Using *Caenorhabditis elegans* germline stem cells to investigate the *in vivo* regulation of mitosis

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i. Abstract

English

Cell proliferation is a tightly regulated process that results in an increase in the number of cells by cell division which is important for proper development. The satisfaction of cell cycle checkpoints prevents the transmission of genetic errors to daughter cells during cell division. The regulation of cell cycle checkpoints, such as the G1/S and G2/M checkpoints, by signaling pathways helps cells to coordinate cell cycle regulation with external conditions. However, less is known about whether these cell cycle-regulating pathways also regulate progression through mitosis.

The *Caenorhabditis elegans* germ line is an excellent model system to study mitosis *in vivo*. *C. elegans* hermaphrodites contain two U-shaped gonad arms which each contain a pool of mitotically dividing germline stem cells (GSCs). GSCs are located within the distal region of the germ line, called the "mitotic zone" (Crittenden et al., 2006; Pazdernik & Schedl. 2012). Similar to stem cells in mammals, *C. elegans* GSCs interpret and respond to physiological changes, such as dietary restriction or growth factor signaling pathways (Hubbard et al., 2013). However, unlike most mammalian stem cells, GSCs are amenable to intravital imaging, making them a powerful model for studying mitosis *in vivo*.

Previous work in our lab has shown that reducing canonical insulin/IGF-1 signaling (IIS) affects GSC mitosis. The knockdown of the IIS receptor DAF-2/IGFR extends the duration of mitosis through a mechanism that requires both DAF-16/FOXO and DAF-18/PTEN (E. Cheng, unpublished). The goal of my research is to investigate if other signaling pathways also affect GSC mitosis. By measuring GSC mitotic duration in RNAi treated worms and in genetic mutants, I found that the reduced activity of the Notch and Transforming Growth Factor beta

(TGF-β) pathways delays GSC mitotic progression. In addition, while dietary restriction results in similar mitotic delays as when IIS is reduced, mitotic delays upon dietary restriction are dependent on DAF-18/PTEN, but not DAF-16/FOXO. These results suggest that both physiological changes and growth factor signaling pathways affect GSC mitosis, and that the mechanisms linking these to GSC mitotic delays are likely to be different.

French

La prolifération cellulaire est l'augmentation du nombre de cellules par division cellulaire. C'est un processus étroitement contrôlé qui est nécessaire pour le bon développement d'un organisme. Les points de contrôle du cycle cellulaire sont nécessaires afin d'assurer la fidélité de la duplication du matériel génétique durant le cycle cellulaire. La régulation de ces points de contrôle, tels que les points de contrôle en G1/S et en G2/M, par des voies de signalisation aide les cellules à adapter la régulation du cycle cellulaire en réponse aux conditions externes. Cependant, nous ne savons pas si ces voies de régulation du cycle cellulaire régulent aussi la progression en mitose.

La lignée germinale de *Caenorhabditis elegans* est un excellent modèle pour étudier la mitose *in vivo*. Les hermaphrodites *C. elegans* possèdent deux gonades, contenant chacune un pool de cellules souches germinales (CSG). Les CSG se divisent par mitose et se situent dans la région distale de la lignée germinale, appelée la « zone mitotique » (Crittenden et al., 2006; Pazdernik & Schedl. 2012). Comme les cellules germinales des mammifères, les CSG de *C. elegans* interprètent et répondent à des changements physiologiques, tels que la restriction alimentaire ou aux voies de signalisation de facteurs de croissance (Hubbard et al., 2013). Contrairement à la plupart des cellules souches des mammifères, les CSG peuvent être visualisées par microscopie intravitale, ce qui en fait un excellent modèle pour étudier de la mitose.

De précédents résultats dans notre laboratoire ont montré qu'une réduction de la signalisation canonique de l'insuline, aussi appelé IIS (Signalisation Insuline/IGF), affectait la mitose des CSG. L'inactivation du récepteur de l'IIS, DAF-2/IGFR, rallonge la durée de mitose par un mécanisme qui requière DAF-16/FOXO et DAF-18/PTEN. L'objectif de ma recherche est de déterminer si d'autres voies de signalisation affectent également la mitose des CSG. En utilisant

de l'ARN interférant et des mutants génétiques et en mesurant la durée mitotique des CSG, j'ai trouvé que l'activité réduite de la signalisation de Notch et du facteur de croissance transformant β (TGF-β) retarde la progression mitotique des CSG. De plus, alors que la restriction alimentaire entraîne des retards en mitose similaires à lorsque IIS est réduit, les retards mitotiques dus à la restriction alimentaire dépendent de DAF-18/PTEN, mais pas de DAF-16/FOXO. Ces résultats suggèrent que les changements physiologiques et les voies de signalisation de facteurs de croissance affectent la mitose des CSG, et que les mécanismes liant ces délais sont suceptibles d'être différents.

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iii. Author's Contributions

Ran Lu carried out all experiments and analyzed data with ImageJ and MATLAB scripts written by Dr. Abigail Gerhold, Réda Zellag, and Eric Cheng. Ran Lu made all figures and wrote the thesis with editorial feedback from Dr. Abigail Gerhold.

iv. Introduction

Cell proliferation is a crucial process that governs developmental events, tissue repair, and growth. The regulation of this process involves intricate signaling networks and environmental cues. Dysregulation of cell proliferation can lead to serious growth defects, such as cancer. Extensive research has been undertaken to unravel the mechanisms underlying cell proliferation regulation. For instance, the G1/S checkpoint, which determines whether the cell is ready to initiate DNA replication, is regulated by cyclin-D-CDK4/6 and cyclin-E-CDK2 in mammalian cells. The G2/M checkpoint, which regulates the entry of cells into mitosis, is regulated by cyclin-B-CDK1 (Duronio & Xiong, 2013). While numerous signaling pathways and environmental cues have been implicated in regulating the cell cycle, it is less well understood how these factors affect mitotic processes and mitotic fidelity.

The aim of this study is to investigate how alterations in signaling pathways and/or environmental factors, such as dietary restriction, affect mitotic processes. The germline stem cells (GSCs) of *Caenorhabditis elegans* (*C. elegans*) serve as an excellent model system to study mitosis *in vivo*. Live imaging of *C. elegans* germlines at the late L4 larval stage is employed as the primary tool in my research. RNA interference and *C. elegans* mutant alleles are used to perform a candidate screen for signaling pathways that potentially impact the mitosis of GSCs. The analysis of mitosis is carried out in terms of the mitotic cell number, mitotic index, and the duration of mitosis, to elucidate how signaling pathways and dietary restriction impact GSC mitosis. My results show that the TGF-β and Notch signaling pathways affect GSC mitosis, with the TGF-β signaling pathway regulating mitotic timing in GSCs dependent on *daf-3* and *daf-5*. Dietary restriction is also found to affect GSC mitosis in terms of mitotic timing, which is dependent on *daf-18*.

1.0. Literature Review

1.1. *Caenorhabditis elegans*

The nematode *Caenorhabditis elegans* (*C. elegans*) has been used as a model organism to study animal development and behavior since 1965 (Brenner, 1975). It is now widely used in the life sciences for many well-known reasons (Zhang et al., 2020). *C. elegans* have a short life cycle, about 3 days for larvae to complete development from embryo to adult under standard conditions (Herndon, 2018; Stiernagle, 2006). Under favorable environmental conditions, *C. elegans* larvae develop through 4 larval stages: L1, L2, L3, and L4. After the fourth larval stage, the animal has a fully developed germ line and enters the reproductive adult stage. The self-fertilizing adult hermaphrodites are about 1mm long with an average lifespan of 18-20 days (Zhang, 2020).

The *C. elegans* germ line is particularly useful to study stem cell regulation *in vivo*. *C. elegans* hermaphrodites have two U-shaped gonadal arms with germ cells organized functionally from the distal to the proximal end (Figure 1A). At the distal end of the gonad, the germ cells are undifferentiated and divided by mitosis. These germ cells are known as germline stem cells or GSCs, and are located in the distal region called the "mitotic zone" or "proliferative zone" (Crittenden et al., 2006; Pazdernik & Schedl. 2012). GSCs are controlled by the somatic distal tip cell (DTC) (Kimble, 2005), which provides the niche at the tip of the mitotic zone and signals to the GSCs through the GLP-1/Notch signaling pathway to regulate the mitosis/meiosis cell cycle decision (Kimble, 2014). As germ cells move proximally along the gonad, they enter meiosis. In hermaphrodites, during the L4 larval stage, a proximal pool of germ cells undergoes meiotic divisions to produce a fixed number of sperm which are stored in the spermatheca. Further meiotic differentiation produces oocytes, which are fertilized as they pass through the spermatheca into the uterus (Kimble, 2011).

The use of the *C. elegans* germ line provides a powerful model for stem cell biology research. *C. elegans* GSCs display many common features with other stem cell systems, such as the capability to self-renew and differentiate. Furthermore, the fast-growing nature of *C. elegans*, as well as the relative ease of genetic and molecular manipulations, makes it an advantageous model for exploring GSC biology. The transparent nature of *C. elegans* also allows direct observation of the mitotic division of GSCs using *in situ* live imaging with fluorescent proteins. Since most mitotic proteins are conserved in *C. elegans* (Pintard & Bowerman, 2019), any insights and findings relating to mitotic processes in this organism are likely to have wider implications and applicability to other biological systems.

1.1.1. *C. elegans* response to unfavorable environmental conditions

C. elegans can sense changes in the environment and adapt accordingly to increase the chance of survival. Under favorable conditions, the animal grows and reproduces rapidly. However, under unfavorable conditions, such as limited food availability, L1 and L2 larvae can shift their developmental program to dauer formation, which is a larval arrested state. *C. elegans* dauer larvae have a distinct morphology and behavior compared to larvae grown under normal environmental conditions (Cassada & Russel, 1975). A crucial feature of the dauer program involves the arrest of germ cell proliferation. This inhibition of germline proliferation is primarily mediated through the regulation of AAK-2/AMPK, which is targeted by TGF-β and insulin-dependent signaling through DAF-18/PTEN (Narbonne & Roy, 2006). The dauer development decision is made in the late L1 larval stage in response to environmental cues including population density, food supply, and temperature (Hu, 2007). Dauer larvae have the capacity to transition from the stress-resistant and non-feeding stage to reproductive adults upon the occurrence of optimal environmental conditions

(Golden & Riddle, 1984). Additionally, germ cell proliferation can rapidly resume when conditions are favorable.

Dietary restriction, which refers to the reduced intake of food without malnutrition, has been linked to a reduced risk of cardiovascular and age-related diseases in humans (Redman & Ravussin, 2011). Similarly, in *C. elegans*, dietary restriction has been found to affect aging and longevity (Walker et al., 2005). The genetic basis of this phenomenon has been elucidated through studies that identified insulin/IGF-1 signaling and the DAF-16/FOXO transcription factor as a modulator of lifespan extension in *C. elegans* by dietary restriction (An et al., 2016; Wu et al., 2021). However, DAF-16/FOXO is not required for the lifespan extension of *eat-2(ad1116)* mutant worms, a genetic way to mimic dietary restriction (Lakowski & Hekimi, 1998; Greer et al., 2009). This suggests that different methods of dietary restriction may have independent genetic mechanisms that contribute to the extension of lifespan (Greer et al., 2009).

During development, GSCs are particularly sensitive to dietary restriction. Dietary restriction has been shown to reduce the number of GSCs, and TOR-S6K signaling plays a critical role in the nutrient-responsive regulation of germline progenitors (Korta et al., 2012). In addition, we have previously reported that dietary restriction mediates a delay in mitotic duration, although the underlying mechanisms remain largely unknown (Gerhold et al., 2015).

1.2. The Cell Cycle

The cell cycle is a series of events that result in the duplication of a cell's DNA and the segregation of the copies into two genetically identical daughter cells (Alberts et al., 2002). The cell cycle consists of four phases: G1, S, G2, and M. G1, S, and G2 phase constitute interphase, which is when the cell duplicates its DNA and grows in size to prepare for division during M phase. Cell cycle progression is tightly regulated to ensure successful cell division. To ensure the

appropriate cell growth and correct duplication and transmission of genetic information, cells have developed cell cycle checkpoints as surveillance mechanisms (Barnum & O'Connell. 2014). The G1/S cell cycle checkpoint prevents cell passage from the G1 phase into S phase in response to errors in cell size and/or DNA integrity. If cells do not satisfy this checkpoint, they may enter a resting state called G0, and they may re-enter the G1 phase once errors are fixed. During S phase, the cell undergoes DNA synthesis to replicate its genetic contents. As a cell progresses to the G2 phase, it grows rapidly and produces proteins necessary for mitosis. Before entering mitosis, the G2/M checkpoint helps maintain genomic stability by checking for and repairing DNA damage and/or incomplete DNA replication (Stark & Taylor, 2004).

1.2.1. Mitosis

After the G2 checkpoint is satisfied, the cell enters mitosis by which the nuclear and cytoplasmic contents are segregated into two daughter cells. During prophase, chromatin condenses into chromosomes, with pairs of replicated sister chromatids joined at the centromere (Rehman et al., 2022). Centrosomes, the microtubule organizing centers for the mitotic spindle, migrate to the opposite sides of the nucleus. After nuclear envelope breakdown, in prometaphase, microtubules grow from the centrosomes and bind to the sister chromatids via a large protein complex called the kinetochore that assembles at the centromere. Prometaphase ends when sister chromatids are aligned at the middle of the cell, which marks the start of metaphase (Malumbres, 2014). Before cells can proceed from metaphase into anaphase, a surveillance mechanism called the Spindle Assembly Checkpoint (SAC) ensures mitotic fidelity by preventing anaphase onset in the presence of improper kinetochore-microtubule attachments (Lara-Gonzalez, 2012). Once the SAC is satisfied, SAC inactivation leads to the cleavage of cohesin to allow for the separation of sister chromatids, and the single chromatids are dragged apart by the mitotic spindle in anaphase

(Silva et al., 2018). After the partitioning of the nuclear contents, the cell is physically divided into two daughter cells during cytokinesis.

1.2.2. Mitotic Fidelity

Mitotic fidelity refers to the ability of cells to divide their genetic material and ensure that each daughter cell receives a complete and identical set of genetic material. The process of mitosis involves a series of tightly regulated events, including chromosome condensation, spindle formation, and chromosome segregation. Failure to correctly execute any of these steps can result in chromosomal abnormalities, such as aneuploidy, which can lead to cell death, genetic disorders, and cancer (Santaguida & Amon, 2015). One of the key factors that contributes to mitotic fidelity is the SAC. Unattached kinetochores promote the assembly of the mitotic checkpoint complex (MCC), which consists of four proteins Mad2, BubR1, Bub3, and Cdc20 (Sudakin et al., 2001). Active MCC mediates the inhibition of the anaphase-promoting complex/cyclosome (APC/C) to stop the degradation of cyclin B and securin, which prevents cells from entering anaphase (Moyel et al, 2014; Henriques et al, 2019; Nasmyth, 2000), thus resulting in prolonged mitotic duration. If the SAC is impaired, cells may enter anaphase prematurely, resulting in chromosome missegregation (Meraldi et al., 2004).

SAC signaling is highly conserved in many organisms. In *C. elegans*, SAC signaling is functionally and structurally conserved, and many studies have reported that disrupting spindle assembly and/or microtubule dynamics activates the SAC and leads to the extension of mitotic duration in *C. elegans* embryos, as in other organisms (Kitagawa, 2009. Encalada et al., 2005). Previous work in our lab also showed that the SAC is important for normal mitotic timing in GSCs. Specifically, targeted RNAi depletion of core SAC components including Mad1, Mad2, and Mad3

led to a marked reduction in the duration of mitosis in GSCs even in the absence of spindle perturbations (Gerhold et al., 2015).

1.3. Cell Signaling

C. elegans GSCs display common features with other stem cell systems that interpret and respond to physiological changes through highly conserved signaling mechanisms (Figure 1B). To survive in response to changes in the environment, cells need to translate extracellular signals into specific cellular responses (Torres, 2006). One of the most extensively studied signaling pathways is the insulin pathway. In humans, elevated levels of blood glucose can prompt insulin to engage with the insulin receptor tyrosine kinase located on the cell surface, triggering a signaling cascade that ultimately targets the Forkhead transcription factor FoxO to regulate a variety of cellular and biological functions (Kramer, 2016; Lee & Dong, 2017). This pathway is highly conserved in *C. elegans* where insulin/IGF-1 signaling regulates lifespan and cell proliferation (Meyts, 2016). In addition to insulin/IGF-1 signaling, many other signaling pathways are functionally and genetically conserved in *C. elegans* including the Notch signaling, MAPK pathway, TGF-β signaling, etc. (Fabian et al., 2021).

