Characterization of signal transduction pathways required for starvation longevity in the model nematode *Caenorhabditis elegans*

by

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Abstract

Growth and development is controlled by specific sets of gene products (proteins and RNAs), forming metabolic and developmental pathways. Mutations in these genes can result in abnormal growth of cells, leading to diseases such as cancer. "Phosphatase and tensin homolog" (PTEN) is one of the main proteins that control developmental and growth processes in humans. In the nematode *Caenorhabditis elegans*, DAF-18 is the homolog of PTEN. At the L1 larval stage, worms use an insulin-like signalling pathway to detect the availability of nutrients. DAF-18 is an antagonist of the insulin signalling pathway and is essential in maintaining quiescence during L1 arrest. Insulin-like peptides bind to the insulin receptor triggering a signal transduction pathway, which inhibits a transcription factor (DAF-16/FOXO) from entering the nucleus and activating the transcription of stress resistance genes. In L1 arrest, wild-type worms can survive up to 22 days without food. We and others have shown that *daf-16* mutants show a decreased survival of 13 days in L1 arrest while *daf-18* mutants can only survive up to 3-4 days. This discrepancy in time suggests that although DAF-18 is upstream of DAF-16, DAF-18 has additional functions essential in maintaining L1 arrest and L1 is, in part, independent of DAF-16 signalling. The aim of this study is to identify a potential alternate DAF-18 pathway, which works independently of DAF-16 and plays a crucial role in L1 survival. I predict that over- or under-expressing genes downstream of *daf-18* in the alternate pathway will allow the *daf-18* mutant worms to live longer than 4 days. I was able to isolate two suppressors through EMS screening, that can enhance the survival up to almost wild type even when DAF-18 is mutated and are possible members of alternate pathway. I also studied the effect of DAF-2 on L1 arrest with somatic *daf-18* rescue. From Yeast Two-Hybrid screens, I isolated multiple possible interactors of DAF-18. The discovery of a new pathway will provide a better understanding of larval growth and development in *C. elegans*. Moreover, these findings could identify genes that play major roles in control of cellular growth and the development of cancer.

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List of Abbreviations

AAK – AMP-activated-kinase

ADE2 – Phosphoribosylaminoimidazole carboxylase encoding gene

AKT – protein kinase B

AGE – ageing alteration

AMP – Adenosine monophosphate

AMPK - AMP-dependent protein kinase

ARD – adult reproductive diapause

BME – Beta Mercaptoethanol

cGMP – cyclic guanosine monophosphate

CGC – Caenorhabditis Genetics Center

CKI – cyclin-dependent kinase inhibitor

Cas-9 – CRISPR-associated protein-9 nuclease

CRISPR – Clustered regularly interspaced short palindromic repeats

DAF– abnormal dauer formation

ddH_2O – double distilled water

dsRNA – double-stranded RNA

DTT – Dithiothreitol

EMS – Ethyl methanesulfonate

FITC – fluorescein isothiocyanate

FOXO – forkhead box protein O

GAL1 – Galactokinase

G2 Phase – gap phase 2

GFP – green fluorescent protein

IC – Ian Chin-Sang lab strain

IGF – insulin-like growth factor

IGFR – insulin-like growth factor receptor

IIS – Insulin and Insulin-like growth factor signaling

IR – Insulin Receptor

L1 – larval stage one

L2– larval stage two

L3– larval stage three

L4 – larval stage four

MAPK – mitogen-actived protein kinase

MEC - mechanosensory

miRNA – microRNA

MosSCI – Mos-1 mediated single copy insertion

mTORC/ TOC– mammalian target of rapamycin complex homologue

MPK – MAP kinase

NGM – nematode growth media

oIC – Ian Chin-Sang lab oligonucleotide

PCR – Polymerase Chain Reaction

PDK1 – 3-phosphoinositide dependent protein kinase-1

PGCs – Primordial germ cells (Z2/Z3)

pIC – Ian Chin-Sang lab plasmid

PI3K – phosphatidylinositol 3-kinases

PIP2 – phosphatidylinositol 4,5-bisphosphate

PIP3 – phosphatidylinositol 3,4,5-triphosphate

PTEN – phosphatase and tensin homolog

RNA seq– RNA sequencing

RISC – RNA induced silencing complex

RNAi – RNA interference

RTK – Receptor tyrosine kinase

S Phase – synthesis phase

siRNA – small interfering RNA

TGF-β – transforming growth factor beta

UAS – upstream activation sequence

Y2H Yeast two-hybrid

Chapter 1

Introduction

The evolution of single-cells to multi-cellular organisms involved complex growth and developmental modifications. Growth and developmental processes are coordinated through timing and cell patterning which ultimately results in specific cell function. Developmental processes are controlled by specific signal transduction pathways. Mutation in these pathways can lead to severe growth deformations. Cancer is one of the phenotypes of such mutations that give rise to uncontrolled growth of cells.

To completely understand the mechanism behind these abnormalities, we need to understand the multitude of genes and pathways that are involved in controlling growth and development. With over three decades of research, many signaling pathways and genes that control these processes have been identified. However, most of these pathways are still not fully understood at the cellular level.

It is very difficult to obtain a comprehensive understanding of all these processes, as redundant pathways may help to overcome mutations in different growth pathways. We cannot mimic these pathways *in vitro* because of the complex cellular environment and the unknown factors that might play a major role in the functioning of these genes. To overcome such issues, model organisms are used. For this study, the nematode *Caenorhabditis elegans* was the preferred model. *C. elegans* is a simple multicellular animal with a transparent body. It has a sequenced genome, a fully annotated cell lineage and neural connectome (Sulston et al. 1983, White et al. 1986). In addition, molecular and genetic tools, which include RNA interference (RNAi) and CRISPR/ Cas9 genome editing, are well established in this model animal (Xu and Kim 2011,

Waaijers and Boxem 2014). Furthermore, the transparent body of *C. elegans* allows visualization of the fluorescent fusion proteins to track gene expression and cellular growth. Many human genes have orthologs in *C. elegans* and signaling pathways are highly conserved across higher organisms. A good example of such conservation is the insulin and insulin-like growth factor signaling (IIS) pathway. This pathway uses the same genetic components to regulate complex processes like metabolism, aging and development in all animals. Thus studying the insulin pathway in model organisms may help us understand how complex regulators of the insulin pathway may function in human diseases (Leung et al. 2008, Shaye and Greenwald 2011).

C. elegans is a free living nematode with an adult body that measures up to 1-2 mm. Its natural habitats include human-made compost heaps and other microbial-rich environments. It feeds on bacteria that are present in these environments, but the nutrient availability varies dependent on habitat (Félix and Braendle 2010). *C. elegans* has developed mechanisms that allow it to survive under scarce nutrient conditions. These mechanisms involve the ability to cease cellular growth at different stages of life. *C. elegans* life cycle consists of four larval stages (L1- L4) before it reaches adulthood. The two main developmental arrests during larval stages are "L1 arrest" and "Dauer". When the worm hatches, it is in the L1 stage and has the ability to assess nutrient availability. If enough nutrients are present, it grows into second larval stage (L2) but if the worm hatches under starvation conditions, it goes into a stress resistant "L1 arrest" state. In L1 arrested state, the worm stops all its energy consuming developmental process until a new food source becomes available. The worm can stay at L1 arrest for 21-22 days which is equivalent to an adult worm's whole life span. (**Figure 1**).

