Characterization of *par-4*-dependent germ line stem cell quiescence in *Caenorhabditis elegans*

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ABSTRACT

Upon encountering harsh environmental conditions, *Caenorhabditis elegans* larvae are able to alter their developmental program and enter the dauer diapause, an alternative developmental stage that enables larvae to endure long periods of stress. During this arrested state, the germ line stem cells, which normally divide during reproductive development, halt their proliferation and are consequently rendered quiescent. Previous work has implicated a role for PAR-4/LKB1 in germ line stem cell quiescence. Inactivating mutations in *par-4* result in aberrant germ line stem cell proliferation during the dauer diapause, suggesting that PAR-4 is required for germ cell cycle arrest. LKB1 is a tumor suppressor protein kinase that is implicated in the rare, autosomal dominant disease Peutz-Jeghers syndrome (PJS). In order to better understand its function in tumorigenesis, we characterized its role in regulating cellular quiescence in developmentally arrested larvae using a genome-wide, RNA interference-based screen to identify suppressors of *par-4*-dependent germ line hyperplasia. We identified 50 genes whose loss-of-function was found to rescue the germ line hyperplasia observed in *par-4* dauer larvae, suggesting that their expression is misregulated in the absence of PAR-4/LKB1. In addition, we demonstrated the importance of *par-4*-dependent germ line arrest by characterizing the post-dauer, reproductive capacity of *par-4* mutants, which was significantly reduced. Future endeavors include the characterization of key candidates—many of which impinge on the cytoskeleton and the extracellular matrix—as well as the post-dauer germ line defects observed in *par-4* animals.

RÉSUMÉ

Lors de conditions environnementales hostiles, la larve *Caenorhabditis elegans* est capable d'altérer son programme de développement. Elle entre en phase dauer diapause, un stade alternative de développement qui permet d'endurer des longues périodes de stress. Durant ce stade, les cellules souches germinales, qui normalement se divisent durant leur développement reproductif, arrêtent leur prolifération et deviennent quiescentes. Plusieurs études ont impliqués PAR-4/LKB1 comme acteur dans l'arrêt du cycle des cellules germinales. Lorsqu'on inactive *par-4* à l'aide d'une mutation, les cellules souches germinales prolifèrent de façon anormales lors de la dauer diapause, ce qui suggère que PAR-4 est nécessaire pour l'arrêt du cycle des cellules germinales. LKB1 est une protéine kinase impliqué dans le syndrome Peutz-Jeghers (PJS). Pour comprendre la fonction de LKB1 dans la tumorogenèse, nous avons caractériser son rôle dans la régulation de la quiescence cellulaire des larves en arrêt développemental, utilisant un crible ARNi global, afin d'identifier les suppresseurs de l'hyperplasie germinale induite par PAR-4. Nous avons identifiés 50 gènes dont la perte de fonction était capable de empêcher l'hyperplasie observée chez les larves dauer *par-4*, suggérant que leur expression est dérégulé en l'absence de PAR-4/LKB1. De plus nous avons démontré l'importance de PAR-4 dans l'arrêt du cycle des cellules germinales en caractérisant la capacité reproductive nettement diminuée des larves mutantes en post dauer. Dans le future, nous recommandons que des recherches supplémentaires caractérisent les gènes clés qui ont le potentiel de empêcher l'hyperplasie observée chez les larves dauer *par-4*.

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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 three 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, Conclusion, Figures and Tables, and Bibliography, in that respective order; Chapter 4 is a general discussion and conclusion of the thesis as a whole. My research supervisor, Dr. Richard Roy, contributed to the research presented in this thesis every step of the way, by suggesting experiments and ideas. I have written the thesis entirely, which was revised by Dr. Richard Roy. Additional contributors are recognized in the Acknowledgement Section.

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CHAPTER 1

Literature review and research motivation

Introduction

Control of cell cycle progression is fundamentally intertwined with metazoan development. Many organisms are capable of altering their developmental state as an adaptation to stressful conditions by entering an arrested phase until the situation improves. Processes such as cell proliferation are consequentially rendered quiescent, illustrating the intimate link between the development and cell division. This environmentally induced cell cycle arrest in such organisms can be studied at the cellular and genetic level to better understand the molecular nature of cell quiescence (Padilla & Ladage, 2012).

Understanding cell quiescence is important for advancing our knowledge of stem cell dynamics (Cheung & Rando, 2013). Stem cells are a unique cell type capable of self-renewal on the one hand, and differentiation on the other—maintaining tissue homeostasis in this way (Weissman, 2000). Certain stem cells are also capable of maintaining a quiescent state, re-entering the cell cycle when prompted by specific developmental contexts and external stimuli (Li & Clevers, 2010). Quiescent stem cell pools in mammalian tissues are important for the proper regulation of tissue dynamics. The loss of stem cell quiescence often leads to the premature exhaustion of the stem cell pool, which can affect tissue homeostasis in the long run (Cheung & Rando, 2013; Orford & Scadden, 2008).

The mis-regulation of proliferation or quiescence in differentiated, non-stem cells can also have severe consequences for any individual. The importance of this is highlighted by disorders such as cancer where pathways that regulate the decision of cell proliferation are often disrupted, resulting in their inappropriate division. Aberrant stem cell proliferation is also associated with the potential for cell transformation, and cancer (Cheung & Rando, 2013; Rossi et al., 2008). Dissecting the signaling pathways responsible for keeping cells in check, when it is neither their time nor place to divide, is thus necessary for understanding the aetiology of such disorders.

The model organism *Caenorhabditis elegans*

The soil-dwelling nematode *Caenorhabditis elegans* possesses several key features that make it suitable for characterizing the molecular genetics of cell cycle quiescence. They are well known for their exceptional amenability to genetic manipulation and developmental analyses in the laboratory. With regards to genetic analysis, *C. elegans* is a great model because they are small (1.5 mm long adults) and have a rapid, 3-4 day reproductive cycle, making them easy to handle and suitable for rapid data acquisition (Jorgensen & Mango, 2002; Riddle et al., 1997). In addition, they exist as both hermaphrodites and males, which facilitates genetic analysis because the self-fertilizing hermaphrodites can also be crossed with the males. With respect to developmental studies, *C. elegans* are useful because they have an invariant somatic cell number, lineage and positioning, rendering their development predictable and easily traceable at the level of organs, tissues, and even single cells (Kipreos, 2005; Riddle et al., 1997; Sulston et al., 1983). Their translucent cuticle, moreover, allows for the live visualization of internal structures, a helpful quality when analyzing the germ line (Riddle et al., 1997). In conjunction with the countless tools available to researchers that facilitate genetic and developmental studies (such as RNA interference and transgenesis), these qualities make *C. elegans* a powerful model to study fundamental biological processes, as well as the many genes and pathways conserved in humans and implicated in diseases (Antoshechkin & Sternberg, 2007; Jorgensen & Mango, 2002; Kaletta & Hengartner, 2006; Labbe & Roy, 2006).

Environmentally induced cell cycle arrest in *C. elegans* – a cell cycle model

A unique aspect of *C. elegans* is their flexible developmental program, which can follow one of two trajectories depending on the environment. In stressful situations, *C. elegans* naturally opt out of normal development and enter a quiescent state as a survival strategy (Cassada & Russell, 1975; Riddle & Albert, 1997). One outcome of such nutritionally and environmentally challenged larvae is

the orchestrated quiescence of their otherwise highly proliferative pool of germ line stem cells (Narbonne & Roy, 2006, 2008). The genetic dissection of this developmental state termed dauer (explained in greater detail in the following section) has been a fruitful area of research, helping our understanding of dauer-like states in parasitic nematodes (Hu, 2007). We may now also take advantage of this knowledge base and use this developmental scenario, in conjunction with genetics, to further our understanding of quiescence, in stem cells and in general.

C. elegans **development and the dauer diapause**

C. elegans development is susceptible to the environment; as a free-living, soil-dwelling animal, the conditions in which *C. elegans* find themselves may take a turn for the worst, which affected animals must cope with (Padilla & Ladage, 2012). As a survival mechanism, *C. elegans* have evolved the ability to circumvent their normal, developmental program and develop into a specialized larva capable of enduring stress (Cassada & Russell, 1975; Hu, 2007; Riddle & Albert, 1997).

The normal developmental program is in effect when the surrounding conditions are favorable for normal growth and reproduction. When they hatch, *C. elegans* normally develop through four larval phases—L1-L4—before reaching adulthood (Figure 1). The newly hatched L1 larvae begin to grow and develop in the presence of food in a well-documented, stereotypical manner, where each larval stage is interjected with a molt. During each molt, a new cuticle is synthesized and the preceding one is shed. After four larval molts, they reach adulthood and sexual maturity (Riddle et al., 1997).

The reproductive cycle in *C. elegans* is launched when somatic and germ line precursor cells found in the L1 hatchling divide and differentiate to form the adult gonad structure and the germ line stem cells from which gametes are derived (Kimble & Hirsh, 1979). Meiosis is initiated during the end of the third larval stage, L3, and first results in the production of sperm that is kept in the spermatheca

(Kimble & White, 1981; Ward & Carrel, 1979). In response to a programmed, developmental switch, gametogenesis switches gear and gives rise to oocytes instead during the final larval stage, L4 (Kimble & White, 1981). The oocytes become fertilized after passing through the spermatheca and the developing eggs pass through the uterus and out through the vulva (McCarter et al., 1997; Ward & Carrel, 1979).

Alternatively, if *C. elegans* are stressed and nutritionally compromised, they execute an alternative developmental program that allows them to survive the harsh conditions or disperse to a new location. This alternative developmental state that arises in response to nutrient deprivation in *C. elegans* is termed the dauer diapause (Cassada & Russell, 1975). During dauer development, larvae undergo an alternative third larval stage. L2 larvae enter a pre-dauer stage, L2d, and then dauer instead of advancing into the L3 stage (Cassada & Russell, 1975; Hu, 2007; Riddle & Albert, 1997). The dauer stage is characterized by global quiescence and altered metabolism that persist until growth conditions improve. The animals remain in a state of complete cell cycle arrest while conserving their lipid stores, or energy (Burnell et al., 2005; Narbonne & Roy, 2009). They remain so for up to four months, or until the environment is favorable again, at which point larva begin to feed, molt into L4, and progress into adulthood without any consequence on health or life span thereafter (Burnell et al., 2005; Hu, 2007; Riddle & Albert, 1997).

Dauer characteristics

Dauer larvae are altered in manners that benefit long-term survival and dispersal. Morphologically, their appearance is distinct from larvae of other developmental stages. They are longer, more slender, and very dense due to compression of the hypodermis and modifications to the cuticle. Their internal architecture is adjusted such that the pharynx and intestinal lumen are constricted, and sensory neurons are reoriented affecting dauer behavior. (Albert & Riddle, 1988; Cassada & Russell, 1975; Riddle & Albert, 1997; Swanson & Riddle, 1981;

Vowels & Thomas, 1992). Naturally, dauer larvae behave in very different ways—they are motionless unless stimulated, possibly a means of saving energy reserves (Riddle & Albert, 1997). Often, they engage in nictation, a behavioral practice where they stand on their tails and project vertically into the air, which is a means of dispersal (Croll & Mathews, 1977; Riddle & Albert, 1997). Importantly, dauer larvae are physiologically and metabolically altered, or quiescent—they have reduced tricarboxylic acid cycle activity (TCA), they do not feed and instead ration through lipid stores that have been accumulated during the L2d stage in order to subsist (Burnell et al., 2005; Riddle & Albert, 1997; Wadsworth & Riddle, 1989). Though transcriptionally inactive for the most part, however, stress resistance, cellular maintenance and detoxification genes are upregulated during the dauer stage, rendering larva more resistant to various types of stresses as a consequence (Dalley & Golomb, 1992; Larsen, 1993; Riddle & Albert, 1997; Vanfleteren & De Vreese, 1995; Wadsworth & Riddle, 1989).

Regulation of dauer entry

Dauer formation relies on the larvae's ability to assess and integrate environmental cues into their developmental program (Golden & Riddle, 1984a; Riddle & Albert, 1997). The environmental signals that govern dauer formation are overcrowding, lack of food and high temperature (Golden & Riddle, 1984a; Riddle & Albert, 1997). Animals sense a growing population density as an increase in the concentration of a dauer-inducing pheromone that is constitutively produced throughout the *C. elegans* life cycle (Jeong et al., 2005). Whereas higher levels of pheromone induce dauer formation, lower concentrations correlate with normal development (Golden & Riddle, 1982, 1984a). If intermediate levels of pheromone are produced and sensed, then the decision to enter dauer is based on nutrient availability and temperature (Golden & Riddle, 1982, 1984a, b, 1985; Riddle & Albert, 1997).

The genetic pathways that convey such environmental parameters have been dissected extensively. Genetic analysis of dauer formation has identified dauer constitutive (Daf-c) mutants and dauer defective (Daf-d) mutants, which execute and fail to execute dauer irrespective of the environment, respectively (Malone & Thomas, 1994; Riddle & Albert, 1997; Swanson & Riddle, 1981). The genetic dissection of Daf-c genes and Daf-d genes has revealed that three, independent signaling pathways regulate dauer: insulin-like signaling, TGF-β, and cGMP (Gottlieb & Ruvkun, 1994; Patterson & Padgett, 2000; Riddle & Albert, 1997; Riddle et al., 1981; Thomas et al., 1993). Each is attenuated in response to adverse conditions, which induces dauer formation by affecting the activity of a downstream nuclear hormone receptor (Antebi et al., 2000; Yeh, 1991).

Insulin-like signaling

Insulin-like signaling (IlS) is a conserved nutrient-sensing pathway involved in regulating cell growth and survival in metazoans (Marshall, 2006). In *C. elegans,* the insulin receptor homolog is encoded by the gene *daf-2*, which regulates life span and dauer diapause (Kenyon et al., 1993; Kimura et al., 1997). Insulin-like molecules bind to the insulin receptor DAF-2 on the plasma membrane and induce its activation (Kimura et al., 1997). The activated insulin receptor initiates a protein kinase cascade that ultimately affects pathways and processes involved in cell growth and cell survival. One important outcome of insulin signaling is the cytoplasmic retention of a FOXO forkhead transcription factor, DAF-16 in *C. elegans* (Lin et al., 2001; Mukhopadhyay et al., 2006).

The activated insulin receptor recruits the phosphoinositide-3-kinase (PI3K) AGE-1 in *C. elegans* to the cell membrane through adaptor proteins, where membrane bound phosphoinositide-3,4-P2 (PIP2) is then converted into phosphoinositide-3,4,5-P3 (PIP3) by PI3K/AGE-1 (Dorman et al., 1995; Morris et al., 1996; Paradis et al., 1999; Tissenbaum & Ruvkun, 1998; Vanhaesebroeck et al., 1997). Increased levels of PIP3 result in the activation of AKT-1 and AKT-2, the

two homologues of the serine-threonine kinase AKT/PKB, which phosphorylate DAF-16/FOXO thereafter, causing it to remain in the cytoplasm (Paradis et al., 1999; Paradis & Ruvkun, 1998). The phosphatase and tensin (PTEN) homologue, DAF-18, antagonizes these insulin-mediated effects by dephosphorylating PIP3 (Gao et al., 2000; Ogg & Ruvkun, 1998).

Under normal growth circumstances in *C. elegans*, insulin like signaling results in the activation of genes required for normal growth and development. Under stressed conditions, insulin signaling is reduced, resulting in a nuclear pool of DAF-16 capable of transcribing genes involved in life-span extension as well as dauer formation (Mukhopadhyay et al., 2006). DAF-2 is thus a Daf-c gene product, while DAF-18 and DAF-16 are Daf-d gene products (Dorman et al., 1995; Gottlieb & Ruvkun, 1994; Riddle & Albert, 1997).

TGF-B signaling

Transforming growth factor β (TGF-β) signaling is equally conserved across species, playing key roles in various aspects of development. Aspects of *C. elegans* hermaphrodite development that are regulated by TGF-β-type signaling include normal body size and dauer formation (Attisano & Wrana, 2002; Patterson & Padgett, 2000; Ren et al., 1996; Riddle & Albert, 1997; Savage et al., 1996; Savage-Dunn, 2005). TGF-β functions in chemosensory ASI neurons and promotes reproductive development (Ren et al., 1996). In TGF-β signaling, ligands belonging to the TGF-β family initiate diverse cellular responses by signaling through two serine/threonine transmembrane receptors (type I and type II), Smad family of mediators, and affecting transcription. Type I and type II receptors form a complex in response to ligand binding, leading to the type II-dependent phosphorylation and activation of the type I receptor. The activated type I receptor recruits and activates receptor-regulated Smad proteins (R-Smad). R-Smad associates with Co-Smad in a complex, which accumulates in the nucleus and regulates target gene transcription (Attisano & Wrana, 2002; Patterson & Padgett, 2000; Savage-Dunn, 2005).

TGF- β pathway components in *C. elegans* are encoded by several gene loci. The main ligand involved in dauer regulation is DAF-7, which binds and activates the type I and II receptors, DAF-4 and DAF-1, respectively (Estevez et al., 1993; Georgi et al., 1990; Ren et al., 1996). DAF-8 and DAF-14, in turn, are the specific R-Smad and Co-Smad cofactors that associate and affect dauer-related transcription in response to DAF-7 signaling (Inoue & Thomas, 2000; Wang & Levy, 2006). Normally, DAF-8/DAF-14 repress the transcription of dauer-specific genes by negatively impinging on DAF-3, an inhibitory Smad, and DAF-5, a member of the Sno/Ski family of oncogene. DAF-3 and DAF-5 are physcially associated with each other, and would otherwise promote dauer development by inducing the transcription of dauer genes (da Graca et al., 2004; Inoue & Thomas, 2000; Patterson et al., 1997). When DAF-7 signaling is reduced in response to stress, DAF-3/DAF-5 is relieved from DAF-8/DAF-14 mediated repression and free to transcribe such genes. Mutations in *daf-7, daf-1, daf-4, daf-8,* and *daf-14* thus result in a Daf-c phenotype, while mutations *daf-3* and *daf-5* yield Daf-d nematodes (Inoue & Thomas, 2000; Riddle & Albert, 1997).

cGMP signaling

The third, parallel pathway, which affects dauer formation is cyclic GMP (cGMP) signaling. cGMP is a secondary messenger molecule that is derived from GTP through a chemical reaction that is catalyzed by transmembrane guanylyl cyclase (TMGC) (Goy, 1991). cGMP is involved in translating extracellular cues received initially by TMGCs into a wide range of cellular responses by affecting various downstream targets, including kinases, other cyclic nucleotides, as well as cGMP-gated channels (Goy, 1991; Vowels & Thomas, 1994). In *C. elegans, daf-11* encodes a transmembrane guanylyl cyclase, mutations in which result in Daf-c larvae. DAF-11 is expressed in several chemosensory neurons, and ultimately affects cGMP-gated channels involved in sensory transdcution. During dauer formation, levels of DAF-11 are reduced, prompting larvae to enter dauer (Birnby et al., 2000; Riddle & Albert, 1997; Vowels & Thomas, 1992, 1994).

The integration of dauer signals

Insulin-like, TGF-B, and cGMP pathways are incorporated through a conserved neuroendocrine pathway that converges on a nuclear hormone receptor DAF-12, a Daf-d gene product (Antebi et al., 2000; Riddle et al., 1981). Nuclear hormone receptors are a class of transcription factors that respond to lipophilic molecules such as steroids. They are homologous to the human Vitamin D and Liver X receptor (Antebi, 2006; Snow & Larsen, 2000). In response to attenuated signaling via these three pathways, DAF-12 regulates *C. elegans* life history, acting like a developmental switch where its ligand-bound form induces dauer and its non-ligand bound form results in reproductive development (Antebi, 2006; Antebi et al., 2000). DAF-12 biosynthesis requires DAF-9, cytochrome p450 (Gerisch & Antebi, 2004; Gerisch et al., 2001; Motola et al., 2006). DAF-9 and DAF-12 thus integrate signals from IlS, TGF-B and cGMP pathways to either mediate developmental arrest or reproductive development (Antebi, 2006).