1.3.1. Insulin/IGF-1 signaling

In *C. elegans*, the Insulin/IGF-1-like signaling (IIS) pathway starts at the receptor DAF-2, which is a homolog of the human insulin receptor. DAF-2 is regulated by the binding of insulin-like peptide ligands. DAF-2 controls the activity of the PI3K/AGE-1 kinase cascade, which catalyzes the conversion of PIP2 to PIP3, which then activates the serine/threonine kinases PDK-1, AKT-1, and AKT-2 (Murphy, 2013). As a result, the phosphorylated DAF-16/FOXO transcription factor is sequestered in the cytoplasm and its translocation to the nucleus is inhibited (Zečić & Braeckman, 2020). The DAF-18/PTEN lipid phosphatase acts as an inhibitor of IIS,

which functions between AGE-1 and AKT-1, 2 to reduce signaling by dephosphorylation of PIP3 (Ogg & Ruvkun, 1998).

In *C. elegans*, the IIS pathway regulates longevity and aging. *daf-2* mutants have a lifespan that is twice as long as wild-type worms (Kenyon et al., 1993). The strong *age-1/PI3K* alleles *mg44* and *m333* exhibit developmental delays and a longer lifespan than wild-type worms (Ayyadevara, 2009). In addition to the regulation of longevity and aging, the IIS pathway is also known to promote germline proliferation. The IIS pathway is required for the accumulation of germ cells during larval development and when DAF-2 activity is partially compromised using the conditional *daf-2(e1370)* allele, adults have fewer germ cells in the distal mitotic zone compared to the wild-type worms (Michaelson, 2014). Our lab has also shown that the IIS pathway affects GSC mitosis. Partial inhibition of *daf-2,* by either mutant allele or RNAi, extends the duration of mitosis, and this regulation is dependent on *daf-18* and *daf-16* (E. Cheng, unpublished).

1.3.2. Notch signaling

The Notch signaling pathway is another highly conserved cell signaling system. Two notch genes in *C. elegans*, *lin-12* and *glp-1*, encode for the notch transmembrane receptors and are best known for their role in mediating cell fate decisions (Artavanis-Tsakonas et al., 1999). The DSL proteins are the ligands of the Notch receptor. In *C. elegans*, the *lag-2* gene, which encodes the DSL protein LAG-2, is expressed in the DTC and drives the activation of Notch signaling via DTC/germline interaction (Henderson et al., 1994). The interaction between the LAG-2 protein and GLP-1/Notch receptor results in the translocation of GLP-1/Notch intracellular domain (NICD) from the cell membrane to the nucleus to regulate the expression of the genes *lst-1* and *sygl-1*, which promote GSC mitotic proliferation (Kershner et al., 2014). In addition, the expression of *fbf-1* represses the meiosis-promoting gene *gld-1,* and the expression of *fbf-2* represses the meiosis-

promoting gene *gld-2*. (Kimble & Crittenden, 2007). GLP-1/Notch signaling is thought to primarily regulate GSCs by regulating the mitosis-meiosis decision, an effect that is distinct from cell cycle progression or checkpoint regulation (Hubbard & Schedl, 2019). The extent to which the GLP-1/Notch signaling pathway regulates GSC mitotic progression remains largely unknown.

1.3.3. Transforming Growth Factor-β

Transforming Growth Factor-β (TGF-β) signaling pathways are required for development and homeostasis in many animals (Savage-Dunn & Padgett, 2017). In *C. elegans*, five genes encoding TGF-β-related proteins have been identified: *dbl-1, daf-7, unc-129, tig-2,* and *tig-3*. Among these, *dbl-1* and *daf-7* act as ligands for two serine/threonine kinase transmembrane receptors, type-1 receptor SMA-6 and type II receptor DAF-4, and regulate different aspects of animal development (Gumienny, 2013; Savage-Dunn & Padgett, 2017). DBL-1 is a bone morphogenetic protein (BMP) homolog in *C. elegans* which targets the type-I receptor SMA-6, type-II receptor DAF-4, and the Smads (R-Smads SMA-2, SMA-3, and Co-Smad SMA-4) and controls body size, male tail development, and innate immune responses (Krishna et al., 1999; Savage-Dunn et al., 2000).

A second TGF-β pathway, the DAF-7 pathway, is known to regulate dauer development. The DAF-7 pathway does not require the type-I receptor SMA-6. Instead, the ligand DAF-7 targets the type-II receptor DAF-4 and type-I receptor DAF-1, which then phosphorylate the R-Smads DAF-8, and DAF-14 (Gumienny, 2013). The upstream R-Smads antagonize the Sno/Ski transcriptional co-factor, DAF-5, which binds to the Co-Smad DAF-3 (da Graca et al., 2004). Mutation in *daf-7* results in a temperature-sensitive dauer phenotype and this phenotype can be rescued by mutations in *daf-3* and *daf-5* (Patterson et al., 1997; da Graca, 2004).

In addition to dauer regulation, recent studies have shown that the DAF-7/TGF- β pathway acts in the GSC niche to control germline development. RNAi depletion or mutation of *daf-7*, *daf-1*, and *daf-4* resulted in a reduced number of GSCs and a reduced brood size. RNAi depletion of *daf-3* and *daf-5* in the *daf-7* or *daf-1* mutant background suppressed the reduced GSC phenotype (Dalfó et al., 2012). The TGF-β receptor and its downstream components act in the DTC to promote *lag-2* expression, suggesting that DAF-7/TGF-β signaling may regulate germline development by modulating Notch activity (Dalfó et al., 2012; Pekar et al., 2017).

1.3.4. The Target of Rapamycin

The Target of Rapamycin (TOR) is critical for development, reproduction, and aging and has been studied extensively in yeast and mammalian cells. Studies have identified two core TOR-binding proteins: Raptor and Rictor, and the interaction of TOR and these TOR-binding proteins forms two functionally different TOR complexes: TORC1 and TORC2 (Blackwell et al., 2019). *C. elegans* has a highly conserved TOR signaling system, and the knockdown of TOR ortholog, *let-363*, by either RNAi depletion or mutation causes developmental arrest in the L3 stage (Long et al., 2002). LET-363/TOR and DAF-15/Raptor together with other proteins form TORC1, which directly phosphorylates the downstream target ribosomal protein S6 kinase (S6K), encoded by *rsks-1* in *C. elegans* (Korta et al., 2012; Pan et al., 2007). Core components of TORC2 include the conserved protein RICT-1/Rictor, TOR, and other proteins. Unlike TORC1, the upstream inputs and downstream targets of TORC2 are not well-understood. Genetic studies have identified several possible downstream targets of TORC2 including SGK-1, SKN-1, DAF-16, and DAF-7 (Blackwell et al., 2019).

Many studies have shown that the TOR pathway is linked to aging. Inhibition of the TOR pathway by RNAi knockdown of *let-363* or *daf-15* extends the *C. elegans* life span (Johnson et al.,

2013; Vellai et al., 2003). In addition, dietary restriction induced lifespan extension is mediated by reduced TORC1 activity (Heintz et al., 2016). TOR signaling also plays an important role in germline development. The loss of *rsks-1* results in ~50% reduction in the number of GSCs, which can be rescued by expressing *rsks-1* in the germ line only, suggesting that *rsks-1* acts germline-autonomously to maintain GSCs (Korta et al., 2014; Roy et al., 2018). In addition, germline defects caused by dietary restriction are similar to those seen in *rsks-1* mutants, and the loss of *rsks-1* results in the germ line being insensitive to dietary restriction, suggesting that TOR-S6K signaling pathway is a key nutrient-responsive regulator of germline progenitors (Korta et al., 2014).

1.3.5. RAS/MAPK

The RAS/MAPK signaling pathway plays a central role in controlling various cellular processes, such as proliferation, differentiation, cell cycle regulation, and apoptosis. It is particularly of interest to many researchers because of its role in tumor formation and growth (Guo et al., 2020). *C. elegans* RAS/MAPK signaling pathway starts at the Receptor Tyrosine Kinase (RTK), LET-23 (EGFR) or EGL-15 (FGFR). Upon growth factor binding, the RTK activates the small GTPase LET-60/Ras. Activated LET-60 then binds to LIN-45/Raf and promotes activity of the MAPK cascade with help from a scaffold protein KSR. The MAPK cascade consists of LIN-45, which phosphorylates MEK-2, and MEK-2 then phosphorylates MPK-1 (Sundaram, 2006).

The RAS/MAPK signaling pathway plays an important role in several developmental processes in *C. elegans.* The *let-60* gene is essential for vulva development, the loss of *let-60* results in a vulvaless phenotype; whereas *let-60* gain of function mutations lead to multivulva development (Beitel et al., 1990; Han et al., 1990). In the germ line, a loss of function temperature sensitive *mpk-1* mutant allele, *mpk-1*(ga111), has a strong pachytene arrest phenotype at the

restrictive temperature that results in complete sterility as germ cells cannot progress through the meiotic cell cycle (Lee et al., 2007). In addition, in adults, the *mpk-1* null mutant also has reduced GSC proliferation (Narbonne et al., 2017). However, this regulation of GSC proliferation is mediated non-autonomously by the soma-specific MPK-1A isoform, while the germline-specific isoform MPK-1B promotes germ cell differentiation (Robinson-Thiewes et al., 2020).

1.3.6. Wnt and Hippo Signaling Pathways

The canonical Wnt pathway controls the expression of target genes through β-catenin. The binding of Wnt to the receptor causes inhibition of β-catenin degradation, which then interacts with transcription factors to induce cell fate specification during *C. elegans* embryonic development by regulating cell polarity (Sawa & Korswagen, 2013; Korswagen et al., 2002; Thorpe et al., 2000). The Wnt/β-catenin pathway also controls mitotic spindle orientation in *C. elegans* embryos (Schlesinger et al., 1999; Walston et al., 2004). In *Drosophila*, the canonical Wnt signaling has been shown to promote germ cell differentiation (Wang et al., 2015). However, if the signaling pathway also affects the *C. elegans* GSC proliferation or differentiation is largely unknown.

The Hippo pathway was first discovered in *Drosophila* in studies to understand the regulation of organ size. The core components of the Hippo pathway, Hippo and Warts, are protein kinases that limit the function of the transcriptional coactivator Yes-associated protein (Yap) to control tissue growth (Li et al., 2019). In multiple studies, Yap has been shown to associate with transcription factors to control cell proliferation and apoptosis (Harvey et al., 2003; Jin et al., 2020). In *C. elegans*, the *yap-1* gene encodes for YAP homolog YAP-1 which is regulated by Warts homolog (WTS-1) encoded by *wts-1* (Yang & Hata, 2013). Studies in mammals and *Drosophila* have shown that there is an interaction between the Hippo pathway and Wnt/ β-catenin through

the Hippo pathway mediator (Lee et al., 2018; Li et al., 2019; Varelas et al., 2010). However, very little is known about the function of the Hippo pathway in *C. elegans*, and the interaction between Hippo and Wnt signaling in *C. elegans* has not been investigated.

In summary, multiple studies have demonstrated that cell cycle progression in *C. elegans* GSCs can be modulated by various external physiological cues, such as temperature, nutrition, and oxidative stress, as well as by intracellular signaling pathways, such as the insulin/IGF-1 signaling pathway. However, whether these factors influence mitotic process specifically, or have an impact on mitotic fidelity, remains largely unknown, particularly in the context of a living organism. The *C. elegans* germline provides an ideal model system for investigating mitosis *in vivo* due to its highly conserved genetics and transparent nature. By utilizing RNAi and genetic alleles, my primary objective was to identify the specific signaling pathways that impact GSC mitosis and to start to elucidate the underlying mechanisms involved. Additionally, I undertook to explore the mechanism by which dietary restriction affects GSC mitosis. Overall, I hope that this study will provide a deeper understanding of the relationship between changes in animal physiology and alterations in signaling pathways and/or environmental factors that impact the mitotic process. Hopefully, the findings of this study may have implications beyond the context of *C. elegans* and may have relevance for other stem cell systems in diverse organisms, particularly humans.

2.0. Methodology

2.1. *C. elegans* Strains and Culture

C. elegans strains were maintained at 20°C on nematode growth media (NGM) plates seeded with OP50 or HT115 *E. coli* as the food source according to standard protocols (Brenner, S. 1974). Temperature sensitive strains (ts) were grown at the permissive temperature of 15°C. Strains used for RNAi screening were ARG50 *(ijmSi7 [pJD348/pSW077; mosI_5'mex-5_GFP::tbb-2; mCherry::his-11; cb-unc-119(+)] I)* and ARG45 *(ijmSi7 [pJD348/pSW077; mosI_5'mex-5_GFP::tbb-2; mCherry::his-11; cb-unc-119(+)] I; [sun-1p::rde-1::sun-1 3'UTR + unc-119(+)] II ; rde-1(mkc36) V)*. Mutant alleles ordered from the *Caenorhabditis* genetics center (CGC) or gifted from other labs were crossed to ARG50 for signaling pathway candidate screening. *eat-2(ad465) II, daf-16(mu86)*, and *daf-18(nr203)* alleles were crossed into UM225 *(ojIs1[unc-119(+) pie-1::GFP::tbb-2] V)* strain for analysis of dietary restriction. All strains and alleles used in this study are listed in Table 2.

2.2. Worm Strain Generation

In order to perform fluorescent imaging, all alleles were crossed to ARG50 to obtain the fluorescent proteins (TBB-2::GFP, HIS-11::mCH) which are located on chromosome I MosSCI - 5.32. Male ARG50 was generated by heat shock at 30°C for 6 hours, and ARG50 males were crossed to late L4 mutant allele hermaphrodites at a ratio of 3:1. Following 24 hours of mating, individual hermaphrodites were carefully transferred onto a fresh OP50 NGM plate. The success of mating was confirmed through observation of approximately 50% of males in the F1 generation. The F1 generation was anticipated to be heterozygous for both fluorescent proteins (FPs) and the mutation under investigation. From this cohort, 5 heterozygous F1s were picked for self-mating. The number of F2 progeny picked for phenotype and/or genotype analysis was based on the

location of the mutation. For example, *daf-8(e1393)* is located at MosSCI 2.97 on the same chromosome as the FPs. The probability of obtaining a homozygous FP and *daf-8* mutation in the F2 generation was approximately 3.6%. Accordingly, 100 F2 L1s were picked individually onto each OP50 plate and were shifted to 25°C to assess for the dauer phenotype, which is an indication of homozygous *daf-8* mutation. Dauer worms were subsequently recovered at 15°C, and the F3 generation was screened for homozygous FPs using an upright epi-fluorescence microscope. In the case of mutant alleles lacking an observable phenotype, primers listed in Table 3 were constructed for the purpose of conducting PCR analysis to validate the efficacy of the cross.

2.3. RNA Interference

To introduce RNAi via feeding, RNAi bacteria cultures were grown overnight in LB with ampicillin (final concentration 0.1 mg/mL) at 37 °C. Precultures were prepared by adding 20 µL of overnight culture to 2 mL of LB with ampicillin and incubating for 4-5 hours at 37 °C or until OD_{600} of ~0.5. 100 µL of preculture was added to 35 mm NGM plates that contain IPTG (0.1mM) and Carbenicillin (0.05 mg/ml). RNAi plates were then incubated at room temperature in the dark for 3 days to induce the expression of double-stranded (ds) RNA. Synchronized L1s were added to the plates and were imaged at the late L4 stage.

To introduce RNAi via soaking, a population of adult worms was washed in sterile M9 solution and bleached for embryo collection. These embryos were subsequently immersed in sterile M9 solution and shaken overnight at a temperature of 15°C to acquire synchronized L1s. To induce RNAi, a fresh soaking solution was prepared consisting of 4 µg of dsRNA, 2 µL of 63nM spermidine, 2 µL of 1.1% gelatine, and 12 µL of 1X soaking buffer. The soaking buffer was prepared from a 3X stock solution containing 32.7 mM Na_2HPO_4, 16.5 mM KH_2PO_4, 6.3 mM NaCl, and 14.1 mM NH_4Cl. 40 synchronized L1s were added into soaking solution and incubated

at 20°C for 24 hours. Subsequently, 40 synchronized L1 worms were added to the soaking solution and incubated at a temperature of 20°C for 24 hours. The treated worms were then transferred to RNAi feeding plates or HT115 plates and grown for 44-48 hours to image at late L4 stage.