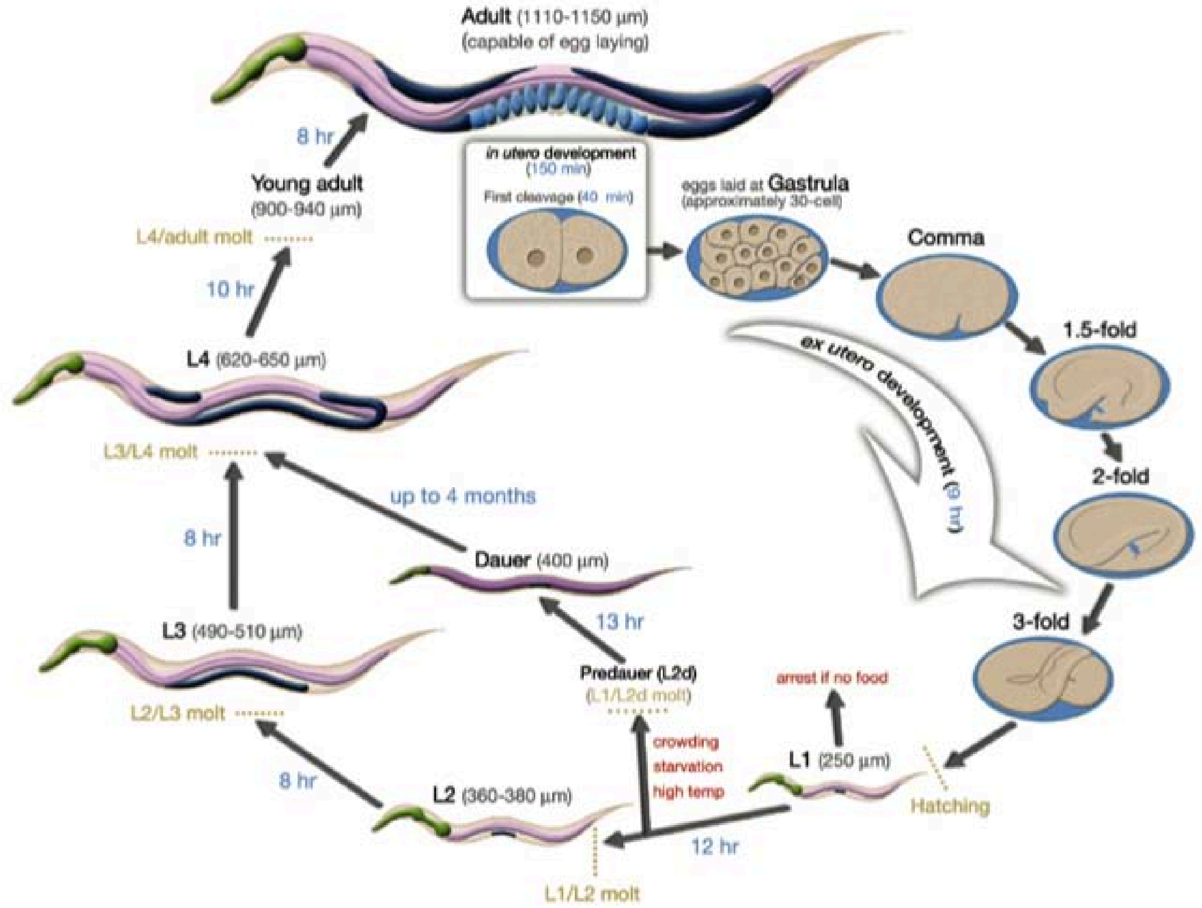


Figure 1: *C. elegans* life cycle at 22° C. Showing the life cycle of *C. elegans* including all developmental stages and time duration required to transition from one stage to the next (wormatlas.org).

The *C. elegans* dauer is a later stage in development where the worm will arrest development in harsh conditions (*i.e*. crowding, starvation or high temperature). The dauer phenotype is studied extensively but knowledge about the L1 arrest mechanisms is limited. However, like dauer regulation, the insulin and insulin-like signaling is involved. Inhibition of IIS leads to the nuclear localization of DAF-16/FOXO which initiates the transcription of genes involved in L1 arrest, dauer formation, lifespan extension and stress resistance. DAF-18/PTEN antagonises AGE-1/PI-

3K resulting the nuclear localization of DAF-16, which otherwise facilitates the phosphorylation of DAF-16 by AKT and inhibits its nuclear localization (**Figure 2**). Previous studies have stated the importance of germline quiescence in L1 arrest. Germline proliferation is mediated by insulin signaling pathway and the arrest of germline proliferation at L1 requires *daf-18* (Fukuyama et al. 2006). In a study from our lab, DAF-18/PTEN was expressed at higher levels in the primordial germ cells Z2/Z3 during L1 stage (Brisbin et al. 2009). These findings pointed towards the importance of germline expression of *daf-18* in maintaining germline quiescence during L1 arrest. DAF-16/FOXO is the ultimate target of the insulin signaling pathway (Ogg et al. 1997) and although it plays a key role in L1 arrest, it does not have a role in germline quiescence during L1 arrest (Fukuyama et al. 2006).

According to previous studies, DAF-18 works independently of DAF-16 to maintain germline quiescence (Fukuyama et al. 2006), however it does need DAF-16/FOXO to control dauer larva formation (Ogg and Ruvkun 1998). Findings from our lab have shown that *daf-16* mutants have decreased L1 survival of 13 days but live longer than *daf-18* mutants, which only survive up to 3-4 days (Zanetti, 2014). This finding suggests a DAF-16/FOXO independent role for DAF-18/PTEN in the L1 arrest longevity. DAF-18 along with the AMPK pathway maintain germline quiescence during L1 starvation (Fukuyama et al. 2012). Moreover, inhibiting TORC1 can restore germline quiescence in *daf-18* mutants but does not fully rescue the longevity of L1 arrested worms. This suggests that *daf-18* maintains L1 arrest longevity through TORC1-independent pathways (Fukuyama et al. 2012). The *daf-18* dauer can be restored by expressing *daf-18(wt)* only in somatic tissues. However neither somatic nor germline *daf-18(wt)* expression can fully restore the L1 arrest the longevity to wild-type, nor the germline proliferation in L1

arrest. This suggests *daf-18* is required both in germline and somatic cells to maintain L1 arrest longevity.