The *C. elegans* gonad

As host to the germ line stem cell population in *C. elegans,* the reproductive organ's response to dauer formation is of great biological interest, raising questions concerning stem cell dynamics and quiescence. Tackling these problems is greatly facilitated by the gonad's simple morphology and spatial design. In hermaphrodites, it is a tubular, curvilinear structure that spans the anterior-posterior axis of mature animals, consisting of two u-shaped gonad arms that are mirror images of other. While one u-shaped gonad is located anteriorly and the other posteriorly, they are joined proximally by a single shared uterus situated near the ventral midline. Each u-shaped arm is divided into an arm that is more distal (or dorsal) to the uterus and vulva, versus that which is more proximal (or ventral) in the L4/adult stage. (Hirsh et al., 1976). The proximal ends of each gonad include a spermatheca, a specialized structure where sperm is stored (Hirsh et al., 1976). In either distal end lies a population of dividing germ line stem cells, which are

enveloped by five pairs of somatic sheath cells and which differentiate into gametes as they are displaced proximally (Hirsh et al., 1976; Kimble & Hirsh, 1979). The developing oocytes inhabit the proximal gonad and are fertilized when they pass through the spermatheca (Hirsh et al., 1976; McCarter et al., 1997; Ward & Carrel, 1979). The gonad's spatial setting is thus polar, where germ cells at either end remain distinct from the more proximal, differentiated cells, enabling the identification of the stem cells and facilitating study of their proliferative dynamics.

Development of the *C. elegans* germ line

From the time larvae hatch to when they reach adulthood, the *C. elegans* gonad primordium develops in order to give rise to a functional reproductive system. Figure 2 illustrates a schematic rendition of this development. The gonad primordium consists of 4 cells, Z1, Z2, Z3, and Z4, which proliferate and differentiate throughout development to give rise to the entire germ cell lineage and somatic gonad (Kimble & Hirsh, 1979). The two inner cells (Z2 and Z3) are the germ line precursor cells, which divide continuously throughout the larval phases in order to generate a pool of stem cells from which gametes are derived. During development, the germ line stem cells occupy the distal end of the gonad and form a syncytium. Cytokinesis is incomplete and therefore the germ cells remain linked to each other through a common cytoplasm. (Hirsh et al., 1976; Hubbard & Greenstein, 2005; Kimble & Crittenden, 2007). The germ cells are eventually displaced from their distal location through time, at which point they enter meiosis, and differentiate into gametes. They enter meiosis during the L4 stage when the length of the gonad arm has substantially expanded anteriorly and posteriorly and begins forming the bend on either side (Hirsh et al., 1976; Kimble & Hirsh, 1979; Kimble & White, 1981). The proximal most stem cells exit the mitotic program and enter meiosis, forming first a population of around 200 sperm that are stored in the spermatheca. At adulthood, germ cells differentiate into oocytes instead and the proximal arm is lined with developing oocytes (Barton & Kimble, 1990; Crittenden et al., 2003; Ellis & Kimble, 1995). A single nematode produces around 300

progeny, given the amount of germ cells and sperm available (Hodgkin & Barnes, 1991).

In the interim, the somatic gonad precursors (Z1 and Z4) follow a developmental path of their own, accumulating in number and undergoing morphogenesis in order to form the somatic features of the adult gonad. By the L4 stage, the main somatic gonad structures are formed and include the distal tip cells (DTCs), found on either distal extremity of the gonad; epithelial sheath cells, which enclose the distal and proximal portion of each gonad arm (where the germ cells and oocytes are found); two spermathecae, and a single uterus where fertilized eggs are held until they exit through the vulva (Kimble & Hirsh, 1979). Throughout germ line development, the DTCs are important for gonad morphogenesis as well as inducing the mitotic program in germ cells (Kimble & White, 1981; McCarter et al., 1997). The cells of other somatic lineages are also necessary for vigorous germ cell proliferation during the L1/L2 stage (McCarter et al., 1997). Finally, the sheath cells that enclose the germ line around meiotic prophase are important for germ line development, exit from pachytene, and oogenesis by communicating with oocytes and germ cells through gap junctions (Grant & Hirsh, 1999; Hall et al., 1999; McCarter et al., 1997, 1999; Seydoux et al., 1990).

During dauer formation, the normally proliferating germ line is instructed to stop dividing. Germ line stem cells slow, and subsequently arrest, proliferation during the pre-dauer and dauer stage respectively (Narbonne & Roy, 2006). The germ line during the dauer phase consists of around 35 germ cell nuclei (Narbonne & Roy, 2006). How these cells adopt an arrested fate is an interesting question that could potentially highlight aspects of the molecular dynamics of stem cell quiescence.

Maintenance of the mitotic germ line

The germ cells' proliferative fate is maintained through their interaction with surrounding cells of the somatic cell lineage during gonad development. Ablation studies have revealed that the DTCs, sheath cells, and spermatheca lineage cells are all important for germ line proliferation (Kimble & White, 1981; McCarter et al., 1997).

The DTCs promote germ cell mitosis throughout all stages of development through the activation of Notch/GLP-1 signaling (Kimble & White, 1981). The GLP-1 ligand, LAG-2/Delta, is expressed on DTCs, while GLP-1 is located on the surface of germ cells (Crittenden et al., 1994; Henderson et al., 1994). Active GLP-1 transduces the DTC signal and instructs the self-renewal of germ cells by activating the appropriate target genes, which include *fbf-2* (Crittenden et al., 2002; Kimble & Crittenden, 2007; Lamont et al., 2004). FBF-2 inhibits translational repressors of mitotic-promoting mRNAs and a translational activator of meiosis-promting mRNAs, respectively (Kimble & Crittenden, 2007; Wickens et al., 2002). The localized expression of LAG-2 on either distal end causes germ cells further away to experience diminished GLP-1/Notch signaling and thus enhanced activity of pro-meiotic genes (Crittenden et al., 2003; Kimble & Crittenden, 2007). DTC-dependent GLP-1 signaling is absolutely required for both proliferation and maintenance of the stem cell pool as germ line precursor cells fail to divide altogether in animals lacking DTCs and differentiate into meiosis in temperature sensitive (ts) *glp-1* mutants shifted to the restrictive temperature (Kimble & White, 1981).

During the early phases of germ line stem cell proliferation, specifically at the L2/L3 molt, sheath and spermatheca lineage cells are also important for germ cell proliferation. The number of mitotic stem cells is reduced if these cells are ablated (McCarter et al., 1997). This interaction is interesting since it occurs when animals are dauer-competent and must decide their developmental fate.

LAG-2/Delta and GLP-1/Notch expression are still present during dauer (Narbonne & Roy, 2006). Nevertheless, coincident with the organism's overall dormant state, the normally proliferating germ line is instructed to stop dividing. Germ line stem cells slow and subsequently arrest proliferation during the pre-dauer and dauer stage respectively, though how this happens remains a mystery (Narbonne & Roy, 2006).

Regulation of dauer-specific germ line quiescence

C. elegans respond to harsh environmental conditions by adapting their developmental program in order to conserve energy. Cell proliferation is an energy-consuming process and thus the ability of germ cells to asses their environment and make the decision to divide or not is essential for their survival (Tobin & Saito, 2012). In aims of identifying genes involved in establishing quiescence in nutritionally and environmentally challenged larvae, a forward genetic screen searching for mutations that cause aberrant, dauer-specific germ cell proliferation, or hyperplasia, was conducted in our laboratory. Through this approach, it was discovered that AMP-activated kinase (AMPK), PAR-4/LKB1, and DAF-18/PTEN are required for quiescence (Narbonne & Roy, 2006).

AMPK is an energy sensing protein conserved throughout eukaryotes, involved in managing diverse cellular processes during times of energetic stress. In energetically depleted cells, AMPK becomes activated and stimulates catabolic process while downregulating anabolic pathways, and in this manner, serves as the molecular link that reacts to metabolic cues. AMPK is crucial in its ability to downregulate energy-consuming pathways to conserve energy (Carling, 2004; Carling et al., 2011; Hardie et al., 2012; Kahn et al., 2005). PAR-4/LKB1 and DAF-18/PTEN are both tumor suppressors, null mutations in which underlie the cancer-predisposing syndromes Peutz-Jeghers (PJS) and Cowden's syndrome, respectively. PAR-4/LKB1 is a serine/threonine kinase, whereas DAF-18/PTEN is a phosphatase (Hemminki et al., 1998; Jenne et al., 1998; Liaw et al., 1997).

PAR-4/LKB1 has been identified as the major upstream kinase regulator of AMPK (Woods et al., 2003). AMPK exists as a heterotrimer, consisting of two regulatory subunits and one catalytic subunit. In response to rising AMP levels, and thus declining ATP, AMP binds to AMPK's regulatory subunits generating its allosteric, conformational change that enhances its interaction with its major upstream kinase, PAR-4/LKB1 (Hardie, 2004; Jansen et al., 2009). To achieve this, PAR-4/LKB1 operates in a heterotrimeric complex as well, bound to the pseudokinase STRAD and scaffolding protein, MO25 (Baas et al., 2003; Boudeau et al., 2003; Hawley et al., 2003). In fact, PAR-4/LKB1 tumor suppressive function has largely been attributed to its ability to activate AMPK (Hardie, 2005; Jansen et al., 2009). Through this signaling mechanism, PAR-4/LKB1 is involved in coupling basic cellular functions to energetic metabolism, protecting cells against aberrant growth and proliferation in this manner. Figure 3 outlines the dynamics and select outputs of the LKB1/AMPK signaling module (Jansen et al., 2009).

AMPK has been shown to be involved in the negative regulation of the core cell growth pathway, mTOR, by phosphorylating and activating an mTOR-antagonizing GAP, tuberous sclerosis 2 (TSC2) (Figure 4) (Hardie, 2004; Inoki et al., 2003; Schmelzle & Hall, 2000). In this particular scenario, PAR/LKB1 tumor suppression lies in its ability to counter this pathway, safeguarding cells from overgrowth in unfavorable conditions (Hardie, 2005).

Nevertheless, though AMPK is one of PAR-4/LKB1's better-characterized downstream targets, in charge of linking PAR-4/LKB1 to the energetic-dependent regulation of cell growth and polarity, it does not entirely account for PAR-4/LKB1-dependent regulation of germ cell quiescence in *C. elegans* (Narbonne & Roy, 2006). Genetic analysis of dauer germ line hyperplasia indicates that AMPK and PAR-4/LKB1 actually cooperate to achieve the proper establishment of cell cycle arrest (Narbonne & Roy, 2006). While either *aak-1* or *par-4* single mutants display certain degrees of hyperplasia based on germ cell nuclei count in dauer larvae, the double mutant displays an enhanced, or additive, phenotype, with twice

the amount of germ cell nuclei compared to either single mutant (Figure 5). This data suggests that in the PAR-4/LKB1-dependent regulation of germ cell quiescence during dauer, PAR-4/LKB1 impinges on downstream targets other than AMPK to achieve cell cycle arrest (Narbonne & Roy, 2006).

Research motivation

The goal of this project was to better characterize the role of PAR-4/LKB1 in regulating dauer-dependent germ line quiescence in *C. elegans,* and uncover novel PAR-4/LKB1-dependent mechanisms of tumor suppression. Since genetic analysis of germ line quiescence reveals that PAR-4/LKB1 must impinge on targets other than AMPK to properly induce cell cycle arrest, we sought to isolate AMPK-independent factors and pathways that govern stem cell quiescence downstream of PAR-4/LKB1. Patients afflicted with PJS retain one functioning copy of LKB1 that is sufficient to activate AMPK, suggesting that LKB1 exerts its tumor suppression independently of AMPK in humans as well (Sakamoto et al., 2005). Using dauer-induced germ cell arrest as a model system, we can genetically dissect the role of PAR-4/LKB1 in regulating hyperplasia and consequently, tumorigenesis. Through this effort, we can participate in understanding stem cell quiescence, and better define the AMPK-independent branch of LKB1/PAR-4 signaling that most likely underlies its tumor suppressive role (Figure 5).

A genome-wide RNAi screen was performed in our laboratory to isolate factors, other than PAR-4/LKB1 and AMPK, required for dauer-dependent germ line quiescence (Wendland, 2010). The aim was to gain further insight into the LKB1/AMPK signaling network involved in this process (Wendland, 2010). Surprisingly, several genes involved in the regulation of cell polarity where discovered to be necessary for germ cell quiescence in *C. elegans* (Wendland, 2010). Indeed, PAR-4 was first isolated in *C. elegans* as a key regulator of cell polarity, required for the first asymmetric cell division in the early embryo, a feat achieved irrespective of AMPK (Kemphues et al., 1988; Narbonne et al., 2010).

These findings bring forth the possibility that LKB1/PAR-4 might be involved in regulating a previously undocumented polarity or related process in germ cells to induce quiescence (Narbonne & Roy, 2008; Wendland, 2010).

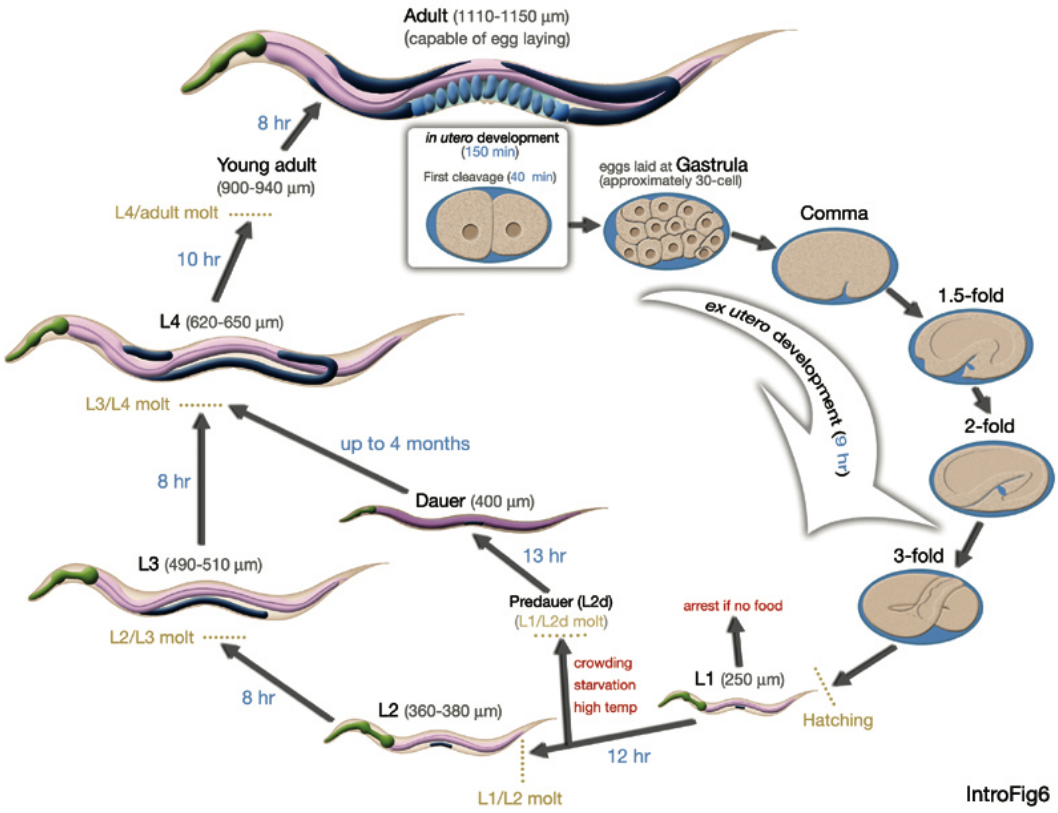

Figure 1 – *C. elegans* life history depends on the environmental conditions

When conditions favor growth and reproductive success, *C. elegans* develop through four larval molts before reaching adulthood. However, if conditions prove suboptimal, an alternative life cycle ensues where L1 larvae enter a developmentally quiescent dauer state instead of L3, and remain so for up to four months, or until conditions improve. During the predauer (L2d) stage, larvae accumulate lipid stores that will sustain them throughout the dormant period. When dauer larvae sense an improving environment, they mold directly into L4. 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.

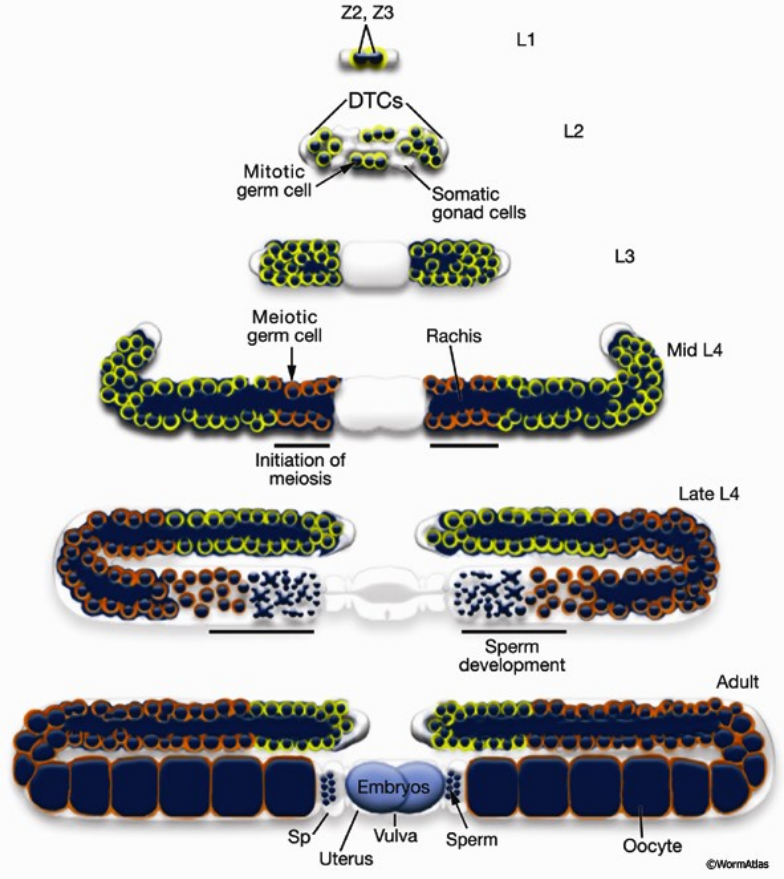

Figure 2 – *C. elegans* germ line development

C. elegans hatch with a gonad primordium that consists of two germline precursor cells (termed Z2 and Z3) that are flanked by two somatic precursor cells (termed Z1 and Z4). Z2 and Z3 proliferate throughout gonad development while inhabiting the distal end of the gonad. Their mitosis is maintained through their interaction with the distal tip cell (DTC) located on either distal extremely, via GLP-1/Notch signaling. As germ cells displace proximally, they experience diminished GLP-1/Notch signaling and differentiate into sperm at first during the L4 stage, then oocytes at adulthood. During the dauer the germline resembles that of L2 larvae, consisting of around 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.

