerous

cellular processes (Hardie *et al.*, 2012). In humans, LKB1 acts as a tumor suppressor, as its misregulation leads to the cancer-predisposing Peutz-Jegher's syndrome (Hemminki *et al.*, 1998). Although loss of LKB1 can have dire consequences for the organism, it is not known how it exerts its tumor-suppressive properties. Understanding the role of AMPK in regulating cell proliferation may also uncover new insights into how LKB1 might block tumor growth.

Cell growth is an energetically demanding process that under certain circumstances is regulated by AMPK. In conditions when cellular energy is low, AMPK acts as a metabolic checkpoint to regulate cellular growth (Mihaylova & Shaw, 2011). In mammals, AMPK phosphorylates the TSC1/TSC2 complex, which acts as a GAP and transforms Rheb-GTP into the inactive Rheb-GDP form. Rheb-GTP can activate the mTOR complex and promote protein synthesis, so inactivation leads to the attenuation of the mTOR activity (Schmelzle & Hall, 2000). In *C. elegans* there have been no TSC1/TSC2 homologues identified as of yet, but recently a novel Ral-GAP has been implicated in the *C. elegans* mTOR signaling (Martin *et al.*, 2014). Additionally, AMPK can directly phosphorylate and inhibit Raptor, a scaffold protein on which the mTOR complex assembles (Gwinn *et al.*, 2008). It is unknown whether AMPK acts via the above effectors and attenuates the mTOR pathway in order to maintain germ cells in a quiescent, non-proliferative state.

Cell proliferation is intimately linked to protein synthesis and consequent cell growth, as cell divisions occur after a cell reaches a certain size to ensure appropriately sized daughter cells (Fingar *et al.*, 2004). Cell polarity and cell divisions are also coupled, as polarity cues dictate the position of the cleavage furrow, which will govern whether the cell will divide symmetrically or asymmetrically, and will determine the content of the daughter cells (Narbonne & Roy, 2008; Yoshimura *et al.*, 2006). Cancer stem cells are known to divide symmetrically and the loss of cell polarity leads to overproliferation and tumor growth (Cicalese *et al.*, 2009; Skinner, 2009). AMPK phosphorylates CLIP-170, a microtubule plus end protein that binds endocytic vesicles, and possibly reduces its affinity for the microtubule (Nakano *et al.*, 2010). Additionally, this study provides a link between AMPK and vesicular transport regulation – a crucial process needed to mobilize and

localize proteins and polarity determinants in order to appropriately polarize the cell. Recently it has been demonstrated that AMPK can differentially regulate VPS-34, a protein required for vesicular trafficking, and can modulate its activity under cellular stress (Kim *et al.*, 2013). These are examples of the broad spectrum of processes that are directly or indirectly controlled by AMPK and could therefore play a role in maintaining *C. elegans* germline stem cell quiescence under compromised growth conditions.

Earlier genetic evidence showed that the absence of both *par-4* and *aak-2*, homologues of mammalian LKB1 and alpha subunit of AMPK, respectively, led to an enhanced hyperplasia, as compared to the loss of either of the genes (Narbonne & Roy, 2006a). This suggests that AMPK and PAR-4 are both necessary for germline stem cell quiescence in dauer, and that PAR-4 may exert its control over cell proliferation through mechanisms other than through AMPK. This underscores the importance of uncovering AMPK-independent function of PAR-4, as they may provide novel insights into mechanisms of its tumor-suppressive function. To address this question, a screen was conducted in our lab, which focused on identifying candidate genes that suppressed the *par-4*-dependent hyperplasia (Chaouni, 2013). In order to differentiate between *aak-2*-dependent and -independent functions of *par-4*, we combined the results of the two screens (Figure 2.2) in order to distinguish the genes that suppressed the hyperplasia in (i) the *aak-2* background only, (ii) in both *par-4* and *aak-2* backgrounds, and (iii) genes that suppressed the hyperplasia only in the *par-4* background.

To better understand and characterize the role of AMPK and its downstream targets that are required for germline quiescence in *C. elegans* dauer we performed a genome-wide RNAi screen, to identify genes that, when compromised, suppress the *aak-2*-dependent hyperplasia observed in dauer larvae. We chose the RNAi approach because it allowed us to identify functional AMPK targets, that act either directly or indirectly downstream of the kinase. The candidates were grouped into functional categories based on their Gene Ontology (GO). This reverse genetics approach gave us a non-biased overview of gene families that may act downstream or in parallel to AMPK to attenuate cell proliferation.

After primary and secondary screens, 59 candidates consistently suppressed the *aak-2*-dependent germline hyperplasia in *daf-2* dauer larvae, suggesting that their expression is misregulated in the absence of AMPK. The candidates were categorized into 9 groups based on their gene ontology and function. Of the 59 genes, 8 candidates are potential AMPK targets based on the bioinformatic analysis. Of particular interest are genes encoding components of the microRNA pathway, specifically core members of the miRNA RISC complex. This suggests that miRNA-mediated silencing may play a role in some aspects of germline quiescence in response to limited energy resources.

Materials and Methods:

Strains and maintenance:

The following strains were used in the screen: MR0998 strain [*daf-2(e1370); aak-1(tm1944); aak-2(ok523); qIs56(lag-2*::GFP*)*] and MR0155 [*daf-2(e1370); qIs56(lag-2*::GFP*)*]. All strains were maintained at 15 °C on NGM plates seeded with OP50 bacteria according to standard protocols (Brenner, 1974).

RNAi library:

We used the RNAi feeding library generated by the Ahringer laboratory that covers 86% of the *C. elegans* genome. The bacterial clones express the dsRNA homologous to a single gene target. Production of dsRNA is triggered by IPTG-inducible T7- RNA polymerase, contained in the *E. coli* clones that carry an L4440 double T7 vector with a fragment corresponding to the gene of interest (Kamath & Ahringer, 2003; Kamath *et al.*, 2001). Bacterial clones were maintained in glycerol stocks in 96-well plates.

Screening strategy and identification of hyperplasia

The RNAi for the genome-wide screen was administered via feeding the animals with bacteria containing a vector expressing double stranded RNA. The MR0998 strain contains an *aak-2(ok524)* mutation. This molecular null allele has a 409 nucleotide deletion in the kinase domain of the alpha catalytic subunit of *C. elegans* AMPK rendering it a molecular null (Apfeld *et al.*, 2004). The strain also harbors a deletion in the second catalytic subunit *aak-1(tm194)*, which results in a more pronounced hyperplasia (Narbonne & Roy, 2006a). We used the temperature sensitive dauer constitutive allele *daf-2(e1370)* which compromises the sole insulin receptor in *C. elegans* (Kimura *et al.*, 1997). Finally, the strain contains a *lag-2::*GFP translational fusion reporter which is expressed in the DTCs and in the IL-2 neuron during dauer (Ouellet *et al.*, 2008). This marker allowed us

to evaluate the distance between the DTCs, and thus indirectly assess the degree of the germline hyperplasia, where a decreased displacement of the DTCs suggested suppression of hyperplasia when arrested in dauer.

The bacteria from the RNAi Ahringer library was cultured overnight at 36 °C in LB medium containing ampicillin. The bacteria was then seeded onto NGM plates containing ampicillin (0.5 ml/L) and IPTG (1 ml/L). The seeded plates were kept at room temperature during the induction of the dsRNA production by IPTG. Synchronized L1 larvae were then put on the IPTG plates and shifted to 25 °C to allow dauer induction. The dauer larvae were subsequently screened, 48 hours after being shifted to a restrictive temperature. During the screen, the distance between the DTCs was evaluated visually by monitoring the *lag-2::*GFP expression, using a fluorescent dissecting microscope (Leica MZ12). The corresponding wells were then matched to the gene IDs. Candidates identified in the primary screen were subjected to secondary and tertiary evaluations.

Dauer germ cell nuclei count

Whole dauer larvae were stained with DAPI as previously described (Narbonne & Roy, 2006a). The total number of dauer germline nuclei was determined by assessing nuclear morphology and their position within the stained gonads. The germline count was performed on *daf-2;aak-1;aak-2* and control *daf-2* strains. The number of germ cell nuclei was then counted in the *aak-2* null strain fed with bacteria containing dsRNA corresponding to the candidate gene, the compromise of which suppressed the *aak-2*-dependent hyperplasia.

Proliferative zone germ cell nuclei count

The *aak-2* null, *daf-2* and N2 animals were synchronized and grown until they reached the adult stage. Their gonads were dissected and mounted on 1% polylysine coated slides and fixed in 4% paraformaldehyde for 5 minutes. The fixation was followed by freeze cracking and a 1 minute fixation in ice-cold methanol. The gonads were then washed in PBST three

times for 10 minutes. 0.01 mg/ml DAPI was applied for 1 minute and then washed with PBST for an additional minute. The gonads were then mounted in the Vectashield to reduce photobleaching.

DIC microscopy

MR0998 larvae were fed with bacteria containing dsRNA for candidate genes involved in the processes of adhesion and extra-cellular matrix formation. Dauer larvae were mounted on 2% agarose pads in a drop of levamisole with a cover slip after which they were imaged using DIC on a Zeiss Imager.21 microscope. The DTC displacement was monitored by *lag-2::*GFP, visualized with a 400nm filter, while the size and morphology of the germline was assessed using DIC.

Results

Identification of candidate genes that suppress the AMPK-dependent dauer germline hyperplasia

The Ahringer RNAi library contains 18, 432 bacterial clones each carrying the L4440 vector with an insert corresponding to a single gene target. Of all the clones available, we screened 17, 606 genes, as 826 of the total number of clones available did not grow when cultured over night at 37 °C. During the first round of the genome-wide RNAi screen, we identified 1,148 candidate genes that rescued the *aak-2*-induced hyperplasia in germline of the dauer larvae, suggesting that in the *aak-2*-depleted strains, expression of these genes is misregulated in the absence of AMPK. In order to confirm and validate the results, two more rounds of screening were conducted. Through secondary and tertiary screens, we narrowed the number of candidates down to 59 genes, depletion of which resulted in a pronounced and reproducible reduction in the *aak-2*-dependent hyperplasia (Table 2.1). Of the 59 genes, 38 had a known function, while 21 were previously uncharacterized (Figure 2.1). The 38 genes were categorized based on Gene Ontology (GO), according to their functional description on WormBase (Figure 2.1)(Table 2.3). A large proportion of the candidates were previously uncharacterized and understanding their role in germline dauer quiescence may help to understand their function. Many genes were involved in metabolic processes and could be fulfilling a housekeeping function. Of particular interest are *alg-1* and *ain-2* genes encoding components of the microRNA pathway, implying that miRNAs may be playing a role in regulating some aspects of germline quiescence in dauer. In addition, genes encoding extracellular matrix (ECM) constituents were also noted, as ECM plays a crucial role in the activity of stem cell niches and therefore may influence the germline proliferation in response to nutrient stress.

Identification of genes that encode components of the miRNA pathway suggests that microRNA-mediated gene silencing may play a role in AMPK-dependent germline quiescence in dauer

The genes *alg-1* and *ain-2*, encode components of the miRNA RISC complex. miRISC is pivotal for the appropriate execution of miRNA-dependent gene silencing. miRISC binding leads to translational repression by blocking steps in initiation as well as elongation. It can also prompt cleavage of the mRNA, deadenylation of the poly-A tail and it can induce mRNA decay (Fabian & Sonenberg, 2012; Pasquinelli, 2012). *alg-1* encodes one of many *C. elegans* Argonaute protein, while *ain-2* encodes one of two orthologs of GW182, Argonaute-binding proteins. Together these components form the core of the miRNA RISC complex (Bouasker & Simard, 2012). Argonaute proteins contain multiple domains, including a PAZ domain, which binds the miRNA in a sequence-independent manner (Yan *et al.*, 2003). AIN-2 bound to ALG-1 is required to assist in blocking the translation initiation, and targets ALG-1 to the processing bodies, sites of mRNA degradation (L. Ding *et al.*, 2005; X. C. Ding & Grosshans, 2009).