Plasmid L4440 was used as an empty RNAi vector control. Other RNAi clones used for screening were listed in Table 1. All RNAi clones, except for *daf-2*, were from the Ahringer library (Kamath et al., 2003) and were gifted from the Labbé or Roy labs. All RNAi clones were validated by sequencing. *gld-2* RNAi targets both *gld-1* and *gld-2*, hereafter referred to as *gld1/2.*

2.4. Dietary Restriction Protocol

Dietary restriction was accomplished by two methods. The first method was by dilution of bacteria (sDR). A fresh HT115 overnight culture was diluted 1:10 in S basal medium and 150 µL of this diluted culture was added to a standard 35 mm NGM plate containing 0.05 mg/mL carbenicillin to prevent additional HT115 growth. The undiluted culture was used as the control. Three adult worms were washed in M9 buffer and then added to the sDR or control plates for egg collection for 2-4 hours. Worms were analyzed at the late L4 stage 72 hours later. For adult analyses, an additional 150µL of diluted bacteria was added after 2 days to prevent complete starvation.

The second method of dietary restriction used an *eat-2* mutant allele. *eat-2(ad465)* animals were raised on HT115 plates containing 0.05 mg/mL carbenicillin to allow for comparison with sDR plates. Worms were synchronized and analyzed following the same protocol as sDR.

2.5. Worm Mounting and Live Imaging

Animals were mounted on a 3% agarose pad following the previously reported protocol (Gerhold et al., 2015; Zellag et al., 2022). In brief, melted 3% agarose was dropped onto a glass slide, followed by the placement of a plastic/silicon groove-molded chip to cover the agarose.

Subsequently, the solidified agarose pad was utilized for the mounting of L4 worms, wherein the worms were immersed in a freshly prepared solution of 0.04% Tetramizole and then carefully placed onto the grooves via a mouth pipette. Finally, a coverslip was added on top, the edges were sealed using Valap, and the area under the coverslip was backfilled with the remaining Tetramizole to prevent desiccation and to inhibit movement during live imaging. Imaging was carried out on a Nikon CSU-X1 spinning disk confocal microscope using an Apo 40×/1.25 numerical aperture (NA) water-immersion objective and Nikon Elements software. Images were taken every 30 seconds for a total of 40 minutes duration. Each frame consisted of 27 0.75μm optical sections through the entire distal gonad. Two-color time-lapse movies (TBB-2::GFP, HIS-11::mCH) were acquired with a laser intensity of 3% and 5% respectively, and 80ms exposure time.

2.6. Image Analysis

Images were processed and analyzed using ImageJ2. The original .nd2 files (Nikon Elements file format) were converted to .tif file format using ImageJ and the movie was corrected for worm movement using in-house ImageJ macros. Centrosomes were tracked and paired following our reported protocol (Zellag et al., 2021). Briefly, tracking and pairing of centrosomes are accomplished using the open-source plug-in Trackmate in Fiji (Schindelin et al., 2012; Tinevez et al., 2017). Trackmate facilitates the automatic detection and tracking of each centrosome across the entire time course, while also providing the option for manual correction of the tracking. The paired centrosome coordinates for every cell are subsequently extracted and exported to MATLAB for the generation of inter-centrosome distance versus time graphs (Figure 1C). This graph can be further utilized for the quantitative analysis of important mitotic features, such as spindle dynamics and mitotic duration. The duration of mitosis was quantified by measuring the time interval between the point at which centrosome-to-centrosome distance reached its minimum (CongS)

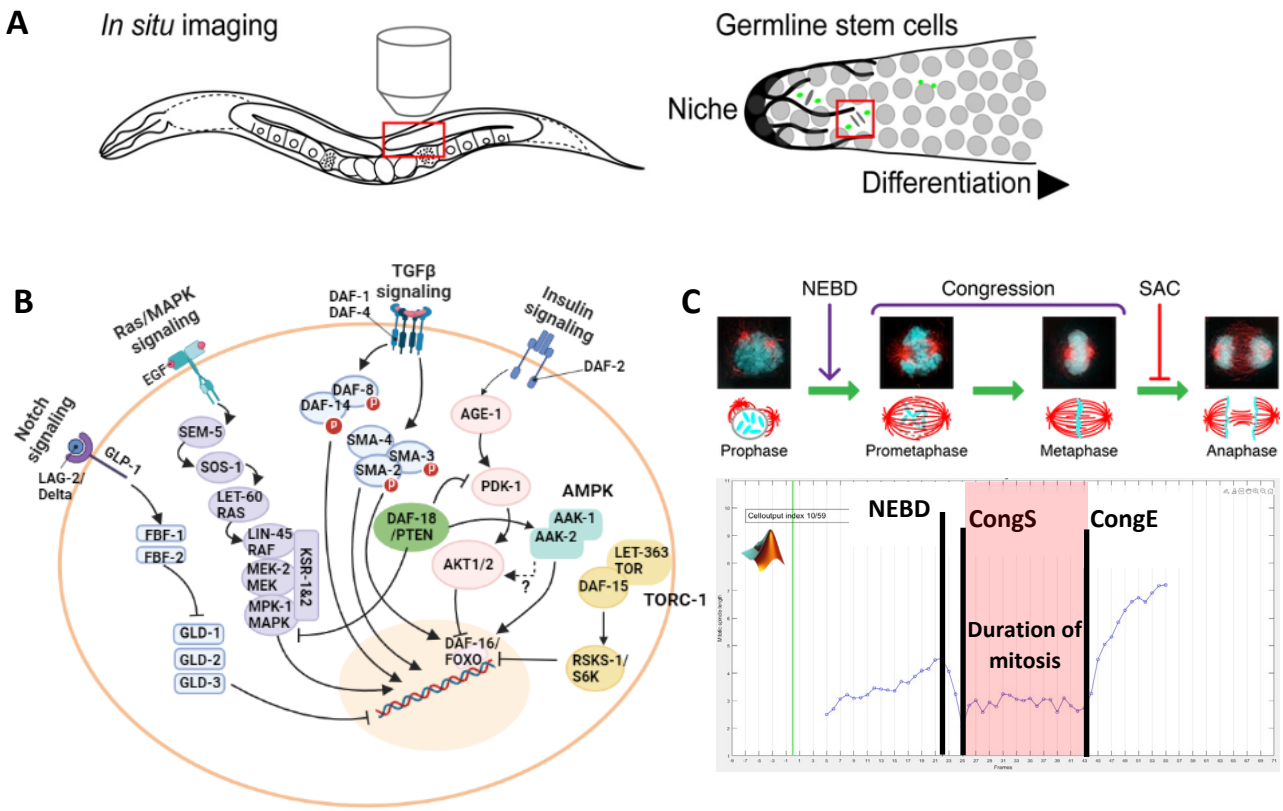
following nuclear envelope breakdown (NEBD) and the onset of anaphase when centrosomes start to separate rapidly (CongE).

The distal mitotic zone was defined as the region from the first cell in the distal tip to the transition zone, marked by the occurrence of meiotic cells featuring crescent-shaped nuclei. Nuclear counts within the mitotic zone were performed in Imaris software using spot detection. First, an ROI was defined to select the entire mitotic zone. Then, the settings for spot detection were adjusted to use a sphere point style and to set the radius scale to 3μm, which corresponds to the average radius of the GSC nuclei. After spot generation, spots were curated manually to add or delete spots as necessary. Total cell count, as found in the statistics tab, was exported to excel. Cells with centrosomes appearing at the opposite side of the cell are counted as mitotic cells. The mitotic index was calculated as the number of mitotic cells divided by the total nuclear count in the mitotic zone.

2.7. Graphing and Statistical Analysis

Statistical analysis was performed in Matlab. One-way ANOVA or Kruskal Wallis test was performed for multiple comparisons. For all statistical tests, *$p \leq 0.05$, **$p \leq 0.01$, p***≤ 0.001, and n.s., $p > 0.05$. Most graphs were generated in Matlab.

Figure 1



(A) A schematic image of the *C. elegans* and the distal tip of the germline. Reproduced from (Gerhold et al., 2015) with permission.

(B) A schematic image of the cell signaling pathways analyzed in this study and their known interactions.

(C) A schematic image of mitotic progression and a sample image of the inter-centrosome distance versus time graphs generated in MATLAB. NEBD is when spindle length rapidly decreases, CongS is when spindle length reached a constant minimum following NEBD, and CongE is when spindle length rapidly increases, marks the onset of anaphase.

3.0. Results

3.1. Candidate screen by RNA interference

RNA interference (RNAi) is a widely used technique to elucidate gene function by introducing double-stranded RNA (dsRNA) into an organism to silence the targeted endogenous gene (De-Souza et al., 2019). In order to generate bacteria that express specific dsRNA, the target DNA fragment is cloned into the multiple cloning site in the L4440 vector, flanked by T7 promoters on either side (Kamath, 2003). RNAi can be delivered to an organism via different routes, including feeding, soaking, or injection. In this study, I employed RNAi by soaking and/or by feeding to perform a candidate gene screen for mitotic phenotypes (Table 1, Figure 2A). The candidate genes that were selected in this study are essential components of signaling pathways that regulate cell proliferation. However, it is not well studied whether the knockdown of these genes has an impact on the progression of mitosis. To ensure the specificity of RNAi, all RNAi sequences were validated by PCR amplification, followed by gel electrophoresis and sequencing.

Because time-lapse live imaging and analysis can be time-consuming, I first sought to identify signaling pathways that are most likely to affect mitosis using single time-point analysis to look for mitotic phenotypes, including the number of mitotic cells in the mitotic zone, mitotic index, and nuclear and/or spindle defects that might indicate mitotic defects. I performed whole-worm RNAi depletion on worms that carry our preferred GSC fluorescent markers: TBB-2::GFP and HIS-11::mCH, to mark mitotic spindles and germ cell nuclei, respectively. My results showed that RNAi-mediated whole worm depletion of cyclin-dependent kinase 1 (*cdk-1*) and the Notch receptor *glp-1* led to abnormal germline development and, in some animals, a complete loss of the germ line. In addition, depletion of the meiosis-promoting genes *gld-1* and *gld-2* resulted in an enlarged germline, and in some worms, tumorous growth was observed (Figure 2B). Conversely,

RNAi depletion of other candidate genes did not result in any noticeable phenotypic changes in the worms.

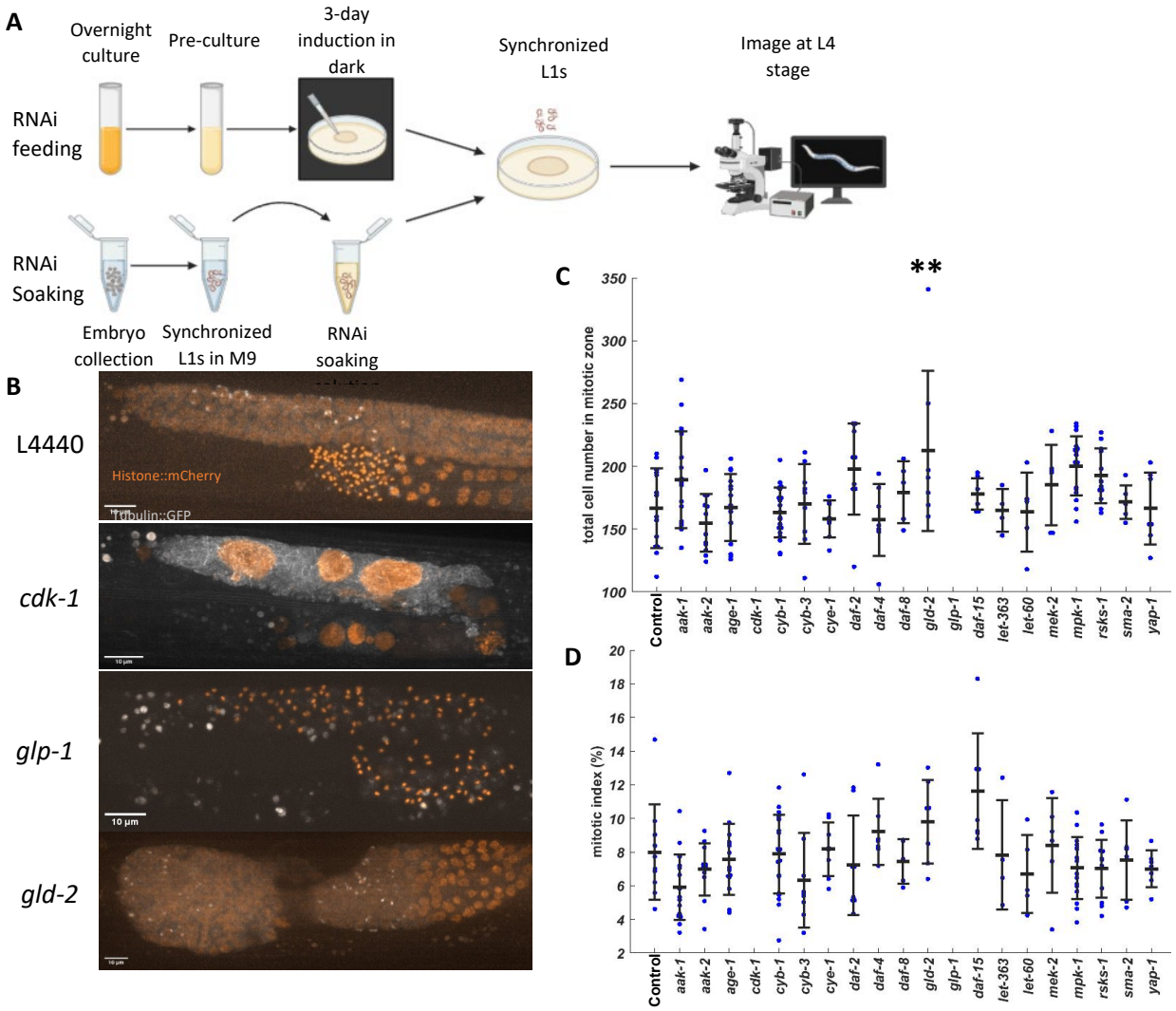
To evaluate the effect of RNAi on GSCs specifically, I quantified the number of mitotic cells and the total number of cells in the germ line mitotic zone to calculate the mitotic index. My data showed that *gld-1/2* RNAi led to a significant increase in the number of germ cells in the mitotic zone, but the mitotic index did not increase (Figure 2C, D). Unexpectedly, RNAi depletion of the RPTOR ortholog *daf-15* led to an apparent, although not statistically significant, increase in the mitotic index (Figure 2D). *Daf-15* RNAi should impair TOR pathway activity and would be expected to reduce cell proliferation (Qi et al., 2017).

To distinguish between cell-autonomous and non-autonomous effects, I also performed germline-specific RNAi depletions. In *C. elegans*, *rde-1* encodes for the Argonaute protein, which performs the crucial role of ensuring RNAi efficiency in a cell-autonomous manner (Tabara et al., 1999). We used a *C. elegans* strain that carries an indel mutation in *rde-1* and a *rde-1* transgene driven by the *sun-1* germline-specific promoter to allow for germline-specific RNAi (Zou et al., 2019), and which also expresses TBB-2::GFP and HIS-11::mCH in the germline. My data showed that the depletion of candidate genes via germline-specific RNAi had analogous outcomes to those attained via whole-worm RNAi depletion (Supplemental Figure S1). The depletion of germline *cdk-1* and *glp-1* RNAi led to significant mitotic phenotypes, suggesting that *cdk-1* and *glp-1* regulate germ cell proliferation in a germline-autonomous manner. Conversely, the lack of notable phenotypic consequences for other candidate genes following germline-specific RNAi depletion may imply a more restricted role in germline cell physiology.

While some of my RNAi treatments produced the expected phenotype (e.g., *glp-1*), other results did not match my expectations. Previous studies have reported that the S6 kinase *rsks-1*

acts germline-autonomously to expand the GSC pool, and loss of *rsks-1* results in a ~50% reduction in the size of the mitotic zone (Korta et al., 2012). RNAi against the mitogen-activated protein kinase *mpk-1* leads to a vulvaless phenotype (Okuyama et al., 2010). Additionally, loss of *mpk-1* leads to sterility due to pachytene arrest (Lee et al., 2007; Yoon et al., 2017). However, none of these phenotypes were observed in our RNAi experiment. Consequently, it remains unclear whether the lack of phenotype following RNAi depletion of the target genes was due to low RNAi efficiency or whether the depletion of the target genes alone is not sufficient to induce changes in GSC mitosis. Therefore, I decided to use *C. elegans* alleles to further examine whether loss or reduction-of-function of candidate genes results in GSC mitosis phenotypes. This approach will allow me to validate the RNAi results and gain a deeper understanding of the genes involved in GSC mitosis.