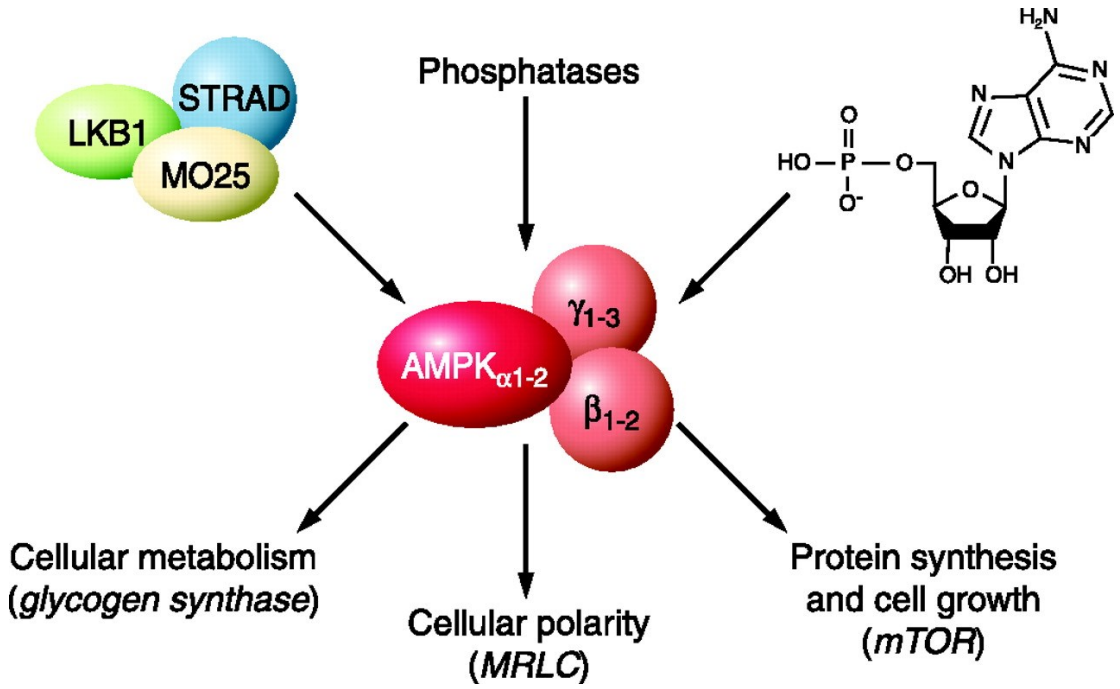

Figure-3 – LKB1 regulates polarization and cell growth via AMPK in an energy-dependent manner

AMPK functions in a heterotrimeric complex, with two regulatory subunits (λ and β) and one catalytic subunit (α). In response to rising AMP levels, AMP binds to the regulatory subunits, conferring an allosteric that enhances LKB1-dependent phosphorylation of AMPK's catalytic subunit. Through its regulation in this manner, LKB1 has been implicated in the regulation of a host of processes such as polarization and cell growth, ensuring their proper regulation in energetically compromised situations. Figure used with permission from Jansen, M., Ten Klooster, J.P., Offerhaus, G.J., Clevers, H., 2009. LKB1 and AMPK family signaling: the intimate link between cell polarity and energy metabolism. Physiological reviews 89, 777-798.

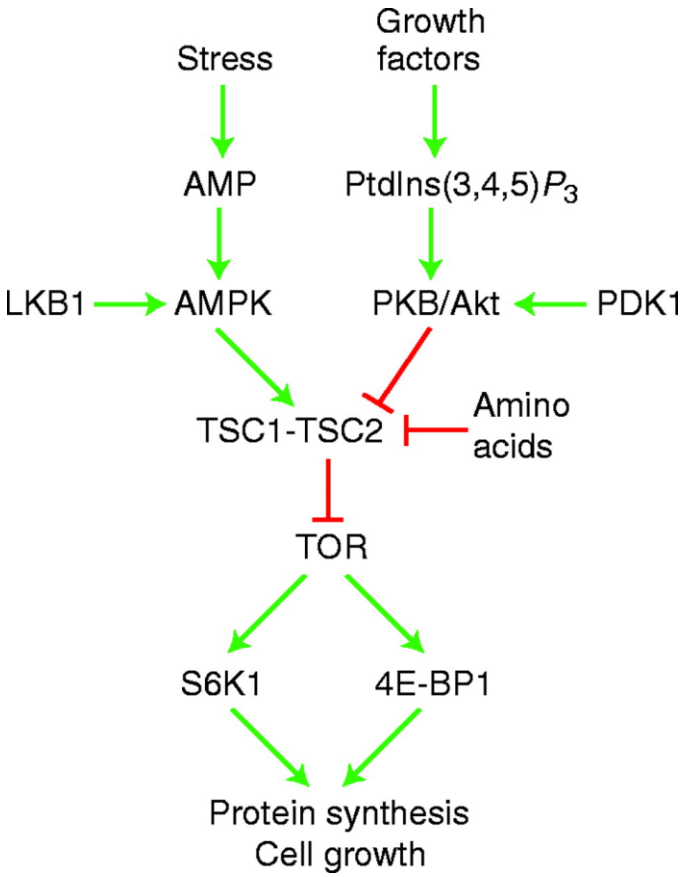

Figure 4 – mTOR model for LKB1-mediated tumor suppression

In response to low energy and LKB1-mediated phosphorylation, activated AMPK has been shown to phosphorylate activate TSC2, a GAP that inhibits TOR through an intermediated stem omitted from this diagram. LKB1's tumor suppressive abilities have traditionally been attributed to its AMPK-dependent ability to counter the mTOR pathway, ensuring its inhibition when there is not sufficient energy to sustain energy-consuming processes such as protein synthesis and cell growth. Figure from Hardie, D.G., 2004. The AMP-activated protein kinase pathway--new players upstream and downstream. Figure used with permission from Hardie, D.G., 2004. The AMP-activated protein kinase pathway--new players upstream and downstream. Journal of cell science 117, 5479-5487.

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(rr48)	96.16±18.06[1] (25)	8.64±1.77[1] (98)
daf-2(e1370); par-4(it57)	148.28±30.90[1] (25)	>15 (60)
daf-2(e1370); par-4(it57); aak-2(rr48)	213.10±38.87[1] (10)	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 5 – AMPK does not fully account for PAR-4/LKB1-mediated germ cell quiescence in *C. elegans*

Since germ cells must sense energy availability and overrule their otherwise programed proliferation during dauer, it is tempting to believe that PAR-4/LKB1 regulates germ cell cycle arrest by impinging on the TOR pathway via AMPK. However, genetic analysis demonstrated in this table reveals that AMPK does not fully account for PAR-4/LKB1-dependent regulation of germ cell cycle arrest. *daf-2 (e1370)* encodes a temperature sensitive allele of the insulin receptor homologue. *daf-2 (e1370)* mutants shifted to the restrictive temperature enter dauer constitutively. *par-4 (it57)* is a temperature sensitive allele of PAR-4 in which its kinase activity is impaired when shifted to the restrictive temperature. Finally, *aak-2 (rr48)* encodes the catalytic subunit of AMPK with a point mutation that impairs catalytic activity. *daf-2 (e1370);par-4 (it57);aak-2 (rr48)* triple mutants contain more germ cells compared to either double mutant, *daf-2 (e1370); par-4 (it57)* and *daf-2 (e1370); aak-2 (rr48)*, suggesting that PAR-4 must be regulating factors in parallel to AMPK. Figure adapted with permission from Narbonne, 2006).

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CHAPTER 2

Global genomic survey for genes that mediate *par-4*-dependent germ line stem cell quiescence in *C. elegans* dauer

Introduction

When faced with harsh environmental conditions, *C. elegans* larvae are able to alter their developmental program and enter the dauer diapause, an alternative developmental stage that enables larvae to endure long periods of starvation and stress. During this arrested phase, the germ line stem cells, which normally divide during reproductive development, halt their proliferation and are consequently rendered quiescent. To investigate the formation of germ cell quiescence, a screen was conducted for dauer-specific, germ line hyperplasia in order to identify genes involved in germ line arrest. The screen identified a mutation in *aak-2,* the homologue of mammalian AMP-activated kinase (AMPK) (Narbonne & Roy, 2006). AMPK is a conserved protein that senses and manages cellular energy levels by inactivating anabolic pathways and stimulating catabolic processes when cellular AMP levels rise (Kahn et al., 2005). Additional experiments revealed that *daf-18* and *par*-4, homologues to the mammalian tumor suppressors PTEN and LKB1, respectively, are also necessary for germ line arrest during the dauer stage (Narbonne & Roy, 2006).

LKB1 is a serine/threonine protein kinase that is misregulated in individuals afflicted with Peutz-Jeghers syndrome, a cancer predisposing disorder (Hemminki et al., 1998; Jenne et al., 1998). One of its best-characterized downstream targets is AMPK (Alessi et al., 2006; Woods et al., 2003). Through its activation of AMPK, LKB1 has been implicated in coordinating the growth, target of rapamycin (TOR) pathway with the metabolic status of the cell, ensuring its inhibition during energetic stress (Hardie, 2005; Woods et al., 2003). When activated by LKB1, AMPK phosphorylates and activates TSC2, a GAP that suppresses TOR signaling through the inhibition of Rheb GTPase (Inoki et al., 2003). It has been suggested that the tumor suppressor function of LKB1 arises from its ability to counter TOR via AMPK, ensuring that cell growth and proliferation occur only during appropriate conditions (Hardie, 2005; Alexander & Walker, 2011) (Figure 4).

Although it is conceivable that LKB1-AMPK could regulate germ line stem cell arrest by antagonizing a potent cell growth pathway such as TOR, there is no known TSC2 homolog in *C. elegans,* suggesting that LKB1 might operate differently when regulating cellular quiescence in the dauer germ line. In fact, it has been demonstrated that *par-4* and *aak-2* cooperate under such nutrient-deficient conditions in order to mediate germ line stem cell quiescence: the knockdown of either gene causes aberrant germ line hyperplasia in dauer larvae, while the inactivation of *par-4* in *aak-2* null mutants causes enhanced or additive hyperplasia, suggesting that AMPK signaling does not fully account for the PAR-4/LKB1-dependent, dauer specific, germ line hyperplasia (Narbonne & Roy, 2006). Therefore, although PAR-4/LKB1 is known to regulate AMPK in *C. elegans* and beyond, genetic analysis suggests that it is unlikely that AMPK is the sole mediator of germ line stem cell quiescence downstream of PAR-4 (Narbonne & Roy, 2006).

That the tumor suppressor function of PAR-4/LKB1's is not exclusively explained by its AMPK-related function is reinforced by additional evidence from mammalian models. First, although hyper-activation of TOR signaling has been associated with PJS, inhibiting TOR via rapamycin in *Lkb1* +/- mice reduces polyp size number, but does not prevent polyp formation altogether, suggesting that the misregulation of TOR is a symptom, but not the underlying cause of the LKB1 aetiology in PJS (Wei et al., 2008; Wendland, 2010). Second, humans with PJS only lack one allele of the LKB1 gene, and the remaining wild-type copy is sufficient to normally regulate most processes downstream of AMPK (Sakamoto et al., 2005). Together with the genetic analysis above, these observations suggest that another molecular function that is AMPK-independent lies at the root of the tumorous growths that arise as a result of LKB1 compromise.

The loss of polarity is intimately linked with tumorigenesis and cancer (Wodarz & Nathke, 2007). PAR-4 has a well-documented role in regulating cell polarity that could reasonably contribute to *par-4*-dependent germ line cell cycle arrest. The role of PAR-4 in regulating cell polarity was discovered in *C. elegans,*

where it was isolated as a factor required for the first asymmetric cell division in the early embryo (Kemphues et al., 1988). In *Drosophila melanogaster*, PAR-4/LKB1 is required to polarize epithelial follicle cells in the oocyte, the disruption of which leads to uncontrolled cell proliferation (Martin & St Johnston, 2003). In mammals, activation of LKB1 in a non-polarized cell line induces the reorganization the actin cytoskeleton and relocalizes junctional protein, suggesting that LKB1 regulates cell polarity in the mammalian system as well (Baas et al., 2004). Importantly, PAR-4/LKB1-dependent polarity is mediated, in part, by AMPK-related kinases such as PAR-1/MARK and SAD/BRSK, conserved kinases that regulate polarity in various systems (Alessi et al., 2006). Perhaps, *par-4*-mediated polarity via such kinases is important in establishing quiescence.

In order to identify PAR-4/LKB1-dependent pathways that are required for cell cycle arrest during dauer, we opted for an unbiased, reverse-genetic approach and conducted a genome-wide, feeding-based RNA interference (RNAi) screen. We searched for genes whose depletion via RNAi rescued the germ line hyperplasia observed in *par-4* dauer larvae, suggesting that these genes' expression is misregulated in the absence of PAR-4 and that in under normal conditions, PAR-4 downregulates their activity in response to dauer.

RNAi analysis involves double-stranded RNA molecules (dsRNA) that trigger sequence-specific degradation of target mRNA transcripts when introduced to cells (Fire et al., 1998; Hammond, 2005; Mello & Conte, 2004; Zamore et al., 2000). Endogenous or exogenously introduced dsRNA molecules cause the RNAi machinery, (which includes Dicer and the RNA induced silencing complex, RISC) to mobilize and knockdown the mRNA targets of dsRNA in a step-by-step, sequence specific manner (Bass, 2000; Bernstein et al., 2001; Hammond, 2005; Hammond et al., 2000; Mello & Conte, 2004; Pham et al., 2004). First, Dicer cleaves dsRNAs into 21-23 nucleotide-long small interfering RNAs (siRNA), which associate with RISC and guide it to the complementary regions of target mRNAs. Then mRNAs are cleaved by RISC, causing a reduction in corresponding

protein levels. (Bass, 2000; Bernstein et al., 2001; Hammond, 2005; Hammond et al., 2000; Mello & Conte, 2004; Pham et al., 2004; Zamore et al., 2000). An RNA-dependent RNA polymerase (RdRP) is important for amplifying the RNAi effect by synthesizing new dsRNA from the siRNAs generated by Dicer (Grishok, 2005; Sijen et al., 2001). RNAi is an effective mode of gene analysis, especially in *C. elegans*, where it is administered fairly easily and results in few off-target effects as compared to other systems (Fire et al., 1998; Kamath et al., 2003; Mohr & Perrimon, 2012). Moreover, an advantage of screening through RNAi is that the identities of positive candidates are known directly (Kamath & Ahringer, 2003; Kamath et al., 2003).

A global RNAi approach to gene analysis, as opposed to targeted, was chosen for this study as it is unbiased and allows for the discovery of genes, or classes of genes, that may not have been predicted to play a role in our process of interest based on the literature beforehand. A genomic survey permits the functional categorization of genes based on their Gene Ontology (GO), yielding a holistic view of the classes of genes that are regulated by PAR-4 during the establishment of dauer-induced germ line quiescence. By using the feeding method for RNAi induction and a bacterial library containing 86% of the 20,000 or so genes in *C. elegans*, analyzing the whole genome in a timely fashion was feasible (Kamath & Ahringer, 2003; Kamath et al., 2003).

We identified a total of 50 genes whose inactivation was found to rescue the germ line hyperplasia observed in *par-4* dauer larvae, suggesting that their expression is misregulated in the absence of PAR-4. Future endeavors include the characterization of key candidates, many of which impinge on the actin cytoskeleton and its regulation. Further understanding of the function of these genes will provide additional insight as to how PAR-4/LKB1 blocks tumorous growth by regulating cell cycle quiescence.

Materials and Methods

Strains and maintenance

The strains used for this screen include the MR0673 strain *[daf-2 (e1370) III; par-4 (it-57); qIs56 (lag-2::GFP) V];* the MR0863 strain *[rrf1 (pk1417) I; daf-2 (e1370) III; par-4 (it-57) V];* and the MR0672 strain *[daf-7 (e1372) III; par-4 (it57) V]*. All strains were maintained at 15°C as described elsewhere, and grown on normal NGM plates seeded with OP50 *E. coli,* unless otherwise indicated (Brenner, 1974)

Screen strategy and analysis of hyperplasia

The genome-wide RNAi feeding screen was performed on the MR0673 *C. elegans* strain, the genotype of which contains a temperature sensitive *par-4 (it57)* mutation affecting its kinase domain once shifted to the restrictive temperature. This temperature sensitive mutation allowed us to evaluate the loss-of-function phenotype of *par-4* during the dauer stage since *par-4* is embryonic lethal. This strain also contains a temperature sensitive Daf-c gene, *daf-2 (e1370)*, which allowed us to induce dauer by shifting larvae to the restrictive temperature. Importantly, this strain carries a *lag-2::GFP* transgene that is co-expressed in the DTCs, marking the distal ends of the germ line. It allowed us to indirectly evaluate the degree of germ line stem cell hyperplasia, where a positive result caused both distally-*lag-2::GFP*-expressing DTCs to shift proximally.

Once the gene candidates where identified through genome-wide screening, they were further tested in two alternative strains, MR0863 and MR0672. Both of these strains lack the *lag-2::GFP* transgene. Hyperplasia in these strains was thus evaluated differently: the gonads of RNAi treated individuals were observed directly through DIC microscopy and individual germ cells counted.

RNAi library

MR0673 larvae were provided with bacterial food containing dsRNA corresponding to specific genes. The RNAi feeding library used was generated by the Ahringer laboratory and contains 86% of the *C. elegans* genome. Bacterial clones are cultured in glycerol, presented in a 384-well format, which we expanded into a 96-well format. Bacterial clones from the library were cultured in separate 96-well plates using a multi-pipette. During this study, 826 of the total available clones could not be cultured for screening due to growth difficulties. Sequence verified RNAi clones include: *cyk-4, pac-1, cdk-1, lam-2, fbl-1, vab-10, let-805, arf-1.1, arf-3, alg-1, div*-1 (Kamath & Ahringer, 2003; Kamath et al., 2003).

Genome-wide RNAi screening protocol

Bacterial clones expressing target-gene dsRNA were transferred from the RNAi library and cultured in LB medium containing ampicillin at 37°C overnight. The bacteria were then seeded onto 12-well plates containing NGM made with ampicillin (0.5ml/L) and IPTG (1ml/L). Seeded plates were incubated at room temperature overnight to induce dsRNA expression. Meanwhile, a population of the MR0673 strain was synchronized and the resulting L1s were incubated at the restrictive temperature (25°C) for 24 hours in order to inactivate PAR-4. The ensuing PAR-4-deficient L1 larvae were added to each RNAi well, and incubated at 25°C for 48 hours in order to induce dauer formation. Following this 48-hour window during which dsRNA is expressed and can initiate an RNAi response to corresponding genes. Worms were subsequently examined for reduced hyperplasia based on the proximal displacement of DTCs.

DIC microscopy

MR0863 and MR0672 dauer larvae were removed from the RNAi wells 48 hours after being added, and mounted on 2% agarose pads covered with 2 ml of levamisole, a paralyzing agent. They were covered with a coverslip and gonads were observed for reduced gonad size, or hyperplasia, using a Zeiss Imager.21 microscope outfitted with DIC optics.

Results

Isolation of genes whose RNAi depletion rescues *par-4*-dependent hyperplasia

Of the 18,432 RNAi bacterial clones available in the RNAi library, a total of 17,606 genes were screened, as 826 of the total number of clones available could not be cultured for screening. Through the genome-wide, primary RNAi screen, we identified a total of 1,812 genes whose loss-of-function via RNAi was found to rescue the germ line hyperplasia observed in *par-4* dauer larvae, suggesting that their expression is misregulated in the absence of PAR-4. Through secondary and tertiary screening of this subset, we narrowed this list down to 50 candidates whose RNAi-mediated depletion reproducibly rescued *par-4*-dependent germ line hyperplasia (Table 1). Of this subset, 45 have known functions and were categorized based on Gene Ontology (GO) (Table 2). These genes where categorized based on their functional description on WormBase (Figure 6).

Primary screen

1,812 primary gene candidates for which RNAi causes reduced dauer specific, *par-4*-dependent germ line hyperplasia represent an excessively high number and possibly reflects an oversight in the method, which was only corrected mid-way through the screen. For nearly half the amount of genes screened, our *C. elegans* strain containing a temperature sensitive form of *par-4* was not incubated at 25°C prior to RNAi feeding, suggesting that these worms still expressed PAR-4 upon being fed target-gene dsRNA. Although they were subsequently incubated at 25°C for 48 hours to induce dauer formation, PAR-4 may not have been depleted in a timely fashion, and may have been active during the pre-dauer stage, inducing germ line quiescence. Thus, the high number of genes likely reflects such instances where PAR-4 itself was not entirely depleted.

Secondary/tertiary screen and control

During the secondary and tertiary screen, a control was used in order to ensure that the reduced hyperplasia phenotype was specifically due to the RNAi knockdown of target genes and not observed in wild-type situations. As a negative control, MR0673 L1 larvae were fed an RNAi bacterial strain containing the empty L4440 expression vector, which lacks the gene-specific DNA-fragment (Kamath & Ahringer, 2003). Approximately 1% of *par-4* dauers reverted to wild type and displayed reduced DTC displacement in the controlled setting. Genes whose RNAi-mediated inactivation resulted in comparable proportions of rescued larvae were discounted from further study, representing situations where reduced hyperplasia was likely an outcome of something other than targeted gene depletion, as it was with the control RNAi vector. Through this process of elimination, the primary gene list was reduced to 50 confirmed candidates, which correspond to RNAi clones that consistently caused $\geq$ 5% of treated larvae to revert back to DTC displacement more typical of wild-type dauers (Table 1, Table 2).