Depleting *alg-1* and *ain-2* results in a global decrease of all miRNA activity because gene silencing cannot occur due to the impaired miRISC. Therefore this allows us to conclude that miRNAs must be involved in regulating the AMPK-dependent germ cell quiescence in dauer, but it provides no hints at which miRNAs might be involved. We speculate that since the depletion of *alg-1* and *ain-2* results in suppression of germline hyperplasia in the absence of AMPK, in the *aak-2* loss of function background certain miRNAs are aberrantly upregulated. This implies that in a wildtype dauer those miRNAs are downregulated and the target mRNAs are transcribed. The knockdown of the miRISC components mimics this donwregulation in animals with depleted AMPK.

Genes encoding the constituents of the extracellular matrix (ECM) may play a role in controlling the dauer germline quiescence

9% of all the genes identified in the screen for which RNAi resulted in suppression of the DTC displacement in *aak-2* dauers, encode proteins that comprise the extracellular matrix or play a role in cell adhesion (Table 4). The ECM is a structural mesh consisting of various insoluble macromolecules that provide an adhesive substrate and structural support to cells, controls spatial distribution of the ECM-bound molecules, presenting them to their receptors, and senses and transduces mechanical signals (J M Kramer, 1994; J. M. Kramer, 2005; Rozario & DeSimone, 2010). Importantly, ECM is a constituent of stem cell niches, and it provides structural and biochemical compartments for the stem cells. Numerous studies show that structural integrity of the ECM is important for proper stem cell maintenance (Bi *et al.*, 2007; Garcion *et al.*, 2004; Kollet *et al.*, 2006; Kurtz & Oh, 2012).

In our screen we identified *lam-2* and *mig-6* genes that encode proteins that are part of the basement membrane, an ECM structure that envelopes the *C. elegans* gonad (J. M. Kramer, 2005). *lam-2* encodes the gamma subunit of the heterotrimeric glycoprotein laminin, which acts as an ECM scaffold and affects the cytoskeleton within the cells of the tissue that it encloses (Rasmussen *et al.*, 2012; Yurchenco, 2011). Early loss of laminin results in embryonic lethality, while partial loss-of-function mutations in subunits alpha and gamma result in disrupted basement membranes, inappropriate adhesion complexes and disorganized cytoplasmic filaments in the gonad and escape of germ cells from the confines of the basement membrane and their proliferation in the adjacent tissues (Miner *et al*, 2004(Huang *et al.*, 2003; Kao *et al.*, 2006). *mig*-6 encodes papillin, a non-competitive inhibitor of ADAMT group of metalloproteases (Fessler *et al.*, 2004; Kramerova *et al.*, 2000). One such metalloprotease is encoded by another candidate *gon-1*, and has an enzymatic activity, allowing it to digest and remodel the basement membrane, allowing expansion of the tissue and extension of the gonad, necessary for the DTC migration (Blelloch, Anna-Arriola*, et al.*, 1999; Blelloch, Newman*, et al.*, 1999; Kawano *et al.*, 2009). The other candidates *vab-10* and *let-805* are components of the hemidesmosome, also required for tissue remodeling and cell adhesion (Bosher *et al.*, 2003; Zahreddine *et al.*, 2010).

The ECM may regulate the germ stem cell dynamics via GLP-1 signaling. Previously it has been shown that the dauer-dependent hyperplasia is regulated in a GLP-1-dependent fashion (Narbonne & Roy, 2006a). A link between *C. elegans* germline GLP-1 and the ECM has been made when it has been shown that mutations in the ECM components rescued the *glp-1* germline mutants in non-dauers, presumably through the interactions with EGF-like repeats in the extracellular domain of the receptor (E M Maine & Kimble, 1993; E.M. Maine & Kimble, 1989; Nishiwaki & Miwa, 1998).

RNAi penetrance and expressivity

The suppressed hyperplasia in the *aak-2*-deficient strain did not display a uniform phenotype with respect to frequency of the suppression within a well, as well as the expressivity of the phenotype. Penetrance is the percentage of animals that exhibit the phenotype in question, while expressivity refers to how pronounced this phenotype is (T. Wang *et al.*, 2005). Thus in order to get a more comprehensive idea of the effect of knocking down each of the genes, we assessed and catalogued the penetrance and expressivity of the 59 shortlisted candidates (Table 2.2). For the penetrance, the categories were assigned as 'low', 'medium' or 'high penetrance, corresponding to the candidates, knockdown of which caused 5-10%, 11-49% or >50% of animals to have a suppressed germline hyperplasia, respectively. As for the expressivity, in some cases, the *C. elegans* displayed full suppression of the hyperplastic germline, where the rescued animals had the DTC distance very similar to the wildtype, while some had only partially reduced germlines when compared to the wildtype dauers. For the purposes of this screen, we rationalized that even if the RNAi of a certain gene only partially reduces the DTC displacement, it must not be discarded as it still indicates that this gene must affect germline quiescence either downstream of AMPK or in a parallel pathway.

RNAi candidate genes with potential AMPK phosphorylation sites

The isolated RNAi candidates suppressed the hyperplasia caused by depletion of AMPK activity in the dauer germline. This suggests that these genes must be epistatic to AMPK. In order to interrogate whether some of these genes may encode a direct phosphorylation target of AMPK we checked for potential consensus AMPK phosphorylation sites using GPS software that allows kinase-specific phosphorylation site prediction. By using the amino acid sequence of a candidate gene, we obtained information about the presence and the number of predicted phosphorylation sites (Table 2.1). Since AMPK is a conserved kinase, we used BLAST to identify the *Drosophila* and the mammalian homologues of our candidates, and then aligned the sequences of the targets that were predicted by GPS in *C. elegans* with those of *Drosophila melanogaster* and *Mus musculus* using ClustalW. From the alignments we looked for the optimal AMPK phospho-motif (Gwinn *et al.*, 2008). This allowed us to identify candidates, which had the optimal AMPK phospho-motif conserved among other model organisms (Table 2.1). These candidates therefore encode proteins that may interact directly with AMPK to control germline stem cell quiescence. Only eight candidates contain a potential AMPK-phosphorylation site that is conserved among other model systems. These genes have not been previously documented to directly interact with AMPK. This may point towards a previously unidentified relationship between AMPK and the proteins, as well as the processes that they act in. According to the gene ontology, these candidates have distinct functions: two of them play a role in protein processing, while the rest are involved in gene expression, metabolism, miRNA, extracellular matrix components and endocytosis. It remains to be identified whether this potential interaction of AMPK and these candidates is responsible for ensuring germ cell quiescence.

RNAi candidates that emerged in both screens for suppressors of AMPK-dependent and PAR-4-dependent germline hyperplasia

Genetic evidence suggested that PAR-4 acts to suppress the germline proliferation, at least in part, independently of AMPK (Narbonne & Roy, 2006a). In order to identify candidates that suppress the *par-4*-dependent germline hyperplasia, a screen was carried to identify targets that mediate the PAR-4 function to control cell proliferation (Chaouni,

2013). In order to determine which of the candidates act independently of AMPK in a PAR-4-defecient background, we have combined the results of the two screens and revealed that 18 candidates were common both to both data sets, implying that PAR-4 mediates its control over cell proliferation via AMPK through these targets (Figure 2.2)(Table 2.3). This implies that the remaining candidates isolated in the screen for suppressors of *par-4*-dependent hyperplasia (Chaouni, 2013), act downstream of PAR-4, and potentially independently of AMPK.

While genes that encoded the components of the miRNA pathway and the ECM emerged in both data sets, genes that regulate polarity and the cytoskeleton only appeared in the screen for suppression of *par-4*-dependent hyperplasia. PAR-4 has been shown to play a direct role in regulating polarity, establishing asymmetry during early embryonic development (Watts *et al.*, 2000). In addition, cytoskeletal regulators emerged from an independent study to identify genes, other than *aak-2, par-4* and *daf-18,* when compromised will also give rise to germline hyperplasia (Wendland, 2010).

RNAi candidates with non-dauer specific phenotypes

The candidates that were identified in our screen could have a role in germline proliferation independently of AMPK or of the dauer context. To verify that the candidates were dauer-specific we tested all 59 candidates in L3 animals in the presence of AMPK. During the L3 stage, the distal mitotic germline undergoes a surge in proliferation, and the gonadal arms extend far enough to begin the formation of the distinctive bend (Korta & Hubbard, 2010). We reasoned, that if a candidate were a cell cycle regulator and affected the proliferation in a general manner, it would suppress the germline of the L3 animals in the presence of AMPK, displaying shorter distance between the DTCs at that stage. Conversely, if the RNAi candidate suppresses the germline proliferation in an AMPK-deficient dauer but not in the L3 animals, then the candidate gene probably acts in an AMPK-dependent and dauer-specific manner.

The disruption of 24 genes led to a reduced germline in the L3 animals (Table 2.5). This suggests that this set of genes promotes cell cycle progression in the germline. A large

proportion of these genes are involved in processes essential for cellular maintenance, such as genes required for ATP production, and genes that are necessary to maintain cellular structure and affect the DTC migration. These general regulators should be further characterized as they may play additional roles in dauer, downstream or independently of AMPK.

AMPK controls germline stem cell proliferation exclusively under the conditions of nutrient stress

The aberrant germline proliferation observed in the *aak-2* null dauer larvae may be a result of active AMPK playing a role in suppressing mitotic proliferation at all stages of the *C. elegans* reproductive development. To determine whether the absence of AMPK affects cell proliferation exclusively under conditions of nutrient stress and not under replete conditions, we examined the proliferative zone of the adult *C. elegans* following reproductive development. The proliferative zone contains actively cycling mitotic cells, as well as cells in the pre-meiotic S phase (S. L. Crittenden *et al.*, 2006; Jaramillo-Lambert *et al.*, 2007), The cells then enter a transition zone where they switch to meiosis (Hubbard, 2007). The nuclear morphology of these regions in *daf-2, daf-2;aak-1;aak-2* and N2 adults were observed using a DAPI staining, and proliferative zone germ cell nuclei were subsequently counted. No difference in the morphology and the number of nuclei were detected between the proliferative zones of the adults, leading us to conclude that the absence of AMPK affects the germline mitotic cell proliferation exclusively under the conditions of nutrient stress (Figure 2.3).

RNAi candidates that encode components of the ECM and that play a role in adhesion

Depleting genes that encode components of the ECM and that influence adhesion may result in reduced distance between the DTCs independently of germ cell proliferation, as RNAi knockdown of these genes may result in DTC migration defects. In order to verify whether the reduced DTC displacement is due to reduced germ cell proliferation or a result of impaired migration, we imaged the *let-805*, *vab-10* and *lam-2* genes in order to

determine whether germline abnormalities were associated with migration, adhesion and ECM defects (Figure 2.4). In Figure 2.4E, the germ cells have progressed beyond the boundary of the GFP-marked DTCs, implying that depletion of *vab-10* does not result in the diminished germline, but rather in DTCs failing to migrate and the aberrantly dividing germ cells escaping the confines of the gonad. To confirm these observations a germ cell nuclei count was performed *daf-2;aak-1;aak-2* animals. In the case of *let-805* and *lam-2*, the reduction in germ cells was significant as compared to the nuclei count in *daf-2; aak-1; aak-2* worms, suggesting that these genes suppressed the germ cell proliferation. *vab-10* depletion did not result in a significant decrease in the germ cell nuclei number, suggesting that it did not suppress the germline hyperplasia *per se*. This suggests that while depleting some genes involved in cell migration, cell adhesion and ECM may result in defects in the somatic cells and structures and may not be directly linked to the germline proliferation, other genes involved in these processes may be involved in suppressing the germ cell divisions. It is unclear how these genes control cell cycle quiescence under the conditions of nutrient stress, but they may simply affect the gonadal expansion, which, in turn, may or may not feed back on germ cell proliferation.