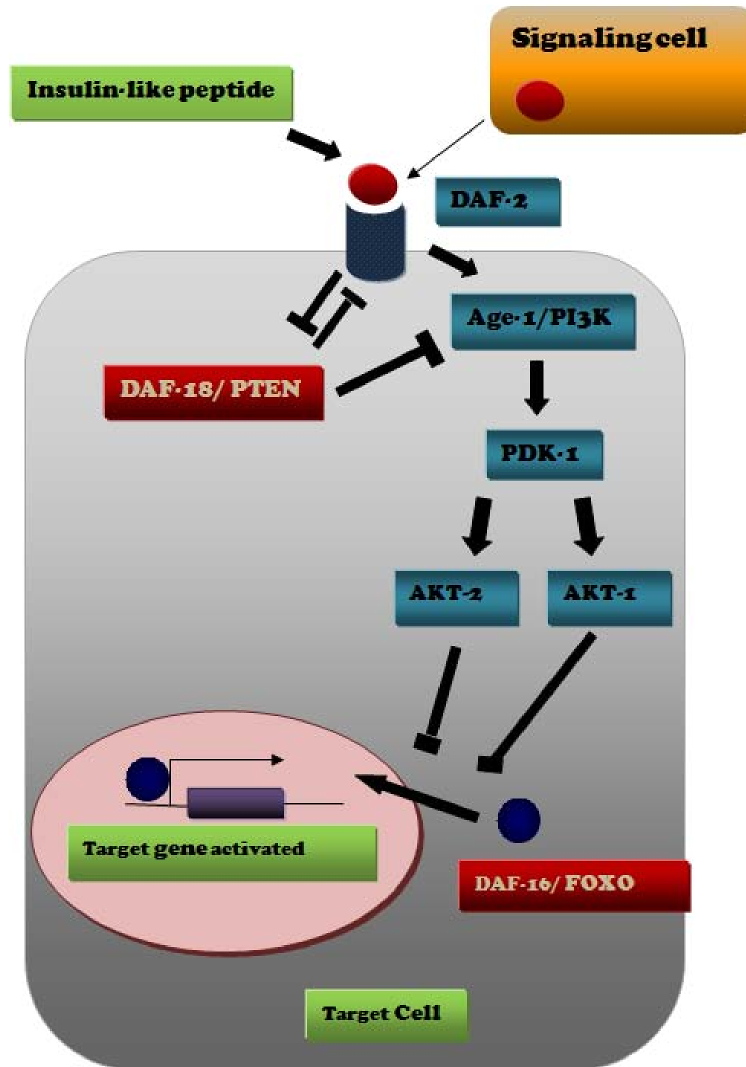


Figure 2: Insulin and insulin-like signaling (IIS) pathway: Insulin- like peptide from signaling cells bind the receptor, which in most case results in activation of IIS pathway. Once activated, the pathway facilitates energy requiring growth and developmental pathways mainly by inhibition of nuclear localization of DAF-16. This pathway is a "protein phosphorylation cascade, which inhibits nuclear localization of DAF-16 by its phosphorylation.

These studies lead to the hypothesis that DAF-18 functions in DAF-16/FOXO independent pathways to maintain L1 arrest and that both somatic and germline DAF-18 expression is necessary for L1 arrest. The objective of this study is to identify the components of pathway through which DAF-18 is maintaining the quiescence in L1 arrest.

Chapter 2

Literature Review

2.1 Natural habitat of *C. elegans*:

The free living nematode was first isolated in soil and was classified as soil nematode. Further studies showed that rather than soil, it natural habitat can be more associated with rotten fruits and plant and other microbe-rich environments.

Its rapid life cycle made *C. elegans* an ideal model to study cellular pathways.

2.1.1 The life cycle of *C. elegans*

The life cycle of *C. elegans* comprises distinct stages before and after hatching. The optimal temperature for worm growth is 20°C. Although worms can grow at 25°C and 15°C as well, the growth at these temperatures is altered as a result of a stress responses. After fertilization, the oocyte takes almost 2.5 hours to complete its *in utero* growth. Once laid, the embryo undergoes further development for another 9 hours before a first larval stage worm is hatched. This larval stage is known as the L1 developmental stage. After hatching, the fate of the worm relies on the availability of a constant nutrient source. If the food is present, the L1 larva molts into an L2 larval stage. At L2 stage, the larva starts developing the reproductive structures. After 8 hours of development at this stage, the worm molts to L3 stage. The next molting takes place at another 8 hour mark and the worm goes into L4 stage. At this stage sexual dimorphism is apparent and a distinguishing feature between the hermaphrodite and male is their tail. *C. elegans* naturally occur in two sexes: males (XO) and hermaphrodites (XX) (**Figure 3**). Hermaphrodite animals self-fertilize which give rise to a higher population of hermaphrodites in nature as compared to the

male population. However in labs, both male and hermaphrodites can be maintained for genetic crosses. The development of oocytes and spermatogenesis is completed at the L4 stage. After 10 hours of further development, the worm finally reaches its adult stage. However, the worm does not start laying eggs right away. It takes another 8 hours to start laying eggs. The molt at the end of each larval stage represents the entry to the next larval stage as the worm forms a new cuticle depending on the size requirement and shedding the old one (Cassada and Russell 1975).

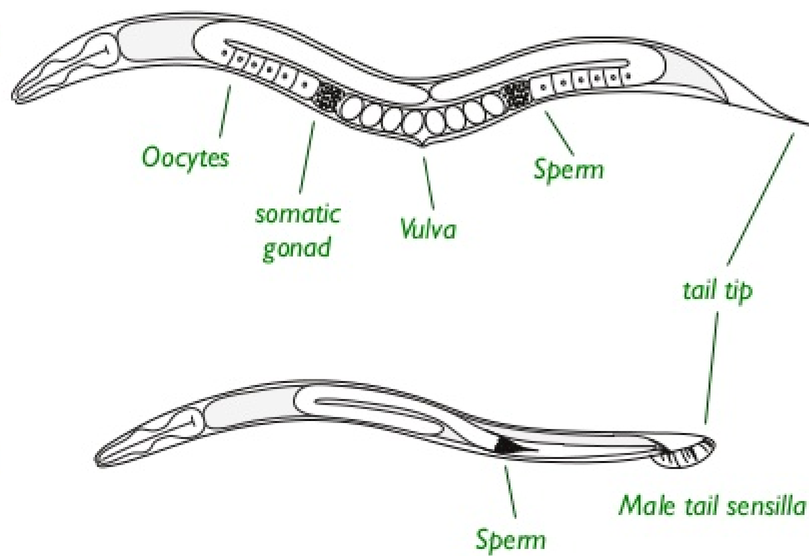


Figure 3: Anatomical differences between *C. elegans* hermaphrodites (XX) and males (XO): Hermaphrodites have a longer and thicker body as compared to males and are present in abundance when compared to male population. The most important visual difference between the two sexes is the tail; males have a fan like tail that facilitates mating (Figure credits: (Portman 2007)).

2.1.1.1 Embryogenesis

The first stage of embryogenesis is proliferation. During this stage the cells divide into 558 non-differentiated cells. During the first 150 minutes of this stage the zygote cells develop into embryonic founder cells. At 150 minutes, the egg is laid and the embryo continues its

development as the cells start migration and the embryo is differentiated into three germ layers (ecto/endo and mesoderm). The second stage of embryogenesis is also known as organogenesis. This stage comes after the 5.5-6 h mark to 12-14 h mark. The cells start their final differentiation. The embryo starts to elongate and becomes three fold thinner. At the end of this stage you can see the larva moving inside the egg along its longitudinal axis. Moreover, the main body plan of the worm doesn't change after embryogenesis is complete.

2.1.1.2 Post-embryonic development:

The development of the larva after hatching mainly depends on the presence of food. The developmental program starts 3 hours after hatching (Ambros 2000). Post-embryonic development comprises of four larval stages (L1 -L4) before the worm reaches its final adult stage. The worm has 671 nuclei at the end of embryogenesis. Out of these 671 nuclei, 113 are programmed to die eventually (Sulston et al. 1983). The worm needs constant supply of food during the post-embryonic development. If the food supply is limited or completely absent, the worm halts its growth until the conditions become favorable.

2.1.1.3 L1 stage

The L1 stage is the first larval stage of post-embryonic development. Most of the nervous system of the worm is completed by the end of L1 stage. In hermaphrodites, the somatic gonad precursor cells, Z1 and Z4, give rise to 12 cells. Then cell division also starts in germline precursor cells, Z2 and Z3, and they continue to divide from L1 throughout the adulthood (Kimble and Hirsh 1979).

2.1.1.4 L2 stage:

After the first molt, the worm enters L2, the second larval stage. There aren't many cell divisions during the L2 larval stage but the germ cells keep on dividing. The somatic and germ cells are still intermingled at this stage.