RNAi candidates that validate the screening strategy

Of the 50 gene loci isolated from the screen, there are several that validate the screening strategy. One such candidate is CDK-1, a cyclin dependent kinase specifically responsible for promoting G2/M phase cell cycle progression (Boxem, 2006). Of all the cell cycle phases, it is important that CDK-1 is involved in promoting M phase entry since this is the cell cycle point at which germ cells arrest specifically during diapause. The isolation of CDK-1 serves as a positive control, demonstrating that by feeding L1 larvae bacterial strains expressing dsRNA, dauer-specific germ line hyperplasia that occurs shortly thereafter can be affected during the window of time available.

The RhoGTPase activating proteins (RhoGAP), CYK-4 and PAC-1, also inspire confidence in the results obtained from the screen. RhoGTPases are signaling molecules that cycle between an active GTP-bound form and inactive GDP bound form. RhoGAPs inactivate RhoGTPases by promoting the hydrolysis of GTP that is bound to RhoGTPases (Lundquist, 2006). The isolation of RhoGAPs is key because it reciprocates the results obtained from an opposite screen conducted in our laboratory, which aimed at identifying factors that phenocopy *par-4*-dependent hyperplasia (Wendland, 2010). The RhoGTPases, RHO-1 and CDC-42, were isolated in this previous screen, as factors required for germ line quiescence (Wendland, 2010). The identification of RhoGAPs as suppressors of hyperplasia indicates that the present screen is in agreement with the reverse study, further validating the screen.

RNAi penetrance and expressivity

Penetrance refers to the proportion of RNAi-treated individuals that display the desired phenotype, while expressivity refers to the phenotypic variance observed with each RNAi clone (Wang et al., 2005). The penetrance and expressivity of the 50 confirmed gene candidates is catalogued in Table 3. In terms of penetrance, 'low', 'medium', and 'high' penetrance where assigned to candidates whose depletion caused approximately 5-10, 11-49, and 50 + percent of tested larvae to display reduced DTC displacement, respectively. In terms of expressivity, candidate-gene RNAi resulted in either full or partial suppression of *par-4*-dependent hyperplasia, where DTC displacement either reverted to distances more typical of wild-type dauers, or was partially reduced compared to wild-type dauers, respectively. In some cases, candidate-gene RNAi affected DTC displacement altogether, where the DTCs of RNAi-treated dauers were more proximal than wild type, suggesting that germ cell divisions were inhibited entirely.

Additional RNAi phenotypes observed in conjunction with reduced hyperplasia include the dumpy (Dpy) phenotype. The genes for which RNAi occasionally caused this phenotype include those involved in the regulation of basement membrane integrity in *C. elegans*, (such as *fbl-1, lam-2,* and, *noah-1*), as well as genes with known roles in morphogenesis and molting (such as *mlt-8*). It seems likely that such genes would cause this effect since they are necessary for maintenance of body morphology and structure (Frand et al., 2005; Kramer, 2005).

RNAi targets with ambiguous phenotypes

During the secondary and tertiary screening process, the RNAi-mediated inactivation of several genes produced an effect that could neither be catalogued as true suppressions, nor ignored. The RNAi phenotypes of 51 loci were identified as ambiguous, and are listed in Table 4. Two major circumstances led to the classification of this subset as ambiguous. The first was when the RNAi treatment had very low penetrance, affecting less than 1% of treated larvae. Though reminiscent of what was occasionally observed when animals were fed control-RNAi, these gene loci were re-tested, and only those targets that reproducibly caused this weak phenotype were considered ambiguous, while the rest were discounted from further investigation. While the penetrance of these RNAi targets is very low, the fact that it is consistent compared to the control might suggest a real suppression.

The second scenario that prompted the classification of an RNAi target as ambiguous was when a large portion of treated larvae did not enter dauer, though the ones that did displayed reduced hyperplasia. One interpretation is that the observed suppression in larvae that entered dauer is a result of other defects caused by RNAi that occurred prior to dauer entry, resulting in secondary and indirect defects in germ cell proliferation upon dauer formation. Another interpretation is that such genes are playing a direct role in regulating dauer germ line quiescence, but also affect some earlier event that interferes with proper dauer development.

Because neither possibility could be ruled out without further analysis, RNAi targets that resulted in this phenotype were classified as ambiguous (Table 4). During the screen, there were instances of the opposite nature, where animals failed to enter dauer after RNAi, and the few that did displayed pronounced hyperplasia instead, highlighting the specificity of the latter case.

RNAi candidates with non-dauer specific phenotypes

Following the isolation of the 50 genes whose inactivation resulted in reduced hyperplasia, we examined whether their role in regulating germ line proliferation was dauer-specific and *par-4*-dependent. The 50 loci were screened for their effect on germ cell proliferation during the L3 stage and in the presence of PAR-4. After synchronization, L1 MR0673 hatchlings were subjected to RNAi without being incubated at the restrictive temperature beforehand in order to conserve PAR-4 function. Larvae were subsequently maintained at the permissive temperature for 48 hours, and the ensuing L3 larvae were examined for reduced DTC displacement using DIC optics. The disruption of 18 gene candidates resulted in reduced germ cell proliferation during the L3 stage, suggesting that this subset promotes cell cycle progression independently of dauer and PAR-4 (Table 5). Many of these genes were found to have a housekeeping function, regulating fundamental processes, such as ATP metabolism and protein processing. It is not surprising that the disruption of such genes would affect the cell cycle, regardless of the germ line's developmental context. The implication of these genes in broader developmental contexts should not disqualify them from further investigation since they may still represent direct or indirect PAR-4 targets in the arrested germ line.

RNAi candidates with germ line autonomous phenotypes

To determine whether the isolated 50 gene loci functioned in a germ line autonomous fashion, they were screened in the MR0863 strain, which contained a loss-of-function mutation in *rrf-1 (pk1417)*. *rrf-1* encodes one of the RNA-

dependent RNAi polymerases in *C. elegans* that acts in a tissue-specific manner where it is required for the amplification of dsRNA in somatic tissues (Grishok, 2005; Sijen et al., 2001). Mutations in *rrf-1* are thought to result in germ line-specific RNAi processing since RNAi is presumably defective in the soma (Grishok, 2005; Sijen et al., 2001). The depletion of 20 of the 50 gene candidates resulted in reduced hyperplasia in MR0863 dauers, suggesting that this subset functions in the germ line (Table 6). Though largely a useful approach, the gene subset identified as germ line autonomous should not be over-interpreted. Since MR0863 larvae lack the *lag-2::GFP* transgene and were analyzed for hyperplasia using DIC optics (see Materials and Method), only 10-20 larvae were observed for hyperplasia per RNAi well, compared to the 100 or so observed when analyzing DTC displacement. It is possible that some candidates could have been overlooked in terms of their germ line-autonomy, given their low RNAi penetrance. Moreover, it has been demonstrated that *rrf-1(pk1417)* mutants are still capable of processing RNAi in the soma (Kumsta & Hansen, 2012), suggesting that some of the gene targets catalogued as germ line autonomous in Table 6 could have in fact been targeted for depletion in somatic tissue, where their function might be important for the regulation of germ line quiescence.

Discussion

Genes encoding components of the extracellular matrix

Many gene loci for which RNAi resulted in reduced DTC displacement in *par-4* dauers include proteins that are involved with various aspects of the extracellular matrix (ECM)—either encoding factors that constitute the ECM, or factors that couple the cell's cytoskeletal network to the overlying matrix (Table 2). The ECM is an adhesive substrate composed of insoluble macromolecules that surround metazoan cells, regulating different aspects of their behavior, including growth, cell proliferation, differentiation, adhesion, and migration. The basement membrane is a specialized matrix, which specifically surrounds epithelial and endothelial cells (Kramer, 2005; Yurchenco, 2011). In *C. elegans,* basement membranes surround the body wall muscles, pharynx, intestine, and gonad (Huang et al., 2003; Kao et al., 2006; Kramer, 2005). The matrix is a major aspect of the stem cell niche, regulating stem cell behavior *in vitro* and *in vivo* (Eshghi & Schaffer, 2008; Guilak et al., 2009). This class of genes is interesting since matrix-based control of germ cells remains largely uncharacterized in *C. elegans*.

One candidate that encodes a basement membrane constituent is *lam-2,* the γ subunit of laminin. Laminin is a conserved heterotrimeric glycoprotein which consists of three subunits: α, β, and γ. There are two α subunits (αA and αB), one β subunit, and one γ subunit in *C. elegans*, which form one of two laminin isoforms, laminin αA and laminin αB. Because there is only one γ subunit, *lam-2* is necessary for the proper function of either isoform (Huang et al., 2003; Kramer, 2005). Laminin is secreted by cells, upon which it subsequently binds through integrin and dystroglycan receptors (Rasmussen et al., 2012). At the cell surface, laminin polymerizes and acts as a scaffold for the assembly of other secreted basement membrane factors, while also affecting cytoskeletal arrangement within respective cells. Laminin is thus the precursor to basement membrane assembly and matrix-induced intracellular modifications (Kramer, 2005; Rasmussen et al., 2012; Yurchenco, 2011).

Laminin plays various essential roles throughout *C. elegans* development, some of which may provide clues into its role in germ cell proliferation during dauer. *C. elegans* lacking laminin arrest development at the embryonic and early larval stage, or develop into sterile adults with basement membrane defects that affect proper adhesion across tissues (Huang et al., 2003; Kao et al., 2006). Because of improper cell-basement membrane adhesion, the gonad is severely affected in *C. elegans* laminin mutants. The somatic gonad (DTCs, sheath cells, and spermatheca) is normally surrounded by a basement membrane, as are germ cells, which are enclosed in a basement membrane upon which sheath cells then reside. As the gonad morphs into its adult form, sheath cells migrate distally on the basement membrane and encapsulate the germ cells (Hall et al., 1999). In laminin mutants, the basement membrane is compromised, leading to aberrant sheath cell (and DTC) migration, unsheathed stem cells, and the invasion of proliferative germ cells into neighboring tissue (Huang et al., 2003; Kao et al., 2006).

One possibility is that the reduced DTC displacement observed in *lam-2* RNAi-fed *par-4* dauer larvae is a consequence of reduced DTC migration rather than rescued germ cell quiescence. In this scenario, mitotic germ cells would continue to accumulate within their non-expanding niche and ultimately 'escape' into neighboring tissue because of the now frail basement membrane. In order to determine if this is the case, it will be important to image *lam-2; par-4* dauer larvae with DIC microscopy and verify whether germ cells escape and divide outside their niche. This finding would discount *lam-2* as a candidate suppressor gene of *par-4*-mediated hyperplasia during the dauer phase, and instead classify it as a gene required for gonad tissue integrity. However, should the dauer germ line of *lam-2; par-4* mutants look normal, this would indicate *lam-2* as an interesting player in the dauer-dependent regulation of germ cell quiescence, downstream of PAR-4. It would suggest that its dauer-dependent, gonad-related role differs from its function outside of dauer.

How might laminin effect germ cell proliferation in *par-4* dauer larvae? One alternative scenario is that the adherence of germ cells to an overlying matrix becomes necessary to sustain their proliferation during dauer hyperplasia. In the absence of PAR-4 and LAM-2, this basement substrate which germ cells must potentially bind to proliferate would thus be perturbed, leading to cell cycle arrest. PAR-4 could influence this process by inhibiting (directly or indirectly) the production and secretion of laminin and/or other basement membrane components in order to jeopardize this contact and ensure proper cell cycle arrest. An influence of the ECM on cell survival and proliferation has been proposed by many studies. Alterations and remodeling of the ECM is an important event during tumorgensis where cell-matrix interactions are modulated, altering cell behavior (Erler & Weaver, 2009; Gao et al., 2010; Kim et al., 2012; Ravichandran et al., 2009). Given the high number of basement membrane genes isolated from the present screen (12%), it is possible that germ line stem cells depend on interactions with the surrounding basement membrane for maintenance, anchoring, and proliferation during hyperplasia.

LKB1 has been shown to negatively impinge on the structural and molecular integrity of the ECM (Carretero et al., 2010; Gao et al., 2010). Lysyl oxidase (LOX) is an enzyme that oxidizes lysine residues in collagen and other ECM components, resulting in their stabilization. Aberrant LOX expression is linked to tumorous growths. LOX has been identified as a target negatively regulated by LKB1 *in vivo*, suggesting that LKB1 alters aspects of the ECM as part of its general role of tumor suppression in at least some cases (Gao et al., 2010). Moreover, the ECM has specifically been implicated in the regulation of stem cell behavior and proliferation through studies in mammals and invertebrates. Human mesenchymal stem cells (MSC) have been shown to enter quiescence when grown on a soft (as opposed to a stiff/elaborate) matrix, suggesting an influence of ECM elasticity on stem cell behavior *in vitro* (Winer et al., 2009). In addition, one population of ovarian stem cells in *Drosophila melanogaster* has been shown to rely on integrin-laminin associations for proliferation control (O'Reilly et al., 2008).

It is thus imaginable that ECM-based modulations and interactions function during hyperplasia, in the absence of PAR-4, to give rise to germ cell over-proliferation.

Interestingly, a relationship between GLP-1, germ cell mitosis, and the basement membrane has previously been documented in *C. elegans.* Mutations in genes involved in matrix integrity have been shown to rescue *glp-1* mutants, suggesting that basement membrane genes influence germ cell fate and behavior at least to some extent in non-dauer *C. elegans* (Maine & Kimble, 1989, 1993; Nishiwaki & Miwa, 1998). Like in non-dauer larvae, dauer-dependent hyperplasia is regulated in a GLP-1 dependent manner (Narbonne & Roy, 2006). One of the gene candidates isolated in the present screen is indeed *glp-1* (Table 1) suggesting that this signaling pathway may be mis-regulated in the absence of PAR-4, and that GLP-1 must be downregulated in order to establish dauer-dependent mitotic arrest. Germ cells likely progress through meiosis in this scenario, but their mitotic division is no longer possible without GLP-1 (Narbonne & Roy, 2006). It is possible, therefore, that the germ cell adhesion to the basement membrane affects GLP-1 activity during dauer (Maine & Kimble, 1993). One proposition that has been made is the EGF-like repeats on the extracellular domain of GLP-1 could interact with components of the basement membrane directly (Maine & Kimble, 1993), influencing its activation thereafter.

In addition to *lam-2,* another interesting ECM gene of interest is *fbl-1*, a member of the fibulin family of ECM proteins (Kramer, 2005). There are two isoforms of FBL-1, both of which are expressed in intestinal cells and localized to the gonadal basement membrane where they assemble (Kubota et al., 2004; Muriel et al., 2005; Wong & Schwarzbauer, 2012). FBL-1 is important for basement membrane integrity in the gonad (like laminin), and the initiation of DTC migration (Hesselson et al., 2004; Kubota et al., 2004; Kubota et al., 2012; Wong & Schwarzbauer, 2012). FBL-1 is a required basement membrane component without which DTCs cannot begin to migrate out (Blelloch et al., 1999; Blelloch & Kimble, 1999; Wong & Schwarzbauer, 2012). *vab-10,* another interesting candidate gene,

has also been shown to be specifically required for proper DTC migration (Kim et al., 2011). VAB-10 is a spectraplakin, a cytoplasmic protein with multiple cellular functions (Brown, 2008; Kim et al., 2011; Roper et al., 2002). Some isoforms are prominent components of the hemidesmosome adhesion complex that tethers intermediate filaments to the ECM, while others crosslink actin, microtubules (MT), and intermediate filaments in the cytoplasm to induce various cellular changes (Bosher et al., 2003; Kim et al., 2011). A previously unidentified isoform of VAB-10, VAB-10B1, has recently been shown to express in the DTCs, where it organizes the cytoskeleton by crosslinking actin and MTs at the leading edge to induce cell protrusions and migration (Kim et al., 2011).

The isolation of DTC migration genes, *fbl-1* and *vab-10*, raises questions regarding stem cell-niche interactions, namely the potential affect of mechanical stress on germ cell behavior and proliferation. During dauer formation, the DTC migration program is likely downregulated. However, in response to germ cell divisions during hyperplasia, the DTC migration program is somehow upregulated, suggesting that DTCs 'sense' germ cell proliferation and act accordingly by migrating proximally and expanding the niche. An interesting hypothesis is that germ cells also sense whether migration is in progress, and adapt their proliferative behavior to some extent. One way in which germ cells could potentially sense migration is through mechanical signals generated by distally-directed DTC migration. Such signals, mechanical in nature, could act in parallel to GLP-1. These signals could alter the cytoskeleton and be translated into biochemical cues that inform stem cells that their niche is growing, and contribute to their regulation of proliferation

Gene candidates that support this hypothesis are *vab-10* and *let-805*. These two factors are components of the hemidesmosome in *C. elegans* (Bosher et al., 2003; Zahreddine et al., 2010). In addition to linking cellular intermediate filaments to the ECM and stabilizing cell-matrix adhesion, hemidesmosomes have recently been implicated in mediating mechanotransductions in *C. elegans* (Borradori &

Sonnenberg, 1999; Labouesse, 2012; Zhang et al., 2011). Specifically, it has been demonstrated that hemidesmosome in the epidermis are remodeled during body-wall muscle contraction; they thus transmit muscle contraction to the epidermis and cuticle for coordinated movement and proper elongation during embryogenesis (Labouesse, 2012; Zhang et al., 2011). It will be interesting to stain for hemidesmosome components in the germ line in order to determine if such complexes are present, possibly at the interface between germ cells and the overlying DTC or basement membrane. *vab-10* was in fact isolated as a germ line autonomous candidate, which supports this hypothesis. PAR-4/LKB1 has no documented role in regulating hemidesmosomes and it would also be interesting to determine whether it affects the expression and/or assembly of this structure.

The link between physical forces and stem cell behavior has emerged from many studies in mammalian stem cell culture (Guilak et al., 2009). In at least one study concerning human embryonic stem cells, it has been shown that mechanical strain was able to inhibit differentiation while promoting self-renewal (Saha et al., 2006). Although the mechanisms governing how these signals are transduced remain poorly understood (Guilak et al., 2009), these findings illustrate that stem cells are exposed to mechanical stimuli that affect their differentiation and proliferation, a phenomenon that may extend to the *C. elegans* germ line.

Cytoskeletal and polarity genes

A number of genes isolated from the present screen also have documented roles in cytoskeleton regulation and cell polarity (Table 2). This gene subset is interesting given that the cytoskeletal profile of mitotic germ cells in *C. elegans* remains elusive, while germ cell polarity has not yet been defined. Of particular interest are *cyk-4* and *pac-1,* two Rho-GAPs that regulate cytoskeletal dynamics by deactivating Rho-GTPases (Lundquist, 2006). Since Rho-GTPases are required for germ line quiescence during the dauer stage (Wendland, 2010), the discovery that their inactivation rescues hyperplasia strongly suggest that a mechanism involving

Rho-family of GTPases is required in the germ line during dauer. Below are analyses of these genes' known functions, and possible questions that can be addressed in aims of characterizing their role downstream of PAR-4 in the dauer germ line.

The RhoGAP CYK-4 is a conserved component of centralspindlin, a complex that lies at the heart of central spindle assembly and cytokinesis in metazoans (Glotzer, 2009; Mishima et al., 2002; White & Glotzer, 2012). During anaphase, the mitotic spindle reorganizes and assembles centrally between the segregating chromosomes (Glotzer, 2009; White & Glotzer, 2012). The central spindle dictates the position of the cleavage furrow and plays an important role in cytokinesis (Bringmann & Hyman, 2005; White & Glotzer, 2012). Central spindle assembly is made possible through an array of microtubule-associated factors, which includes the centralspindlin complex (Glotzer, 2009; Mishima et al., 2002; White & Glotzer, 2012). Centralspindlin accumulates in the region of central spindle formation where it first promotes microtubule bundling, (Mishima et al., 2002), and then induces the activation of RHO-1 required for the contractile ring (Kamijo et al., 2006; Miller & Bement, 2009; Nishimura & Yonemura, 2006; White & Glotzer, 2012; Yuce et al., 2005). Since CYK-4 is essentially a RhoGAP, it achieves this by binding the RhoGEF ECT-2 and relieving it from autoinhibition (Kim et al., 2005; White & Glotzer, 2012).