Figure 2: Candidate signaling pathways screen by RNAi



(A) A schematic outline of the RNAi feeding and soaking protocol.
(B) Maximum intensity projections of representative germ lines following L4440, *cdk-1*, *glp-1*, and *gld-2* whole worm RNAi. Germ cell nuclei (HIS-11::mCH; orange) and microtubules/spindles (TBB-2::GFP; white) were visualized using the strain ARG50. Scale bar = 10µm
(C) Beeswarm plot showing the total number of cells in the mitotic zone per gonad of worms following RNAi treatment. Control = ARG50 following L4440 RNAi.
(D) Beeswarm plot showing the mitotic index of the germline mitotic zone in worms following RNAi treatment. The mitotic index was calculated as the number of mitotic cells divided by the total number of cells in the mitotic zone. Control = ARG50 following L4440 RNAi.
For (C) and (D), each dot represents data for one germ line. Black bars represent the mean and error bars represent the standard deviation. Data were compared using a one-way ANOVA (**: $p \leq 0.01$)

3.2. Candidate screen using *C. elegans* mutant alleles

Temperature-sensitive mutations facilitate detailed analysis of gene function by allowing for a reduction in gene activity with a simple temperature shift during the course of cell division or development (Varadarajan et al., 1996). For example, a temperature-sensitive allele of the TGF-β ligand *daf-7(e1372)* at 15°C behaves comparably to the wild type allele. Shifting *daf-7(e1372)* worms from the permissive temperature (15°C) to the restrictive temperature (25°C) results in 100% dauer formation. Whereas shifting the animals from 15°C to the semi-permissive temperature (20°C) instead leads to worms with smaller body size and a smaller pool of GSCs (Dalfó et al., 2012). The semi-permissive temperature thus serves as a critical tool for studying gene function by allowing the study of gene function at a reduced level rather than complete knockdown. This is particularly advantageous when examining genes that are essential for cell survival or development.

To determine whether reducing the activity of candidate genes affected GSC mitosis, I selected core components of each signaling pathway and obtained *C. elegans* mutant alleles from either the CGC or other laboratories (Table 2). For fluorescent microscopy, all strains, except JK2879, were crossed with our control strain ARG50, which harbors the fluorescent markers TBB-2::GFP and HIS-11::mCH for the visualization of mitotic centrosomes and chromosomes, respectively. Each of these strains was then used for live-cell imaging of GSC mitosis to look for differences in mitotic timing and/or mitotic defects, and to determine the number of mitotic cells per germ line as a proxy for the mitotic index.

My findings indicate that GSCs in animals bearing a loss-of-function allele of *daf-7* (*daf-7(e1037)* exhibit a longer duration of mitosis compared to wild type (Figure 3A). Furthermore, *daf-7(e1037)* animals have fewer mitotic cells per gonad (Figure 3B), supporting the previously reported finding that DAF-7/TGF-β signaling plays a role in regulating the GSC mitosis/meiosis

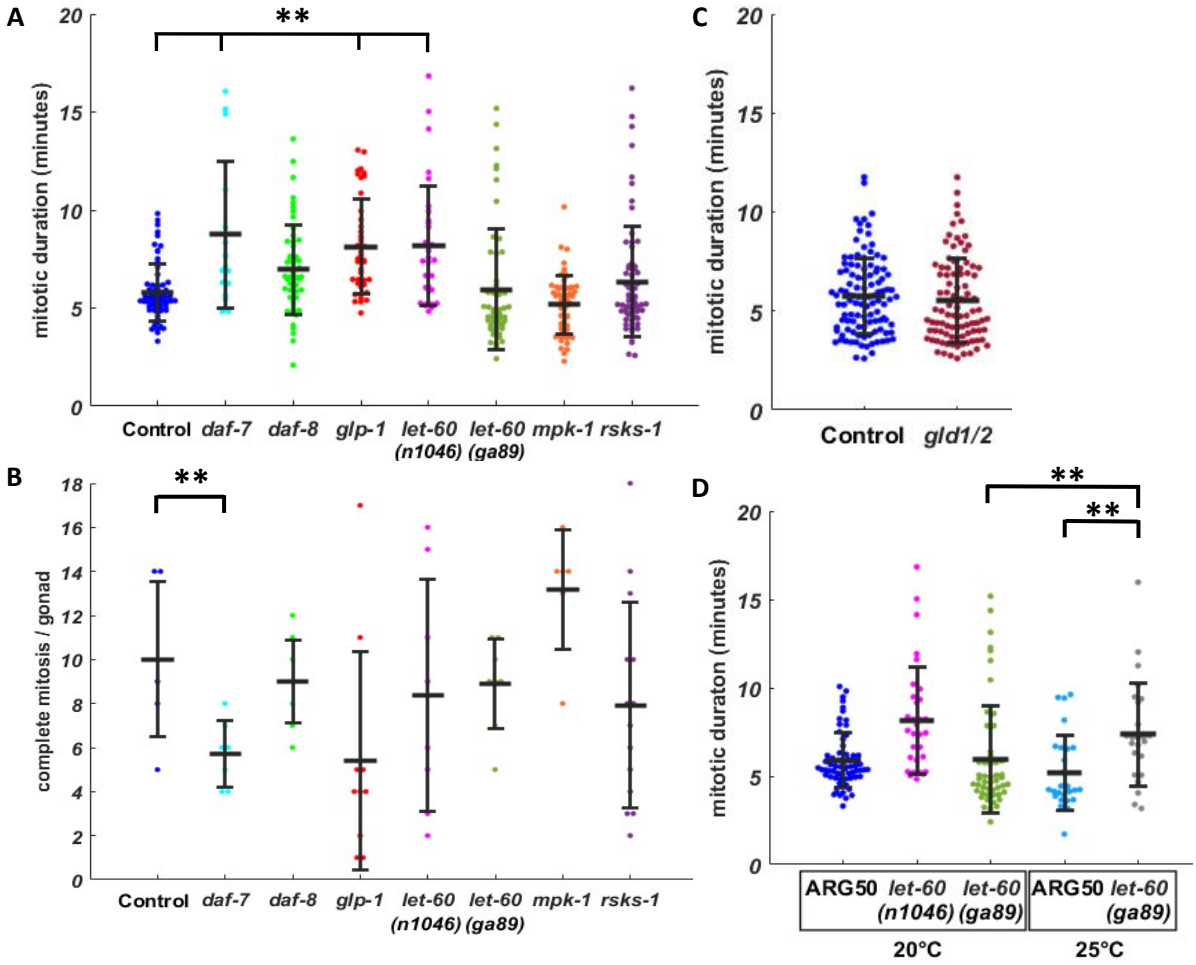
decision (Dalfó et al., 2012). Interestingly, GSCs in animals bearing a loss-of-function allele of *glp-1* (*glp-1(e4037)*) also showed longer durations of mitosis. This finding contradicts previous reports showing that *glp-1* works in the Notch signaling pathway to regulate the mitosis-meiosis decision, but not cell proliferation/cell cycle progression (Hubbard & Schedl, 2019). A *gld-1(q485)* and *gld-2(q497)* double mutant did not exhibit a significantly different duration of mitosis from that of the wildtype (Figure 3C).

In addition, GSCs in animals bearing a gain-of-function allele of the RAS *let-60* (*let-60(n1046)*) had longer durations of mitosis, while GSCs in animals bearing a second gain-of-function allele of *let-60* (*let-60(ga89)*) exhibited a normal mitotic duration at the semi-permissive temperature (Figure 3A). To investigate whether the different results between *let-60(n1046)* and *let-60(ga89)* were due to the nature of the mutant allele or to differences in the level of gene reduction, I imaged GSCs in *let-60(ga89)* worms grown at the fully restrictive temperature of 25°C. GSCs in these worms exhibited significantly longer mitotic durations compared to cells in wild type animals grown at either 20°C or 25°C, suggesting that hyperactivation of LET-60/RAS delays mitosis generally. However, a loss-of-function allele of *mpk-1(ga111)*, which should disrupt Ras/MAPK signaling downstream of LET-60 did not affect GSC mitotic duration (Figure 3A, D). *rsks-1(ok1255)* mutants displayed a reduction in the size of the mitotic zone that aligns with the observations in prior studies (Data not shown) (Korta et al., 2012). However, GSC mitotic duration was not significantly different from that of wildtype. All other alleles examined in this study did not exhibit any obvious mitotic phenotypes in the GSCs.

Altogether, I have found that the knockdown or reduction of several candidate genes affects GSC mitosis, while others have little to no effect. Specifically, the DAF-7/TGF-β and Notch signaling pathways have a strong and consistent effect on GSC mitosis. Moreover, I observed that

let-60/RAS signaling is important for proper mitotic progression in GSCs, and this may or may not involve the known downstream effector MPK-1.

Figure 3: Candidate signaling pathways screen using mutant allele



(A) Beeswarm plot of showing GSC mitotic duration in minutes in *C. elegans* animals bearing the indicated mutant alleles. Control = ARG50 (n = 56(8), 19(5), 54(12),42(5), 32(6), 52(8), 57(6), 67(12), GSCs (germ lines), respectively). Each dot represents a single cell.

(B) Beeswarm plot showing the number of cells per germ line that completed mitosis during the 40-minute live-cell imaging acquisition period. Control = ARG50. (n = 6, 7, 9, 10, 8, 8, 6, 14). Each dot represents one germ line.

(C) Beeswarm plot showing GSC mitotic duration in minutes in *gld-1 & gld-2* double mutant animals as compared to the control strain UM225. (n = 110(15), 94(12), GSCs (germ lines), respectively). Each dot represents a single cell.

(D) Beeswarm plot showing GSC mitotic duration in minutes in *let-60* mutant alleles. 20°C data are reproduced from (A). (n = 56(8), 32(6), 52(8), 25(4), 26(6), GSCs (germ lines), respectively). Each dot represents a single cell.

In all plots, black bars represent the mean and error bars represent the standard deviation. Data were compared using a one-way ANOVA (**: $p \leq 0.01$; ***: $p \leq 0.001$)

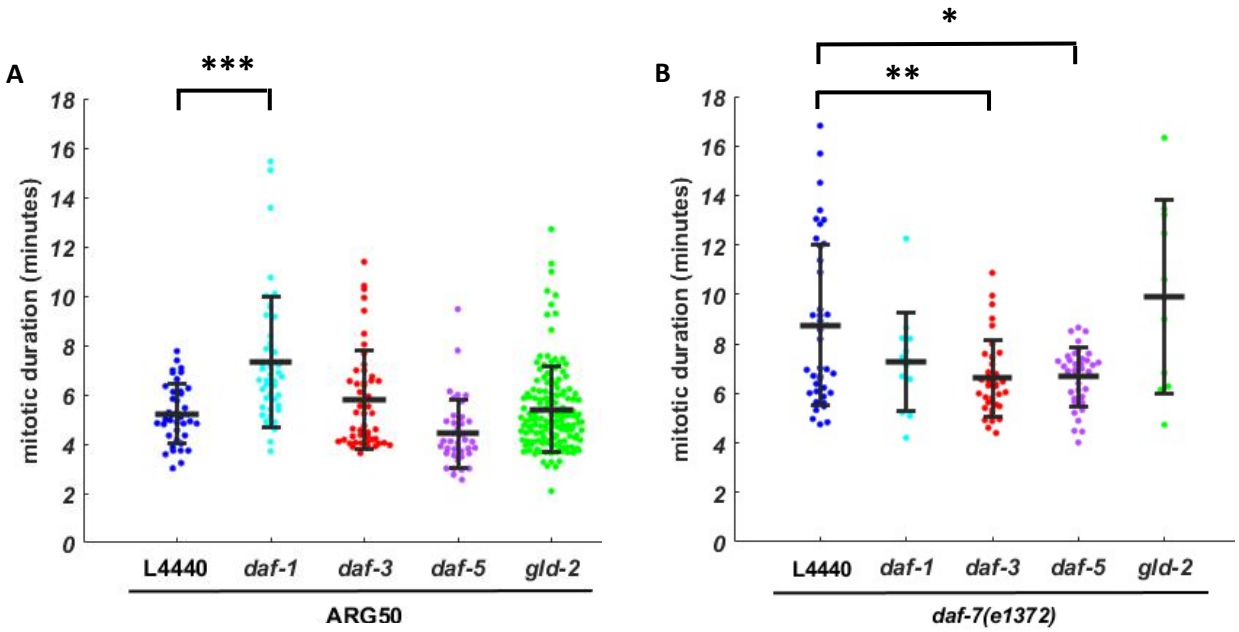
3.3. DAF-7/TGF-β signaling regulates mitotic timing in GSCs dependent on *daf-3* and *daf-5*

The DAF-7/TGF-β signaling pathway is known to play a role in the regulation of GSC proliferation during germline development in *C. elegans*. Specifically, the downregulation of DAF-7/TGF-β signaling has been associated with a reduced proliferation zone, an observation that can be rescued by reducing the activity of the downstream targets co-Smad *daf-3* and Ski oncoprotein homolog *daf-5* through RNAi (Dalfó et al., 2012). Accordingly, I sought to investigate the role of *daf-3* and *daf-5* in GSC mitotic regulation in the context of the DAF-7/TGF-β signaling pathway. My data showed that reduced *daf-3* and *daf-5* activity did not have a significant effect on GSC mitosis in wild-type worms (Figure 4A). However, both *daf-3* and *daf-5* RNAi in the *daf-7* mutant background suppressed the extension of mitotic duration normally seen in *daf-7* mutants (Figure 4B), suggesting that *daf-3* and *daf-5* are required downstream of *daf-7* to regulate GSC mitosis. In addition, *daf-1* RNAi resulted in a similar mitotic delay as observed in *daf-7* RNAi and *daf-7* mutants. However, *daf-1* RNAi did not induce further extensions of mitotic duration in the *daf-7* mutant (Figure 4A, B), suggesting that *daf-1* acts in the same pathway as *daf-7* to promote GSC mitotic progression. Taken together, these results suggest that DAF-7/TGF-β signaling plays a role in the regulation of GSC mitosis in *C. elegans*, and this pathway may act through downstream targets *daf-3* and *daf-5*.

Previous reports demonstrated that the DAF-7/TGF-β pathway acts in the DTC to promote the expression of *lag-2*, which encodes the ligand of the GLP-1/Notch signaling receptor (Dalfó et al., 2012; Pekar et al., 2017). Based on this evidence, it was proposed that normal TGF-β signaling activates GLP/Notch signaling, which subsequently inhibits the expression of *gld1/2* to regulate the mitosis-meiosis decision in GSCs. In *daf-7* mutants, decreased TGF-β levels are

expected to enhance the expression of *gld1/2*. As GSCs in *glp-1(e4037)*) mutants also showed longer durations of mitosis (Figure 3A), it is possible that Notch signaling also acts downstream of DAF-7/TGF-β to govern mitosis in GSCs. If so, depletion of *gld-1/2* by RNAi should restore the duration of mitosis in *daf-7* mutants. However, contrary to this prediction, RNAi depletion of *gld-1/2* in *daf-7(e1372)* mutants did not rescue the duration of GSC mitosis (Figure 4C). Although the sample size is small and the data is preliminary, these findings suggest that Notch signaling may not be required for the regulation of GSC mitosis by TGF-β.

Figure 4: *daf-3* and *daf-5* RNAi rescue mitotic duration of *daf-7* mutant



(A) Beeswarm plot of showing GSC mitotic duration in minutes in ARG50 worms following RNAi treatment. (n = 40(4), 43(8), 48(6), 38(5), 140(12), GSCs (germ lines), respectively). Each dot represents a single cell.

(B) Beeswarm plot of showing mitotic duration in minutes in ARG75 *daf-7(e1372)* worms following RNAi treatment. n = 53(12), 14(4), 35(11), 37(5), 10(4), GSCs (germ lines), respectively. Each dot represents a single cell.

In all plots, black bars represent the mean and error bars represent the standard deviation. Data were compared using a one-way ANOVA (*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$).

3.4. Dietary restriction delays mitotic progression in GSCs and this delay is independent of DAF-16/FOXO

My results so far indicate that several major signaling pathways may play a previously unappreciated role in regulating mitotic duration in GSCs. Many of these signaling pathways serve to link cell behavior with environmental changes. We have shown previously that changing food availability or dietary intake, and thereby producing a state of dietary restriction, also extends mitotic duration in GSCs (Gerhold et al., 2015). However, whether this effect involves cell signaling pathways is not known. Therefore, another goal of my project was to determine how dietary restriction regulates GSC mitosis by investigating the role of the insulin pathway, a crucial signaling pathway that mediates nutrient-dependent growth and development (Murphy & Hu, 2013).