2.1.1.5 L3 larval stage:

At the L3 stage of larval development, the somatic gonad precursor cells give rise to the anterior and posterior gonadal sheaths, the uterus and the spermathecae (Kimble and Hirsh 1979). 16 sex muscle cells are generated by the division of the two sex myoblasts which are also known as egg-laying and sex-muscles. The proper positioning of these sex muscles allows egg laying through the vulva.

2.1.1.6 L4 larval stage:

The gonad development is completed at this stage. In the hermaphrodite, at the end of L4, the production of mature sperm stops and the remaining germ cells continue meiosis to give rise to oocytes. The formation of the egg laying apparatus is also completed .

2.1.1.7 Adult stage:

When growing at 22° - 25°C, the hermaphrodite lays its first egg after 45-50 h post hatching (Lewis and Fleming 1995). This completes the reproductive life cycle of *C. elegans* in 3 days (Byerly et al. 1976). The mature sperms are already present in the spermathecae. The adult hermaphrodite requires another 4 days to make the oocytes and these oocytes are continuously fertilized by the already stored sperm. At the end of its 3-4 day fertile period, the hermaphrodite lays almost 300 eggs. However if there is external sperm supply as a result of mating, the laid

eggs number reach to 1200 -1400 (Hodgkin and Barnes 1991). The worm lives up to 10 -15 days after this fertile period of 3-4 days making its life cycle about 22 days. The adult hermaphrodite comprises of 959 somatic nuclei and the adult male has a total of 1031 somatic nuclei (White 1988).

2.2 Survival adaptations during external stresses

Nature is full of hardships. It is not easy for any organism to survive unless they have adopted certain protective mechanisms. Survival of the fittest is the foundation stone of evolution. Although *C. elegans* seem very delicate, they have established many developmental, metabolic and behavioral protective mechanisms that help them to survive extreme conditions. These mechanisms are not limited to adults, as the freshly hatched fragile L1 larvae are also resistant to many environmental stresses.

2.2.1 L1 Larval arrest:

The egg hatches after 9 h of *ex utero* development. The L1 larva starts looking for food immediately and if there is no food present, it halts its growth and goes into an L1 arrest stage. During this stage cellular growth is completely shut down until a food source is detected again. L1 arrest is distinct from dauer formation as there are no morphological changes involved and it is solely because of nutrient deficiency. Although there are no morphological modifications during L1 arrest, the L1 arrested larvae are resistant to many environmental stresses and can even be frozen at extreme cold temperatures (-80°C) for laboratory studies. Stress resistance genes are observed to be up-regulated when larvae are in L1 arrested state (Baugh et al. 2009). The L1 arrested larva can survive up to 22 days in minimal media (e.g. M9 buffer) at 20°C. In wild-type

L1 arrested worms, the post embryonic-development like germline proliferation, neuronal development etc, is completely paused.

2.2.1.1 Insulin-like signaling controlling L1 arrest:

Insulin and insulin-like growth factors signaling plays a major role in most of the developmental process by assessing nutrient availability. Mutants that either have a strong loss of function or a null allele of insulin-like receptor DAF-2/InsR, result in constitutive L1 arrest and embryonic lethal phenotype respectively (Gems et al. 1998b, Patel et al. 2008). L1 arrest survival is increased significantly more than wild-type in *daf-2* hypomorphic mutants (Baugh and Sternberg 2006). To further support the evidence that insulin-like signaling regulates L1 arrest, it was observed that mutants lacking insulin-like peptide secretions displayed a constitutive L1 arrest phenotype (Kao et al. 2007).

A major effector of insulin-like signaling is the transcription factor, DAF-16/FOXO, which also plays a key role in L1 arrest by activation of stress resistance genes (Weinkove et al. 2006). *daf-16* mutants are sensitive to nutrient stress and exhibit a short life-span during L1 arrest (Baugh and Sternberg 2006). These mutants also show postembryonic development, which normally occur in nutrient abundant conditions, showing that *daf-16* controls the L1 arrest by regulating development (Baugh and Sternberg 2006).

An important interactor of insulin-like signaling is AMP-activated kinase (AMPK). Mutations in the α subunit of AMPK (*aak-2*) also result in a defective L1 arrest characterized by post embryonic development (Baugh and Sternberg 2006). The cyclin-dependent kinase inhibitor *cki-1* and the microRNA (miRNA) *lin-4* are important components of cell cycle arrest and are downstream of Insulin-like signaling pathway. Mutations in these genes also results in defects of

L1 arrest because of uncontrolled postembryonic development (Baugh and Sternberg 2006). Another important miRNA associated with developmental control in L1 arrested worms is *mir235*. The expression of *mir235* is up-regulated during L1 arrest by DAF-16/FOXO (Kasuga et al. 2013). Insulin-like signaling works cell non-autonomously to control developmental arrest (Kasuga et al. 2013).

Another important aspect of L1 arrest is germline arrest. L1 larvae have two primordial germ cells (PGCs) called Z2 and Z3 (Sulston and Horvitz 1977). In wild type worms, these germ cells do not proliferate during L1 arrest. This germline arrest is controlled by DAF-18 and α subunits of AMPK encoded by *aak-1* and *aak-2* (Ogg and Ruvkun 1998, Fukuyama et al. 2006). In *daf-18* mutants, the germ line cells further proliferate even in L1 arrest, which could be one of the reasons of short lived L1 arrest in *daf-18* mutants (Fukuyama et al. 2006). This germline proliferation in *daf-18* mutants is independent of *daf-16,* suggesting that *daf-18* controls L1 arrest through a different pathway (Baugh and Sternberg 2006).

In addition to insulin-like peptides, genes involved in secretion of Insulin-like peptides also play roles in controlling developmental arrest during L1 arrest. Mutation in *unc-31*/CAPS which is a calcium-activated regulator of dense-core vesicle release, results in an increase of L1 starvation, in a DAF-16/FOXO dependant manner (Lee and Ashrafi 2008). Moreover, mutation in *asna-1,* that encodes a conserved ATPase required for Insulin-like peptide secretion, leads to a constitutive L1 arrest phenotype (Kao et al. 2007). In addition to insulin signaling, autophagy in the pharynx also plays an important role in recovering from L1 arrest and mutants that have extensive autophagy in pharynx during L1 arrest have defective recovery from L1 arrest (Kang et al. 2007).

Among miRNA that control L1 arrest survival, mutation in *ain-1,* which encodes a component of RNA-induced silencing complex, results in sensitivity towards starvation and a reduced L1 arrest survival (Zhang et al. 2011). Similarly MIR-71, which is upstream of Insulin-like signaling pathway, also has roles in starvation induced L1 arrest survival and mutants tend to have defective vulva after they come out of L1 arrest (Zhang et al. 2011).

Genes involved in caloric restrictions and pharynx and intestine formation have also been seen to affect starvation survival. Over-expression of *pha-4/*FOXA, the fork-head transcription factor, results in an increase in starvation survival (Zhong et al. 2010). Similarly over-expression of bZip transcription factor *skn-1*/Nrf, also extend L1 arrest survival (Paek et al. 2012).

2.2.1.2 Environmental factors controlling L1 arrest survival:

Other than genetic factors, environmental factors also contribute towards stress resistance of L1 arrested worms. High temperatures are not favorable during L1 arrest and larvae tend to have a shorter L1 arrest at high temperatures (Lee et al. 2012). Density of the worms in the incubation tube also affects the L1 survival longevity and larvae tend to survive longer when incubated at a higher worm density (Artyukhin et al. 2013). The composition of the buffer used for incubation also affects the longevity of L1 arrested worms as worms tend to survive longer in S. basal buffer as compared to M9 buffer. Addition of ethanol in the media also significantly increases the survival of L1 arrested worms by providing a carbon source for the production of fatty acids and amino acids (Castro et al. 2012, Patananan et al. 2015).