Due to its role in cytokinesis, RNAi-mediated suppression of *cyk-4* may have resulted in reduced hyperplasia in *par-4* dauer larvae because of germ cells failing to separate. Germ cells normally arrest before M phase (G2/M) during dauer, suggesting that PAR-4 is required around this time to induce cell quiescence (Narbonne & Roy, 2006). Should RNAi of *cyk-4* rescue hyperplasia by impinging on cytokinesis, this would indicate that CYK-4 is an unlikely target downstream of PAR-4. It will be key to examine the germ cell nuclei in these genetically compromised larvae in order to determine the stage of their germ cell cycle arrest.

Importantly however, centralspindlin has demonstrated roles in other biological processes that are unrelated to cytokinesis. Since CYK-4 is a central component of this complex, it is important to consider these alternative roles when interpreting *cyk-4* mutant phenotypes. One non-cytokinetic role of centralspindlin is its regulation of transcription (Lyberopoulou et al., 2007; White & Glotzer, 2012). The mammalian homologue of *cyk-4,* MgcRacGAP, has been shown to interact with the transcription factor hypoxia inducible factor 1 (HIF-1) (Lyberopoulou et al., 2007). HIF-1 regulates the expression of different classes of genes that help cells adapt to various stress (Lyberopoulou et al., 2007; Semenza, 2002). Though this interaction has yet to be investigated in *C. elegans,* it is relevant since the germ-cell-response to stress-induced dauer is essentially quiescence, and it would be interesting to investigate whether HIF-1 were involved in this response. Given the high number of cytoskeletal genes isolated in the screen, it could potentially represent one way in which these actin-associated factors may affect dauer-dependent stem cell quiescence (Lyberopoulou et al., 2007).

Another *cyk-4*-mediated function outside of cytokinesis is its recently demonstrated role in maintaining germ line architecture in *C. elegans* (Zhou et al., 2013). Germ line mitosis is incomplete, resulting in cup-shaped germ cells that open to a common cytoplasm called the rachis (Hall et al., 1999; Maddox et al., 2005; Zhou et al., 2013). Intercellular bridges (or rachis bridges) are formed and stabilized along the distal gonad arm, between the individual germ cells and the rachis, until the loop region where cells cellularize and expand in volume to form oocytes. Molecularly, rachis bridges resemble the mitotic cleavage furrow and actomyosin ring, retaining many of the factors found in these structures (Hime et al., 1996; Zhou et al., 2013). In a recent study, centralspindlin has been revealed to localize to the rachis bridge and regulate microtubule bundling at this site in order to stabilize the bridge and maintaining gonad integrity (Zhou et al., 2013).

This centralspindlin-dependent role in the rachis bridge is distinct from its role during cytokinesis (Zhou et al., 2013). While centralspindlin remains important

for microtubule bundling in both scenarios, it does not regulate the localization of actomyosin ring components to this rachis-based location, nor promote the contraction of the actomyosin ring as it does during cytokinesis (Zhou et al., 2013). In fact, it is postulated that a regulatory mechanism *inhibiting* contraction of actomyosin ring at the rachis bridge is required for stabilizing this structure during development. In other words, this actomyosin ring at the rachis bridge exists in a relaxed, or stabilized, state during normal germ line development, enlarging (rather than contracting) around the loop region as meiotic germ cells mature so as to facilitate the influx of cytoplasm into developing oocytes (Zhou et al., 2013). One possibility is that the contractility of the rachis-actomyosin ring is modulated in response to dauer, affecting germ cell function and proliferation in this manner.

An RNAi target that attests to this possibility is *mel-11,* a target gene classified as ambiguous due to low penetrance. It encodes a myosin phosphatase in charge of inhibiting contraction by de-phosphorylating non-muscle myosin light chain (MLC-4) that is required for contraction (Gallagher et al., 1997; Karess et al., 1991; Piekny & Mains, 2002). It has been implicated in the negative regulation of contraction during *C. elegans* development (Piekny & Mains, 2002; Piekny et al., 2000; Wissmann et al., 1999; Wissmann et al., 1997). In the adult germ line, it is epistatic to *cyk-4* and implicated in regulating oocyte cellularization (Piekny & Mains, 2002). It would be interesting to examine the *mel-11* localization pattern in the dauer germ line and determine whether it localizes to the rachis bridge. Moreover, it could also be interesting to determine whether its expression or activation (phosphorylation) is *par-4*-dependent.

ANI-2 is a cytoskeletal binding protein that localizes to the rachis bridge, where it is required for the recruitment of CYK-4 and rachis bridge integrity (Maddox et al., 2005; Zhou et al., 2013). Interestingly, ANI-2 has been shown to inhibit actin contraction by antagonizing non-muscle myosin heavy chain (NMY-2), required for contraction, in the early embryo (Chartier et al., 2011). ANI-2 is downregulated in a *par-4*-dependent manner to enable the contraction of

actomyosin at the cell cortex (Chartier et al., 2011). Though ANI-2 was not isolated as a gene candidate in this screen, its role in the dauer germ line rachis would be interesting to explore since it not only regulates the localization of CYK-4 to the rachis, but has also been implicated in a *par-4*-dependent pathway. (Chartier et al., 2011; Zhou et al., 2013). Based on its antagonistic relationship to NMY-2 in the embryo (Chartier et al., 2011), it is imaginable that the relaxed, stable state of the actomyosin ring at the rachis is attributed to ANI-2's presence there, along with CYK-4. It would be interesting to examine whether PAR-4 impinges on ANI-2 to promote actin contractility upon dauer formation. Since ANI-2 functions upstream of CYK-4 (Zhou et al., 2013), this *par-4*-dependent mechanism would simultaneously impinge on CYK-4 and account for an alternative, non-cytokinetic way in which *cyk-4* RNAi rescues *par-4*-dependent hyperplasia. With both ANI-2 and CYK-4 presumably absent from this area upon dauer, NMY-2 and RHO-1 could then accumulate in this region and induce contraction (Zhou et al., 2013). One direct way to test this would be to examine the expression patterns of ANI-2, RHO-1, NMY-2 and actin during dauer formation.

How might the contraction of the actomyosin ring, present at the rachis bridge, inform germ cell function and affect proliferation? In general, the physical properties of the cytoskeleton can have a profound influence on cell function and therefore contribute to proliferative behavior (Guilak et al., 2009). One possibility is that 'basally' localized actomyosin contraction could set up a polarity or cellular orientation that is necessary for the proper establishment of cell quiescence. Genes with demonstrated roles in polarity are required for proper germ line stem cell arrest (Wendland, 2010), and the asymmetric contraction of actin at the rachis bridge could potentially contribute to this polarization. During dauer formation, GLP-1 disappears from the germ cell membranes and accumulates in the rachis in a PAR-4/AMPK dependent manner (Wendland, 2010). The localized contraction of actin at the rachis could somehow play a role in guiding this polarized process.

The Rho-GTPases RHO-1 and CDC-42 are required for germ line quiescence during dauer diapause since their RNAi-mediated inactivation results in hyperplasia (Wendland, 2010). While CYK-4 is implicated in the regulation of RHO-1, another Rho-GAP identified through the screen, PAC-1, is linked to the regulation of CDC-42 (Anderson et al., 2008). PAC-1 has been shown to regulate radial polarity in the 4-cell stage embryo by negatively impinging on CDC-42 (Anderson et al., 2008). PAC-1 is localized to regions of cell contact where it locally inactivates CDC-42, which causes 'inner-outer asymmetry' since active CDC-42 then accumulates in the cell regions that are contact free (Anderson et al., 2008). It will be important to examine the expression pattern of CDC-42 in the dauer germ line, as well as determine whether CYK-4 and PAC-1 regulate RHO-1 and CDC-42 in a respective manner, through further genetic analysis.

Genes with roles in morphogenesis and molting

Several candidates from other functional groups (namely, Gene Expression Signal Transduction, and microRNAs) have been shown to influence processes regarding morphogenesis and molting. These genes include the transcription factor *vab-23,* two secreted signaling peptides *mlt-8* and *pan-1*, and one argonaute, *alg-1*. *vab-23* is a conserved zinc finger transcription factor that plays an essential role in epithelial and vulval morphogenesis (Pellegrino et al., 2011; Pellegrino et al., 2009); *mlt-8* and *pan-1* are signaling peptides essential for molting (Frand et al., 2005); *alg-1* regulates elongation (Vasquez-Rifo et al., 2012). In conjunction with *vab-10* and *let-805,* these genes raise the question of how morphogenetic processes relate to germ line proliferation. While some genes might function germ line autonomously (*pan-1, vab-10)* helping to reconcile the matter, the rest do not, bringing forth the possibility that morphogenesis is itself important for germ cell proliferation. We must determine whether these set of genes directly impinge on germ cell proliferation, or whether their role in morphogenesis somehow regulates germ line proliferation during the dauer stage. Interestingly, *alg-1* has a dual role in regulating germ line proliferation (Bukhari et al., 2012) and elongation morphogenesis, suggesting that the other genes might also function in this manner.

Conclusion

General summary:

Through genome-wide screening, 50 genes from various functional categories were identified as potential factors that function downstream of PAR-4 in the regulation of dauer-specific germ line quiescence. Roughly 24% of genes from this subset have roles in regulating the structural aspects of cell biology, impinging on ECM integrity and also cytoskeletal dynamics. This discovery is interesting since aspects of PAR-4/LKB1 biology that regulate such processes—and the influence of such processes on germ line stem cell quiescence in *C. elegans*—has yet to be explored. While the molecular cues governing germ line stem cell proliferation in *C. elegans* have been widely studied, factors that are more 'physical' in nature have up until recently escaped attention.

Future directions:

Although we reduced the initial number of hits from 1,862 to 50, much work remains to further characterize their roles with regard to PAR-4 mediated, dauer-specific germ cell quiescence. To begin the characterization process, it will be important to generate strains with mutations in candidate gene loci. Since off-target gene silencing is a potential source of error with any RNAi experiment, generating target-gene, loss-of-function mutants in a *par-4* background, and quantifying degrees of hyperplasia through germ cell nuclei counts, would be the best way of confirming that their inactivation rescues *par-4*-mediated hyperplasia. Subsequently, it will be important to determine the germ-line specific, dauer dependent expression pattern of the isolated genes in question in the presence and absence of PAR-4. The 50 candidates are presumably upregulated in the absence of PAR-4 and it will be important to stain for some of the genes deemed significant to determine whether their expression pattern is *par-4*-dependent. In all, further understanding of the function of these genes will provide additional insight as to how PAR-4/LKB1 blocks tumor growth by regulating cell cycle quiescence.

Table 1: RNAi candidates that suppress *par-4*-dependent hyperplasia during dauer

Chr.	Gene	Locus	Brief description
I	H26D21.1	*hus-1*	DNA damage checkpoint protein required for DNA damage-induced cell cycle arrest in *C. elegans*
I	C43E11.6	*nab-1*	Neurabin ortholog that regulate interactions between actin and microtubules during cell division, migration and growth cone guidance
I	F27C1.7	*atp-3*	Subunit of mitochondrial ATP synthase, which regulates growth rate, body size, and ageing
I	C34G6.6	*noah-1*	ECM component required for molting, development, vulval development, and normal body morphology
I	H15N14.1	*adr-1*	Adenosine deaminase that acts on RNA by deaminating adenosines and generating inosines in dsRNA; protects transgenic RNA from RNAi silencing
I	C54G4.8	*cyc-1*	Subunit of complex III cytochrome c reductase required for normal ATP production
I	F26E4.9	*cco-1*	Subunit of cytochrome c oxidase-1, a component the electron transport chain in mitochondria.
I	E03H4.8		Uncharacterized
I	H28O16.1		Uncharacterized
I	M01D7.1		Uncharacterized
I	ZK1151.1	*vab-10*	A spectraplakin, component of the hemidesmosome in *C. elegans;* required for transducing mechanical signals from muscle cells to epidermis, and DTC migration
I	B0414.2	*rnt-1*	Transcription factor required for seam cell proliferation; interacts with SMA-4 and regulates expression of CDK-inhibitor *cki-1*
II	T15H9.3	*hlh-6*	Helix loop helix transcription factor

II	C47D12.6	*tars-1*	Threonyl amino-acyl tRNA synthetase
II	ZK930.3	*vab-23*	Transcription factor involved in ventral closure, elongation, and vulval morphogenesis that acts downstream of LET-60
II	W03C9.3	*rab-7*	Rab-GTPase required for endosome to lysosome trafficking
II	R09D1.7	*chil-20*	Chitinase-like protein
II	W08F4.6	*mlt-8*	Putative signaling peptide secreted from cells involved in the L2/L3 molting process
II	F59G1.3	*vps-35*	Vacuolar protein sorting factor
II	C41C4.4	*ire-1*	Transmembrane serine/threonine kinase and endoribonuclease necessary for unfolded protein response (UPR).
II	F10B5.1	*rpl-10*	Ribosomal subunit L10
II	W09B6.3	*eri-3*	RNA-dependent RNA polymerase required for RNA interference
III	T05G5.3	*cdk-1*	Cyclin-dependent kinase required for cell cycle progression through the G2/M checkpoint
III	R01H10.1	*div-1*	DNA polymerase subunit required for normal interphase timing and asymmetric distribution of PIE-1 and P granules
III	K12H4.4		Uncharacterized
III	C04D8.1	*pac-1*	Rho-GAP involved in establishing radial asymmetry during *C. elegans* development by regulating the spatial localization of CDC-42
III	F58A4.8	*tbg-1*	γ-tubulin
III	K08E3.6	*cyk-4*	Rho-GAP and member of the centralspindlin complex required for cytokinesis; also factor localized to intercellular bridge in the rachis required for gonad structural integrity
III	R10E11.2	*vha-2*	V-ATPase subunit involved in protein sorting and receptor mediated endocytosis

III	H19M22.2	*let-805*	Myotactin; subunit of the hemidesmosome complex
III	F26F4.10	*rars-1*	Arginyl-tRNA synthetase
III	F02A9.6	*glp-1*	Notch receptor homologue required for germline stem cell mitotic proliferation
III	M88.6	*pan-1*	Transmembrane protein required for completion of larval molts; expression enriched in the germline
III	B0336.2	*arf-1.2*	ADP-ribosylation factor homolog, a GTPase that regulates intracellular trafficking and the actin cytoskeleton
III	T12A2.2	*stt-3*	Yeast oligosaccharyltransferase subunit homolog
III	F37C12.4	*rpl-36*	Large ribosomal subunit L36
III	F58A4.11	*gei-13*	Predicted DNA binding protein involved in body shape regulation, cuticle synthesis, and locomotion
IV	C46A5.2	*del-7*	Degenerin-like protein; degenerin family of proteins are sodium ion channels essential for homeostasis and involved in mechanotransduction
IV	F56H11.1	*fbl-1*	Fibulin, a component of the extracellular matrix required for DTC migration initiation
IV	F56H9.5	*lin-25*	Subunit of the Mediator complex, which functions downstream of LET-60 to regulate differentiation of a number of cell types
IV	F57H12.1	*arf-3*	ADP-ribosylation factor homolog, a GTPase that regulates intracellular trafficking and the actin cytoskeleton
IV	R13H7.1	*srx-20*	Serpentine receptor, class X
V	K06A4.3	*gsnl-1*	Gelsolin-related protein predicted to function as an actin regulatory protein, capping barbed ends of actin filaments
V	Y49A3A.2		Uncharacterized
V	F33E11.1	*nhr-15*	A nuclear hormone receptor
V	E02C12.3	*srx-47*	Serpentine receptor, class X

X	F53A9.10	*tnt-2*	Troponin, a tropomyosin binding protein
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

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

Table 2: Categorization of 45/50 RNAi targets with known functions

Cell cycle	Cytoskeleton & polarity	Cell growth & metabolism	ECM & adhesion	Gene expression regulation	Intracellular trafficking	Protein processing	Signal transduction	Micro RNAs
hus-1	*nab-1*	*atp-3*	*noah-1*	*adr-1*	*rab-7*	*stt-3*	*gei-13*	*alg-1*
hsr-9	*pac-1*	*cyc-1*	*let-805*	*hlh-6*	*arf-1.2*	*ire-1*	*srx-20*	*eri-3*
cdk-1	*tbg-1*	*cco-1*	*lam-2*	*tars-1*	*arf-3*	*rpl-10*	*srx-47*	
div-1	*del-7*	*vha-2*	*vab-10*	*rars-1*	*vps-35*	*rpl-36*	*mlt-8*	
	gsnl-1		*chil-20*	*nhr-15*			*pan-1*	
	cyk-4		*fbl-1*	*rnt-1*			*glp-1*	
	tnt-2			*lin-25*				
				vab-23				

The RNAi targets with known function identified as suppressors of *par-4*-mediated germ line hyperplasia were categorized based on their demonstrated roles and functions. Descriptions of each functional group are presented in Figure 7, while gene descriptions, obtained from WormBase, are presented in Table 1.

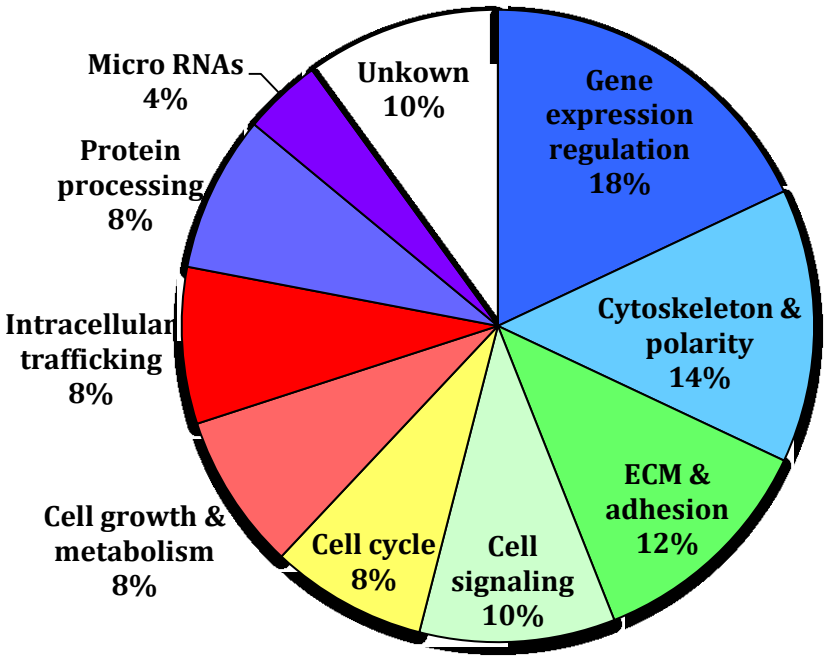

Figure 6: Categorization of RNAi candidates into functional groups

The RNAi targets identified as suppressors of *par-4*-dependent germ line quiescence were categorized based on their functional description on WormBase. The functional groups assigned include genes with known functions in the regulation of gene expression, at the level of DNA and RNA; in cytoskeletal regulation and cell polarity; in the regulation of cell-ECM adhesion and ECM integrity; cell signal transduction; cell cycle progression; cell growth signaling and metabolism; intracellular trafficking; protein processing; and finally in the regulation of micro-RNAs. A portion of candidate genes is uncharacterized and falls into the Unknown functional group.