In *C. elegans*, dietary restriction can be induced by either reducing the concentration of bacteria to curtail food availability (sDR) or by using *eat-2* mutant worms with reduced pharyngeal pumping to decrease food intake (Greer et al., 2007; Korta et al., 2012). Under both conditions, GSCs exhibited significantly longer mitotic durations in comparison to those that were fed ad libitum (Figure 5A, B). Specifically, the average congression duration under sDR or in an *eat-2* mutant background was 8.91+/- 5.3 and 8.24 +/- 3.42 minutes, respectively, as compared to the relevant controls (5.03 +/- 1.41 and 5.65 +/- 1.84 minutes, respectively). My findings align with previously reported data, indicating that dietary restriction leads to a delay in GSC mitotic progression (Gerhold et al., 2015).

Previous studies conducted in our lab have demonstrated that reduced insulin/IGF-1 signaling, using a temperature-sensitive allele of *daf-2* (*daf-2(e1370)*) at the semi-permissive temperature, significantly prolongs mitotic duration in GSCs. This delay was rescued by null

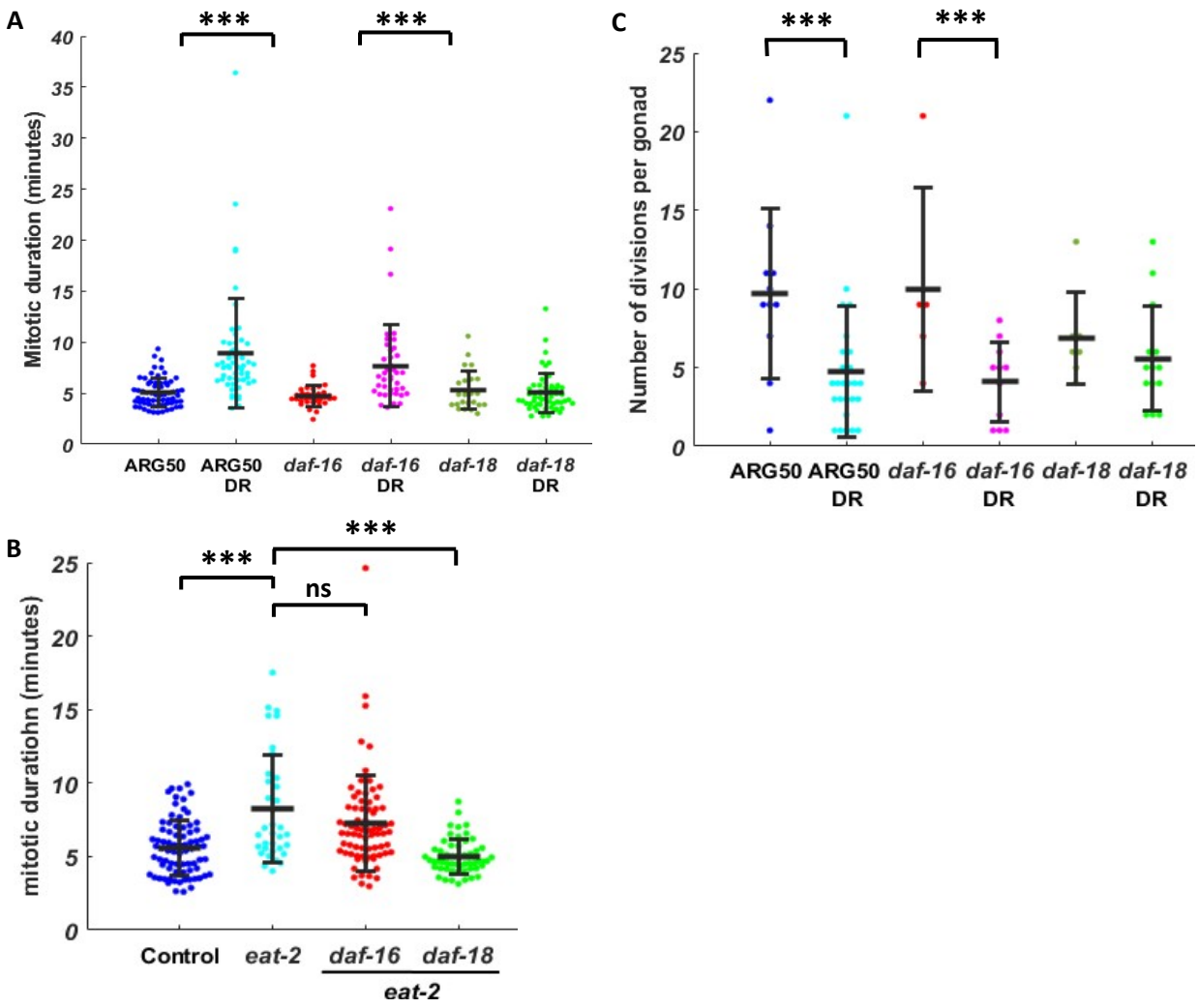
mutations in the downstream negative regulators of the pathway, *daf-16(mu86)* and *daf-18(nr2037),* in the *daf-2* mutant background. Furthermore, *daf-2* delays in mitosis were also rescued by a gain-of-function mutation in *akt-1(mg144)*. These findings suggest that the canonical insulin pathway regulates mitotic progression in GSCs (E. Cheng, unpublished).

Given that reduced insulin/IGF-1 signaling yields a similar delay in GSC mitosis to that observed upon dietary restriction, I hypothesized that mitotic delays upon dietary restriction may be dependent on the insulin/IGF-1 signaling pathway. Specifically, the knockdown of *daf-16* and *daf-18* should rescue mitotic duration under dietary restriction. To test this hypothesis, I subjected *daf-16(mu86)* and *daf-18(nr2037)* worms to sDR and used strains bearing *eat-2(ad465)* and either *daf-16(mu86)* or *daf-18(nr2037)* mutations to investigate whether they could rescue the duration of mitosis under both modes of dietary restriction. I found that GSCs in *daf-16* and *daf-18* mutants fed ad libitum had normal mitotic timing (Figure 5A). Interestingly, dietary restriction still led to GSC mitotic delays in *daf-16* mutants, suggesting that *daf-16* is not responsible for these delays. Conversely, GSCs in *daf-18* mutants subjected to dietary restriction showed an average mitotic duration similar to controls and to *daf-18* mutants fed ad libitum, indicating that knocking out *daf-18* fully rescues the mitotic delays caused by dietary restriction (Figure 5A). sDR treatment also led to a reduced number of mitotic cells, indicative of an overall slowing of the GSC cell cycle, and this phenotype was also rescued by mutation of *daf-18* but not *daf-16* (Figure 5C). Similarly, GSCs in *eat-2, daf-16* double mutants exhibited a longer mitotic duration compared to controls, whereas delays were rescued in the *eat-2, daf-18* double mutant (Figure 5B).

These results demonstrate that GSC mitotic delays following dietary restriction may be dependent on the insulin/IGF-1 signaling pathway, but that they involve *daf-18* specifically, rather than both *daf-18* and *daf-16*, as was observed for *daf-2*-dependent mitotic delays. These

observations imply that although signaling pathways and environmental factors may produce similar outcomes, their underlying mechanisms could be distinct, and they might operate via different downstream targets.

Figure 5: Dietary restriction extends mitotic duration in GSCs is dependent on *daf-18*, but not *daf-16*



(A) Beeswarm plots showing mitotic duration in minutes in worms that were either well-fed or following sDR treatment. Control = ARG50. (n = 63(8), 54(14), 33(5), 40(11), 26(6), 56(14), GSCs (germ lines), respectively). Each dot represents a single cell.

(B) Beeswarm plots showing mitotic duration in minutes in worms that are either well-fed or dietary restricted by *eat-2* mutation. Control = UM225. (n = 99(16), 41(11), 81(24), 54(12), GSCs (germ lines), respectively). Each dot represents a single cell.

(C) Beeswarm plot showing the number of cells per germ line that completed mitosis during the 40-minute live-cell imaging acquisition period. Control = ARG50. (n = 11, 26, 5, 11, 7,18, respectively). Each dot represents one germ line.

In all plots, black bars represent the mean and error bars represent the standard deviation. Data were compared using a one-way ANOVA (***: $p \leq 0.001$)

4.0. Discussion

The goal of my study was to explore the molecular mechanisms that govern GSC mitosis and to understand the effects of dietary restriction on this process. Through the use of RNAi and *C. elegans* alleles, I was able to characterize the mitotic index and mitotic duration in GSCs to provide insight into how the depletion or reduction of specific genes may affect mitosis. My results revealed that the TGF-β and Notch signaling pathways play important roles in GSC mitosis, and efforts were made to unravel the molecular mechanisms underlying the impact of TGF-β signaling on this process. Furthermore, my study confirmed that dietary restriction affects *C. elegans* GSC mitosis and uncovered the role of insulin/IGF-1 signaling in mediating dietary restriction-induced mitotic delay.

4.1. Candidate screening by RNAi was inconclusive and may be due to inefficient gene depletion

In this study, I employed RNAi by soaking as well as RNAi by feeding to perform a candidate screen for mitotic phenotypes in the *C. elegans* germ line. The results showed that RNAi-mediated depletion of *cdk-1*, *glp-1*, and *gld-2* led to abnormal germline development, while depletion of other candidate genes did not result in any noticeable phenotypic changes. These findings align with the known roles for *cdk-1*, *glp-1*, and *gld-2* in the germ line. For example, *cdk-1* RNAi injection hinders mitotic and meiotic divisions in both GSCs and the one-cell embryo (Boxem et al., 1999). Furthermore, previous studies have indicated that *glp-1* is required for GSC proliferation, and its loss causes all GSCs to undergo meiosis and to differentiate into sperm (Austin & Kimble, 1987). However, the depletion of other candidate genes did not result in any phenotypic changes in the worms is unexpected, given that previous studies have reported noticeable phenotypic alterations. For instance, Korta et al. (2012) observed that loss of *rsks-1*

gene function leads to a substantial (~50%) reduction in the size of the mitotic zone, while van der Voet et al. (2009) demonstrated that RNAi depletion of *cyb-1* triggers aneuploidy, and inhibition of *cyb-1* and *cyb-3* induces M phase arrest. Thus, it remains unclear whether the lack of phenotype that I observed was due to low RNAi efficiency or whether depletion of the target genes alone was insufficient to induce GSC mitosis changes.

While phenotypic analysis remains the prevailing approach to confirm RNAi knockdown in *C. elegans*, complementary molecular techniques can provide valuable insights into the efficacy of RNAi. Among these techniques, RT-PCR has proven to be a reliable and sensitive method to assess the degree of RNAi knockdown (Holmes et al, 2010). In this regard, RNAi treated worms can be used to isolate total RNA, followed by cDNA synthesis. Subsequently, gene-specific primers can be used to amplify the target gene of interest, enabling the detection and quantification of its transcript levels via RT-PCR. By providing a direct measurement of the impact of RNAi at the molecular level, RT-PCR offers a powerful tool to complement phenotypic observations. Thus, to determine whether target gene depletion was achieved using my experimental conditions, RT-PCR should be used to determine target RNA levels.

The quality and purity of dsRNA used for RNAi are critical for efficient gene knockdown and accurate interpretation of the resulting phenotypic changes. The dsRNAs used in the present study underwent sequencing, and BLAST results indicated the accuracy of the sequences. I purified the dsRNAs and ran gel electrophoresis to verify the correct sequence size. However, the resulting gel exhibited several non-specific bands alongside those of the correct size (Supplemental Figure S2). This observation suggests that the purified dsRNAs might have been contaminated or degraded, leading to the presence of non-specific bands, and these non-specific bands raise concerns about the accuracy and reliability of the RNAi process in this study. Further

investigations may be necessary to identify the cause of the non-specific bands and their impact on RNAi efficiency and subsequent phenotypic changes. For my study, I decided to use *C. elegans* mutant alleles as an alternative approach to investigate the effect of candidate genes on GSC mitosis.

4.2. Candidate signaling pathway screening using *C. elegans* alleles identified TGF-β and Notch signaling pathway to regulate GSC mitosis

The use of mutant alleles can provide more definitive evidence of the role of a candidate gene in GSC mitosis regulation, as it eliminates concerns about the quality and purity of dsRNA and the efficiency of the RNAi process. The results presented in this study using *C. elegans* mutant alleles demonstrate that the knockdown or reduction-of-function of candidate genes affects GSC mitosis, providing insight into the complex regulation of germ cell development and the factors that influence mitotic progression in GSCs. Specifically, I found that DAF-7/TGF-β and Notch signaling pathways play important roles in promoting GSC mitosis. My results indicate that reduced TGF-β signaling, as seen in the *daf-7(e1037)* allele, leads to a longer duration of mitosis and fewer mitotic cells per gonad, while reduced Notch signaling, as seen in the *glp-1(e4037)* allele, also results in delayed mitotic progression. My findings are consistent with the observations reported by Dalfó et al. (2012), which demonstrated that the *daf-7(e1037)* exhibits a reduced number of GSCs in the mitotic zone, indicating the role of TGF-β signaling in the accumulation of GSCs. However, my results suggest that in addition to cell cycle regulation, the TGF-β signaling pathway also plays a previously unappreciated role in ensuring normal mitotic duration.

Moreover, we observed that LET-60/RAS, which is upstream of the MPK-1, is important for proper mitotic progression in GSCs. Specifically, gain-of-function alleles of *let-60* resulted in delayed mitotic progression, suggesting a critical role for this pathway in regulating mitosis.

However, I did not observe any mitotic phenotypes in the *mpk-1(ga111)* loss-of-function allele. This could mean that LET-60 affects GSC mitosis by affecting other signaling pathways or cellular processes. Alternatively, reducing RAS/MAPK signaling may not be sufficient to generate mitotic phenotypes in GSCs. Therefore, further studies are needed to fully understand the molecular mechanisms underlying this process and the role of MPK-1 and other downstream targets in this regulation. Depletion of *mpk-1* by RNAi in the context of *let-60(gf)* mutants can be performed to investigate whether the observed mitotic delay in the *let-60(gf)* mutants is dependent on the presence of *mpk-1*. *let-60* gain of function alleles are expected to elicit a corresponding elevation in *mpk-1* activity. In this case, if *mpk-1* is required for the delay of mitosis, depletion of *mpk-1* through RNAi would be expected to suppress the effects of the *let-60* gain of function mutation.

Overall, my study highlights the value of using temperature-sensitive alleles in *C. elegans* as a tool for investigating gene function and understanding complex biological processes. The results presented in this study identify potentially novel regulators of GSC mitosis and pave the way for future studies aimed at investigating the molecular mechanisms underlying this regulation.

4.3. The impact of DAF-7/TGF-β signaling on GSC mitosis is modulated by *daf-3* and *daf-5*

In investigating the mechanism by which reducing TGF-β signaling delays GSC mitosis, I have determined that *daf-3* and *daf-5* are required. Specifically, *daf-3* and *daf-5* RNAi in the *daf-7* mutant background led to a suppression of the *daf-7* extension of mitotic duration. These results are in line with previous studies that have implicated the TGF-β signaling pathway in the regulation of stem cell proliferation and differentiation (Dalfó et al., 2012). Previous studies showed that this pathway regulates cell cycle progression or the mitosis-meiosis decision in GSCs (Dalfó et al., 2012). My results add a new role for the TGF-β signaling pathway in regulating events that occur

during mitosis and determine the duration of mitosis. In order to further investigate the relationship between DAF-7/TGF-β signaling and GSC mitosis, future experiments could involve examining the expression levels and activity of downstream targets of this pathway in the germline, such as *daf-3* and *daf-5*, in the context of wild-type and *daf-7* mutant worms. Extended mitotic duration may be caused by compromised spindle assembly which activates the SAC. Alternatively, it could also be caused by a decrease in the activity of the APC/C and/or an increase in SAC strength. To test whether the mitotic delay observed in *daf-7(e1372)* mutants is dependent on the SAC, removal of core checkpoint components via RNAi or by genetic null mutants in a *daf-7(e1372)* mutant background can be performed to determine the SAC dependence. If mitotic delays in *daf-7(e1372)* mutants are SAC dependent, loss of core checkpoint components should fully suppress the delays.

In addition, it has been reported that TGF-β promotes the expression of *lag-2* in the DTC, which in turn modulates the activity of the germline GLP-1/Notch receptor to regulate the mitosis-meiosis decision in GSCs (Pekar et al., 2017). Therefore, it will be worth investigating the potential relationship between DAF-7/TGF-β signaling and Notch signaling in regulating GSC mitosis. Future experiments could include testing whether mitotic delays in the *glp-1(e4037)* loss-of-function and *daf-7(e1037)* loss-of-function alleles are additive and whether a *glp-1(ar202)* gain-of-function allele can suppress delays in *daf-7(e1037)* loss of function mutants. Furthermore, these alleles could be combined with RNAi to also test whether the mitotic delays exhibit additivity. Specifically, such experiments could involve the administration of *glp-1* RNAi to *daf-7(e1037)* mutants, or *daf-7* RNAi to *glp-1(e4037)* mutants, to see if the mitotic duration is further extended. These experimental manipulations would allow for the examination of whether the TGF-β signaling pathway regulation of GSC mitosis is dependent on Notch signaling.