2.2.1.3 Developmental arrest at L1 stage in response to stress other than nutrient availability

C. elegans larvae can arrest at L1 because of factors other than nutrient deficient environment. However, starvation-induced L1 arrest is reversible on encountering a new food source. Larvae that hatch under high temperature (*i.e*., 30°C) undergo developmental arrest (Muñoz and Riddle 2003, Baugh and Sternberg 2006). Larvae that hatch in 200 mM NaCl also go under arrest but the can continue growing after they adapted themselves to high salt concentrations (Baugh 2013). The progeny produced by these adapted worms do not arrest development at 200 mM NaCl (Baugh 2013). *C. elegans* larvae also stop feeding when exposed to toxic compounds like heavy metals, salicylate and alcohols temporarily (Jones and Candido 1999). Feeding is also stopped upon heat shock (Jones and Candido 1999). Worms also arrest development when exposed to diacetyl or when they experience anoxia (Padilla et al. 2002, Hoffmann et al. 2010).

2.2.2 L1 aggregation during starvation

Among other survival adaptations of *C. elegans* larvae, there is the presence of circular aggregation of L1 larvae on starved plates. The circular aggregates formed are due to the presence of small amounts of ethanol or acetate. The ethanol is metabolized using functional alcohol dehydrogenase *sodh-1*. The acetate formed during this process is used in *de novo* fatty acid synthesis. Fatty acids are further derivatized to glycerophosphoethanolamides. When these compounds are released into the surrounding medium, they possibly act as aggregation cues. The worms then aggregate which possibly assists in a longer survival time (Artyukhin et al. 2015).

2.2.3 Dauer Stage:

Dauer larval stage is the most studied stage in *C. elegans* starvation responses. This stage is characterized by increased stress resistance, altered body morphology and prolonged survival (Hu 2007). After L1 larval stage, the larva prepares itself to go into dauer stage in response to environmental stresses like high population density, poor nutrition availability and increased temperatures (Golden and Riddle 1984). The worms are actively looking for food throughout this stage.

After getting cues from the environment, certain pathways are altered in the worm body which changes the worm physically. the cuticle thickens and the body is elongated. In natural environment the worm can survive up to a period of few months as dauer.

The three most important and well-studied pathways for dauer regulation are:

- Guanylyl cyclase pathway
- TGFβ-like signaling pathway
- Insulin and insulin-like factor signaling pathway

2.2.4 Checkpoint Arrests between L3/L4 Developmental Stages:

Each larval stage is followed by a cuticle molt before the worm enters into the next stage. Before each molt, there are developmental checkpoints that determine whether the worm should halt its developmental or continue to molt and enter into the next larval stage (Schindler et al. 2014). The decisions to enter into the next larval stage depends on the nutrition availability in the environment. The worm must feed for a continuous 30 minute period to pass these checkpoints. The checkpoint is mainly controlled by DAF-2, the insulin/insulin-like growth factor receptor, and mutations in certain downstream genes can alter the duration of this feeding duration. For example, mutations in *daf-2* result in an increase in feeding duration and mutations in *daf-16* will

result in a decrease of this time duration (Schindler et al. 2014). The checkpoint decisions are based solely on the presence or absence of food and not on the energy levels of the worm (Schindler et al. 2014). In the absence of food, the larvae arrest their development at precise checkpoint in early L3 or L4 stages and if the worm passes through one checkpoint, the arrest takes place at the next one, and not in any middle stage (Schindler et al. 2014). The results also showed that hypodermis is the key site for Insulin signaling pathway to control the checkpoints at L3 and L4 stages (Schindler et al. 2014). Moreover DAF-9, works downstream of DAF-16, resulting in steroid mediated hormone signaling to control the L3/ L4 checkpoint, but does not involve DAF-12 for the arrests (Schindler et al. 2014).

2.2.5 Oogenic Germline Starvation Response/ Adult Reproductive Diapause

The food supply is not a constant factor in natural environments. In times of starvation, *C. elegans* employs certain mechanisms to ensure the safety of the next generation. These mechanisms include a delay in reproductive maturity, a decrease in number of developing embryos, shrinkage of germline cells and retention of embryos *in utero* (Waggoner et al. 2000, Angelo and Van Gilst 2009, Seidel and Kimble 2011). The choice of mechanism depends on the point of L4 stage at which the worm is starved (Seidel and Kimble 2011). Once any food source is available, worms reverse these conditions and continue their normal growth. When the worms are starved at a very early stage of L4, they tend to delay the molt into adulthood, onset of oogenesis and embryo development and tend to produce very few embryos (Seidel and Kimble 2011). When the worms are starved at a later stage of L4, they tend to retain the progeny within and don't lay the eggs. These eggs hatch within the parent hermaphrodite, resulting in the death of the hermaphrodite. The parent body then acts as a food source for the newly hatched worms. This processes is classified as "bagging" or "facultative vivipary" (Chen and Caswell-Chen 2004). The

offsprings grow up to a more resistant, long lived dauer stage, hence surviving until they come across the next food source. If the hermaphrodites had laid these eggs under extreme starvation conditions, they would not have any chance to survive (Chen and Caswell-Chen 2004). The worms starved at early L4 do not show bagging phenotype as they do not have enough resources to produce even one viable offspring (Seidel and Kimble 2011). Another commonly observed phenomenon is germline shrinkage which occurs regardless of the age of the worm at which it was starved. However, the onset of oogenesis is required for the germline shrinkage phenotype (Seidel and Kimble 2011). The germline shrinkage phenotype is reversed when the worm starts feeding again (Angelo and Van Gilst 2009). The above mentioned starvation responses are not affected by crowding as believed previously, in fact all worms show the same starvation responses (Seidel and Kimble 2011).

2.2.6 Autophagy and lipolysis responses during environmental stresses:

Autophagy is a catabolic process that breaks down certain cytoplasmic organelles in double-membrane vesicles known as phagosomes. The organelles are broken down into fundamental building blocks and help to recycle the cellular proteins. *C. elegans* use autophagy in multiple developmental processes like cell survival, cell death, stress responses, aging and various other pathologies (Kang et al. 2007, Kang and Avery 2008, Samara et al. 2008, Tavernarakis et al. 2008, Lu et al. 2013).

Autophagy protects *C. elegans* from hypoxia-induced neuronal and myocyte injury, and mutants that lack autophagy become more sensitized to hypoxic cellular injuries (Samokhvalov et al. 2008). Autophagy plays a very crucial role as a defense mechanism against intracellular pathogens and other bacterial infections (Jia et al. 2009, Curt et al. 2014, Visvikis et al. 2014). Mutations in autophagy genes can result in drastic changes in overall life span (Toth et al. 2008,

Tian et al. 2009, Tian et al. 2010, Lu et al. 2011, Yang and Zhang 2011, Liang et al. 2012, Wu et al. 2012, Zhang et al. 2013a). Starvation responses like L1 arrest and dauer formation are mediated by autophagy and mutation in the protein L-isoaspartyl-O-methyltransferase gene, *pcm-1* results in a reduced L1 arrest and dauer diapause (Melendez et al. 2003, Gomez et al. 2007, Gomez and Clarke 2007). Autophagy can have both prodeath and prosurvival roles under starvation. If there is insufficient amount of autophagy, as in the case of *bec-1* mutants, starvation will lead to death (Kang et al. 2007). However in case of starvation sensitive mutants (*gpb-2* (a G-protein α subunit involved in Regulator of G protein signaling (RGS) mediated inhibition of the Gqα pathway)) (You et al. 2006), the knockdown of *bec-1* rescues starvation-induced death (Kang et al. 2007).