Table 3: Penetrance and expressivity of RNAi candidates

Chr.	Gene	Locus	Penetrance and expressivity
I	H26D21.1	*hus-1*	Medium penetrance; full suppression
I	C43E11.6	*nab-1*	Medium penetrance; full suppression
I	T05F1.6	*hsr-9*	Encodes a protein that regulates the cell cycle checkpoint in response to DNA damage
I	F27C1.7	*atp-3*	High penetrance; partial suppression
I	C34G6.6	*noah-1*	High penetrance; full suppression; relatively immobile
I	H15N14.1	*adr-1*	High penetrance; full suppression
I	C54G4.8	*cyc-1*	Medium penetrance; full suppression; occasionally dumpy phenotype
I	F26E4.9	*cco-1*	Medium penetrance; partial suppression
I	E03H4.8		High penetrance; partial suppression; dumpy
I	H28O16.1		High penetrance; full suppression; occasionally dumpy phenotype
I	M01D7.1		High penetrance; partial suppression; displaced germline
I	ZK1151.1	*vab-10*	Low penetrance; partial suppression; occasionally dumpy
I	B0414.2	*rnt-1*	High penetrance; partial suppression
II	T15H9.3	*hlh-6*	Low penetrance; full suppression
II	C47D12.6	*tars-1*	High penetrance; partial suppression

II	ZK930.3	*vab-23*	Medium penetrance; full suppression
II	W03C9.3	*rab-7*	Medium penetrance; full suppression; occasionally partial suppression
II	R09D1.7	*chil-20*	High penetrance; full suppression
II	W08F4.6	*mlt-8*	Low penetrance; partial suppression; dumpy
II	F59G1.3	*vps-35*	High penetrance; full suppression
II	C41C4.4	*ire-1*	High penetrance; full suppression; partial suppression
II	F10B5.1	*rpl-10*	High penetrance; full suppression
II	W09B6.3	*eri-3*	Low penetrance; full suppression
III	T05G5.3	*cdk-1*	Medium penetrance; No DTC displacement
III	R01H10.1	*div-1*	High penetrance; partial suppression
III	K12H4.4		High penetrance; full suppression
III	C04D8.1	*pac-1*	Low penetrance; full suppression
III	F58A4.8	*tbg-1*	Low penetrance; full suppression
III	K08E3.6	*cyk-4*	High penetrance; full suppression
III	R10E11.2	*vha-2*	High penetrance partial suppression
III	H19M22.2	*let-805*	High penetrance; full suppression
III	F26F4.10	*rars-1*	Highly penetrant; partial suppression

III	F02A9.6	*glp-1*	Low penetrance; full suppression
III	M88.6	*pan-1*	Medium penetrance; full suppression
III	B0336.2	*arf-1.2*	High penetrance; partial suppression
III	T12A2.2	*stt-3*	High penetrance; partial suppression; occasionally full suppression
III	F37C12.4	*rpl-36*	Low penetrance; full suppression
III	F58A4.11	*gei-13*	Medium penetrance; full suppression
IV	C46A5.2	*del-7*	Medium penetrance; partial suppression
IV	F56H11.1	*fbl-1*	Low penetrance; full suppression; occasionally dumpy phenotype
IV	F56H9.5	*lin-25*	High penetrance; full suppression
IV	F57H12.1	*arf-3*	High penetrance; full suppression; occasionally partial suppression
IV	R13H7.1	*srx-20*	Medium penetrance; full suppression; occasionally partial suppression
V	K06A4.3	*gsnl-1*	Low penetrance; full suppression; occasionally partial suppression
V	Y49A3A.2		High penetrance; partial suppression; occasionally full suppression
V	F33E11.1	*nhr-15*	High penetrance; full suppression
V	E02C12.3	*srx-47*	High penetrance; full suppression; occasionally partial suppression
X	F53A9.10	*tnt-2*	Low penetrance; partial suppression
X	C54D1.5	*lam-2*	High penetrance; full suppression; occasionally dumpy

X	F48F7.1	*alg-1*	High penetrance; full suppression; occasionally dumpy

The gene identity of each RNAi clone was determined by the database available with the commercially available Ahringer RNAi library. The brief descriptions for each gene locus found in Table 1, derived from WormBase or determined according to their functional homology, (www.wormbase.org).

Table 4: RNAi candidates with ambiguous phenotypes

Chr.	Gene	Locus	Brief description
I	W10C8.5		Uncharacterized
I	M01D7.1		Uncharacterized
I	C01H6.5	*nhr-23*	Nuclear hormone receptor required for expression of *acn-1* and *dpy-7* in the hypodermis
I	DY3.2	*lmn-1*	Nuclear lamin homolog
I	F26E4.5		Uncharacterized
I	C47B2.4	*pbs-2*	Proteasome beta subunit
I	K05C4.1	*pbs-5*	Proteasome beta subunit
I	F48C1.1	*aman-3*	Co(II)-activated alpha-mannosidase II which is predicted to remov e mannose residues from oligosaccharides of glycoproteins
I	ZK430.1	*toe-1*	Target of ERK MAPK
I	F29D11.2	*capg-1*	Subunit of condensin which organizes chromosomal structure and regulates chromosome segregation
II	C09E8.1		Uncharacterized
II	T19D12.5		Uncharacterized
II	T14B4.6	*dpy-2*	A cuticle-localized collagen required for maintaining body morphology and length
II	F10E7.1		Uncharacterized
II	C09H10.3	*nuo-1*	Mitochondrial complex I subunit required for normal developmental progression

II	F43G6.1	*dna-2*	Replication helicase subunit
II	C16A11.1	*gpa-11*	G protein alpha subunit expressed in ADL and ASH amphid neurons
II	Y53C12.3	*nos-3*	Nanos homologue which promotes developmental switch from sperm to oocyte differentiation
II	K05F1.7	*msp-63*	Major sperm protein
II	ZK1248.6	*msp-64*	Major sperm protein
II	C27A2.6	*dsh-2*	Dishevelled homolog
II	T0915.10	*lin-5*	Novel spindle component required for chromosome and spindle movement during the cell cycle
III	H19M22.1		Uncharacterized
III	F26A1.14		Uncharacterized
III	F43C1.2	*mpk-1*	Mitogen-activated-protein kinase (MAPK)
III	C27D11.1	*egl-45*	Subunit of translation initiation factor 3
III	C07A9.11	*ncx-7*	Ion exchange channel
III	F11H8.4	*cyk-1*	Formin homologue required for cytokinesis
III	T20G5.1	*chc-1*	Clathrin heavy chain ortholog important for yolk uptake during oogenesis
III	F57B9.6	*inf-1*	Eukaryotic initiation factor 4
III	C29E4.3	*ran-2*	RanGAP
III	F31E3.1	*ceh-20*	Homeodomain protein required for embryonic viability, vulval development, and Q neuroblasts migration

III	T12D8.6	*mlc-5*	Myosin light chain ortholog
III	K10D2.6	*emb-8*	Cytochrome P450 reductase
III	K04H4.1	*emb-9*	Type IV collagen
IV	F45E4.1	*arf-1.1*	ADP-ribosylation factor homolog, a GTPase that regulates intracellular trafficking and the actin cytoskeleton
IV	F26D10.10	*gln-5*	Glutamine synthetase
IV	F32B6.6	*msp-77*	Major sperm protein
IV	ZK792.3	*inx*	
IV	F42G8.9	*irld-8*	Insulin/EGF receptor L domain
IV	ZK792.2	*inx-8*	Integral transmembrane protein ion channel
IV	ZK792.3	*inx-9*	Integral transmembrane protein ion channel
IV	F19B6.2	*ufd-1*	Member of the Cdc48/Ufd-1/Npl-4 complex which mediates degradation of proteins associated with the ER
V	C49G7.8	*cyp-35*	Member of the cytochrome P450 family of proteins
V	C49G7.6		Unchatacterized
V	K07C11.1	*air-1*	Centrosome-localized serine/threonine kinase that regulates recruitment of centrosomal γ tubulin
V	C18G1.9		Uncharacterized
V	T04C12.5	*act-2*	An invertebrate-specific actin
V	T05H4.11		Uncharacterized

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

Table 5: RNAi candidates with a non-dauer-specific phenotype

Chr.	Gene	Locus	Brief description
I	F27C1.7	*atp-3*	Subunit of mitochondrial ATP synthase, which regulates growth rate, body size, and ageing
I	C34G6.6	*noah-1*	ECM component required for molting, development, vulval development, and normal body morphology
I	H15N14.1	*adr-1*	Adenosine deaminase that acts on RNA by deaminating adenosines and generating inosines in dsRNA; protects transgenic RNA from RNAi silencing
I	C54G4.8	*cyc-1*	Subunit of complex III, cytochrome c reductase, required for normal ATP production
I	F26E4.9	*cco-1*	Subunit of cytochrome c oxidase-1, a component the electron transport chain in mitochondria.
I	E03H4.8		Uncharacterized
I	H28O16.1		Uncharacterized
I	B0414.2	*rnt-1*	Transcription factor required for seam cell proliferation; interacts with SMA-4 and regulates expression of CDK-inhibitor *cki-1*
II	C47D12.6	*tars-1*	Threonyl amino-acyl tRNA synthetase
II	F10B5.1	*rpl-10*	Ribosomal subunit L10
III	H19M22.2	*let-805*	Myotactin; subunit of the hemidesmosome complex
III	F26F4.10	*rars-1*	Arginyl-tRNA synthetase
III	B0336.2	*arf-1.2*	ADP-ribosylation factor homolog, a GTPase that regulates intracellular trafficking and the actin cytoskeleton
III	F02A9.6	*glp-1*	Notch receptor homologue required for germline stem cell mitotic proliferation
III	F58A4.8	*tbg-1*	γ-tubulin

III	F37C12.4	*rpl-36*	Large ribosomal subunit L36
III	T05G5.3	*cdk-1*	Cyclin-dependent kinase required for cell cycle progression through the G2/M checkpoint
III	R10E11.2	*vha-2*	Subunit c of the vacuolar proton translocating ATPase

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

Table 6: RNAi candidates that function germ line autonomously

Chr.	Gene	Locus	Brief description
I	C43E11.6	*nab-1*	Neurabin ortholog that regulate interactions between actin and microtubules during cell division, migration and growth cone guidance
I	H15N14.1	*adr-1*	Adenosine deaminase that acts on RNA by deaminating adenosines and generating inosines in dsRNA; protects transgenic RNA from RNAi silencing
I	ZK1151.1	*vab-10*	A spectraplakin, component of the hemidesmosome in *C. elegans;* required for transducing mechanical signals from muscle cells to epidermis, and DTC migration
I	B0414.2	*rnt-1*	Transcription factor required for seam cell proliferation; interacts with SMA-4 and regulates expression of CDK-inhibitor *cki-1*
II	W03C9.3	*rab-7*	Rab-GTPase required for endosome to lysosome trafficking
II	W09B6.3	*eri-3*	RNA-dependent RNA polymerase required for RNA interference
III	T05G5.3	*cdk-1*	Cyclin-dependent kinase required for cell cycle progression through the G2/M checkpoint
III	K12H4.4		No description
III	F58A4.8	*tbg-1*	γ-tubulin
III	C04D8.*1*	*pac-1*	Rho-GAP involved in establishing radial asymmetry during *C. elegans* development by regulating the spatial localization of CDC-42
III	K08E3.6	*cyk-4*	Rho-GAP and member of the centralspindlin complex required for cytokinesis; also factor localized to intercellular bridge in the rachis required for gonad structural integrity
III	F26F4.10	*rars-1*	Arginyl-tRNA synthetase
III	F02A9.6	*glp-1*	Notch receptor homologue required for germline stem cell mitotic proliferation

III	M88.6	*pan-1*	Transmembrane protein required for completion of larval molts; expression enriched in the germline
III	B0336.2	*arf-1.2*	ADP-ribosylation factor homolog, a GTPase that regulates intracellular trafficking and the actin cytoskeleton
III	F37C12.4	*rpl-36*	Large ribosomal subunit L36
IV	C46A5.2	*del-7*	Degenerin-like protein; degenerin family of proteins are sodium ion channels essential for homeostasis and involved in mechanotransduction
IV	F57H12.1	*arf-3*	ADP-ribosylation factor homolog, a GTPase that regulates intracellular trafficking and the actin cytoskeleton
V	K06A4.3	*gsnl-1*	Gelsolin-related protein predicted to function as an actin regulatory protein, capping barbed ends of actin filaments
X	F48F7.1	*alg-1*	Argonaute ortholog required for RNA interference

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

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CHAPTER 3

Characterization of the reproductive defects in post-dauer *par-4* mutant *C. elegans*

Introduction

Stem cells are characterized by their self-renewal capacity and ability to differentiate into various cell types (Weissman, 2000). The germ line lineage of various sexually reproducing organisms contains a population of stem cells specifically dedicated to the production of gametes throughout the animal's lifetime (Lin, 1997). Cells of the germ line are thus the link between generations (Hubbard & Greenstein, 2005). To generate haploid gametes, germ line stem cells exit the mitotic program required for their self-renewal, and initiate meiosis (Hubbard & Greenstein, 2005; Lin, 1997). The integrity of germ line stem cell proliferation, and balance between germ cell proliferation and meiotic differentiation, is important for maintaining reproductive success since the fitness of many organisms, including *C. elegans,* relies on the number of gametes produced (Hodgkin & Barnes, 1991; Weir, 1973).

In *C. elegans,* germ line stem cells proliferate throughout larval development and adulthood to increase in number and ultimately maintain a steady supply of stem cells from which gametes are continuously derived (Hubbard & Greenstein, 2005; Kimble & Crittenden, 2007; Narbonne & Roy, 2006b). Their proliferation, however, is susceptible to the environmental conditions that larvae may encounter during their development (Narbonne & Roy, 2006b, 2008). This correlation is highlighted by the dauer-specific, germ cell quiescence discussed in the previous chapter, but also observed in at least two other points during *C. elegans* development: during the L1 diapause, which occurs when larvae hatch directly into nutrient-deficient conditions, and during adult reproductive diapause, which occurs if late L4 larvae are deprived of food (Angelo & Van Gilst, 2009; Fukuyama et al., 2006; Fukuyama et al., 2012; Narbonne & Roy, 2008; Subramaniam & Seydoux, 1999). In L1 diapause, the two, germ line precursor cells arrest at the G2 phase of the cell cycle until food is reintroduced. During adult reproductive diapause, the germ line stem cells arrest division until feeding resumes as well, at which point they regenerate a germ line capable of producing

progeny at wild-type levels (Angelo & Van Gilst, 2009; Fukuyama et al., 2006; Fukuyama et al., 2012).

The regulation of germ cell cycle arrest in at least the L1 and dauer diapauses occurs through an active mechanism that involves energy sensing and cell cycle control. Studies have implicated AMPK, as well as bona fide tumor suppressors, in the regulation of germ cell quiescence in both contexts (Fukuyama et al., 2006; Fukuyama et al., 2012; Narbonne & Roy, 2006a, b, 2008). Germ cells in both diapause states arrest in a *daf-18*/PTEN and AMPK dependent manner, while dauer-specific germ cell quiescence requires *par-4*/LKB1, in addition (Fukuyama et al., 2006; Fukuyama et al., 2012; Narbonne & Roy, 2006a). Mutations in these respective genes result in aberrant germ cell division, or hyperplasia, during diapause, as is illustrated with the studies of the previous chapter (Fukuyama et al., 2006; Narbonne & Roy, 2006a).

The existence of various potential instances of germ cell quiescence during *C. elegans* development—coupled to the identification of tumor suppressor genes as necessary for germ cell arrest during the L1 and dauer diapause states—highlights the importance of coordinating the germ cell cycle with energy deprivation (Fukuyama et al., 2012). Germ cells must arrest to conserve energy since completing the cell cycle comes at a high energetic cost (Tobin & Saito, 2012). Moreover, the harsh conditions that larvae are exposed to during L1 and dauer diapause, could effect the integrity of germ cell division and influence fitness reproduction when larvae recover from their arrested states, suggesting that quiescence is also a protective strategy (Narbonne & Roy, 2006b, 2008).

Here, we examined the significance of *par-4*-dependent germline quiescence during the dauer stage. We determined the fitness recovery of adults that had experienced dauer-specific germ line hyperplasia. Their reproductive output was found to be significantly reduced compared to post-dauer control adults, whose germ cells had arrested during dauer stage. Through DIC optics, it was

observed that the germ lines of post-dauer, *par-4* adults were disorganized compared to the control animals. Though preliminary, the data suggest that germ cell hyperplasia in *par-4* dauers affects subsequent germ line development and reproduction, highlighting the importance of *par-4*-mediated, germ cell quiescence during dauer. Future work aims at characterizing the observed defects to better understand the post-dauer recovery of *par-4* mutants.

Materials and Methods

Strains and maintenance

The strains used for this study include the MR0673 strain *[daf-2 (e1370) III; par-4 (it-57); qIs56 (lag-2::GFP) V]* and MR0155 strain *[daf-2 (e1370) III; qIs56 (lag-2::GFP) V]*. All strains were maintained at 15°C as described elsewhere, and grown on normal NGM plates seeded with OP50 *E. coli,* unless otherwise indicated (Brenner, 1974).

Inducing dauer-specific, *par-4*-dependent hyperplasia

The MR0673 and MR0155 strains contain a temperature sensitive Daf-c gene, *daf-2 (e1370),* which allowed us to induce dauer by shifting larvae to the restrictive temperature (25°C). MR0673 larvae additionally contain a temperature sensitive *par-4 (it57)* mutation affecting its kinase domain once shifted to the restrictive temperature (25°C). Since *par-4* loss-of-function mutations are embryonic lethal, the *par-4 (it57)* allele allowed us to inactivate *par-4* during the dauer stage and induce hyperplasia. Both strains express a *lag-2::GFP* transgene that co-expresses in the DTCs, marking the distal ends of the germ line. *lag-2::GFP* expression enabled us to indirectly evaluate the degree of hyperplasia. The *lag-2::GFP* signal expressed in both DTCs of MR0673 larvae are further apart than those of MR0155 larvae due to hyperplasia.

Assessing the total reproductive output of larvae following dauer formation

A population of the MR0673 strain was synchronized, and the resulting L1s were incubated at the restrictive temperature (25°C) for 24 hours in order to inactivate PAR-4. The ensuing PAR-4-deficient L1 larvae were added to normal NGM plates seeded with *E. coli*, and incubated at 25°C for 48 hours in order to induce dauer formation. Following this 48-hour window during which dauer is

formed and germ cells proliferate aberrantly, larvae were shifted to the permissive temperature for 48 hours to allow dauer exit, PAR-4 recovery, and development into adulthood. The adults were picked and individually isolated onto separate, plates. They were transferred at 16 to 24 hour intervals thereafter. The brood size of each animal was the sum of non-hatched and hatched progeny of each individual. The same procedure was followed for MR0155 animals, except that synchronized larvae were not incubated at the restrictive temperature prior to inducing dauer.

Assessing the germ line following dauer recovery

MR0673 and MR0155 dauer larvae were removed from the restrictive temperature and shifted to the permissive temperature for 72 hours to allow dauer exit, PAR-4 recovery, and development into the young adulthood. Young adults were individually observed for any germ line defects through imaging of DAPI-stained dissected gonads as well as DIC optics.

Gonad dissections, DAPI staining, and fluorescent microscopy

Larvae that had recovered from dauer were placed in a drop of PBST (1 x PBS, 0.1% Tween-20) on microscope slides that were coated with a poly-lysine mixture consisting of 210 µl of distilled water, 25 µl of 1% poly-lysine, 12.5 µl of 2% gelatin, and 2.5 µl of 1% ChromAlum. The heads of larvae were cut in order to extrude the gonad. The extruded gonads were fixed in 1% PFA for 30 minutes, freeze-cracked, and dipped into cold methanol for 1 minute. Slides were washed three times with PBST, 3 minutes for each wash. Gonads were stained with 20 µl of 0.7 µl/ml DAPI in 1% BSA to stain the nuclei of extruded gonads, and then washed for 1 minute with PBST. 8 µl of Vectashield was then added to the slide, which was subsequently sealed with a coverslip and nail polish. DAPI stained gonads were observed using a Zeiss Imager.21 microscope.

DIC microscopy

Larvae that had recovered from dauer were mounted on 2% agarose pads covered with 2 ml of levamisole, a paralyzing agent as described elsewhere. They were covered with a coverslip and gonads were observed for abnormalities using a Zeiss Imager.21 microscope equipped with DIC optics. 10-20 larvae were mounted per slide.

Results

Post-dauer reproductive fitness is significantly reduced in *par-4(it57)* mutants

To evaluate the consequence of dauer-specific, *par-4*-dependent hyperplasia on subsequent germ line development, we first determined the post-dauer reproductive output of *daf-2(e1370);par-4(it57);lag-2::GFP* adults—henceforth referred to as *par-4(it57)*. As a control, the brood size of post-dauer *daf-2(e1370);lag-2::GFP* adults (or, *daf-2(e1370)*) was scored. This control was used to ensure the post-dauer reproductive capacity of *par-4(it57)* adults was due to *par-4*-dependent hyperplasia and not the inactivation *daf-2(e1370)*. After shifting *par-4(it57)* larvae to the restrictive temperature to induce dauer hyperplasia, the mutants were allowed to develop into adulthood, and the number of hatched and non-hatched embryos of 120 individuals, was scored. Embryos were considered non-hatched if they remained unchanged within 24 hours. Compared to controls, the total reproductive output of *par-4(it57)* adults, that had undergone dauer-specific germ line hyperplasia, was significantly reduced (Figure 7). While post-dauer controls laid around 209.58 ± 10.95 embryos in total, recovered *par-4(it57)* adults laid 72.43 ± 10.95 (Figure 7). Of the total eggs laid in control and *par-4(it57)* adults, 10.95 ± 1.98 and 23.28 ± 2.37 were unhatched, respectively (Figure 7). This data suggests that dauer hyperplasia caused by aberrant PAR-4 signaling impacts germ line integrity and influences reproductive capacity post-dauer.