Discussion

An RNAi screen covering approximately 86% of the *C. elegans* genome was conducted in three rounds, yielding a list of 59 candidate genes that reproducibly suppressed the *aak-2*-dependent dauer germline hyperplasia. From the primary screen, we identified 1,148 suppressors, which is a rather large number of candidates, which prompted us to conduct secondary and tertiary screens in order to eliminate false positive hits. The false positives could arise due to several reasons such as the off-target RNAi effects, as well as occasions when sample number of worms was low, thus increasing the relative percentage of worms with reduced hyperplasia. For the secondary and tertiary screens a stringent threshold was applied where the gene candidate would qualify for the next round only if more than 1% of worms possessed the reduced DTC displacement, as it has been found that when the strain was fed with an empty vector, approximately 1% of animals appeared to have a reduced hyperplasia, as compared to the rest of the population.

The multiple rounds of screening allowed us to minimize the rate of occurrence of false positive hits. When conducting the screen, we were also mindful of the potential presence of false-negatives, genes that may be playing a role in the germline proliferation control but that could not be detected. False negatives, when using the RNAi feeding method, occur at varying rates, depending on the tissue (Kamath & Ahringer, 2003; Kamath *et al.*, 2001; Tabara *et al.*, 1998). For instance, when the RNAi is delivered by feeding, the neuronal tissues appear to be refractory to its effect (Tavernarakis *et al.*, 2000). In this case genes that are expressed in those tissues and are targeted by the RNAi will not be silenced. The other set of genes that would not be detected in the screen are genes that are required early in development. The maternally contributed mRNA is deposited in the oocytes so that upon fertilization its translational products can act in the early events of the embryogenesis (Anderson & Kimble, 1997). Depleting these genes by RNAi at the L1 stage may result in the depletion of the newly transcribed mRNA, but it will not have an effect on the proteins that have been translated earlier. This may be the explanation for detecting only one gene, *cyb-2.1,* which is involved in the general cell cycle progression, as many cell

cycle regulators are maternally supplied (Bao *et al.*, 2008). Feeding RNAi to the adult worms in this case will result in embryonic lethality if we deplete the mothers of mRNA necessary for early embryonic development. Another consideration that was kept in mind during the screen is that the accuracy of the RNAi Ahringer library is attenuated due to occasional mis-annotation and cross-RNAi (Qu *et al.*, 2011). As a result the final number of candidates is a significant underestimation of all genes involved in this process. Overall, however, the RNAi feeding method allowed us to conduct a high-throughput screen that permitted to scan the entire *C. elegans* genome, providing a broad overview of gene families that could potentially be involved in mediating germ stem cell quiescence.

Of the 59 loci that suppressed the *aak-2*-dependent germline hyperplasia in dauer, candidates belonging to gene families that participate in energy metabolism and cell signalling were the most abundant. This is not surprising as these genes have a housekeeping function and are necessary for general cell maintenance. The largest group of candidates was the unknown genes with no functional description on WormBase. Further characterization of these must be done as this may lead to a discovery of novel regulators and provide missing links in previously characterized pathways. An unexpectedly large number of candidates emerged in the ECM and adhesion category of genes. These are genes that encode the components of the ECM, intermediates between cytoskeletal network and the matrix and proteins that play a role in adhesion. Another intriguing finding was the discovery of *alg-1* and *ain-2* genes that encode central component of the microRNA pathway, pointing towards involvement of miRNA in regulating AMPK-dependent quiescence.

microRNAs have been known to play an important role in nematode development (Ambros, 2003; Lee *et al.*, 1993). Due to the imperfect binding to a short region on the mRNA homologous to the seed, miRNAs can affect multiple targets, while mRNA have the capacity of being regulated by multiple miRNAs (Ibanez-Ventoso & Driscoll, 2009; Zhang *et al.*, 2011). This makes these small regulators good candidates to coordinate various developmental events and changes, but it also implies that that deletion of one miRNA may not produce a phenotype, as miRNAs often act in networks to downregulate gene

expression. In our work we observe suppression of AMPK-dependent germline hyperplasia in *alg-1* and *ain-2* depleted animals, which impairs the miRNA-mediated gene silencing machinery. This suggests that certain miRNAs must be downregulated in order to establish the dauer-dependent germ cell cycle arrest, however it is unclear which miRNAs or miRNA families are involved.

In *C. elegans,* the expression of miRNAs differs depending on the developmental stage of the animal (Karp *et al.*, 2011). Of particular interest to us are miRNA changes that occur when the worms enter the dauer state. Entry into dauer seems to alter the miRNA circuitry, impacting gene expression under the control of those miRNAs (Karp & Ambros, 2012). A study by (Zhang *et al.*, 2011) also revealed that miRNAs modulate the starvation response during the L1 diapause, an alternative quiescent state that the L1 larvae enters after hatching, and thus play a critical role in the life history of the worm in response to nutritional challenges. Interestingly, miRNA activity has been implicated in various stages of germline development (Cook & Blelloch, 2013). In *C. elegans* it has been documented that loss of function of *alg-1* and *alg-2* resulted in reduced proliferative zone and early meiosis entry in adult *C. elegans,* suggesting that miRNAs are necessary for regulation of normal germline development in worms (Bukhari *et al.*, 2012). Given the documented role of miRNAs in response to nutrient deprivation and their role in germline development, miRNAs may regulate dauer germline quiescence in response to nutrient stress.

Although antisense technologies are available to deplete miRNAs, emergence of *alg-1* from the screen does not shed light on which miRNA/miRNAs may play a role in establishing the germline quiescence in dauer, therefore we cannot use a targeted approach to reveal which miRNAs are differentially expressed in AMPK dauer mutants. Another reason why this approach is not ideal lies in the fact that the antisense reagents are usually administered through gonadal injections causing the F1 progeny to be miRNA-deficient from the beginning of their larval development. We are interested in looking at the miRNA activity only in dauer and would like to understand which miRNAs are inappropriately expressed in the absence of AMPK at that stage. Therefore, the first step towards addressing the question of which miRNAs are involved in the AMPK-dependent

quiescence, would be to use a large-scale transcriptomics approach using RNA Sequencing (RNA-Seq). RNA-Seq can generate a snapshot of the RNA expression at a given time, detecting the abundance of RNAs and small RNAs (Hoen T. *et al.*, 2013). Comparing the miRNA expression profiles of dauer larvae between active AMPK and AMPK null animals, will allow us to detect the overall changes in miRNA expression in all tissues and will provide insights into which of the miRNAs are differentially expressed in the absence of AMPK in dauer. Alternatively an anti-sense probe, may be useful to test whether certain normally downregulated miRNAs are inappropriately upregulated in our AMPK mutant strain using Northern blot analysis (Karp *et al.*, 2011; Reinhart *et al.*, 2000). This may be the first step towards the identification of the miRNA network that regulates dauer germline quiescence.

The emergence of candidates that encode the ECM components is interesting, as their role has not been studied extensively in the *C. elegans* germline and not at all in the dauer germline, and how AMPK may impinge on these components is unclear. Based on the previous findings, we proposed that the ECM might directly interact with GLP-1 signaling to regulate germ cell quiescence (E M Maine & Kimble, 1993; E.M. Maine & Kimble, 1989; Nishiwaki & Miwa, 1998). This interaction may also be indirect and can be mediated by integrin signaling as it has been previously shown that integrins can modulate Notch activity (Campos *et al.*, 2006). Studying the ECM genes and their role in the germline quiescence will help to better understand the interactions between the niche and the stem cell population, and the orchestration of germline proliferation with DTC migration. The germline hyperplasia must be coordinated with the DTC movement and the gonad extension to allow the tissue expansion. It may also be a combination of the GLP-1 signaling and movement that influence cell proliferation. It will also be interesting to understand whether it is the niche and the ECM that grow and signal to the germline to proliferate, or whether it is the growth and multiplication of the germ stem cells that instruct the ECM to alter its size to accommodate this expansion, and whether AMPK plays a role in these interactions.

Interestingly, when we compared the lists of suppressors of *aak-2*- and *par-4*-dependent hyperplasia, genes encoding cytoskeletal regulators suppressed exclusively the *par-4*-dependent proliferation. These are interesting findings as polarity in the germ stem cells has not been extensively studied, and the findings from the screen allow us to hypothesize that PAR-4 may play a previously undocumented role in germ stem cell polarity, and by doing so control the germ stem cell quiescence in an AMPK-independent manner.

Overall our work provided an overview of the gene families that may be involved in regulation of germline quiescence downstream or in parallel of AMPK in response to nutrient stress. It will be necessary to further investigate the previously undocumented roles of miRNAs in dauer germline quiescence, the ECM components in the niche-germline interactions, as well as of other candidates that emerged from the screen. This work may be a stepping stone to uncovering novel pathways in the AMPK-dependent coordination of stem cell dynamics in response to environmental challenges.

Tables and Figures

Table 2.1: RNAi candidates that suppressed AAK-2-dependent hyperplasia during dauer

Chr.	Gene	Locus	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 onRNA 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
I	I-6 Q3 G2*		
I	I-6 Q3 H8*		
I	I-8 Q2 A12*		
I	B0041.2	*ain-2*	Encodes an orthologue of the GW182 protein in mammals; required for miRNA-mediated gene silencing and is recruited to the RISC complex by direct binding to the Argaonaute

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_143.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
X	F48F7.1§§	*alg-1*	Argonaute ortholog required for RNA interference

*****These wells did not appear to be in the Ahringer library database. The RNAi clones from these wells were sequenced and sequences were compared to other species to identify potential homologues.
§Candidates with GPS-predicted AMPK phosphorylation sites
§§Candidates with GPS-predicted AMPK phosphorylation sites, conserved with *Drosophila melanogaster* and *Mus musculus*.

Table 2.2: RNAi candidates that suppressed AAK-2-dependent hyperplasia during dauer display different penetrance and expressivity

Chr.	Gene	Locus	Penetrance and Expressivity of the RNAi candidates
I	T12F5.4	*lin-59*	Low penetrance; partial suppression
I	H15N14.1	*adr-1*	Medium penetrance; partial suppression
I	C54G4.8	*cyc-1*	Medium penetrance; partial suppression
I	F26E4.9	*cco-1*	Low penetrance; partial suppression
I	F35C12.1		Full penetrance; full suppression
I	I-5 Q4 G9		Full penetrance; full suppression; dauer head signal is present but faint
I	E03H4.8		Full penetrance; partial suppression
I	H28O16.1		Medium penetrance; almost full suppression
I	Y18D10A.13	*pad-1*	Medium penetrance; full suppression
I	I-6 Q3 G2		Full penetrance; partial suppression
I	I-6 Q3 H8		Full penetrance; partial suppression
I	I-8 Q2 A12		Full penetrance; full suppression
I	B0041.2	*ain-2*	Low penetrance; partial suppression
II	T08E11.5	*fbxc-19*	Low penetrance; partial suppression
II	B0281.6		Low penetrance; partial suppression

II	F42G2.4	*fbxa-182*	High penetrance; full suppression
II	K02F6.1		High penetrance; almost full suppression
II	T24E12.9		Medium penetrance; partial suppression
II	F29G1.3	*vps-35*	High penetrance; partial suppression
II	T02G5.9	*kars-1*	Medium penetrance; partial suppression
II	F22B5.2	*eif-3.G*	Low penetrance; partial suppression
II	C50E10.2		Medium penetrance; partial suppression
II	C50E10.3	*sre-53*	Medium penetrance; partial suppression
II	Y53F4B.g		High penetrance; partial suppression
III	H19M22.2	*let-805*	High penetrance; full suppression
III	T20B6.3		Low penetrance; partial suppression
III	F26A1.13		Medium penetrance; partial suppression
III	B0336.2	*arf-1.2*	High penetrance; partial suppression
III	C28H8.11	*tdo-2*	Low penetrance; partial suppression
III	T12A2.2	*stt-3*	Medium penetrance; partial suppression
III	C18F10.4	*srg-1*	Low penetrance; partial suppression
III	F23F12.6	*rpt-3*	Medium penetrance; partial suppression