2.3 Insulin-like signaling pathway in *C. elegans*:

The insulin signaling pathway plays a major role in controlling many complex pathways in all animals. In mammals, activation of the insulin receptor leads to autophosphorylation which results in the activation of multiple downstream pathways. These pathways regulate processes like glucose uptake and storage, protein synthesis, regulation of lipid synthesis and mitogenic responses. The insulin signaling pathway is negatively regulated by phosphatase and tensin homologue on chromosome 10 (PTEN, a 3' phosphatase) and the family of SRC homology 2 proteins which include inositol 5'-phosphatase (SHIP, a 5' phosphatase) (Rauh et al. 2003, Sasaoka et al. 2006).

The *C. elegans* insulin and insulin-like growth factor signaling (IIS) pathway controls development, growth, longevity, metabolism as well as behavior, depending on the nutrient availability. The insulin/IGF-1 transmembrane receptor (IGFR) ortholog in worms is called DAF-2 and is regulated by the binding of insulin-like peptide ligands. Activation of DAF-2/IGFR

results in activation and recruitments of the downstream phosphoinositide 3-kinase AGE-1/PI3K (**Figure 4**). Once activated, AGE-1/PI3K phosphorylates phosphatidylinositol ($PI_{(4,5)}P2$) into phosphatidylinositol (3,4,5)-trisphosphate ($PI_{(3,4,5)}P3$) (Toker and Cantley 1997). Once formed, PIP3 further activates the serine/threonine kinases PDK-1, AKT-1, and AKT-2 (Downward 1998). AKT, a protein kinase, inhibits the ability of a FOXO transcription factor, DAF-16 to enter the nucleus by phosphorylation, promoting growth and development (Ogg et al. 1997). DAF-16/FOXO is the terminal regulator of this pathway controlling important functions of this pathway (Accili and Arden 2004).

2.3.1 DAF-18 as an antagonist of Insulin-like signaling pathway:

DAF-2/IGFR regulates the activity of DAF-16/FOXO by phosphorylating phosphoinositide 3-kinase (PI3K)/AKT kinase cascade (Taniguchi et al. 2006). DAF-18 antagonizes the activity of AGE-1/PI3K through its phospholipid phosphatase activity converting $PI_{(3,4,5)}P3$ to $PI_{(4,5)}P2$ and thereby promoting the nuclear localization of DAF-16/FOXO (Ogg and Ruvkun 1998). DAF-18 has been shown to have important roles in dauer formation, longevity and developmental processes (Gottlieb and Ruvkun 1994, Mihaylova et al. 1999). DAF-18 mutants also show a defect in larval growth and development (Riddle et al. 1981).

2.3.2 PTEN: An important tumor suppressor:

"Phosphatase and tensin homolog" (PTEN) is an important protein that functions as a phosphatase for the 3' position of inositol ring of phosphatidylinositol phosphate (PIP) (Maehama and Dixon 1998). $PI_{(3,4,5)}P3$ is the most important substrate of PTEN and levels of $PI_{(3,4,5)}P3$ at the plasma membrane regulate the PI3K-AKT pathway (Iijima and Devreotes 2002).

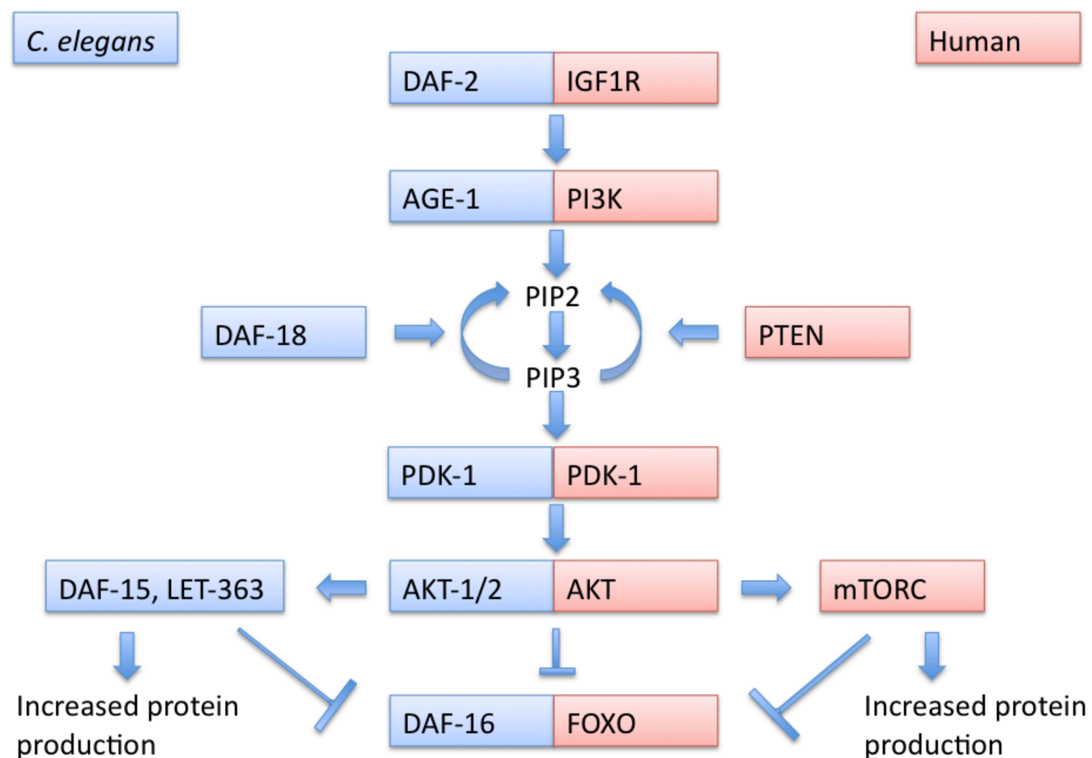


Figure 4: Insulin IGF signaling (IIS) pathway. The high conservation of insulin IGF signaling (IIS) pathway between *C. elegans* (Blue) and Human (Red) can be seen (Chamberlain, 2014).

By down-regulating levels of $PI_{(3,4,5)}P3$, PTEN essentially controls downstream pathways involving transcription, cell proliferation, cell survival and angiogenesis. Increased levels of $PI_{(3,4,5)}P3$, due to mutations in PTEN, result in a hyperactive AKT. Activation of AKT results in activation of downstream biosynthetic pathways leading to a broad spectrum of cancers (Cully et al. 2006). The positive feedback loop between p53 and PTEN, also makes PTEN an important tumor suppressor as increased levels of one, increase the levels of other and mutation in one can also decrease the levels of other protein (Cully et al. 2006). In addition to that, the PTEN C'

terminal plays an important role in genome stability and mutations in PTEN C' terminal lead to formation of spontaneous tumors (Sun et al. 2014).