DAPI-stained germ cell nuclei of post-dauer *par-4(it57)* mutants seem normal

Since the reproductive fitness of *par-4(it57)* adults that had undergone dauer hyperplasia was significantly reduced compared to the control animals, we examined the germ lines of the affected individuals to catalogue any observed defects. The germ line of wild-type adult animals is divided into distinct regions based on chromosome morphology as germ cells progress from mitosis through meiosis with respect to the position of the DTC (Kimble & Crittenden, 2007).

There were no gross differences in chromosome morphology along the distal length of the extruded gonads of *par-4(it57)* adults versus the *daf-2(e1370)* controls (Fig. 8+9). When germ cells exit pachytene and enter diplotene in the loop region, some undergo physiological apoptosis, while others begin to grow and cellularize. One possibility is that the reduced reproductive output of post-dauer *par-4(it57)* adults is due to increased apoptosis (Gumienny et al., 1999). Germ cells undergoing apoptosis in this region are highly condensed compared to their neighbors (Gumienny et al., 1999). Incidences of apoptosis did not seem greater in post-dauer *par-4(it57)* adults compared to the control (Fig. 8+9). Based on these qualitative analyses of, meiotic progression and apoptosis in post-dauer *par-4(it57)* hermaphrodites seem normal. In addition to these qualitative data, it will be important to quantify the number of germ cells found in each cell cycle phase.

Gonad morphology of post-dauer *par-4(it57)* mutants is compromised

When the gonads of post-dauer *par-4(it57)* adults were observed using DIC microscopy, it was apparent that they was disorganized compared to post-dauer control individuals (Figure 10). The observed defects seemed to reflect problems with late oogenesis. In the control individuals, the germ cells that progress from diplotene to diakinesis after the loop region are organized in a single file line (Figure 10, A); they also grow in size as they were displaced proximally (Figure 10, C)(McCarter et al., 1999). In many post-dauer *par-4(it57)* adults, the oocytes were not organized in the same linear fashion, but rather disorganized and stacked on top of one another (Figure 10, B). Moreover, some oocytes appeared smaller in size compared to the oocytes in the control individuals (Figure 10, D). Oocytes of older adults sometimes appear smaller since there is essentially more of them in the proximal arm of the gonad (Kim et al., 2013); however, the individuals analyzed were young adults, the same age as the control animals, suggesting that the smaller oocytes phenotype is an actual abnormality. The defects in oocyte organization and size might be secondary to defects that occurred prior to late oogenesis; however, germ line development leading up to this point seemed normal.

Discussion

Reduced brood size is likely due to compromised sperm and late oogenesis

Brood size analysis of adult worms that had either experienced hyperplasia during the dauer stage or not revealed that coordinating germ line proliferation with developmental status is important for optimal reproductive capacity. The reproductive success of *par-4* adults that had experienced dauer hyperplasia had a significantly reduced reproductive output compared to adults that had not, suggesting the importance of PAR-4 signaling in the dauer, germ line stem cells.

During development, germ cells first differentiate into sperm during the L4 stage, and then switch to oocyte differentiation at adulthood, suggesting that the sperm of post-dauer *par-4* animals is likely derived from the germ cells that had undergone aberrant dauer hyperplasia (Crittenden et al., 2003). Since sperm is the limiting factor in terms of the total reproductive capacity of self-fertile hermaphrodites, one defect that could explain the reduced brood size of post-dauer, *par-4* animals is reduced viable sperm (Hodgkin & Barnes, 1991). Although sperm is visible through DIC microscopy, the harsh conditions germ cells are exposed to during the dauer stage could compromise their genomic integrity, resulting in a reduction of viable sperm and a reduced progeny count (Narbonne & Roy, 2006b).

When the germ lines of adults with reduced offspring were analyzed, it was noticed that the oocytes were disorganized compared to the recovered controls; they were either relatively small or stacked on top of one another instead of being arranged in a linear fashion, suggesting late oogenesis events were affected in animals that experienced dauer-specific, *par-4*-dependent hyperplasia. Because cellularization, maturation, and ovulation must occur in a highly organized fashion to produce viable progeny (Kim et al., 2013), these defects might also account for the reduced post-dauer brood size of *par-4* hermaphrodites, affecting embryogenesis and reproductive output in general.

Events of late oogenesis

When transitioning from pachytene to diplotene around the loop region, germ cells begin to grow and cellularize (Gumienny et al., 1999; Wolke et al., 2007). By the time they reach diakinesis in the proximal arm (oviduct), oocytes are fully enclosed within a plasma membrane and arranged in a single file line (Maddox et al., 2005) (Hall et al., 1999; McCarter et al., 1999). Approximately 5, fully cellularized oocytes are normally found in an adult oviduct at any given time.

The proximal most oocyte undergoes maturation in response to a sperm-derived cue, where its nuclear envelope is broken down (allowing for chromosome separation), and the cell adopts a rounded morphology (McCarter et al., 1999; Miller et al., 2001). Ovulation occurs concurrently, which is when the oocyte physically enters the spermatheca where it is fertilized (McCarter et al., 1999). During ovulation, the proximal sheath cells contract while the distal spermatheca dilates to allow passage of the mature oocyte (McCarter et al., 1999). Preceding oocytes are subsequently displaced, with the proximal-most oocyte initiating maturation and ovulation around 23 minutes later (McCarter et al., 1999).

The sperm signal necessary for oocyte growth, maturation and ovulation includes members of the major sperm proteins (MSPs) (Miller et al., 2001). Sperm-secreted MSPs bind to G-protein coupled receptors on sheath cells to elicit two parallel responses: they stimulate the Gα-acetylate cyclase protein kinase A (PKA) pathway required for oocyte growth and maturation, and the EGL-30/Gαq pathway important for sheath cell contraction (Kim et al., 2013). The Gα-acetylate cyclase PKA pathway is relayed from sheath cells to the underlying oocytes/germ line via gap junctions (promoting their growth and maturation) while the EGL-30/Gαq pathway induces sheath-cell specific cytoskeletal changes necessary for cell contraction (Govindan et al., 2006; Govindan et al., 2009; Kim et al., 2013). The sheath cells are thus key in that they communicate the presence of MSP signals to the underlying oocytes/germ line.

Possible explanations for the small oocyte phenotype

Defects that have previously been shown to result in small oocytes are those related to sperm, cytoplasmic flow, and cellularization (Kim et al., 2013). In the total absence of sperm and MSP signaling, oocytes continue to be produced without interruption, though their ability to mature and ovulate is compromised, resulting in a greater number of oocytes in the proximal arm, that are all arrested in diakinesis. They appear smaller than usual while maintaining their linear arrangement (McCarter et al., 1999). The post-dauer *par-4* animals probably have at least some viable sperm since they were not completely sterile (and sperm was visible through DIC microscopy), though the fact that the morphological appearance of their proximal germ line resembles that of sperm mutants suggests that the observed defects might also be sperm related. This would suggest that in addition to limiting the absolute number of possible progeny, sperm-related defects might also account for the small oocyte phenotype in post-dauer *par-4* hermaphrodites.

In fact, sperm induce the cytoplasmic flow that drives oocyte growth (Govindan et al., 2009; Kim et al., 2013). During normal germ line development, there is a current of cytoplasm that flows through the syncytial gonad, in an actomyosin dependent manner, which moves cytoplasmic material into the enlarging oocytes, defects in which have been shown to result in small oocytes (Wolke et al., 2007). The MSP signal activates the Gα-acetylate cyclase PKA pathway in the sheath cells, which, in addition to promoting the maturation of the proximal most oocyte, results in a global increase of phosphorylated myosin light chain (pMLC) in the germ line (Govindan et al., 2009). In conjunction with the intracellular bridges present in the germ cells and oocytes, this pMLC accumulation generates the force necessary for cytoplasmic streaming and uptake (Govindan et al., 2009). The small oocytes observed in post-dauer *par-4* adults could thus reflect defects in this process, at the level of either steps—the sperm derived MSP signaling, or the ability of germ cells to respond correctly by activating MLC and modulating their cytoskeletal network accordingly to generate the physical force.

How might *par-4*-dependent dauer-specific hyperplasia impact this actomyosin dependent process? One answer could be that the harsh conditions that the germ cells are exposed to during the dauer stage could compromise their genomic integrity, resulting in defective sperm and sperm-induced MSP signaling. With a reduced amount of viable sperm, the amount of MSPs and the whole cytoplasmic response might be diminished, resulting in reduced cytoplasmic uptake and smaller oocytes. In addition to this, another possibility might be that the dauer-specific misregulation of cytoskeletal genes in the absence of PAR-4 could compromise the cytoskeletal framework in germ cells necessary for cytoplasmic streaming. Cytoplasmic uptake requires the proper regulation of actin cables, without which oocytes fail to grow (Kim et al., 2013; Wolke et al., 2007). One candidate to potentially consider is CYK-4, which localizes to the intracellular bridge of germ cells and developing oocytes where it is important for actin filament and microtubule bundling (Zhou et al., 2013).

To determine which of these processes is disrupted, it will be necessary to cross post-dauer *par-4* hermaphrodites with healthy, *par-4* males and determine whether exogenous sperm is able to rescue the small oocytes phenotype and reduced brood size. Should exogenous sperm successfully restore germ line integrity, it will be important to then determine whether the problem rests in general sperm availability or compromised MSP signaling. One way to address this question would be to a) directly count the total sperm available in post-dauer *par-4* animals and b) to inject MSPs into the affected hermaphrodites to asses if this specific addition restores the small oocytes defect (Kim et al., 2013). To assess if the germ line response to MSP signaling is intact, it will be important to assess the expression pattern of pMLC in post-dauer *par-4* animals. The accumulation of pMLC seems to be the primary germ line response to MSP signaling and if the levels are reduced or expression pattern skewed, this could suggest that the germ cells and oocytes themselves are incapable of properly adjusting their cytoskeletal framework for cytoplasmic streaming. Actin staining could also help reveal if the cytoskeletal organization of the germ cells impaired in some way.

Interestingly, it has recently been demonstrated that a GLP-1 function that is independent from its role in regulating germ cell mitosis is responsible for countering the growth promoting cytoplasmic current that is initiated by MSP signaling. The oocytes in *glp-1* mutants were shown to grow abnormally large, suggesting that GLP-1 functions to modulate the response of germ cells to MSP-derived signal and ensure that they do not overgrow (Nadarajan et al., 2009). This balance between both signaling mechanisms on either end of the germ line, is thus important to attain oocytes of the right size (Nadarajan et al., 2009). One hypothesis to also consider is that aberrant GLP-1 activity during the dauer stage in *par-4* larvae not only results in the over-proliferation of germ cells, but also somehow compromises this balance upon dauer recovery. One way to test this possibility would be to determine if GLP-1 expression levels are elevated in post-dauer *par-4* hermaphrodites compared to post-dauer controls, perhaps owing to its aberrant dauer-specific activity.

In addition to sperm and cytoplasmic flow, the small oocytes phenotype might also reflect defects with cellularization. Though oocyte cellularization in post-dauer *par-4* animals is intact, the timing may be compromised. The spacing of each cellularization event might be off, resulting in membranes forming too close together and oocytes appearing more slim than normal. The mechanisms governing oocyte cellularization are incompletely understood, though it is known to involve MLC-4, MEL-11, and CYK-1. *mlc-4* and *mel-11* encode the regulatory myosin light chain subunit and its phosphatase, respectively, while *cyk-1* encodes an actin regulator (Piekny & Mains, 2002; Shelton et al., 1999; Swan et al., 1998). *mel-11* was isolated as an ambiguous RNAi candidate (Table 4). Though inactivating mutations in *mel-11* have not been associated with abnormal oocyte size (Piekny & Mains, 2002), the effect of its overexpression has not yet been characterized. If *mel-11* is indeed misregulated in the absence of PAR-4, it is possible that its overexpression during the dauer stage results in secondary defects during the post-dauer cellularization process. To assess this possibility, we can assess whether the dauer-specific inactivation of *mel-11* rescues the observed post-dauer defects.

Possible explanation for the stacked oocyte phenotype

In addition to the small oocyte phenotype, another defect observed in post-dauer *par-4* hermaphrodites was that the oocytes in the oviduct were stacked on top of one another. Defects that have previously been demonstrated to cause stacked oocytes within a swollen oviduct are those related to ovulation and sheath cell contractility, specifically (Iwasaki et al., 1996; Kim et al., 2013; McCarter et al., 1999; Myers et al., 1996; Rose et al., 1997). When oocyte development and maturation are able to occur, but the passing of oocytes into the spermatheca (ovulation) is impaired, developing oocytes accumulate in the proximal arm. The oocytes undergo multiple rounds of maturation as a consequence, and animals are rendered infertile (Kim et al., 2013; McCarter et al., 1999). Since post-dauer *par-4* hermaphrodites were not completely sterile and still had some offspring, maturation and ovulation must have occurred properly to some degree; one explanation for the stacked-oocyte phenotype in these individuals could therefore be that ovulation occurred less frequently than normal. An imaginable scenario could be that the sperm-derived cue necessary for maturation and ovulation is intact and able to signal to the sheath cells. The sheath cells however, while capable of relaying the maturation cue to the underlying oocyte, are unable to contract at a normal rate. The oocytes would thus mature, but their physical passage into the spermatheca would occur less frequently owing to sheath cell contractility defects. Due to untimely sheath cells contraction, some oocytes would maturate several times as a consequence of not ovulating (becoming unviable) while others would manage ovulate on time.

Many gene loci for which RNAi-mediated depletion resulted in reduced *par-4*-dependent, dauer hyperplasia discussed in the previous chapter encode ECM components and cytoskeletal regulators—factors that are, in general, involved in contractile events. Several candidates from this subset were found to act germ line non-autonomously, suggesting they function in somatic tissue, which could include the gonadal sheath cells. Their *par-4*-dependent misregulation during the dauer

stage could affect the cytoskeletal framework and gonad morphology necessary for proper contraction after dauer, leading to aberrant ovulation and oocyte stacking. Some interesting candidates to potentially consider are *let-805*, a myotactin, and *tnt-2,* troponin T. In addition to functioning in a germ line non-autonomous fashion, these factors have demonstrated and/or predicted roles in regulating contraction, respectively (Myers et al., 1996; Zahreddine et al., 2010; Zhang et al., 2011). To date, there are no accounts of *let-805* functioning in the somatic gonad, but it has been shown to regulate muscle contraction. A paralog of *tnt-2* on the other hand*, mup-2,* has in fact been demonstrated to regulate sheath cell contraction and result in oocyte stacking when mutated (Myers et al., 1996).

To examine whether post-dauer *par-4(it57)* hermaphrodites suffer from ovulation defects, it will be necessary to perform time-lapse microscopy and observe whether sheath cell contractions and ovulation occur infrequently compared to control individuals. In parallel, we must also analyze the nuclei of the stacked oocytes more closely since multiple rounds of maturation are often associated with ovulation defects. Moreover, since defects in contraction are likely a result of an impaired cytoskeletal framework, it will be important to visualize the actin cytoskeleton in the sheath cells and proximal gonad. Actin filaments normally extend laterally across the length of the sheath cells in an organized bundles; it will be interesting to see if these actin conformations are compromised in post-dauer *par-4* hermaphrodites (Myers et al., 1996). Finally, since *let-805* and *tnt-2* seem like the most probable candidates to be involved in this process due to their non-germline-autonomous function and roles in the regulation of the cytoskeleton and contraction, it will be necessary to analyze a) their expression pattern to determine whether they are expressed in the sheath cells and b) to determine if their dauer-specific inactivation rescues the post-dauer defects observed in *par-4* individuals (Myers et al., 1996; Zahreddine et al., 2010).

Conclusion

In this chapter, we examined the reproductive consequence of aberrant germ line proliferation during the dauer stage. The post-dauer reproductive capacity of *par-4* adults was significantly reduced compared to controls, suggesting that the absence of PAR-4 in the dauer germ cells leads to germ line defects at adulthood. The morphological events of late oogenesis were compromised in post-dauer *par-4* adults, where the oocytes were smaller than those of control hermaphrodites, or stacked on top of one another in a disorganized fashion. Based on the literature, these defects seem to reflect problems with sperm, as well as the actomyosin dependent processes of cytoplasmic flow, cellularization, and ovulation.

par-4-dependent dauer hyperplasia might have disrupted these developmental events in various ways. First, the proliferation of germ cells during dauer could compromise their genomic integrity and result in defective sperm upon recovery. Defective sperm would not only result in morphological defects, but would also limit the total number of progeny possible. Second, because sperm-induced cytoplasmic flow is offset by GLP-1 activity, dauer-specific GLP-1 signaling in PAR-4 deficient germ lines could install an imbalance upon recovery and result in smaller oocytes. Third, due to the many cytoskeletal candidates that are presumably up-regulated in the absence of PAR-4 during dauer, the cytoskeletal profile of post-dauer *par-4* germ lines and sheath cells might be compromised, leading to defects in cytoplasm uptake, cellularization, and ovulation.

Future research entails the characterization of the observed defects, mainly through mating, analysis of the actomyosin framework in the germ and sheath cells, and time-lapse imaging to directly determine if these postulated defects, morphological in nature, are true. In all, the results obtained from this preliminary effort to examine the importance of PAR-4 mediated germ line quiescence during dauer suggest that it is in fact a process necessary for germ line integrity and reproductive success.

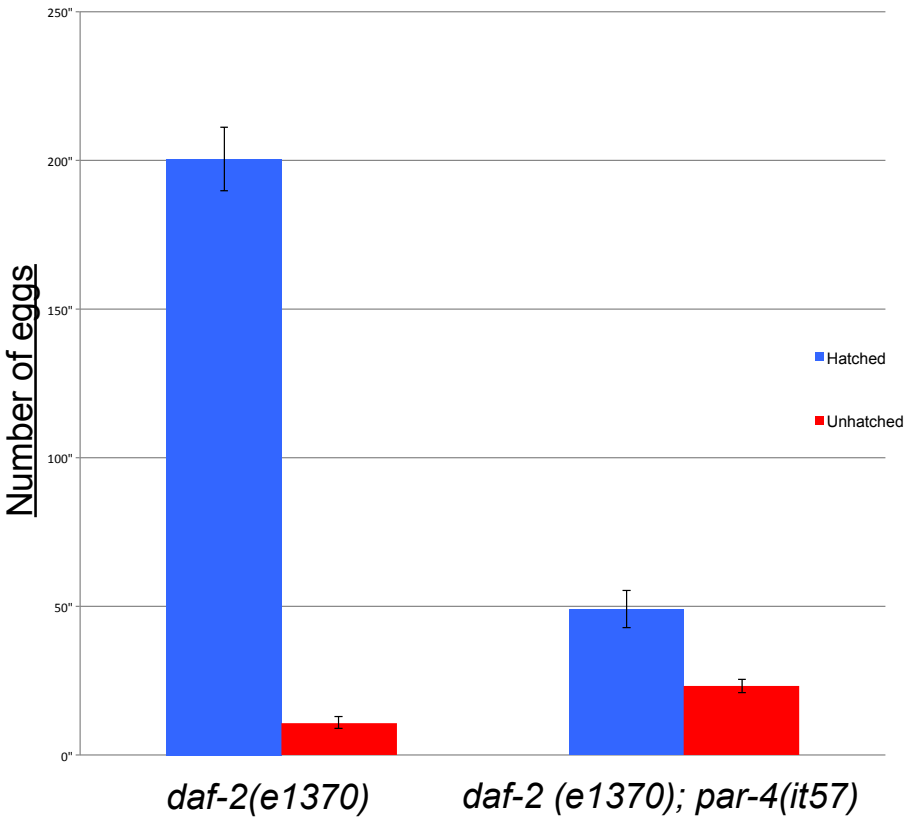

Figure 7: Post-dauer reproductive output is significantly reduced in *par-4(it57)* mutants

daf-2(e1370) and *par-4(it57)* larvae were shifted to the restrictive temperature to induce dauer hyperplasia; the mutants were then allowed to develop into adulthood, and the number of hatched and non-hatched embryos of 120 individuals, was scored. Embryos were considered non-hatched if they had not done so within 24 hours. All animals contained a *lag-2::GFP* transgene.