III	R13A5.7		Low penetrance; partial suppression
III	K12H4.4		High penetrance; full suppression
III	F02A9.6	*glp-1*	Low penetrance; full suppression
III	F54G8.1	*irld-34*	Low penetrance; partial suppression
III	R10E11.2	*vha-2*	High penetrance; partial suppression
IV	F47C12.6		Medium penetrance; partial suppression
IV	W03B1.6	*oac-51*	Medium penetrance; partial suppression
IV	F57H12.1	*arf-3*	High penetrance; partial suppression
IV	Y43E12A.1	*cyb-2.1*	Medium penetrance; full suppression
IV	C28c12.11		Medium penetrance; partial suppression
IV	K07F5.7		High penetrance; partial suppression
IV	C04G2.11	*irld-21*	Medium penetrance; partial suppression
IV	T17B5.1		Medium penetrance; partial suppression
IV	F25H8.3	*gon-1*	High penetrance; partial suppression; extra GFP signal between the two DTCs
V	F33E11.1	*nhr-15*	High penetrance; partial suppression
V	F48G7.11	*nhr-190*	High penetrance; partial suppression
V	C24B9.7	*srg-59*	Medium penetrance; partial suppression

V	F35F10.3		Low penetrance; partial suppression
V	F32D1.2	*hpo-18*	High penetrance; full suppression; the animals look sick
V	T24A6.12	*srbc-69*	Low penetrance; partial suppression
V	Y47D7A_143.d		Medium penetrance; partial suppression
V	C10G8.8		High penetrance; partial suppression
V	F07G11.4		High penetrance; partial suppression
V	C54F6.10	*str-31*	High penetrance; partial suppression
V	C12D5.7	*cyp-33A1*	High penetrance; partial suppression
V	C37C3.6	*mig-6*	High penetrance; partial suppression
X	C54D1.5	*lam-2*	High penetrance; full suppression
X	F48F7.1	*alg-1*	High penetrance; partial suppression

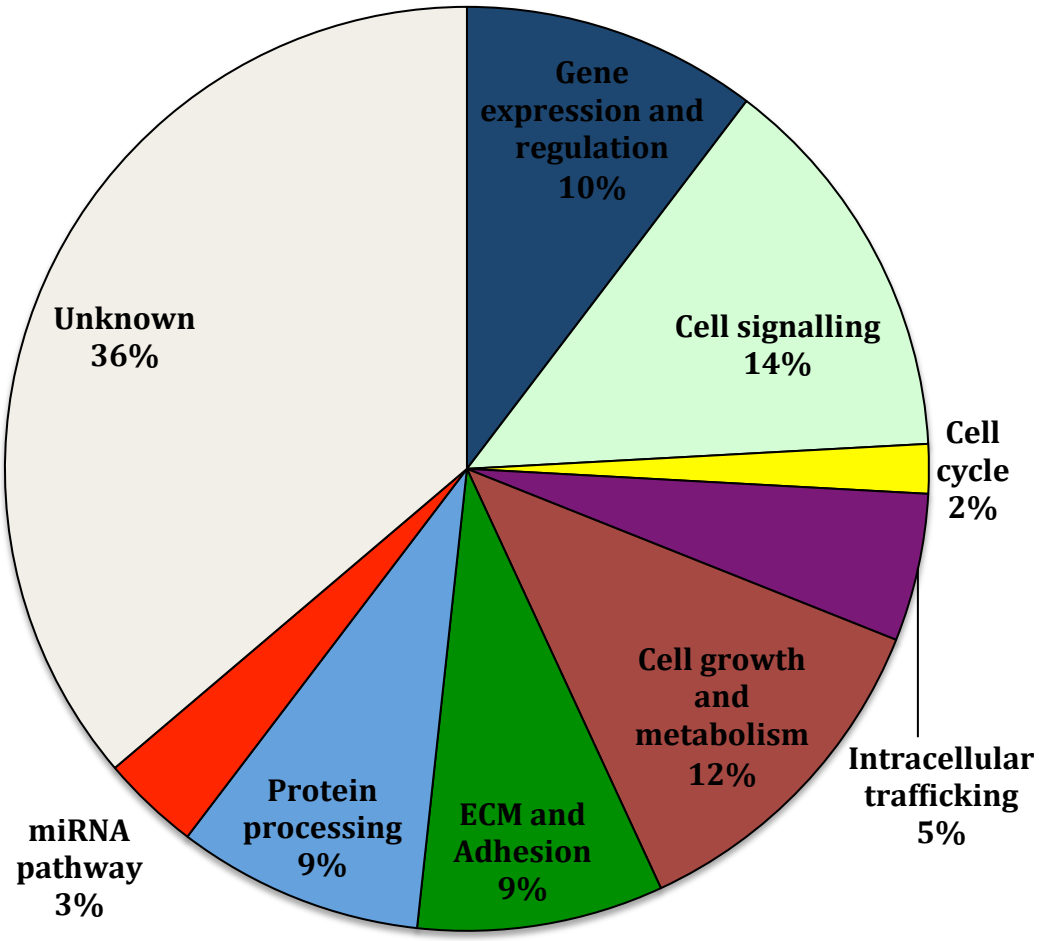


Figure 2.1: Categorization of 59 RNAi candidates into functional groups

The 59 gene candidates, depletion of which suppressed the AAK-2-dependent germline hyperplasia, were categorized based on their functional description on WormBase. The functional groups that were assigned included genes that played a known role in transcriptional and translational regulation of gene expression; in the regulation of extracellular matrix structure and adhesion; in processes responsible for cell growth, protein synthesis and metabolism; in cell cycle progression; in intracellular trafficking; in protein processing; and in regulation of the microRNA pathway. The grey slice represents genes that had no description on WormBase.

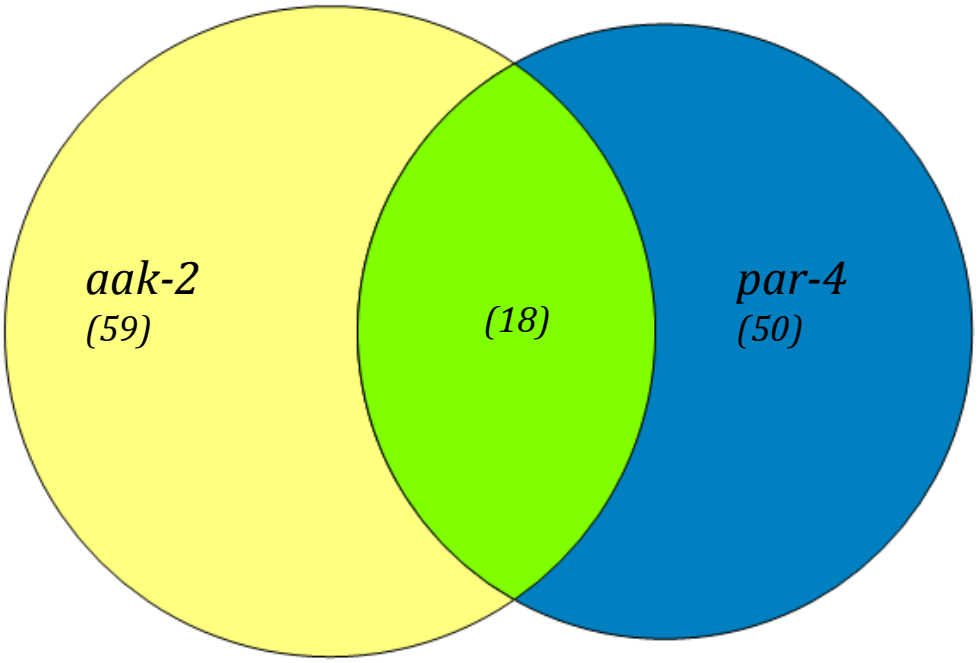


Figure 2.2 - A Venn diagram to represent candidates that suppress *aak-2*- and *par-4*-dependent hyperplasia in the dauer germline

The Venn diagram illustrates the total number of RNAi candidate genes that suppress the hyperplasia in the *aak-2*- and *par-4*-deficient backgrounds. The overlap (green) represents the number of candidates that suppressed the hyperplasia in both scenarios, implying that the candidates are acting downstream of PAR-4 in an AAK-2-dependent fashion.

Table 2.3: RNAi candidates that suppressed the *par-4*- and *aak-2*-dependent hyperplasia

Chr.	Gene	Locus	Brief Description
I	H15N14.1§	*adr-1*	Adenosine deaminase that acts onRNA 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	E03H4.8		Uncharacterized
I	H28O16.1		Uncharacterized
I	ZK1151.1	*vab-10*	A spectraplakin, component of the hemidesmosome in *C. elegans;* required for transducing mechanical signals from the muscle cells to epidermis
II	F29G1.3§	*vps-35*	Vacuolar protein sorting factor
III	H19M22.2§	*let-805*	Myoactin; subunit of the hemidesmosome complex
III	B0336.2§	*arf-1.2*	ADP-ribosylation factor homologue, a GTPase that regulates intracellular trafficking and the actin cytoskeleton
III	T12A2.2§	*stt-3*	Yeast oligosaccharyltransferase subunit homologue

III	K12H4.4		Uncharacterized
III	F02A9.6§	*glp-1*	Notch receptor homologue required for germline stem cell mitotic proliferation
III	R10E11.2	*vha-2*	V-ATPase subunit involved in protein sorting and receptor-mediated endocytosis
IV	F57H12.1	*arf-3*	ADP-ribosylation factor homologue, a GTPase that regulates intracellular trafficking and the actin cytoskeleton
V	F33E11.1§	*nhr-15*	A nuclear hormone receptor
V	F32D1.2	*hpo-18*	Hypersensitive to POre-forming toxin
X	C54D1.5	*lam-2*	Subunit of laminin required for basement membrane integrity and gonad morphology
X	F48F7.1	*alg-1*	Argonaute ortholog required for RNA interference