2.3.3 DAF-18 as a homolog for PTEN in *C. elegans*

PTEN is mutated in most forms of cancer such as glioblastoma multiform (You et al. 2007, Xu et al. 2014), breast (Baig et al. 2011, Davis et al. 2014), endometrial carcinoma (Norimatsu et al. 2007, Mackay et al. 2010), skin (Romano and Schepis 2012) and prostate (Patel et al. 2013). Statistically, PTEN is the second most mutated tumor suppressor gene after p53 (Yin and Shen 2008). DAF-18 is the worm homolog of human PTEN. The structure of both proteins is also similar and both contain a phosphatase domain, a C2 domain, and a PDZ binding domain. The mutations that alter PTEN function also have protein variants in DAF-18. Moreover, human PTEN has the ability to rescue null *daf-18* phenotypes (Solari et al. 2005). Null mutants of PTEN are embryonic lethal in mouse, fruit flies and zebra fish. However, despite being short lived, null mutants of Daf-18/PTEN in *C. elegans* are viable and fertile (Stambolic et al. , Suzuki et al. , Huang et al. 1999, Faucherre et al. 2007, Liu and Chin-Sang 2015, Zheng and Chin-Sang 2016). This makes *C. elegans* a model organism to study PTEN function.

2.3.4 Expression and regulation of DAF-18/PTEN:

DAF-18/PTEN plays a crucial role in many cellular processes by catalyzing the conversion of phosphatidylinositol-3,4,5-trisphosphate to phosphatidylinositol-4,5-bisphosphate (Maehama and Dixon 1998, Leevers et al. 1999). DAF-18/PTEN antagonizes the functions of IIS pathway, mammalian target of rapamycin complex 1 (mTORC1) pathway and mitogen activated protein kinase (MAPK) pathway. Interestingly, DAF-18 antagonizes vulval development through MAPK pathway independent of its PIP_3 lipid phosphatase activity and the downstream components *i.e.,*

AKT and DAF-16 of the IIS pathway (Nakdimon et al. 2012). The most studied roles of DAF-18/PTEN include antagonizing PI3K, AKT and mTOR signaling which plays an integral part in regulation of glucose metabolism, nutrient response, cell proliferation and survival. DAF-18 is expressed in most of the tissues of *C. elegans*. DAF-18 is also reported to modulate PI3K signaling pathway to control the developmental neurite outgrowth by activating DAF-16/FOXO (Christensen et al. 2011). The *daf-18* promoter reporters show transcripts in neurons, intestines, body wall muscles and epidermis (Masse et al. 2005). RNA Seq analysis has shown high expression levels in early embryos and adults (Hillier et al. 2009).

The expression, localization and activity of human PTEN is controlled at post-transcriptional and post-translational levels by mono- or poly-ubiquitination, deubiquitination, acetylation, phosphorylation and expression regulation by non-coding RNAs (Wang and Jiang 2008, Fata et al. 2012, Song et al. 2012, Zhang et al. 2013b). Micro-RNAs e.g. miR-141-3p show an inverse correlation with regulation of PTEN (Jin et al. 2016). Moreover, PTEN also shows auto-inhibition through C-tail phospho-cluster, which interacts with C2 and catalytic domains resulting in the phosphorylation of Ser/Thr residues (amino acids 380-385) on the C-terminal tail (Chen et al. 2016). Post-translational modifications play a major role in regulating DAF-18/PTEN levels and stability (Song et al. 2012). The main post-translational negative regulators of DAF-18/PTEN include the insulin receptor (IR) (Liu et al. 2014), and Eph receptor tyrosine kinase (RTK) (Brisbin et al. 2009). E3 ubiquitin ligase is also a negative regulator of human PTEN but it hasn't been recognized as a negative regulator of DAF-18 (Guo et al. 2012). RNA in-situ hybridization has also shown that DAF-18/PTEN interacts with *ceh-18* and *mec-8* in oocyte maturation and embryonic elongation, respectively (Davies et al. 1999, Suzuki and Han 2006). *C. elegans* retinoblastoma homolog, *lin-35* has shown functional redundancy with DAF-18/PTEN in

controlling cell proliferation (Fay et al. 2002). As mentioned earlier, previous work from our lab has suggested that the IR negatively regulates DAF-18/PTEN by phosphorylation human PTEN at Y27 and Y174 (Liu et al. 2014).

2.4 Regulation of L1 arrest through DAF-18 independent of DAF-16

Dauer phenotype of *C. elegans* is studied extensively but knowledge about the L1 arrest mechanisms is limited. Previous studies have stated the importance of germline quiescence in L1 arrest. Germline proliferation is mediated by the insulin signaling pathway and quiescence requires the action of the insulin signaling antagonist *daf-18* (Fukuyama et al. 2006). In a study from our lab, Brisbin *et al.* (2009) showed DAF-18 is expressed at higher levels in the primordial germ cells Z2/Z3 during L1 stage (Brisbin et al. 2009). These findings pointed towards the importance of germline expression of *daf-18* in maintaining quiescence during L1 arrest. Interestingly, although *daf-16* is the ultimate target of the insulin signaling pathway (Ogg et al. 1997) it does not appear to have a role in maintaining the germline quiescence during L1 arrest. DAF-16 does play a role in L1 arrest longevity as *daf-16* mutants are short lived in L1 arrest but there must be DAF-16 independent roles for L1 arrest longevity as *daf-16* mutants do not rescue the longevity of *daf-18* mutants (Baugh and Sternberg 2006). Nuclear localization of DAF-16/FOXO initiates the transcription of genes involved in L1 arrest, dauer formation, lifespan extension and stress resistance. DAF-18 antagonizes the expression of AGE-1/PI-3K by de-phosphorylation and hence facilitates the nuclear localization of DAF-16 (**Figure 2**). However, given that *daf-18* mutants have a shorter L1 arrest longevity than *daf-16* mutants suggests there is a branch in the IIS pathway where DAF-18/PTEN functions independently of DAF-16.

L1 arrest involves a variety of genes that monitor different aspects of arrest. *daf-16* is required for starvation survival and mutation in *daf-16* results in reduced L1 arrest longevity (Baugh and

Sternberg 2006). *daf-18* also plays a key role in L1 arrest by maintaining germline quiescence (Fukuyama et al. 2006) . However, *daf-18* maintains the germline quiescence independent of *daf-16*, indicating that *daf-18* maintains L1 arrest independent of *daf-16* (Fukuyama et al. 2012). Previous work from our lab revealed that *daf-16* mutants show a decreased survival of 13 days which is a less severe phenotype than *daf-18* mutants, which only survive up to 3-4 days (Zanetti, 2014). DAF-18 along with AMPK pathway maintain germline quiescence during starvation. The viability of *aak-1; daf-18(nr2037); aak-2* triple mutants is reduced (5 days) relative to *daf-18 (nr2037)* (7 days) and ampk mutants (*aak-1* = 20 days *aak-2*= 15 days) showing that *daf-18* and *aak* genes act in parallel pathways to maintain survival during L1 diapause (longevity extended depending on the M9 media composition) (Fukuyama et al. 2012). Moreover, suppressing TORC1 to maintain germline quiescence using *let-363* (RNAi) and *daf-15* (RNAi) does not fully rescue the longevity of L1 arrested worms. This suggests that *daf-18* maintains L1 arrest longevity functions through TORC1-independent pathways (Fukuyama et al. 2012). This results also shows that the pathway controlling germline quiescence and L1 arrest longevity are separate as TORC1 mutants suppress germline proliferation but not the longevity of L1 arrested worms (Fukuyama et al. 2012). Intestinal and hypodermal expression of *daf-18* rescues survival to some extent and somatic expression of *daf-18* increases the longevity to some extent in *daf-18 (ok-480)* worms. Dauer and neuronal phenotypes can be restored with somatic rescue. However neither somatic nor germline *daf-18* expression in *daf-18 (ok-480)* mutants rescues the longevity to wild-type thus suggesting that *daf-18* is required both in germline and somatic cells to maintain L1 arrest. Human retinoblastoma tumor suppressor, *lin-35/Rb,* has also been reported to be an essential part of L1 arrest. It was suggested by the global gene expression analysis that *Rb*

suppresses the "re-feeding-induced' transcriptome while expressing the "starvation-induced" transcriptome (Cui et al. 2013).