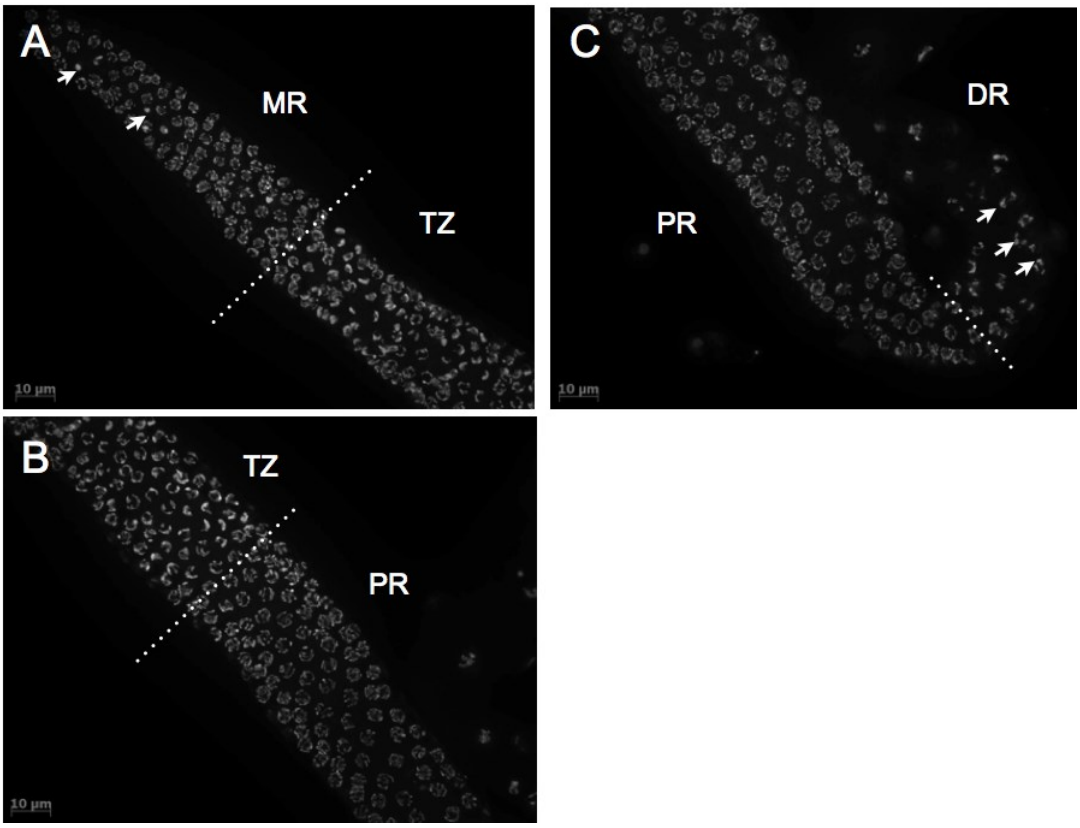

Figure 8: DAPI-stained distal germ cell nuclei of post-dauer *daf-2(e1370)* animals

daf-2(e1370) control larvae were removed from the restrictive temperature (25°C) and transferred to the permissive temperature (15°C) to allow dauer exit and development into adult. The distal ends of the germ lines were dissected out and stained with DAPI. Dotted lines delineate the mitotic region (MR), transition zone (TZ), pachytene region (PR), and diplotene region DR. **A)** The MR-TZ boundary. Note the actively dividing germ cell nuclei (arrowheads point to condensed chromosomes during metaphase) and condensed, crescent shape nuclei in the TZ. **B)** The TZ-PR boundary. Note the condensed strands of chromatin in the PR. **C)** The PR-DR boundary. Arrowheads indicate apoptotic nuclei.

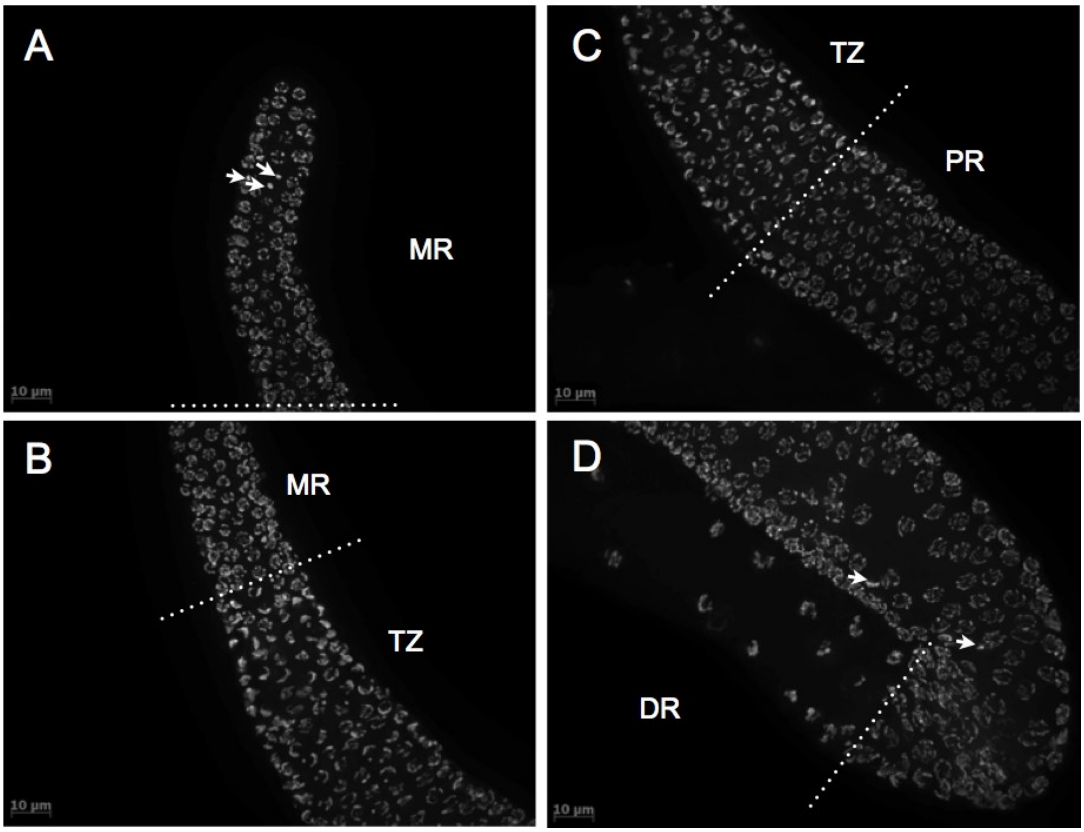

Figure 9: DAPI-stained distal germ cell nuclei of post-dauer *par-4(it57)*animals

par-4(it57) larvae were removed from the restrictive temperature (25°C) and transferred to the permissive temperature (15°C) to allow dauer exit, PAR-4 recovery, and development into adult. The distal ends of the germ lines were dissected out and stained with DAPI. Dotted lines delineate the mitotic region (MR), transition zone (TZ), pachytene region (PR), and diplotene region DR. **A)** The MR. Note the actively dividing germ cell nuclei (arrowheads point to condensed chromosomes during metaphase); chromosome morphology is similar to what is observed in *daf-2(e1370)* controls in Figure 9, A. **B)** The MR-TZ boundary. Note the condensed, crescent shape nuclei in the TZ; chromosome morphology is identical that found in TZ of *daf-2(e1370)* controls in Figure 9, A and B. **C)** The TZ-PR boundary. Note the condensed strands of chromatin in the PR. **D)** The PR-DR boundary. Arrowheads indicate apoptotic germ cell nuclei.

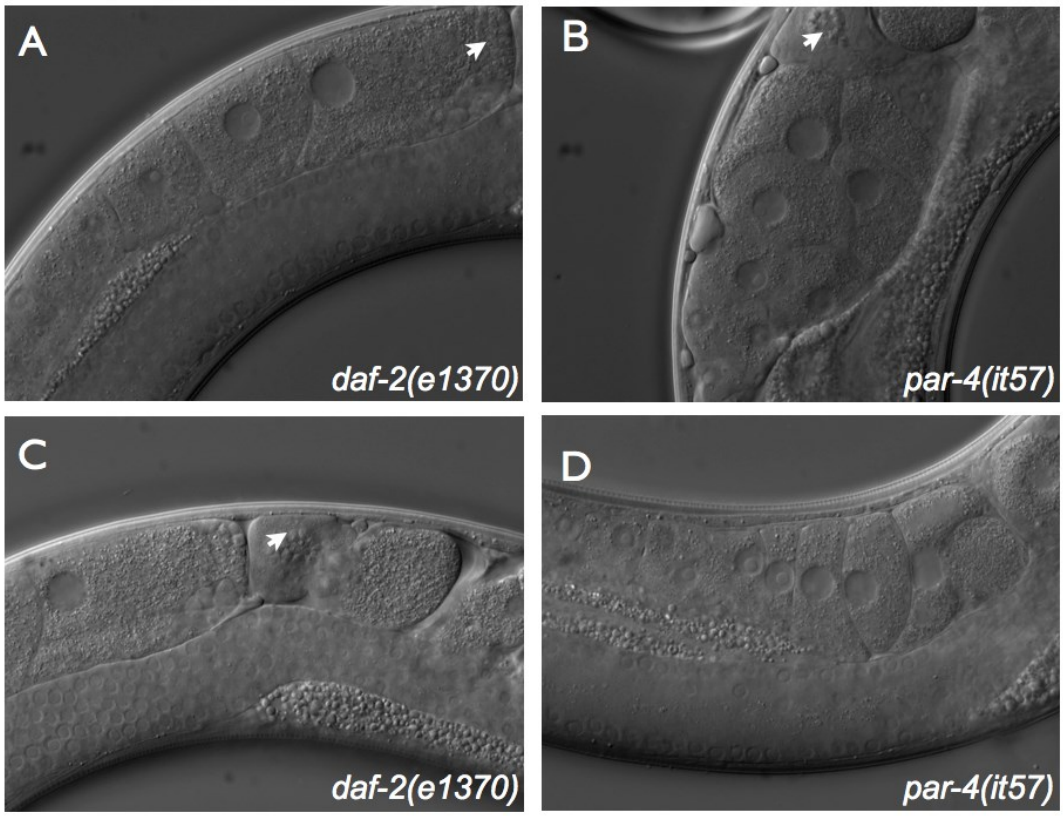

Figure 10: Germ line morphology of post-dauer *par-4(it57)* animals is compromised

daf-2 (e1370 and *par-4(it57)* larvae were removed from the restrictive temperature and shifted to the permissive temperature to allow dauer exit and development into adulthood. The adult germ lines of both genotypes were observed using DIC microscopy. (A, C) The oocytes of post-dauer control *daf-2(e1370)* adults are arranged in a single file line and attain a normal size by the time they reach the spermatheca. (B, D) The oocytes of post-dauer *par-4(it57)* adults were frequently observed to stack in the oviduct, and sometimes observed to be much smaller in size. White arrowheads indicate discernable sperm in A, B, and C. Though sperm was present in all individuals analyzed, it was sometimes not captured with DIC due to the imaged focal plane.

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CHAPTER 4

General discussion and conclusion

Introduction

The dauer diapause is an alternative developmental stage that enables *C. elegans* larvae to endure long periods of stress. As part of this arrested phase, the germ line stem cells, which normally divide throughout reproductive development and adulthood, enter cell cycle quiescence (Narbonne & Roy, 2006). Previous work has revealed that PAR-4/LKB1 and AMPK cooperate under such nutrient-deficient conditions to mediate this germ line stem cell cycle arrest (Narbonne & Roy, 2006). The knockdown of either gene causes dauer-specific, germ line hyperplasia, while the inactivation of *par-4* in *aak-2*/AMPK null mutants causes enhanced hyperplasia. Although PAR-4/LKB1 is known to regulate AMPK in *C. elegans,* our genetic analyses suggest that AMPK is unlikely the sole mediator of germ cell quiescence downstream of PAR-4 (Narbonne & Roy, 2006).

LKB1 is a tumor suppressor protein kinase that is implicated in the cancer-predisposing syndrome Peutz-Jeghers syndrome (PJS) (Hemminki et al., 1998; Jenne et al., 1998). To better understand its function in tumorigenesis, we characterized its role in regulating cell quiescence in developmentally arrested larvae using a genome-wide, feeding based, RNAi screen to identify suppressors of *par-4*-mediated germ line hyperplasia. We identified 50 genes whose loss-of-function was found to rescue the germ line hyperplasia observed in *par-4* dauer larvae, suggesting that their expression is misregulated in the absence of PAR-4/LKB1. Many of these genes impinge on the ECM, as well as the actin cytoskeleton and its regulation. Some of these include the ECM components laminin and fibulin, *lam-2* and *fbl-1*, respectively*;* components of the hemidesmosome adhesion complex, *vab-10* and *let-805*; and the Rho-GAP, *cyk-4*. The isolation of such genes suggests that ECM- and cytoskeletal- based, *par-4*-dependent mechanisms are necessary for preventing aberrant germ line hyperplasia during the dauer stage.

Summary of ECM-based model of germ line stem cell proliferation

The germ line is enclosed within a basement membrane, with which both, the germ line stem cells and DTCs are in direct contact (Kramer, 2005). Dauer-dependent remodeling of the extracellular matrix may convey important cues necessary for stem cell cycle arrest. The ECM may signal to the germ line stem cells directly, where the EGF-like repeats on the extracellular domain of GLP-1 could interact with components of the basement membrane (Maine & Kimble, 1993), affecting its activity. Alternatively, changes in ECM composition may affect the migration of DTCs, which could in turn affect germ line stem cell proliferation. Hemidesmosomes anchor cells to the matrix and have recently been shown to be involved in mechanotransduction; the identification as *vab-10* as a germ line autonomous gene may suggest that a process involving mechanosensing—between the DTCs and the germ cells—is important for germ cell cycle regulation; germ line stem cell proliferation may be coordinated with DTC migration, or lack thereof.

Summary of cytoskeletal-based model of germ line stem cell proliferation

In addition to being enclosed within a basement membrane, the germ cells are linked to the rachis through intracellular bridges: cytoskeletal structures located between the germ cells and rachis. Intracellular bridges resemble the cytokinetic, actomyosin ring, though they exist in a non-contractile state during normal germ line development (Zhou et al., 2013). One possibility is that the contractile state of this cytoskeletal ring is also modulated in response to dauer, where its contraction at the germ cell/rachis boundary is important for the regulation of germ line stem cell quiescence. The Rho-GAP CYK-4 localizes to the bridges of germ cells where it is hypothesized to inhibit actin contraction (Zhou et al., 2013). By impinging on CYK-4, PAR-4 might be involved in promoting a localized, actomyosin-dependent contraction, which could in turn be responsible for setting up a polarity that is necessary for the establishment of cell cycle quiescence. GLP-1 relocalizes to the

rachis in a PAR-4/AMPK dependent manner during dauer (Wendland, 2010); the localized contraction of actin at the rachis could somehow play a role in guiding this polarized process. Preliminary data from our laboratory illustrates a dauer-dependent accumulation of actin in the rachis, which further supports this model. Germ line imaging, and determining the expression patterns of the potentially involved factors, will be key in testing this hypothesis.

Role of AMPK in *par-4*-dependent germ line stem cell quiescence

A genome-wide, feeding-based RNAi screen, aimed at identifying factors involved in AMPK-dependent germ line stem cell quiescence, is currently in progress in our laboratory. Comparing the genes isolated from the ongoing, AMPK suppressor screen with the candidates described in this study will allow us to distinguish between factors that function downstream of the LKB1-AMPK pathway, versus those that function downstream of LKB1, independently of AMPK. Based on the results obtained thus far, the RNAi-mediated inactivation of *lam-1* (the α subunit of laminin) and *vab-10* was able to rescue AMPK-dependent germ line hyperplasia, suggesting that these two factors function downstream of LKB1-AMPK. Interestingly, *cyk-4* was not identified as a candidate gene downstream of AMPK. This is intriguing because it is a component of centralspindlin, a factor that is necessary for cytokinesis. The fact that it was not isolated through the AMPK suppressor screen either suggests that it was overlooked—or—that its non-cytokinetic role at the rachis bridge precedes M phase, and is regulated in a *par-4*-dependent manner, independently of AMPK. This preliminary data might point to a model where AMPK is necessary downstream of PAR-4 in regulating ECM-based mechanisms of germ cell quiescence; however, *cyk-4*-dependent regulation of the actin cytoskeleton at the germ cell/rachis boundary occurs independently of AMPK. A potential model could be that PAR-4 is responsible for conferring a certain 'initial' germ cell polarity, or orientation, by promoting the localized contraction of actin at the germ cell/rachis boundary. The localized contraction of actin at the rachis would then

play a role in guiding the polarized relocalization of GLP-1 from the membrane to the rachis. AMPK would be necessary for the endocytic process in itself, but not for the early polarity establishment. The AMPK-dependent branch of *par-4*-mediated signaling would thus entail a) regulation of the ECM and b) GLP-1 endocytosis, while the AMPK-independent branch would consist of regulating CYK-4 (and other polarity factors) to achieve dauer-specific germ cell polarity important for their cell cycle arrest.

Post-dauer reproductive capacity and germ line morphology

In addition, we investigated the reproductive consequence of aberrant germ line proliferation during the dauer stage by characterizing the post-dauer reproductive capacity of *par-4* mutants. The post-dauer reproductive output of *par-4* adults was found to be significantly reduced compared to controls, suggesting that the absence of PAR-4 in the dauer germ cells leads to germ line defects at adulthood. The post-dauer, germ lines of *par-4* adults were morphologically impaired, displaying small oocytes or oocyte stacking. While, small oocytes may reflect defects with sperm, cytoplasmic flow, and cellularization timing, oocyte stacking seems to reflect defects with ovulation (Govindan et al., 2009; Kim et al., 2013; McCarter et al., 1999; Myers et al., 1996; Nadarajan et al., 2009; Rose et al., 1997; Wolke et al., 2007). Overall, these observed outcomes seem to suggest three separate models.

The reduced brood size and small oocytes phenotype of post-dauer *par-4* hermaphrodites suggests a model where sperm is compromised. Sperm determines the total number of possible progeny and also control oocyte growth by providing the impetus for cytoplasmic flow. Because the sperm in post-dauer *par-4* hermaphrodites is, in all likelihood, derived from aberrantly proliferating dauer-germ cells, it might have acquired deleterious mutations, rendering it defective or in less abundance. With defective sperm signaling, one would expect reduced progeny and as well as defects associated with oocyte growth and development.

The small oocytes phenotype also suggests a model where persistent dauer-specific GLP-1 activity offsets the otherwise balanced interplay between sperm-induced oocyte growth and GLP-1-dependent growth restriction. Finally, the small oocytes and stacked oocyte phenotypes suggest a model where the *par-4*-dependent misregulation of the actin cytoskeleton during the dauer stage alters the cytoskeletal profile in such a way where even though PAR-4 is recovered post-dauer, the actin framework necessary to drive cytoplasmic flow, cellularization, and/or sheath cell contraction is compromised.

Closing statement:

This study has contributed greatly to our understanding of *par-4*-dependent germ line stem cell quiescence. We have identified 50 candidates whose inactivation resulted in reduced *par-4*-dependent germ line hyperplasia. These candidates represent many possible directions for future projects. Their characterization will provide additional insight as to how PAR-4/LKB1 blocks tumorous growth by regulating cell cycle arrest. Moreover, we discovered that the reproductive capacity of post-dauer, *par-4* animals was significantly reduced. Dauer-specific, *par-4*-dependent germ line hyperplasia seems to impact post-dauer germ line integrity and morphology at the level of late oogenesis. We have identified two major phenotypes, oocyte stacking and small oocytes, which may now be taken on as new projects and further characterized.

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