Table 2.4: Categorization of 35 RNAi candidates based on their cellular function

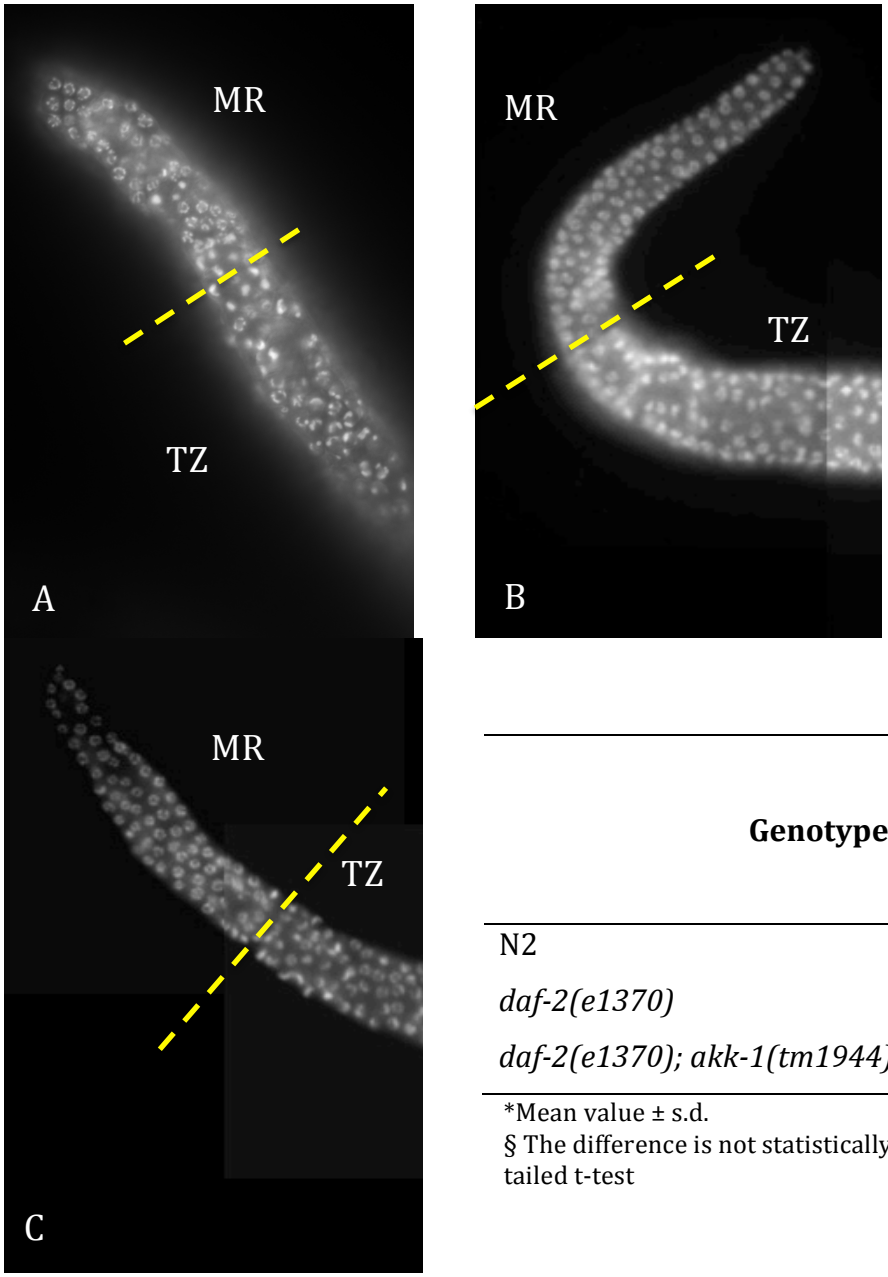
Gene Expression and regulation	Signal transduction	Cell Cycle	Endocytosis	Cell growth and Metabolism	ECM and adhesion	Protein processing	miRNA
adr-1	*irld-21*	*cyb-2.1*	*arf-1.2*	*cyc-1*	*vab-10*	*stt-3*	*alg-1*
eif-3.G	*srg-1*		*arf-3*	*tdo-2*	*let-805*	*fbxa-19*	*ain-2*
glp-1	*srg-59*		*vps-35*	*oac-51*	*lam-2*	*rpt-3*	
nhr-190	*srbc-69*			*cyp-33A1*	*mig-6*	*fbxa-182*	
nhr-15	*str-31*			*cco-1*	*gon-1*	*kars-1*	
*lin-59**	*irld-34*			*vha-2*			
	sre-53			*hpo-18*			

35 RNAi targets with a known, previously documented function, identified as suppressors of AAK-2-mediated dauer germline hyperplasia were categorized based on their known function. Descriptions of each functional group are presented in figure 2.1. Table 1 provides brief functional descriptions of each gene, collected from WormBase. In green are 12/18 genes with known function that appeared to suppress the hyperplasia in both *aak-2* and *par-4* deficient background.

Table 2.5: RNAi candidates that suppress the germline outside of the dauer stage

Chr.	Gene	Locus	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 onRNA 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	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
II	T08E11.5	*fbxc-19*	F-box C protein
II	B0281.6§		Uncharacterized
II	K02F6.1		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	B0336.2§	*arf-1.2*	ADP-ribosylation factor homologue, a GTPase that regulates intracellular trafficking and the actin cytoskeleton

III	C18F10.4§	*srg-1*	Serpentine Receptor, Class G
III	R13A5.7§		Uncharacterized
III	F02A9.6§	*glp-1*	Notch receptor homologue required for germline stem cell mitotic proliferation
IV	W03B1.6	*oac-51*	O-ACyltransferase homologue
IV	Y43E12A.1	*cyb-2.1*	Medium penetrance; full suppression
IV	K07F5.7		Uncharacterized
IV	F25H8.3	*gon-1*	A metalloprotease involved in ECM degradation; controls gonadal morphogenesis
V	F48G7.11§	*nhr-190*	A nuclear hormone receptor
V	C24B9.7	*srg-59*	Serpentine Receptor, Class G
V	F32D1.2	*hpo-18*	Hypersensitive to POre-forming toxin
V	T24A6.12	*srbc-69*	Serpentine Receptor, Class BC
V	C54F6.10	*str-31*	Seven-transmembrane G-protein coupled receptor
V	C37C3.6	*mig-6*	Similar to extracellular matrix proteins papilin and lacunin; required for DTC migration at all phases



Genotype	Number of germ cell nuclei in proliferative zone* (n=9)
N2	222.33±19.85
daf-2(e1370)	197.78±26.24§
daf-2(e1370); akk-1(tm1944); aak-2(ok524)	201.50±10.50§

*Mean value ± s.d.
§ The difference is not statistically significant when measured with the two-tailed t-test

Figure 2.3 - DAPI-stained distal germ cell nuclei in adult *C. elegans*
The size of the proliferative zone of young *C. elegans* adults was evaluated in order to verify that the AMPK-dependent germline proliferation is dauer-specific, and does not affect mitotic division during replete conditions. Germ cell nuclei at the distal part of the gonad in (A) N2 wildtype adults (B) *daf-2* hermaphrodites (C) *daf-2;aak-1;aak-2* adults. The animals were allowed to develop normally at 15C until adulthood and their germlines were dissected out and stained with DAPI. The punctate yellow line marks the end of the mitotic

region (MR) with distinct circular nuclei and the beginning of the transition zone (TZ) with condensed crescent shaped nuclei. The table shows the germ cell nuclei count of the proliferative zone.

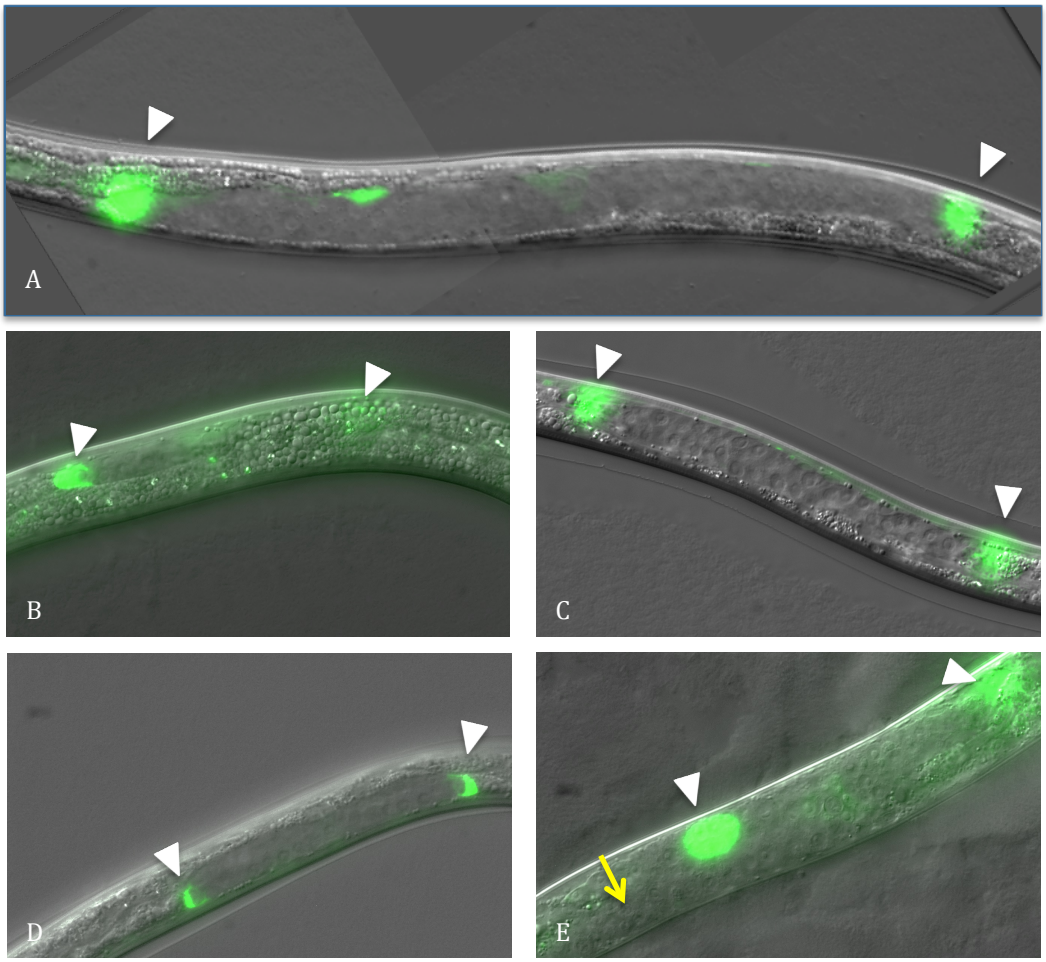


Figure 2.4 – Germline morphology of *aak-2* dauer larvae following the suppression of DTC displacement with RNAi for candidates encoding the components of ECM and cell adhesion

Dauer germlines were visualized using DIC optics in (A) *daf-2;aak-1;aak-2* animals fed with bacteria containing an empty vector, (B) *daf-2* control animals, and in *daf-2;aak-1;aak-2* animals fed with RNAi for genes that encode regulators of ECM and adhesion: (C) *daf-2;aak-1;aak-2;lam-2(RNAi),* (D) *daf-2;aak-1;aak-2;let-805(RNAi)* and (E) *daf-2;aak-1;aak-2;vab-*

10(RNAi). The arrowheads point to the GFP signal coming from the DTCs that express *lag-2::*GFP. In (D), the yellow arrow indicates the germ cell nuclei that extend beyond the DTC

Table 2.4: The reduced DTC displacement following the RNAi for certain candidates encoding the components of the ECM and cell adhesion in *aak-2* dauer larvae may be due to defects in DTC migration and not germ cell number

Genotype	Number of germ cell nuclei in dauer * (n=30)
daf-2(e1370)	35.44±3.85
daf-2(e1370); akk-1(tm1944); aak-2(ok524)	132.49 ± 29.20§
daf-2(e1370); akk-1(tm1944); aak-2(ok524); lam-2(RNAi)	63.53 ± 18.76
daf-2(e1370); akk-1(tm1944); aak-2(ok524); let-805(RNAi)	61.63 ± 19.81
daf-2(e1370); akk-1(tm1944); aak-2(ok524); vab-10(RNAi)	107.60 ± 29.58§

*Mean values ± s.d.
§ The difference is not statistically significant when measured with the two-tailed t-test

The germ cell nuclei count of in the *aak-2* dauer worms was performed following a DAPI staining. The effect on the germ cell number was evaluated in *aak-2* animals fed with RNAi for genes encoding the components of the ECM and cell adhesion in order to verify that the reduced DTC displacement observed was due to the decrease in germ cell proliferation as opposed to defects in DTC migration. The RNAi for *vab-10* produced no significant decrease in the number of germ cells as compared to the *aak-2* mutants, suggesting that it did not truly suppress the hyperplasia. The RNAi for other genes belonging in this category exhibited significantly reduced number of germ cell nuclei compared to *aak-2* null worms.