4.4. The timing of mitosis is delayed by dietary restriction, and this delay is dependent on DAF-18/PTEN, but not DAF-16/FOXO.

The results of my study are consistent with our prior findings, as reported by Gerhold et al. (2015), which revealed that dietary restriction delayed mitotic progression in *C. elegans* GSCs. Specifically, I observed that sDR and the *eat-2* mutant allele both induced an extension in the duration of mitosis. Previous studies have suggested that DAF-16/FOXO, which is the major target of insulin/IGF-1 signaling in *C. elegans*, is located in the cytoplasm under conditions of abundant food. In contrast, nutrient deprivation triggers the nuclear localization of DAF-16, indicating that nutrient stress may be linked to IIS in *C. elegans* (Henderson & Johnson, 2005). My data indicate that a *daf-18* null mutation completely ameliorates the delayed mitosis induced by dietary restriction treatment. In contrast, a *daf-16* null mutation does not rescue these delays, implying that mitotic delay mediated by dietary restriction is dependent on *daf-18*, but not *daf-16*. Unpublished work from our lab has shown that reducing IIS using a conditional *daf-2* allele leads to mitotic delays in GSCs that require both *daf-18* and *daf-16*. This implies that dietary restriction may act in parallel to the IIS pathway to regulate GSC mitosis, with both requiring *daf-18*.

Future investigation should focus on understanding the molecular mechanism by which DAF-18 regulates the mitotic delay induced by dietary restriction in GSCs. Previous studies have revealed that DAF-18/PTEN is a convergence point for TGF-β and insulin/IGF-1 signaling, which suppress GSC proliferation during dauer development (Narbonne & Roy, 2006). Subsequent research indicated that DAF-18/PTEN modulates GSC proliferation by transmitting signals through the AMPK pathway via its upstream activator LKB1 to repress MPK-1/MAPK signaling (Narbonne & Roy, 2006; Narbonne et al., 2017). Taken together, these findings suggest that, in addition to the insulin/IGF-1 signaling pathway, DAF-18 can also modulate AMPK signaling.

These results open up new avenues for exploring the specific mechanisms by which DAF-18 regulates dietary restriction-induced mitotic delay in GSCs, particularly through AMPK signaling. As such, future investigations should investigate the interplay between DAF-18/PTEN, AMPK signaling, and GSC mitosis under dietary restriction to gain a comprehensive understanding of the molecular mechanisms that govern this process.

4.5. Exploring the mechanism of cell signaling pathways and environmental factors in regulating mitotic fidelity

The findings presented in this study offer compelling evidence for the role of cell signaling pathways and environmental factors in regulating mitosis, particularly in terms of their impact on mitotic timing. Additionally, the study has identified the mechanism by which DAF-7/TGF-β signaling regulates GSC mitosis, adding to the existing knowledge on the regulation of this critical cellular process. However, the results presented here raise further questions regarding the underlying mechanisms through which changes in these pathways or environmental factors affect mitotic timing. Specifically, it is necessary to investigate the specific cellular processes that these factors are regulating, which ultimately lead to mitotic delays in GSCs. Previous studies have shown that longer mitotic duration resulting from compromised spindle assembly is dependent on the SAC, and that dietary restricted GSCs are more susceptible to chromosome segregation errors in the absence of the SAC, indicating reduced mitotic fidelity (Gerhold et al., 2015). Thus, it is important to explore whether the SAC is responsible for the observed mitotic delay in response to reduced cell signaling. It is also crucial to investigate whether alterations in these factors affect mitotic fidelity.

In *C. elegans*, the *mdf-1*/MAD-1 and *mdf-2*/MAD-2 are core components of SAC. These proteins form a complex that localizes to unattached kinetochores, where it facilitates the efficient binding of MAD2 to CDC20 (Kitagawa, 2009). If the mitotic delays observed in response to reduced cell signaling are SAC-dependent, it is hypothesized that knockdown of *mdf-1* through RNAi or utilizing a genetic null mutant of *mdf-2(lt4)* would lead to complete suppression of the observed delays. However, if these delays are SAC-independent, it is expected that the loss of *mdf-1* or *mdf-2* would have minimal effect on the mitotic delays.

In order to investigate whether the reduced cell signaling and/or dietary restriction may lead to compromised mitotic fidelity, a GFP reporter (*xol-1::GFP*), which is expressed specifically in XO embryos resulting from meiotic X chromosome segregation errors in non-mated hermaphrodites, can be used to quantify segregation defects (Kelly et al., 2000). By quantifying the number of GFP-positive embryos, it would be feasible to determine whether reduced TGF-β signaling results in increased segregation errors. In addition, the fluorescently labelled histone (HIS-11::mCherry) can also be used for visualizing any segregation defects in the germline in real time. These proposed experiments may be useful in elucidating the underlying mechanisms by which cell signaling pathways and environmental factors affect mitotic fidelity.

5.0. Conclusion

In conclusion, my study aimed to identify the signaling pathways that potentially affect GSC mitosis in *C. elegans*. Through the *C. elegans* mutant allele approach, the TGF-β and Notch signaling pathways were identified. Further investigation of TGF-β signaling in GSC mitosis revealed that *daf-3* and *daf-5* play critical roles in this process. While previous studies have demonstrated that DAF-7/TGF-β promotes *lag-2* expression and subsequently targets Notch signaling to regulate the GSC mitosis-meiosis decision, my preliminary data suggests that Notch signaling is not involved in the regulation of GSC mitosis by DAF-7/TGF-β signaling. Further investigation is necessary to confirm these findings.

In addition to identifying the signaling pathways that affect GSC mitosis in *C. elegans*, my study also investigated the relationship between dietary restriction and GSC mitosis. My results confirmed previous reports that dietary restriction extends the mitotic duration of GSCs. Furthermore, I found that dietary restriction-induced mitotic delay is dependent on *daf-18*, but not *daf-16*, indicating that the regulation of GSC mitosis by dietary restriction may be independent of insulin/IGF-1 signaling. Altogether, this work contributes to the continuously growing understanding on the regulation of mitotic progression *in vivo*, particularly with respect to the impact of signaling pathways and environmental factors.

6.0. Appendix

Table 1: Candidate genes for RNAi screening

Candidate Gene	Sequence	Source	Gene Product	Molecular Function
aak-1	PAR2.3	Ahringer library	AMP-activated protein kinase	metabolic regulation (Lee et al., 2008)
aak-2	T01C8.1	Ahringer library	AMP-activated protein kinase	metabolic regulation (Lee et al., 2008)
age-1	B0334.8	Ahringer library	PI3K	activates AKT1/2, lifespan regulation (Murphy & Hu, 2013)
cdk-1	T05G5.3	Ahringer library	Cyclin-dependent kinase	cell cycle regulation
cyb-1	ZC168.4	Ahringer library	B-type cyclin	cell cycle regulation
cyb-3	T06E6.2	Ahringer library	B-type cyclin	cell cycle regulation
cye-1	C37A2.4	Ahringer library	Cyclin E	cell cycle regulation
daf-2	Y55D5A.5	Gerhold lab	Insulin/IGF-1 receptor	germline proliferation regulation, lifespan regulation (Murphy & Hu, 2013)
daf-4	C05D2.1	Ahringer library	TGF-β type II receptor	inhibit dauer larva formation (Estevez et al., 1993)
daf-8	R05D11.1	Ahringer library	R-smad	Inhibits Co-smad, dauer regulation
daf-15	C10C5.6	Gerhold lab	Raptor	lifespan regulation (Vellai et al., 2003)
daf-16	R13H8.1	Ahringer library	FOXO transcription factor	aging and longevity (Sun et al., 2017)
gld-2	ZC308.1	Ahringer library	STAR RNA-binding protein	promotes meiotic entry (Kimble & Crittenden, 2007)
glp-1	F02A9.6	Ahringer library	Notch receptor	germline development (Qiao et al., 1995)
let-363	B0261.2	Gerhold lab	TOR	lifespan regulation (Vellai et al., 2003)
let-60	ZK792.6	Ahringer library	RAS	vulva development (Beitel et al., 1990)
mek-2	Y54E10BL.6	Gerhold lab	Map kinase	activate MAP kinase, control vulval differentiation (Wu et al., 1995)
mpk-1	F43C1.2	Ahringer library	Map kinase	GSC proliferation regulation (Narbonne et al., 2017)

rsks-1	Y47D3A.16	Ahringer library	S6K	GSC maintenance (Roy et al., 2018)
sma-2	ZK370.2	Ahringer library	R-Smad	Aging and longevity (Gumienny, 2013)
wts-1	T20F10.1	Ahringer library	Warts	regulate body length (Cai et al., 2009)
yap-1	F13E6.4	Ahringer library	Yes-associated protein	thermotolerance and aging (Iwasa et al., 2013)

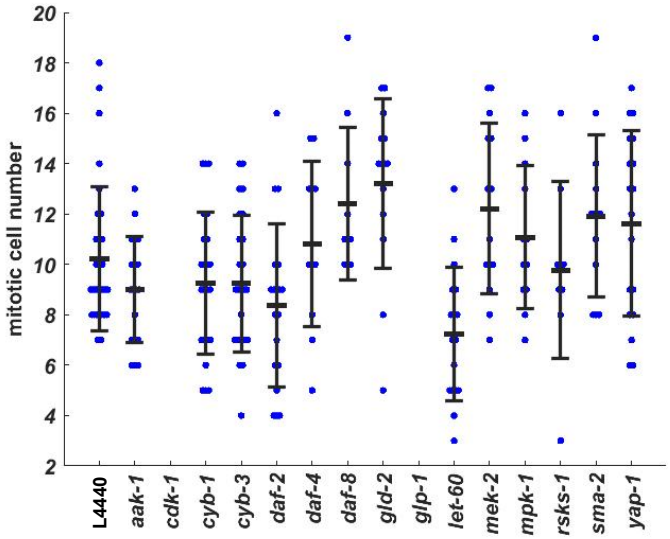
Table 2: *C. elegans* strains and alleles

Strain	Allele/Genotype	Source	Comments
CB1364	*daf-4(e1364) III*	CGC	Temperature sensitive dauer constitutive
CB1372	*daf-7(e1372) III*	CGC	Temperature sensitive dauer constitutive
CB1393	*daf-8(e1393) I*	CGC	Temperature sensitive dauer constitutive
CB4037	*glp-1(e4037) III*	CGC	Temperature sensitive, sterile at 25°C
GC833	*glp-1(ar202) III*	Labbe Lab	Temperature sensitive; tumorous germline at 25°C
JK2879	*gld-2(q497) gld-1(q485) I/ hT2[qIs48] (I;III)*	Labbe Lab	Segregates WT GFP+ heterozygotes, non-GFP sterile gld-2 gld-1 homozygotes
MR1175	*aak-1(tm1944) III; aak-2(ok524) X*	Roy lab	Dauer recoveries are sterile
MT2124	*let-60(n1046) IV*	CGC	Gain of function; multivulva
RB1206	*rsks-1(ok1255) III*	CGC	
RB2286	*sma-2(ok3109) III*	CGC	
SD551	*let-60(ga89) IV*	Roy lab	Temperature sensitive; gain of function; Muv at 25°C and sterile
SD939	*mpk-1(ga111) unc-79(e1068) III*	CGC	Temperature sensitive; sterile at 25°C
ARG50	*mosI_5'mex-5_GFP::tbb-2; mCherry::his-11; cb-unc-119(+)]I*	Gerhold Lab	
ARG45	*mosI_5'mex-5_GFP::tbb-2; mCherry::his-11; cb-unc-119(+)]I; sun-1p::rde-1::sun-1 3'UTR; rde-1(mkc36)V*	Gerhold Lab	Germline specific RNAi machinery
UM225	*ojIs1[unc-119(+) pie-1::GFP::tbb-2] V*	Gerhold Lab	
UM255	*gld-2(q497) gld-1(q485) I/ hT2[qIs48](I;III); ojIs1[unc-119(+) pie-1::GFP::tbb-2] V*	Labbe Lab	Tumorous germline
UM272	*eat-2(ad465) II; ojIs1[unc-119(+) pie-1::GFP::tbb-2] V*	Gerhold Lab	Abnormal feeding; Slow pumping
UM362	*daf-16(mu86) I; eat-2(ad465) II; ojIs1[unc-119(+) pie-1::GFP::tbb-2] V*	Gerhold Lab	
UM363	*eat-2(ad465) II; daf-18(nr2037) IV; ojIs1[unc-119(+) pie-1::GFP::tbb-2] V*	Gerhold Lab	

ARG73	*glp-1(e2141) III. His-11::mCH; TBB-2::GFP*	Gerhold Lab	Temperature sensitive, sterile at 25°C
ARG74	*rsks-1(ok1255) III; His-11::mCH; TBB-2::GFP*	Gerhold Lab	
ARG75	*daf-7(e1372) III; His-11::mCH; TBB-2::GFP*	Gerhold Lab	Temperature sensitive dauer constitutive
ARG76	*mpk-1(ga111) unc-79(e1068) III; His-11::mCH; TBB-2::GFP*	Gerhold Lab	Temperature sensitive; sterile at 25°C
ARG82	*let-60 (n1046) IV; His-11::mCH; TBB-2::GFP*	Gerhold Lab	Gain of function; multivulva
ARG84	*let-60 (ga89) IV; His-11::mCH;TBB-2::GFP*	Gerhold Lab	Temperature sensitive; gain of function; Muv at 25°C and sterile
ARG86	*daf-8(e1393) I; His-11::mCH;TBB-2::GFP*	Gerhold Lab	Temperature sensitive dauer constitutive
ARG101	*glp-1(ar202) III; His-11::mCH;TBB-2::GFP*	Gerhold Lab	Temperature sensitive; tumorous germline at 25°C

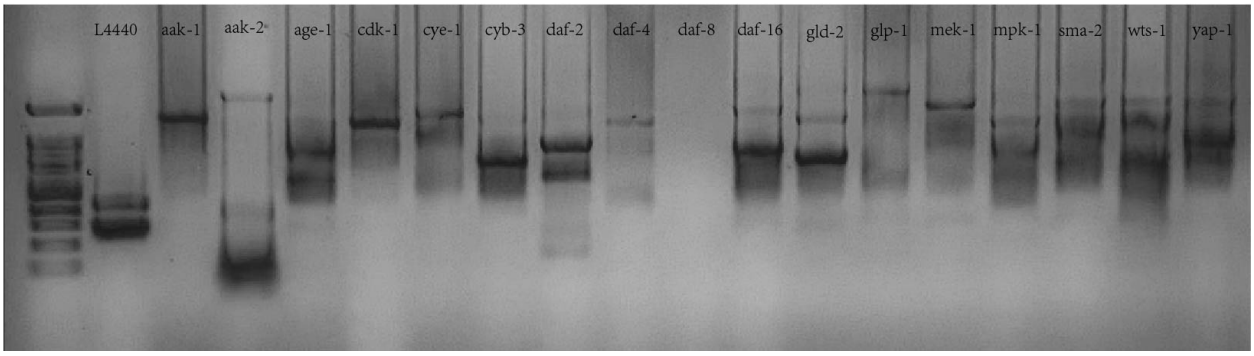
Table 3: Primers

Primer	sequence
T7_L4440_F1	TAATACGACTCACTATAGGGCGAA
*let-60*_T7_Fwd	TAATACGACTCACTATAGGGGAGACATGCCTCCTCGACAT
*let-60*_T7_Rev	TAATACGACTCACTATAGGGCAGACCGGGTGTCGTATTTT
*mek-2*_f_SacI	TAAGCAGAGCTCTGAGAACTGCGACCAATCAG
*mek-2*_r_NcoI	TAAGCACCATGGTCTGAGCTTTGAACCTCCAT
*daf-15*_f_SacI	TAAGCAGAGCTCAAATGTGGTGCACACAAAGC
*daf-15*_r_NcoI	TAAGCACCATGGCCGGAAATTCCAACTTTCAA
*let-363*_f_SacI	TAAGCAGAGCTCGGTGTTGAATGGCTTGTCCT
*let-363*_f_NcoI	TAAGCACCATGGCGCAGTTTCGGAATTCTCAT
*ok1255*_external_f	GAGATGCGGAAGCTATGCTC
*ok1255*_external_b	GTTGAATTCCTGCTCCTCCA
*ok1255*_internal_f	ATTCAACTGTGTGCCAGTGC
*ok1255*_internal_b	TGGGGCTTCACTATTTGGTC
*aak-1*_rr1514	TTCGCGTCCAGAAGAAGATT
*aak-1*_rr1515	GTGAAACCGAAACGGAAAAA
*aak-2*_rr1104	TTGGAATCCATGAGACAACTC
*aak-2*_rr1105	AAGACTTGGCACGTGCTC



Supplemental Figure S1: Candidate signaling pathways screen by germline-specific RNAi

Beeswarm plot showing the mitotic cell number in the mitotic zone per gonad of worms following RNAi treatment. Control = ARG45 following L4440 RNAi.