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Chapter 3

Characterization of post dauer reproductive defects in the recovered AMPK-deficient *C. elegans* hermaphrodites

Introduction

In response to limited growth conditions, *C. elegans* can arrest their reproductive development and enter an alternative L3 stage referred to as "dauer". A remarkable feature of the dauer diapause is the ability of the larvae to exit this enduring stage once conditions improve in order to resume their reproductive development. While the processes that are involved in the dauer entry are well documented, little is known about the factors involved in dauer recovery (Ouellet *et al.*, 2008; Tissenbaum *et al.*, 2000). Remarkably, under most circumstances, larvae recovers from dauer with no reproductive consequences (Ouellet *et al.*, 2008).

In dauer, concurrent with the general cell cycle arrest, the germline stem cell population remains quiescent until the reproductive development is resumed (Narbonne & Roy, 2006a). Germline stem cells (GSC) are a population of mitotically dividing totipotent stem cells that have the capacity to self-renew or undergo meiosis in order to give rise to haploid gametes. In *C. elegans*, GSC proliferate throughout the life of the organism providing a population of cells from which the gametes are derived (Narbonne & Roy, 2006c). The germline is responsive to fluctuations in the environment, particularly to nutrient availability, as demonstrated by the germ stem cell quiescence during dauer, the L1 diapause and in the nutrient-deprived L4 animals (Fukuyama *et al.*, 2006; Fukuyama *et al.*, 2012). If the animal hatches into an environment lacking adequate nutrient resources it will execute the L1 diapause, during which two germline precursor cells arrest at the G2 phase of the cell cycle (Fukuyama *et al.*, 2006). If the larva detects low nutrient availability during the later stages of larval development, it enters a starvation-induced adult reproductive diapause (ADR), during which the germline arrests cell division and undergoes apoptosis, reducing the number of germ cells per gonadal arm. With the re-introduction of food, the germline proliferates and recovers to resemble the germline of the young, non-starved animals (Angelo & Van Gilst, 2009).

The regulation of germline stem cell quiescence during the L1 diapause and the dauer stage requires the cellular energy sensor AMPK, as well as the activity of the tumor suppressor *daf-18/*PTEN (Fukuyama *et al.*, 2006; Fukuyama *et al.*, 2012; Narbonne & Roy, 2006a). In the case of dauer, *par-4/*LKB1 also appears to be necessary to maintain germ stem cell quiescence (Narbonne & Roy, 2006a, 2006c). Mutations in these genes lead to inappropriate germ cell proliferation during compromised growth conditions (Narbonne & Roy, 2006a). Maintaining cells in a quiescent state during environmentally unfavorable conditions is advantageous for the animal's survival, presumably because it lowers the energetic cost of completing the cell cycle in times when energy is limited (Tobin & Saito, 2012). Moreover, environmental stress experienced by the germ cells during the L1 and dauer diapauses may affect the germline integrity after the animal recovers from dauer, and the quiescent state of the germline may serve a protective role (Narbonne & Roy, 2006c).

Previous work has supported the role of AMPK in maintaining germ stem cell quiescence, so we wanted to explore the consequences of the *aak-2*-dependent germline hyperplasia on *C. elegans* post dauer reproductive development. We assessed the brood size of *aak-2* animals that were allowed to recover from dauer and found that in the absence of AMPK, the post dauer recovered hermaphrodites were sterile as compared to the post dauer recovered controls. We then proceeded to determine whether the AMPK-dependent mechanisms of germline quiescence and of germline post dauer recovery might be related, by testing candidate suppressors of *aak-2*-dependent hyperplasia for their role in post dauer recovery.

Materials and Methods

Strains and maintenance:

The following strains were used in the study: MR 0998 strain [*daf-2(e1370); aak-1(tm1944); aak-2(ok523); qIs56(lag-2*::GFP*)*] and MR 0155 [*daf-2(e1370); qIs56(lag-2*::GFP*)*]. All strains were maintained at 15 °C on NGM plates seeded with OP50 bacteria according to standard protocols (Brenner, 1974).

RNAi library:

We used the RNAi feeding library generated by the Ahringer laboratory and that covers 86% of the *C. elegans* genome (Kamath & Ahringer, 2003; Kamath *et al.*, 2001). The bacterial clones express the dsRNA homologous to a single gene target. Production of dsRNA is triggered by IPTG-inducible T7-polymerase, contained in the *E. coli* clones that carry an L4440 double T7 vector with a fragment corresponding to the gene of interest (Kamath & Ahringer, 2003; Kamath *et al.*, 2001). Bacterial clones were cultured in glycerol in 96-well plates.

Assessing the total reproductive output of larvae following dauer formation

A population of the MR0998 strain was synchronized, and the resulting L1 larvae were transferred to NGM plates seeded with *E. coli* OP50 and incubated at 25°C for 48 hours. The MR0998 strain contains the dauer constitutive temperature sensitive allele *daf-2 (e1370)* of the sole insulin receptor in *C. elegans* (Kimura *et al.*, 1997). After 48 hours, dauer larvae with aberrant germline proliferation were formed. The larvae were individually transferred onto separate NGM plate with *E. coli*, and shifted to the permissive temperature - 15°C. Larvae were allowed to recover for 48 hours, after which they were transferred to new plate. This was repeated every 24 hours. The brood size of each animal was the sum of hatched and unhatched progeny of the individuals (Beanan & Strome,

1992). In parallel the same procedure was followed for the MR0155 animals that contained only the *daf-2 (e1370)* allele.

For subsequent analysis of the effects of RNAi on candidate genes that suppressed the germline hyperplasia on the post dauer reproductive output, the plates on which we transferred the L1 larvae following synchronization contained IPTG (1ml/L) and ampicillin (0.5 ml/L) to allow the production of the double stranded RNA. The plates were seeded with RNAi bacterial cultures on which the L1 larvae were subsequently incubated at the restrictive temperature for 48 hours. The dauer larvae were transferred to NGM plates and allowed to recover at permissive temperature. The brood size of the recovered animals was scored.

Assessing the germline following dauer recovery using DIC optics

The MR0998 and the control MR0155 strain were allowed to recover for 3 days at 15°C. Following recovery, 10 – 20 worms were mounted on slides containing 2% agarose pads and were immobilized with 10 micrcoL of levamisole (Shaham, 2006). Animals were observed for germline abnormalities using a Zeiss Imager 2.1 microscope equipped with DIC optics.

Results

Absence of AMPK leads to sterility of the post dauer recovered adults

In order to examine the consequences of dauer-specific, AMPK-dependent hyperplasia on the germline development following post dauer recovery, we first examined the brood size of the *daf-2(e1370); aak-1(tm1944); aak-2(ok523); qIs56(lag-2*::GFP*)* adults (from here on referred to as *aak-2*). As a control we examined the *daf-2(e1370); qIs56(lag-2*::GFP*)* (from here on referred to as *daf-2)* to ensure that any observed differences in the brood size were due to the AMPK-dependent hyperplasia in dauer, as opposed to the mutation in the *daf-2* gene. We recorded the brood size, defined as the number of hatched and non-hatched progeny, from 120 individual animals (Beanan & Strome, 1992). The control *daf-2* animals had an average brood size of 170 (Figure 3.1). The average for the animals containing the *daf-2 (e1370)* mutation is lower than the brood size of the wild type N2 strain that did not undergo dauer, which is approximately 250-350 animals (Hodgkin & Barnes, 1991). The decreased brood size is consistent with previously documented data, where animals containing a *daf-2 (e1370)* allele give rise to 191±44 animals when maintained at permissive temperature of 15°C (Gems *et al.*, 1998). This suggests that the animals containing the *daf-2* mutations that recover from dauer do not have a significantly different brood size from animals with the same genotype that have never entered dauer. Strikingly, animals that possessed the AMPK null mutation, when recovered from dauer, laid, on average, 0.43 embryos. The contribution of outliers when calculating the average brood size for the AMPK-deficient animals skewed the average and resulted in the value greater than zero. If we do not take into account the 2 outliers that have produced very few progeny, the post dauer animals were essentially sterile. This suggests that the dauer hyperplasia, caused by the loss of AMPK signaling, impacts the germline integrity and influences the reproductive fitness of the post-dauer adults.

Suppression of the AMPK-dependent germline hyperplasia does not rescue the post dauer sterility phenotype

The drastic reduction in the brood size of AMPK mutant animals recovered from dauer, suggests that AMPK is necessary for post dauer reproductive recovery. Since it is also necessary for the germline stem cell arrest in dauer, we wanted to examine whether the same, AMPK-dependent mechanism controls the dauer germline quiescence and post dauer recovery of the reproductive capacity. We have recently described the results of the screen that generated a list of 59 candidates, depletion of which suppresses the AMPK-dependent aberrant dauer GSC proliferation. Knockdown of these genes resulted in suppression of the germline, implicating the role of these genes as targets of AMPK that are necessary to mediate the germline quiescence during the conditions of nutrient stress. Using RNAi, we knocked down these genes in the *aak-2* dauers, and then allowed the animals with the reduced hyperplasia to recover by shifting them to the permissive temperature. Each gene was tested on 10 animals and their brood size was scored. Out of the 59 candidates that were tested, only the depletion of 6 genes: *irld-2, nhr-15, nhr-190, srbc-69, arf-3* and T17B5.1 resulted in the animals laying embryos. The average number of embryos laid was low, with most animals laying no embryos, and with exception of one animal per group that seemed to produce a considerably larger brood size as indicated by the outlier points on the chart (Figure 3.1). In order to get a better idea of whether this increase in brood size is by chance or whether it is due to the depletion of these genes, a larger number of animals will have to be tested. The fact that only a small fraction of the genes that consistently suppressed the germline hyperplasia in dauer laid embryos following the recovery suggests that suppression of the germline hyperplasia in the absence of AMPK does not rescue the sterility phenotype of post-dauer recovered animals. Therefore we can conclude that although AMPK is necessary for both the regulation of germline quiescence and post dauer reproductive recovery, the AMPK-dependent mechanisms that regulate these two processes are independent.

The post-dauer recovered *aak-2* animals display gross morphological germline defects

In order to understand the cause of the post-dauer sterility exhibited by the recovered AMPK null hermaphrodites, we examined the germline for any visible morphological defects that could cause this phenotype. We noted that the architecture of the adult hermaphrodite gonad is grossly disrupted, appearing smaller, and lacking a characteristic gonadal bend (Figure 3.2). The oocyte morphology also appeared abnormal with very few cells that lacked the typical, file-like organization typical of the recovered *daf-2* animals (Figure 3.2).

In the majority of animals, the gonad appeared to be underdeveloped, displaying less germ cells than the *daf*-2 gonad. In 87% of the cases there was a profound gonadal asymmetry with animals either lacking an entire gonadal arm or displaying striking morphological differences between the two arms, such as the absence of a characteristic gonadal bend on one side (Table 3.1). This suggests that the development of the two gonadal arms is not coordinated properly in these mutants. The germlines of most of the adult animals contained cells that were reminiscent of oocytes, although there were fewer than in the wild type and they were irregularly shaped and did not form a file of stacked cells like in the wild type or *daf*-2 animals (Table 3.1). In order to confirm that the cells are, in fact, oocytes, a staining with a specific oocyte marker such as anti-RME-2 should be performed (Bachorik & Kimble, 2005). It is also unclear at what stage the gamete development is perturbed in order to give rise to the abnormal oocyte phenotype. Overall, it can be concluded that the underdeveloped gonad, the asymmetry, and the presence of fewer disorganized oocytes is consistently displayed by the *aak-2* mutant hermaphrodites recovered from dauer. The severe defects in the germline in animals lacking AMPK indicate that the germline integrity of these animals is severely compromised and that this aberrant germline development during post-dauer is most likely the cause of the hermaphrodite sterility.

In order to better understand the nuclear architecture of the germline, we stained the gonad of post-dauer recovered *aak-2* animals with DAPI. The germline of the adult wild type animals is divided into distinct regions based on chromosome morphology as germ cells progress from the proliferative region through meiosis towards differentiation (J. Kimble & Crittenden, 2007). Unfortunately due to the previously described structural defects, it was impossible to dissect and extract a full gonad from the recovered mutants in a manner that would be interpretable. Instead, we performed a whole-worm DAPI staining, which confirmed the architectural defects of the gonad, however was not informative of chromosome morphology (data not shown). In order to give a better idea of the cellular events in the compromised germline, whole-worm antibody staining should be performed for meiotic markers anti-REC-8 and anti-HIM-3 (Pasierbek *et al.*, 2001; Zetka *et al.*, 1999). Many germ cells are eliminated from the germline through apoptotic cell death during meiotic maturation at the pachetyne – diplotene stages (T.L. Gumienny *et al.*, 1999). It will also be necessary to examine the germline for the markers of apoptosis and to determine if the upregulation of programmed cell death may result in fewer oocytes.

The germline defects in the post-dauer recovered *aak-2* animals are accompanied by defects in somatic structures

The germline defects in the recovered AMPK null mutants were accompanied by defects in the somatic structures of the reproductive system. The worms exhibited the protruding vulva (Pvl) and multi-vulva (Muv) phenotypes (Table 3.2). The gonad was often extruded, which may be indicative of defective vulval muscles or overall vulval differentiation. The appearance of animals with extruded gonads was more frequent with later transfers, and thus could also be age-related. Previously, we showed that the mechanism by which AMPK regulates the post-dauer germline recovery is different from its control of GSC quiescence in dauer. These somatic changes in the recovered animals may provide insights into which pathways are not properly regulated in the absence of AMPK in the post-dauer animals and whether their misregulation also leads to the germline phenotypes that we observe. The Muv phenotype is often indicative of aberrant LIN-12/Notch signalling or defective regulation of Ras signalling. Further studies will

determine whether these molecular signalling cascades are aberrantly regulated in the absence of AMPK and if this misregulation is also responsible for the observed germline phenotypes.

Discussion

Under the conditions of nutrient stress, the *C. elegans* larva arrests its reproductive development and enters a quiescent state. The L1 diapause, the dauer diapause and the starvation-induced adult reproductive diapause (ADR) are all physiological states that allow *C. elegans* to withstand unfavorable growth conditions (Angelo & Van Gilst, 2009; Fukuyama *et al.*, 2006). These quiescent states are accompanied by the arrest of germ cell divisions. Cellular energy sensor AMPK is implicated in the regulation of this germline quiescence during the L1 diapause and dauer (Fukuyama *et al.*, 2012; Narbonne & Roy, 2006a). In the absence of AMPK, the dauer germline experiences hyperplasia, yet the post-dauer effects of this aberrant germline proliferation have not been documented (Narbonne & Roy, 2006a). In this work we show that post-dauer recovered AMPK null hermaphrodites are sterile and display gross morphological germline defects, often accompanied by aberrant protruding vulva and multivulva (Muv) phenotypes. Although both the germline hyperplasia in dauer and the post-dauer sterility are AMPK-dependent, our data suggest that the mechanisms that regulate these processes are different.

RNAi of the candidate genes that suppressed the AMPK-dependent germline hyperplasia did not restore the sterility of the post-dauer recovered worms, leading us to believe that AMPK may regulate a different molecular mechanism to mediate the recovery of post-dauer germline. These results pose a question of what are the molecular processes that lie under the control of AMPK, required to regulate this post-dauer germline recovery. To begin to answer this question, we should look at the somatic vulval defects that occurred following the post-dauer recovery as the molecular signalling involved in the patterning and development of the vulva has been extensively studied and it may be the similar mechanisms that ensure successful germline recovery from dauer.

During vulval development, the fates of vulval precursor cells (VPCs) are determined by the inductive growth factor LIN-3 produced by the anchor cell (AC), which activates the Ras signalling cascade, which impinges on MAPKs in the cell that will adopt

the primary fate. The lateral inhibition of primary fate of the adjacent cells occurs via the LIN-12/Notch signalling by the binding of the product of the MAPK signaling, which instructs the cells to adopt a secondary fate (Greenwald *et al.*, 1983). The abnormal signaling of these two pathways may be responsible for the multivulva phenotype that accompanies the *C. elegans* post dauer sterility. Particularly it has been shown that gain of function mutations in *lin-12* can produce a multivulva phenotype (Greenwald *et al.*, 1983). This suggests that during normal germline development this *lin-12* signal is attenuated, but in the AMPK mutants *lin-12* may be overexpressed, leading to the phenotype described. The development of multivulva may also occur in response to the elevated Ras-MAPK signaling, as demonstrated by overexpression of *let-60*, *let-23* and/or the inductive signal *lin-3* (Aroian *et al.*, 1990; Han *et al.*, 1990; Hill & Sternberg, 1992). This may indicate that AMPK is needed to downregulate the activity of the Ras pathway. Whether AMPK regulates vulval development at the level of *lin-12* or the Ras pathway remains to be determined. Interestingly, downregulation of Ras is also required for the germline to transition from mitosis into meiosis and to undergo differentiation (Cha *et al.*, 2012). The Ras/MAPK upregulation by depletion of Ras/MAPK germline inhibitor PUF-8 results in sterility and germline that consists of predominantly mitotic cells (Vaid *et al.*, 2013). How AMPK may affect the Ras pathway is not clear, but it would be important to test for over activation of the components of the Ras/MAPK pathway in wildtype and AMPK null post dauer recovered animals.

Overall, the results of this work highlight the importance of arresting the germline stem cell divisions during the conditions of nutrient stress. We demonstrate that continuous cell proliferation during dauer has detrimental consequences for the post-dauer recovered animals, severely compromising their reproductive capacity and therefore reducing the fitness of the animal. The AMPK-dependent mechanism responsible for appropriate germline recovery after dauer remains to be determined, but the mechanism is most probably independent of that which mediates dauer germline quiescence.

Figures and Tables

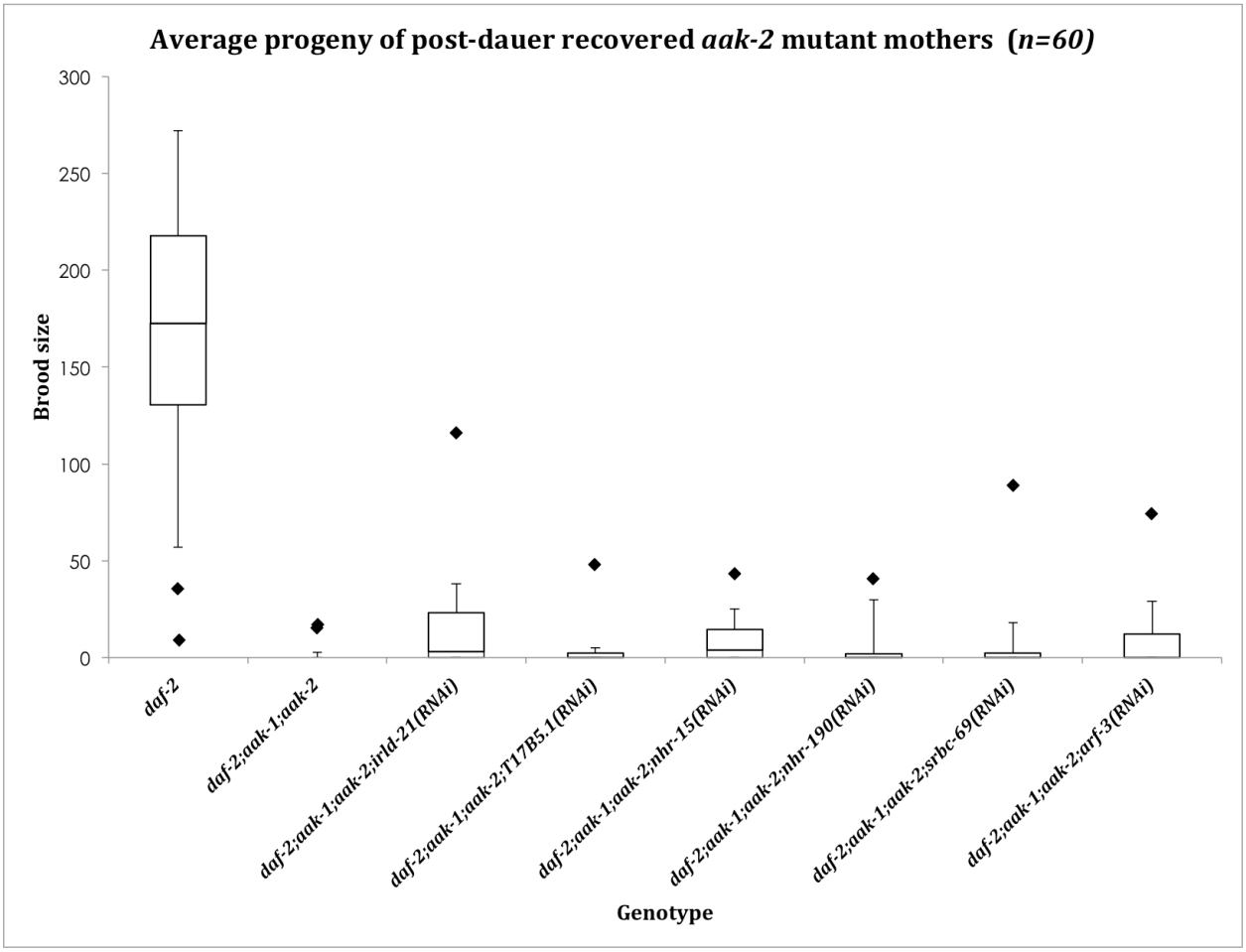

Figure 3.1 – Average progeny per post dauer recovered adult animal

The graph represents the number of progeny laid by *daf-2* and *daf-2; aak-1; aak-2* animals that were recovered from dauer and allowed to develop at permissive temperature. The number of hatched and non-hatched embryos from 120 post-dauer hermaphrodites was scored. Subsequently, RNAi for genes that rescued the germline hyperplasia (see Chapter 2) were tested in order to determine whether they could also rescue the post-dauer sterility phenotype. Above are 6/59 genes that were tested and which, when knocked down in dauer in the AMPK null animals, resulted in post-dauer recovered adults laying embryos.