Supplemental Figure S2: Image of the gel for RNAi sequences

7.0. References

1. Alberts B, Johnson A, Lewis J, et al. Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002. An Overview of the Cell Cycle. Available from: https://www.ncbi.nlm.nih.gov/books/NBK26869/
2. Albert Hubbard, E. J., & Schedl, T. (2019). Biology of the *Caenorhabditis elegans* germline stem cell system. *Genetics*, 213(4), 1145–1188. https://doi.org/10.1534/genetics.119.300238
3. An, S. W., Artan, M., Park, S., Altintas, O., & Lee, S.-J. V. (2016). Longevity regulation by insulin/IGF-1 signaling. *Healthy Ageing and Longevity*, 63–81. https://doi.org/10.1007/978-3-319-44703-2_4
4. Artavanis-Tsakonas, S., Rand, M. D., & Lake, R. J. (1999). Notch signaling: Cell Fate Control and Signal Integration in development. *Science*, 284(5415), 770–776. https://doi.org/10.1126/science.284.5415.770
5. Austin, J., & Kimble, J. (1987). GLP-1 is required in the germ line for regulation of the decision between mitosis and meiosis in *C. elegans*. *Cell*, 51(4), 589–599. https://doi.org/10.1016/0092-8674(87)90128-0
6. Ayyadevara, S., Tazearslan, Ã. Ã., Bharill, P., Alla, R., Siegel, E., & Shmookler Reis, R. J. (2009). *Caenorhabditis elegans* PI3K mutants reveal novel genes underlying exceptional stress resistance and lifespan. *Aging Cell*, 8(6), 706–725. https://doi.org/10.1111/j.1474-9726.2009.00524.x
7. Barnum, K. J., & O'Connell, M. J. (2014). Cell cycle regulation by checkpoints. Methods in Molecular Biology, 29–40. https://doi.org/10.1007/978-1-4939-0888-2_2

8. Beitel, G. J., Clark, S. G., & Horvitz, H. R. (1990). *Caenorhabditis elegans ras* gene *let-60* acts as a switch in the pathway of vulval induction. *Nature*, 348(6301), 503–509. https://doi.org/10.1038/348503a0
9. Blackwell, T. K., Sewell, A. K., Wu, Z., & Han, M. (2019). TOR signaling in *Caenorhabditis elegans* development, metabolism, and aging. *Genetics*, 213(2), 329–360. https://doi.org/10.1534/genetics.119.302504
10. Boxem, M., Srinivasanxs, D. G., & van den Heuvel, S. (1999). The *Caenorhabditis elegans* gene *ncc-1* encodes a *cdc2*-related kinase required for M phase in meiotic and mitotic cell divisions, but not for S phase. *Development*, 126(10), 2227–2239. https://doi.org/10.1242/dev.126.10.2227
11. Brenner, S. (1974). The genetics of *caenorhabditis elegans. Genetics*, 77(1), 71–94. https://doi.org/10.1093/genetics/77.1.71
12. Brenner, S. (1973). The genetics of behavior. *British Medical Bulletin*, 29(3), 269–271. https://doi.org/10.1093/oxfordjournals.bmb.a071019
13. Cassada, R. C., & Russell, R. L. (1975). The dauerlarva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. *Developmental Biology*, 46(2), 326–342. https://doi.org/10.1016/0012-1606(75)90109-8
14. Cai, Q., Wang, W., Gao, Y., Yang, Y., Zhu, Z., & Fan, Q. (2009). *CE-wts-1* plays important roles in *Caenorhabditis elegans* development. *FEBS Letters*, 583(19), 3158–3164. https://doi.org/10.1016/j.febslet.2009.09.002
15. Crittenden, S. L., Leonhard, K. A., Byrd, D. T., & Kimble, J. (2006). Cellular analyses of the mitotic region in the *Caenorhabditis elegans* adult germ line. *Molecular Biology of the Cell*, 17(7), 3051–3061. https://doi.org/10.1091/mbc.e06-03-0170

16. da Graca, L. S., Zimmerman, K. K., Mitchell, M. C., Kozhan-Gorodetska, M., Sekiewicz, K., Morales, Y., & Patterson, G. I. (2004). Daf-5 is a ski oncoprotein homolog that functions in a neuronal TGFβpathway to regulate *C. elegans* dauer development. *Development*, 131(2), 435–446. https://doi.org/10.1242/dev.00922
17. Dalfó, D., Michaelson, D., & Hubbard, E. J. A. (2012). Sensory regulation of the *C. elegans* germline through TGF-β-dependent signaling in the niche. *Current Biology*, 22(8), 712–719. https://doi.org/10.1016/j.cub.2012.02.064
18. De Meyts P. The Insulin Receptor and Its Signal Transduction Network. [Updated 2016 Apr 27]. In: Feingold KR, Anawalt B, Blackman MR, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK378978/
19. De-Souza, E. A., Camara, H., Salgueiro, W. G., Moro, R. P., Knittel, T. L., Tonon, G., Pinto, S., Pinca, A. P. F., Antebi, A., Pasquinelli, A. E., Massirer, K. B., & Mori, M. A. (2019). RNA interference may result in unexpected phenotypes in *Caenorhabditis elegans*. *Nucleic Acids Research*, 47(8), 3957–3969. https://doi.org/10.1093/nar/gkz154
20. Duronio, R. J., & Xiong, Y. (2013). Signaling pathways that control cell proliferation. *Cold Spring Harbor Perspectives in Biology*, 5(3). https://doi.org/10.1101/cshperspect.a008904
21. Encalada, S. E., Willis, J., Lyczak, R., & Bowerman, B. (2005). A spindle checkpoint functions during mitosis in the early *Caenorhabditis elegance* mbryo. *Molecular Biology of the Cell*, 16(3), 1056–1070. https://doi.org/10.1091/mbc.e04-08-0712
22. Estevez, M., Attisano, L., Wrana, J. L., Albert, P. S., Massagué, J., & Riddle, D. L. (1993). The *daf-4* gene encodes a bone morphogenetic protein receptor controlling *C. elegans* dauer larva development. *Nature*, 365(6447), 644–649. https://doi.org/10.1038/365644a0

23. Fabian, D. K., Fuentealba, M., Dönertaş, H. M., Partridge, L., & Thornton, J. M. (2021). Functional conservation in genes and pathways linking ageing and Immunity. *Immunity & Ageing*, 18(1). https://doi.org/10.1186/s12979-021-00232-1

24. Gerhold, A. R., Ryan, J., Vallée-Trudeau, J.-N., Dorn, J. F., Labbé, J.-C., & Maddox, P. S. (2015). Investigating the regulation of stem and progenitor cell mitotic progression by in situ imaging. Current Biology, 25(9), 1123–1134. https://doi.org/10.1016/j.cub.2015.02.054

25. Gerhold, A. R., Poupart, V., Labbé, J.-C., & Maddox, P. S. (2018). Spindle Assembly checkpoint strength is linked to cell fate in the *Caenorhabditis elegans* embryo. *Molecular Biology of the Cell*, 29(12), 1435–1448. https://doi.org/10.1091/mbc.e18-04-0215

26. Golden, J. W., & Riddle, D. L. (1984). The *Caenorhabditis elegans* dauer larva: Developmental effects of pheromone, food, and temperature. *Developmental Biology*, 102(2), 368–378. https://doi.org/10.1016/0012-1606(84)90201-x

27. Greer, E. L., Dowlatshahi, D., Banko, M. R., Villen, J., Hoang, K., Blanchard, D., Gygi, S. P., & Brunet, A. (2007). An AMPK-FOXO pathway mediates longevity induced by a novel method of dietary restriction in C. elegans. *Current Biology*, *17*(19), 1646–1656.

28. Greer, E. L., & Brunet, A. (2009). Different dietary restriction regimens extend lifespan by both independent and overlapping genetic pathways in *C. elegans*. *Aging Cell*, 8(2), 113–127. https://doi.org/10.1111/j.1474-9726.2009.00459.x

29. Gumienny, T. L. (2013). TGF-β signaling in *C. elegans*. *WormBook*, 1–34. https://doi.org/10.1895/wormbook.1.22.2

30. Guo, Y. J., Pan, W. W., Liu, S. B., Shen, Z. F., Xu, Y., & Hu, L. L. (2020). Erk/MAPK signaling pathway and tumorigenesis (review). *Experimental and Therapeutic Medicine*. https://doi.org/10.3892/etm.2020.8454

31. Han, M., Aroian, R. V., & Sternberg, P. W. (1990). The *let-60* locus controls the switch between vulval and nonvulval cell fates in *Caenorhabditis elegans*. *Genetics*, 126(4), 899–913. https://doi.org/10.1093/genetics/126.4.899

32. Harvey, K. F., Pfleger, C. M., & Hariharan, I. K. (2003). The drosophila MST ortholog, Hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell*, 114(4), 457–467. https://doi.org/10.1016/s0092-8674(03)00557-9

33. Henderson, S. T., Gao, D., Lambie, E. J., & Kimble, J. (1994). LAG-2 may encode a signaling ligand for the GLP-1 and Lin-12 receptors of *C. elegans*. *Development*, 120(10), 2913–2924. https://doi.org/10.1242/dev.120.10.2913

34. Henderson, S. T., & Johnson, T. E. (2005). DAF-16 integrates developmental and environmental inputs to mediate aging in the nematode *Caenorhabditis elegans*. *Current Biology*, 15(7), 690. https://doi.org/10.1016/j.cub.2005.03.045

35. Henriques, A. C., Ribeiro, D., Pedrosa, J., Sarmento, B., Silva, P. M. A., & Bousbaa, H. (2019). Mitosis inhibitors in anticancer therapy: When blocking the exit becomes a solution. Cancer Letters, 440-441, 64–81. https://doi.org/10.1016/j.canlet.2018.10.005

36. Herndon, L. A., Wolkow, C., & Hall, D. H. (2018). Wormatlas Aging Handbook - Introduction to aging in *C. elegans*. *WormAtlas*. https://doi.org/10.3908/wormatlas.8.4

37. Heintz, C., Doktor, T. K., Lanjuin, A., Escoubas, C. C., Zhang, Y., Weir, H. J., Dutta, S., Silva-García, C. G., Bruun, G. H., Morantte, I., Hoxhaj, G., Manning, B. D., Andresen, B. S., & Mair, W. B. (2016). Splicing factor 1 modulates dietary restriction and TORC1

pathway longevity in *C. elegans*. *Nature*, 541(7635), 102–106. https://doi.org/10.1038/nature20789

38. Holmes, K., Williams, C. M., Chapman, E. A., & Cross, M. J. (2010). Detection of siRNA induced mRNA silencing by RT-qPCR: Considerations for Experimental Design. *BMC Research Notes*, 3(1). https://doi.org/10.1186/1756-0500-3-53
39. Hu, P. J. (2007). Dauer. *WormBook*. https://doi.org/10.1895/wormbook.1.144.1
40. Hubbard, E. J., Korta, D. Z., & Dalfó, D. (2012). Physiological control of Germline Development. *Germ Cell Development in C. elegans*, 101–131. https://doi.org/10.1007/978-1-4614-4015-4_5
41. Hubbard, E. J., & Schedl, T. (2019). Biology of the *Caenorhabditis elegans* germline stem cell system. *Genetics*, 213(4), 1145–1188. https://doi.org/10.1534/genetics.119.300238
42. Iwasa, H., Maimaiti, S., Kuroyanagi, H., Kawano, S., Inami, K., Timalsina, S., Ikeda, M., Nakagawa, K., & Hata, Y. (2013). Yes-associated protein homolog, *yap-1*, is involved in the thermotolerance and aging in the nematode *Caenorhabditis elegans*. *Experimental Cell Research*, 319(7), 931–945. https://doi.org/10.1016/j.yexcr.2013.01.020
43. Jin, J., Zhao, X., Fu, H., & Gao, Y. (2020). The effects of yap and its related mechanisms in central nervous system diseases. *Frontiers in Neuroscience*, 14. https://doi.org/10.3389/fnins.2020.00595
44. Johnson, S. C., Rabinovitch, P. S., & Kaeberlein, M. (2013). MTOR is a key modulator of aging and age-related disease. *Nature*, 493(7432), 338–345. https://doi.org/10.1038/nature11861
45. Kamath, R. (2003). Genome-wide RNAi screening in *Caenorhabditis elegans*. *Methods*, 30(4), 313–321. https://doi.org/10.1016/s1046-2023(03)00050-1

46. Kamath, R. S., Fraser, A. G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., Welchman, D. P., Zipperlen, P., & Ahringer, J. (2003). Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature*, 421(6920), 231–237. https://doi.org/10.1038/nature01278

47. Kelly, K. O., Dernburg, A. F., Stanfield, G. M., & Villeneuve, A. M. (2000). *Caenorhabditis elegans msh-5* is required for both normal and radiation-induced meiotic crossing over but not for completion of meiosis. *Genetics*, *156*(2), 617–630. https://doi.org/10.1093/genetics/156.2.617

48. Kenyon, C., Chang, J., Gensch, E., Rudner, A., & Tabtiang, R. (1993). A *C. elegans* mutant that lives twice as long as wild type. *Nature*, 366(6454), 461–464. https://doi.org/10.1038/366461a0

49. Kershner, A. M., Shin, H., Hansen, T. J., & Kimble, J. (2014). Discovery of two GLP-1/notch target genes that account for the role of GLP-1/Notch signaling in stem cell maintenance. *Proceedings of the National Academy of Sciences*, 111(10), 3739–3744. https://doi.org/10.1073/pnas.1401861111

50. Kimble, J. (2005). Germline proliferation and its control. WormBook. https://doi.org/10.1895/wormbook.1.13.1

51. Kimble, J., & Crittenden, S. L. (2007). Controls of germline stem cells, entry into meiosis, and the sperm/oocyte decision in *Caenorhabditis elegans*. *Annual Review of Cell and Developmental Biology*, 23(1), 405–433. https://doi.org/10.1146/annurev.cellbio.23.090506.123326

52. Kimble, J. (2011). Molecular regulation of the mitosis/meiosis decision in multicellular organisms. *Cold Spring Harbor Perspectives in Biology*, 3(8). https://doi.org/10.1101/cshperspect.a002683

53. Kimble, J. (2014). *C. elegans* germline stem cells and their niche. *StemBook*. https://doi.org/10.3824/stembook.1.95.1

54. Kitagawa, R. (2009). The spindle assembly checkpoint in *Caenorhabditis elegans*: One who lacks mad1 becomes mad one. *Cell Cycle*, 8(3), 338–344. https://doi.org/10.4161/cc.8.3.7448

55. Korswagen, H. C. (2002). Canonical and non-canonical Wnt Signaling Pathways in *Caenorhabditis elegans*: Variations on a common signaling theme. *BioEssays*, 24(9), 801–810. https://doi.org/10.1002/bies.10145

56. Korta, D. Z., Tuck, S., & Hubbard, E. J. (2012). S6K links cell fate, cell cycle and nutrient response in *C. elegans* germline stem/progenitor cells. *Development*, 141(1), 236–236. https://doi.org/10.1242/dev.106021

57. Kramer, I. J. M. (2016). Signaling through the insulin receptor. *Signal Transduction*, 849–886. https://doi.org/10.1016/b978-0-12-394803-8.00016-4

58. Krishna, S., Maduzia, L. L., & Padgett, R. W. (1999). Specificity of TGFB signaling is conferred by distinct type I receptors and their associated SMAD proteins in *Caenorhabditis elegans*. *Development*, 126(2), 251–260. https://doi.org/10 .1242/dev.126.2.251

59. Lakowski, B., & Hekimi, S. (1998). The genetics of caloric restriction in *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences*, 95(22), 13091–13096. https://doi.org/10.1073/pnas.95.22.13091

60. Lara-Gonzalez, P., Westhorpe, F. G., & Taylor, S. S. (2012). The spindle assembly checkpoint. *Current Biology*, 22(22). https://doi.org/10.1016/j.cub.2012.10.006