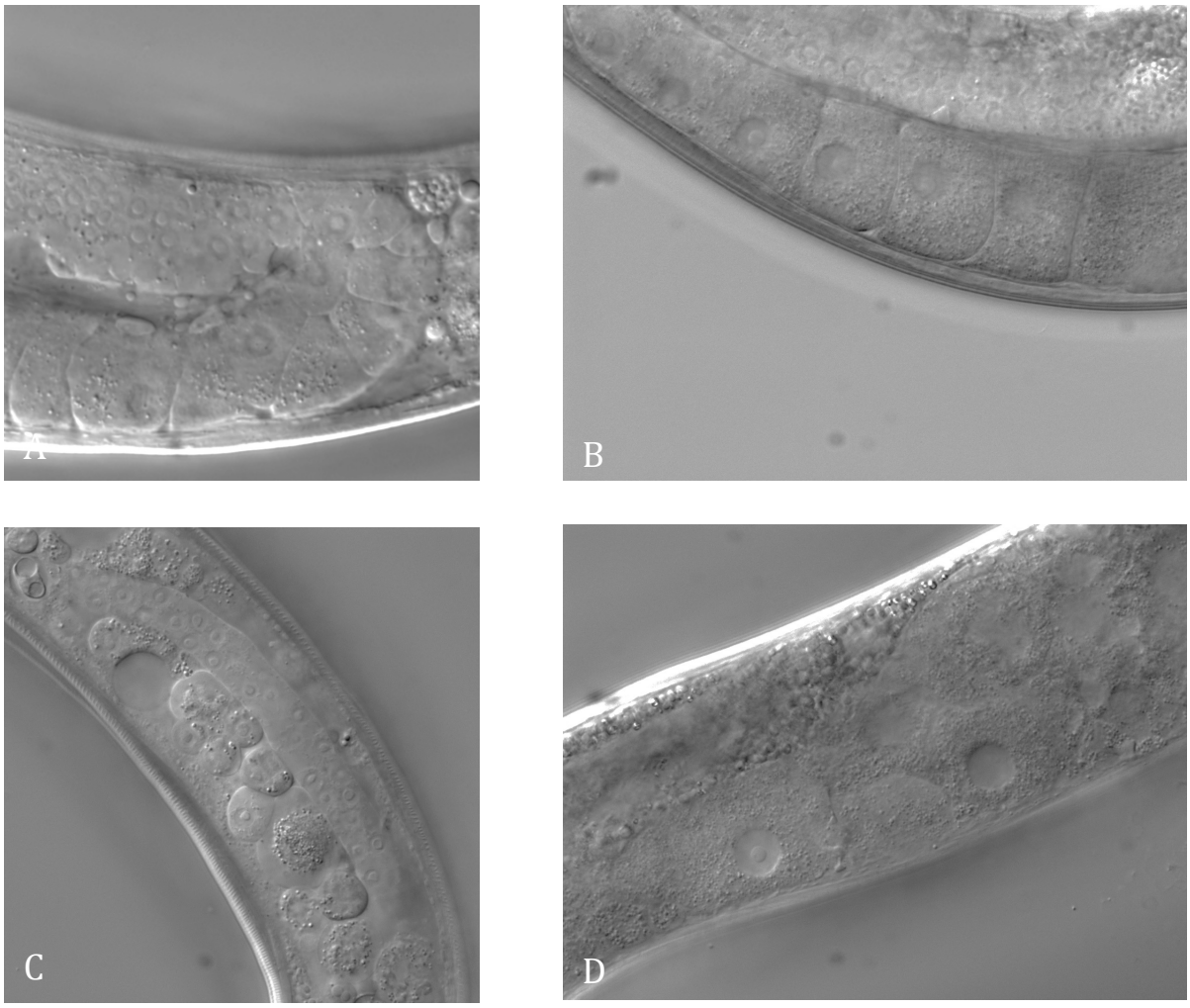

Figure 3.2 – Post dauer recovered *aak-2* adults display severe morphological germline defects as compared to the recovered *daf-2* animals.

daf-2 and *aak-2* dauer larvae were kept at 25°C for 24 hours and subsequently recovered by placing them at 15°C. The adult germline was observed using the DIC optics. (A) and (B) represent the bend and the oocyte regions in the *daf-2* hermaphrodite recovered form dauer, respectively. (C) and (D) represent the corresponding regions in the *aak-2* mutants. The germline in the *aak-2* mutant contained less nuclei and lacked the gonadal arm bend (C), displaying less oocytes that were mostly disorganized (D), and not stacked in files like in the *daf-2* animals (B).

Germline phenotype		Percentage (%) post-dauer recovered *aak-2* mutants displaying germline phenotypes (n=45)
Disorganized, irregular shaped oocytes		89%
Absent oocytes		11%
Underdeveloped gonad*		69%
Asymmetric gonad**		87%
Absence of a bend in the gonadal arms:	1 arm	33%
	2 arms	44%

* An underdeveloped gonad refers to gonad that appeared to be thinner than usual, a about 3-4 cell wide, and which contains less cells than the recovered *daf-2* gonad
** An asymmetric gonad refers to a gonad that is either missing a gonadal arm or had a significant difference in architecture and development of the arms

Table 3.1 – The morphological defects of the gonad reveals persistent defects in oocyte organization and in the gonadal symmetry

The recovered post-dauer *aak-2* hermaphrodite germline was examined under the DIC optics to determine if there were germline features that could explain the post dauer sterility. The *aak-2* germline exhibited gross morphological defects that were quantified. We found that among the most persistent aberrations, the oocytes, if present, were abnormally organized and the number was reduced. Most of the germlines appeared underdeveloped. Underdeveloped germlines were those that contained less cells, appeared thinner and shorter, lacking the complete extension and bend of the normal germline. Asymmetry in the development of the gonadal arms was also present in most of the recovered hermaphrodites.

Somatic phenotype	Percentage (%) post-dauer recovered *aak-2* mutants displaying somatic phenotypes (n=60)
Multivulva (*muv*)	20%
Protruding vulva (*pvl*)	13%
Burst vulva*	15%
Dauer	18%
Dead	13%
Recovered**	21%

* With subsequent transfers many of the animals exhibiting the Pvl and the Muv phenotypes were found with their gonads extruded, so with time the percentage increased
** Refers to animals that recovered from dauer without exhibiting any gross somatic defects but that were sterile

Table 3.2 – Non-germline defects exhibited by post-dauer recovered hermaphrodites

In addition to profound morphological germline defects, the *aak-2* post-dauer recovered mutants display somatic developmental phenotypes. These phenotypes were recorded after the dauer larva was allowed to recover at permissive temperature for 3 days. In total, 48% of animals exhibited vulval defects (multivulva, protruding vulva and burst vulva), suggesting that the processes that are involved in appropriate regulation and specification of vulva development lie under the control of AMPK. Other phenotypes included animals that never recovered from dauer and animals that were dead. The recovered animals were those that displayed no obvious somatic phenotype, however they laid no eggs and were sterile.

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Chapter 4

General Discussion

Discussion

Results presented in this thesis are a stepping-stone towards understanding the role of AMPK in establishing germline stem cell quiescence in *C. elegans* dauer. Our screen revealed different cellular processes that may play a role in AMPK-dependent germline response to the unfavorable growth conditions. Some of these processes such as miRNA activity or cell adhesion have not been previously reported to lie under the control of AMPK. Therefore our work may provide novel insights into how AMPK regulates cell cycle quiescence, specifically in the context of germline development, and how it can do so independently of the general cell cycle arrest associated with entry into dauer.

Organisms must sense and react to changes in the environment in order to survive and successfully propagate (Sommer & Ogawa, 2011). In order to withstand long periods of environmental stress, the nematode *C. elegans* can enter an alternative developmental stage, called "dauer" (Golden & Riddle, 1982). The L3 larva arrests its reproductive development during an environmental duress, but when conditions improve, *C. elegans* can resume the program with no morphological or reproductive consequences (Ouellet *et al.*, 2008; Riddle & Albert, 1997). The dauer state is accompanied by global somatic cell cycle arrest presumably as a consequence of coordinating available energy resources in the environment to an energetically costly process of cell proliferation (Kipreos, 2005; Tobin & Saito, 2012). The germline stem cells also arrest cell divisions and remain quiescent until normal reproductive development is resumed (Narbonne & Roy, 2006a). Previous work has revealed that AMPK and PAR-4/LKB1 mediate this germline stem cell cycle arrest (Narbonne & Roy, 2006a). The disruption of either of the genes causes a dauer-specific germline hyperplasia, while depletion of both causes an enhanced hyperplasia, suggesting that the two act in parallel pathways to regulate germline quiescence. In light of PAR-4/LKB1 regulating AMPK, the findings suggest that AMPK may not be the sole mediator of the PAR-4/LKB1 activity in controlling germline cell division (Narbonne & Roy, 2006a).

AMPK is a protein kinase that is activated by low cellular energy levels and coordinates a metabolic response by phosphorylating specific targets and thereby

regulating numerous processes (Carling *et al.*, 2012; Carling *et al.*, 1987). We sought to characterize the role of AMPK in maintaining germline stem cell quiescence by identifying potential AMPK targets using a reverse genetic approach. We used a genome-wide RNAi screen to identify 59 genes, loss of which reproducibly suppressed the germline hyperplasia typical of AMPK mutants, suggesting that their expression may be misregulated in the absence of AMPK. Characterization of all the candidates may provide us with valuable insight into AMPK-dependent regulation of cell cycle events.

Particularly promising are the candidates that encode essential components of the miRNA pathway, *alg-1* and *ain-2,* due to their previously documented role in modulating the response to starvation-induced L1 diapause, as well as their role in germline development (Bukhari *et al.*, 2012; Zhang *et al.*, 2011). The compromise of these core miRNA RISC components attenuates miRNA-mediated gene silencing in the AMPK-depleted animals. The relationship between AMPK and miRNA should be further investigated in order to determine the previously unreported link between this metabolic regulator and the miRNA network as both play a known role in cell proliferation and malignant growth (Mihaylova & Shaw, 2011)(Hwang *et al.,* 2006) . Our findings from the screen lead us to propose a model where in order to achieve germline stem cell quiescence in dauer, certain miRNAs must be downregulated in the presence of AMPK, leading to expression of certain genes. In the AMPK null dauer larvae, these miRNAs are aberrantly upregulated, leading to inappropriate silencing of those particular genes, but compromising the components of the miRNA pathway leads to a reduced miRNA-dependent gene silencing, allowing the expression of those genes. To test this model, we will perform a Northern blot analysis to test whether the expression of certain miRNAs in dauer is perturbed in the AMPK null animals, as well as RNA-seq in order to determine the differential expression of miRNAs in that background (Karp *et al.*, 2011).

Combining the results of the present screen and the screen for suppressors of PAR-4-dependent hyperplasia we found that 18 candidates were common to both data sets, which implies that PAR-4 impinges upon these genes in order to suppress the germline hyperplasia via AMPK (Chaouni, 2013). This implies that the remaining PAR-4 candidates

may act independently of AMPK. Among these candidates are genes encoding cytoskeletal and polarity regulators (Chaouni, 2013). 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 can propose a model where PAR-4 maintains germline quiescence by regulating germline cell polarity (Watts *et al.*, 2000; Wendland, 2010).

During the *C.* elegans life cycle there are several instances where germline development is arrested under the conditions of nutrient stress (Angelo & Van Gilst, 2009; Fukuyama *et al.*, 2006; Fukuyama *et al.*, 2012; Narbonne & Roy, 2006a). The maintenance of germline stem cell quiescence under unfavorable growth conditions in dauer might act as a protective mechanism, as environmental stress may compromise the integrity of the germline (Narbonne & Roy, 2006c). To explore this further, we evaluated the reproductive capacity of post-dauer recovered hermaphrodites that experienced the AMPK-dependent hyperplasia, and found that AMPK null post-dauer animals are sterile and exhibit profound morphological germline defects. This highlights the importance of AMPK in post-dauer recovery in order to preserve the fitness of the animals. Although the regulation of germline quiescence and reproductive recovery is AMPK-dependent, depletion of genes that suppress the germline hyperplasia do not rescue the post dauer sterility, suggesting that the mechanisms that control the two processes are different. In addition to the germline defects, we identified somatic vulval phenotypes that may involve Ras signaling in post-dauer recovery. To test whether this is true, we will evaluate the activation of Ras signaling components in recovered post dauer wild type and AMPK null hermaphrodites. The relationship between Ras signalling and AMPK has previously been reported, where AMPK gets activated by Ras (Mihaylova & Shaw, 2011). Our speculations that Ras signaling may be increased in the absence of AMPK in the dauer animals are novel and intriguing as they may point towards a new relationship between the two signaling pathways. The first step would be to do a Western blot analysis in order to test whether the components of the Ras signalling cascade are phosphorylated in the absence of AMPK.

Closing statement

This work has contributed to our understanding of AMPK-dependent germline stem cell quiescence. We found 59 genes that could act as potential targets of AMPK to promote germline stem cell cycle arrest. These candidates represent different avenues for future projects, with characterization of the role of miRNAs in establishing germline quiescence under nutrient stress being a particularly promising one. Understanding the mechanism of AMPK-dependent post-dauer reproductive recovery is also very intriguing and may be taken on as a new project, with particular attention being paid to establishing a link between Ras signaling and AMPK during germline recovery.

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