61. Lee, H., Cho, J. S., Lambacher, N., Lee, J., Lee, S.-J., Lee, T. H., Gartner, A., & Koo, H.-S. (2008). The *Caenorhabditis elegans* amp-activated protein kinase AAK-2 is phosphorylated by LKB1 and is required for resistance to oxidative stress and for normal motility and foraging behavior. *Journal of Biological Chemistry*, 283(22), 14988–14993. https://doi.org/10.1074/jbc.m709115200

62. Lee, H., Kang, J., & Lee, J. (2018). Involvement of YAP-1, the homolog of yes-associated protein, in the Wnt-mediated neuronal polarization in *Caenorhabditis elegans*. *G3 (Bethesda)*, 8(8), 2595–2602. https://doi.org/10.1534/g3.118.200325

63. Lee, M.-H., Ohmachi, M., Arur, S., Nayak, S., Francis, R., Church, D., Lambie, E., & Schedl, T. (2007). Multiple functions and dynamic activation of MPK-1 extracellular signal-regulated kinase signaling in *Caenorhabditis elegans* germline development. *Genetics*, 177(4), 2039–2062. https://doi.org/10.1534/genetics.107.081356

64. Lee, S., & Dong, H. H. (2017). Foxo integration of insulin signaling with glucose and lipid metabolism. *Journal of Endocrinology*, 233(2). https://doi.org/10.1530/joe-17-0002

65. Li, N., Lu, N., & Xie, C. (2019). The hippo and Wnt signaling pathways: Crosstalk during neoplastic progression in gastrointestinal tissue. *The FEBS Journal*, 286(19), 3745–3756. https://doi.org/10.1111/febs.15017

66. Long, X., Spycher, C., Han, Z. S., Rose, A. M., Müller, F., & Avruch, J. (2002). Tor deficiency in *C. elegans* causes developmental arrest and intestinal atrophy by inhibition of mRNA translation. *Current Biology*, 12(17), 1448–1461. https://doi.org/10.1016/s0960-9822(02)01091-6

67. Malumbres, M. (2014). Control of the cell cycle. *Abeloff's Clinical Oncology*. https://doi.org/10.1016/b978-1-4557-2865-7.00004-7

68. Meraldi, P., Draviam, V. M., & Sorger, P. K. (2004). Timing and checkpoints in the regulation of mitotic progression. *Developmental Cell*, 7(1), 45–60. https://doi.org/10.1016/j.devcel.2004.06.006

69. Michaelson, D., Korta, D. Z., Capua, Y., & Hubbard, E. J. (2014). Insulin signaling promotes germline proliferation in *C. elegans*. *Development*, 141(1), 237–237. https://doi.org/10.1242/dev.106013

70. Moyle, M. W., Kim, T., Hattersley, N., Espeut, J., Cheerambathur, D. K., Oegema, K., & Desai, A. (2014). A BUB1–mad1 interaction targets the MAD1–mad2 complex to unattached kinetochores to initiate the spindle checkpoint. *Journal of Cell Biology*, 204(5), 647–657. https://doi.org/10.1083/jcb.201311015

71. Murphy, C. T. & Hu P. J. (2013). Insulin/insulin-like growth factor signaling in *C. elegans*. *WormBook*. 1-43. doi: 10.1895/wormbook.1.164.1. PMID: 24395814; PMCID: PMC4780952.

72. Nasmyth, K., Peters, J.-M., & Uhlmann, F. (2000). Splitting the chromosome: Cutting the ties that bind sister chromatids. *Science*, 288(5470), 1379–1384. https://doi.org/10.1126/science.288.5470.1379

73. Nair, A., Chauhan, P., Saha, B., & Kubatzky, K. F. (2019). Conceptual evolution of Cell Signaling. *International Journal of Molecular Sciences*, 20(13), 3292. https://doi.org/10.3390/ijms20133292

74. Narbonne, P., Maddox, P. S., & Labbé, J.-C. (2017). DAF-18/PTEN signals through AAK-1/AMPK to inhibit MPK-1/MAPK in feedback control of germline stem cell proliferation. *PLOS Genetics*, 13(4). https://doi.org/10.1371/journal.pgen.1006738

75. Narbonne, P., & Roy, R. (2006). Inhibition of germline proliferation during *C. elegans* dauer development requires PTEN, LKB1 and AMPK signaling. Development, 133(4), 611–619. https://doi.org/10.1242/dev.02232

76. Ogg, S., & Ruvkun, G. (1998). The *C. elegans* PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway. *Molecular Cell*, 2(6), 887–893. https://doi.org/10.1016/s1097-2765(00)80303-2

77. Pan, K. Z., Palter, J. E., Rogers, A. N., Olsen, A., Chen, D., Lithgow, G. J., & Kapahi, P. (2007). Inhibition of mRNA translation extends lifespan in *Caenorhabditis elegans*. *Aging Cell*, 6(1), 111–119. https://doi.org/10.1111/j.1474-9726.2006.00266.x

78. Patterson, G. I., Koweek, A., Wong, A., Liu, Y., & Ruvkun, G. (1997). The DAF-3 SMAD protein antagonizes TGF-β-related receptor signaling in the *Caenorhabditis elegans* dauer pathway. *Genes & Development*, 11(20), 2679–2690. https://doi.org/10.1101/gad.11.20.2679

79. Pazdernik, N., & Schedl, T. (2012). Introduction to germ cell development in *Caenorhabditis elegans*. *Germ Cell Development in C. elegans*, 1–16. https://doi.org/10.1007/978-1-4614-4015-4_1

80. Pekar, O., Ow, M. C., Hui, K. Y., Noyes, M. B., Hall, S. E., & Hubbard, E. J. (2017). Linking the environment, DAF-7/TGFB signaling and *lag-2*/DSL ligand expression in the germline stem cell niche. *Development*, 144(16), 2896–2906. https://doi.org/10.1242/dev.147660

81. Pintard, L., & Bowerman, B. (2019). Mitotic cell division in *Caenorhabditis elegans*. *Genetics*, 211(1), 35–73. https://doi.org/10.1534/genetics.118.301367

82. Qiao, L., Lissemore, J. L., Shu, P., Smardon, A., Gelber, M. B., & Maine, E. M. (1995). Enhancers of *glp-1*, a gene required for cell-signaling in *Caenorhabditis elegans*, define a set of genes required for germline development. *Genetics*, 141(2), 551–569. https://doi.org/10.1093/genetics/141.2.551

83. Okuyama, T., Inoue, H., Ookuma, S., Satoh, T., Kano, K., Honjoh, S., Hisamoto, N., Matsumoto, K., & Nishida, E. (2010). The Erk-MAPK pathway regulates longevity through SKN-1 and insulin-like signaling in *Caenorhabditis elegans*. *Journal of Biological Chemistry*, 285(39), 30274–30281. https://doi.org/10.1074/jbc.m110.146274

84. Redman, L. M., & Ravussin, E. (2011). Caloric restriction in humans: Impact on physiological, psychological, and behavioral outcomes. *Antioxidants & Redox Signaling*, 14(2), 275–287. https://doi.org/10.1089/ars.2010.3253

85. Rehman I, Gulani A, Farooq M, et al. Genetics, Mitosis. [Updated 2022 Mar 26]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK482449/

86. Robinson-Thiewes, S., Dufour, B., Martel, P.-O., Lechasseur, X., Brou, A. A., Roy, V., Chen, Y., Kimble, J., & Narbonne, P. (2020). Non-autonomous regulation of germline stem cell proliferation by somatic MPK-1/MAPK activity in *C. elegans*. https://doi.org/10.1101/2020.08.24.265249

87. Roy, D., Kahler, D. J., Yun, C., & Hubbard, E. J. (2018). Functional interactions between *rsks-1/*S6K, *glp-1/*Notch, and regulators of *Caenorhabditis elegans* fertility and germline

stem cell maintenance. G3 (Bethesda), 8(10), 3293–3309. https://doi.org/10.1534/g3.118.200511

88. Santaguida, S., & Amon, A. (2015). Short- and long-term effects of chromosome mis-segregation and aneuploidy. *Nature Reviews Molecular Cell Biology*, 16(8), 473–485. https://doi.org/10.1038/nrm4025

89. Sawa, H., & Korswagen, H. C. (2013). Wnt signaling in *C. elegans*. *WormBook*, 1–30. https://doi.org/10.1895/wormbook.1.7.2

90. Savage-Dunn, C., Tokarz, R., Wang, H., Cohen, S., Giannikas, C., & Padgett, R. W. (2000). SMA-3 SMAD has specific and critical functions in DBL-1/SMA-6 TGFB-related signaling. *Developmental Biology*, 223(1), 70–76. https://doi.org/10.1006/dbio.2000.9713

91. Savage-Dunn, C., & Padgett, R. W. (2017). The TGF-β family in *caenorhabditis elegans*. *Cold Spring Harbor Perspectives in Biology*, 9(6). https://doi.org/10.1101/cshperspect.a022178

92. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis. *Nature Methods*, 9(7), 676–682. https://doi.org/10.1038/nmeth.2019

93. Schlesinger, A., Shelton, C. A., Maloof, J. N., Meneghini, M., & Bowerman, B. (1999). Wnt pathway components orient a mitotic spindle in the early *Caenorhabditis elegans* embryo without requiring gene transcription in the responding cell. *Genes & Development*, 13(15), 2028–2038. https://doi.org/10.1101/gad.13.15.2028

94. Silva, R. D., Mirkovic, M., Guilgur, L. G., Rathore, O. S., Martinho, R. G., & Oliveira, R. A. (2018). Absence of the spindle assembly checkpoint restores mitotic fidelity upon loss of sister chromatid cohesion. *Current Biology*, 28(17). https://doi.org/10.1016/j.cub.2018.06.062

95. Stark, G. R., & Taylor, W. R. (2004). Analyzing the G2/M checkpoint. *Checkpoint Controls and Cancer*, 051–082. https://doi.org/10.1385/1-59259-788-2:051

96. Stiernagle, T. (2006). Maintenance of *C. elegans*. *WormBook*. https://doi.org/10.1895/wormbook.1.101.1

97. Sudakin, V., Chan, G. K. T., & Yen, T. J. (2001). Checkpoint inhibition of the APC/C in Hela cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. *Journal of Cell Biology*, 154(5), 925–936. https://doi.org/10.1083/jcb.200102093

98. Sun, X., Chen, W.-D., & Wang, Y.-D. (2017). DAF-16/FOXO transcription factor in aging and longevity. *Frontiers in Pharmacology*, 8. https://doi.org/10.3389/fphar.2017.00548

99. Sundaram, M. (2006). RTK/Ras/MAPK signaling. *WormBook*. https://doi.org/10.1895/wormbook.1.80.1

100. Swift, L. H., & Golsteyn, R. M. (2016). The relationship between checkpoint adaptation and mitotic catastrophe in genomic changes in cancer cells. *Genome Stability*, 373–389.

101. Tabara, H., Sarkissian, M., Kelly, W. G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., & Mello, C. C. (1999). The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. *Cell*, 99(2), 123–132. https://doi.org/10.1016/s0092-8674(00)81644-x

102. Tinevez, J.-Y., Perry, N., Schindelin, J., Hoopes, G. M., Reynolds, G. D., Laplantine, E., Bednarek, S. Y., Shorte, S. L., & Eliceiri, K. W. (2017). TrackMate: An open and extensible platform for single-particle tracking. *Methods*, 115, 80–90. https://doi.org/10.1016/j.ymeth.2016.09.016

103. Torres, M., & Forman, H. J. (2006). Signal transduction. *Encyclopedia of Respiratory Medicine*, 10–18. https://doi.org/10.1016/b0-12-370879-6/00351-3

104. van der Voet, M., Lorson, M., Srinivasan, D. G., Bennett, K. L., & van den Heuvel, S. (2009). *C. elegans* mitotic cyclins have distinct as well as overlapping functions in chromosome segregation. *Cell Cycle*, 8(24), 4091–4102. https://doi.org/10.4161/cc.8.24.10171

105. Varadarajan, R., Nagarajaram, H. A., & Ramakrishnan, C. (1996). A procedure for the prediction of temperature-sensitive mutants of a globular protein based solely on the amino acid sequence. *Proceedings of the National Academy of Sciences*, 93(24), 13908–13913. https://doi.org/10.1073/pnas.93.24.13908

106. Varelas, X., Miller, B. W., Sopko, R., Song, S., Gregorieff, A., Fellouse, F. A., Sakuma, R., Pawson, T., Hunziker, W., McNeill, H., Wrana, J. L., & Attisano, L. (2010). The Hippo pathway regulates Wnt/β-catenin signaling. *Developmental Cell*, 18(4), 579–591. https://doi.org/10.1016/j.devcel.2010.03.007

107. Vellai, T., Takacs-Vellai, K., Zhang, Y., Kovacs, A. L., Orosz, L., & Müller, F. (2003). Influence of tor kinase on lifespan in *C. elegans*. *Nature*, 426(6967), 620–620. https://doi.org/10.1038/426620a

108. Walker, G., Houthoofd, K., Vanfleteren, J. R., & Gems, D. (2005). Dietary restriction in *C. elegans*: From rate-of-living effects to nutrient sensing pathways.

Mechanisms of Ageing and Development, 126(9), 929–937. https://doi.org/10.1016/j.mad.2005.03.014

109. Walston, T., Tuskey, C., Edgar, L., Hawkins, N., Ellis, G., Bowerman, B., Wood, W., & Hardin, J. (2004). Multiple Wnt signaling pathways converge to orient the mitotic spindle in early *C. elegans* embryos. *Developmental Cell*, 7(6), 831–841. https://doi.org/10.1016/j.devcel.2004.10.008

110. Wang, S., Gao, Y., Song, X., Ma, X., Zhu, X., Mao, Y., Yang, Z., Ni, J., Li, H., Malanowski, K. E., Anoja, P., Park, J., Haug, J., & Xie, T. (2015). Wnt signaling-mediated redox regulation maintains the germ line stem cell differentiation niche. *ELife*, 4. https://doi.org/10.7554/elife.08174

111. Wu, Y., Han, M., & Guan, K. L. (1995). MEK-2, a *Caenorhabditis elegans* map kinase kinase, functions in Ras-mediated vulval induction and other developmental events. *Genes & Development*, 9(6), 742–755. https://doi.org/10.1101/gad.9.6.742

112. Wu, Z., Isik, M., Moroz, N., Steinbaugh, M. J., Zhang, P., & Blackwell, T. K. (2021). Dietary restriction extends lifespan through metabolic regulation of innate immunity. *Cell Metabolism*, 33(10), 2090. https://doi.org/10.1016/j.cmet.2021.08.016

113. Yang, Z., & Hata, Y. (2013). What is the Hippo pathway? Is the Hippo pathway conserved in *Caenorhabditis elegans*? *Journal of Biochemistry*, 154(3), 207–209. https://doi.org/10.1093/jb/mvt060

114. Yoon, D. S., Alfhili, M. A., Friend, K., & Lee, M.-H. (2017). MPK-1/ERK regulatory network controls the number of sperm by regulating timing of sperm-oocyte switch in *C. elegans* germline. *Biochemical and Biophysical Research Communications*, 491(4), 1077–1082. https://doi.org/10.1016/j.bbrc.2017.08.014

115. Zellag, R., Zhao, Y., & Gerhold, A. (2022). Live-cell imaging and analysis of germline stem cell mitosis in *Caenorhabditis elegans*. *BIO-PROTOCOL*, 12(1). https://doi.org/10.21769/bioprotoc.4272

116. Zellag, R. M., Zhao, Y., Poupart, V., Singh, R., Labbé, J.-C., & Gerhold, A. R. (2021). Centtracker: A trainable, machine-learning–based tool for large-scale analyses of *Caenorhabditis elegans* germline stem cell mitosis. *Molecular Biology of the Cell*, 32(9), 915–930.

117. Zečić, A., & Braeckman, B. P. (2020). DAF-16/FOXO in *Caenorhabditis elegans* and its role in metabolic remodeling. *Cells*, 9(1), 109. https://doi.org/10.3390/cells9010109

118. Zhang, S., Li, F., Zhou, T., Wang, G., & Li, Z. (2020). *Caenorhabditis elegans* as a useful model for studying aging mutations. *Frontiers in Endocrinology*, 11. https://doi.org/10.3389/fendo.2020.554994

119. Zou, L., Wu, D., Zang, X., Wang, Z., Wu, Z., & Chen, D. (2019). Construction of a germline-specific RNAi tool in *C. elegans*. *Scientific Reports*, 9(1). https://doi.org/10.1038/s41598-019-38950-8

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