2.4.1 DAF-2 suppression of *daf-18* L1 arrest phenotype

When the worms hatch under normal feeding conditions, the insulin receptor DAF-2 is activated resulting in normal growth of the animal. The *daf-2* mutants lack the proper insulin signaling which results in constitutive L1 arrest phenotype or are embryonically lethal (Baugh and Sternberg 2006). Previous work from our lab has shown that *daf-2*, as well as *age-1* mutants show an increased L1 arrest survival, where *daf-2* mutants can survive as long as almost 31 days (Zanetti 2014). The *daf-2(e1370); daf-16(mu86)* double-mutants were able to rescue the L1 arrest phenotype to almost wild-type. However, *daf-2(e1370); daf-18(ok480)* double-mutants can only increase the L1 arrest survival by 3 days than that of *daf-18* null mutants (Zanetti 2014). This result suggest that DAF-18/PTEN may function independently of the insulin receptor.

These studies lead to the hypothesis that DAF-18 works in an independent pathway that requires the insulin receptor DAF-2 and DAF-18/PTEN but branches between PIP3 production and the DAF-16/FOXO transcription factor to maintain L1 arrest. Alternatively, DAF-18/PTEN may function downstream of other receptors for L1 arrest longevity, which could explain why DAF-2 does not fully suppress L1 longevity in *daf-18* mutants as it does for *daf-16* mutants.

The objective of this study is identify the components of pathway through which DAF-18 is maintaining the quiescence in L1 arrest and the connection between other L1 arrest maintaining genes and DAF-18.

Chapter 3

Materials and Methods

3.1 Experimental Strains

All strains were maintained and manipulated at 20°C unless mentioned otherwise as explained by Brenner (1974). N2 Bristol was used as wild type *C. elegans* for comparisons. The following strains are used during this research:

CGC Strains: RB712 – *daf-18 (ok480) IV*

IC Strains: IC314 – *zdIs5 (Pmec-4::GFP) I*; *him-5 (e1490) V*, **IC722** – *mIs11 (Pmyo-2::gfp) IV; him-5 (e1490) V*, **IC748** – *quIs18 (daf-18 genomic; pRF4) II,* **IC756** – *him-5 (e1490) V; oxIs12 [unc-47::GFP] X,* **IC817** – *quIs18 (daf-18 genomic; pRF4) II; daf-18(ok480) IV*, **IC1015** – *zdIs5 (Pmec-4::GFP) I; daf-18 (ok480) IV*; *him-5 (e1490) V*, **IC1127:** *zdIs5 (Pmec-4::GFP) I ; daf-2 (e1370) III ; daf-18 (ok480) IV,* **IC1304** (*mIn1 [dpy-10(e128); mIs14[myo-2::GFP]] II; daf-18(ok480) IV;him-5(e1490) V*), **IC1578:** *zdIs5(Pmec-4::GFP) I; daf-2 (e979) III; daf-18 (ok480) IV*, **IC1589** – *quIs24 (Ppie-1::daf-18cDNA::pie-1 3'UTR) II*; *daf-18 (ok480) IV; him-5 (e1490) V*, **IC1620** – *age-1(m333) II ; daf-18 (ok480) IV*, **IC1675** – *quIs18 (daf-18 genomic; pRF4) II; quIs24 [Ppie-1::daf-18cDNA::pie-1 3'UTR] II ;daf-18 (ok480) IV*, **IC1731** – *quIs24 [Ppie-1::daf-18cDNA::pie-1 3'UTR] II; daf-18 (ok480) IV,* **IC1787** – *quIs18 (daf-18 genomic; pRF4) II; daf-18 (ok480) IV; him-5 (e1490) V,* **IC1828** – *sup-17 (n1260) I; daf-18(ok480) IV; him-5 (e1490) V,* **IC1829** *–sup (qu44) (suppressor of IC817 (quIs18 (daf-18 genomic; pRF4) II)) Line E7.7 II; daf-18(ok480) IV,* **IC1830** – *sup (qu45) suppressor of IC817 (quIs18 (daf-18 genomic;*

pRF4) II) Line E1.1 II; daf-18(ok480)), **IC1851** – *quIs18 (daf-18 genomic; pRF4) II ; daf-18 (ok480) IV ; quEx693 [Prgef-1::ins-34; odr-1::RFP],* **IC1852** – *zdIs5 (Pmec-4::GFP) I; quIs18 (daf-18 genomic; pRF4) II; daf-18 (ok480) IV; him-5 (e1490) V; quEx693 [Prgef-1::ins-34; odr-1::RFP],* **IC1853** – *zdIs5; daf-18 (ok480) IV; quIs18 (daf-18 genomic; pRF4) II; quEx680 [Prgef-1::ins-39; odr--1::RFP],* **IC1854** – *daf-18 (ok480) IV; quIs18 (daf-18 genomic; pRF4) II; quEx680 [Prgef-1::ins-39; odr--1::RFP],* **IC1855** – *daf-18 (ok480) IV; quEx680 [Prgef-1::ins-39; odr-1::RFP],* **IC1858:** *quIs18 (daf-18 genomic; pRF4) II ; daf-2 (e1370) III; daf-18 (ok480) IV,* **IC1865** – *quIs18 (daf-18 genomic; pRF4) II; daf-18(ok-480); unc-25::GFP (juIs73) III; him-5(e1490) V,* **IC1885:** *zdIs5 (Pmec-4::GFP) I; daf-18 (ok480) IV; quIs18 (daf-18 genomic; pRF4) II; daf-2 (e979) III,* **IC1887** – *quIs18(daf-18 genomic; pRF4) sup (qu44) II; daf-18(ok480)IV; him-5 (e1490) V,* **IC1888** – *quIs18 (daf-18 genomic; pRF4) sup (qu45) (outcross line 1)II; daf-18(ok480)IV; him-5 (e1490) V,* **IC1889** – *quIs18 (daf-18 genomic; pRF4) sup (qu45) (outcross line 2) II; daf-18(ok480)IV; him-5 (e1490) V,* **IC1903** – *daf-18 (ok480) mIs11[myo2-GFP] IV; him5-(e1490) V,* **IC1904** – *daf-18(ok480) IV; oxIs12 [unc-47::GFP] X,* **IC1905** – *daf-18 (ok480) IV; mIs10 [myo-2-gfp] V ,* **IC1939** – *daf-18 (ok480) him-8 (e1489) IV; mIs10 [myo-2-gfp] V,* **IC1940** – *quIs18 (daf-18 genomic; pRF4) II; daf-18 (ok480) mIs11 [myo2-GFP] IV; him5-(e1490) V,* **IC2010** – *quIs18 (daf-18 genomic; pRF4) II; daf-18(ok-480) IV; him-5(e1490) V; oxIs12 (unc47::GFP) X.*

Crosses performed for linkage mapping:

To map the location of suppressors to a specific location, chromosomal linkage analysis was performed. For this purpose, the L4 hermaphrodites from suppressor strains were crossed to males from strains with GFP markers on specific chromosomes. If the suppressor was present on

that specific chromosome, the F1 generation would have a suppressor/GFP marker genotype. F1 Hermaphrodites at L4 stage with GFP were picked to separate plates. F2 will have either suppressor / suppressor, suppressor / GFP marker or GFP marker / GFP marker. L4 hermaphrodites showing strong GFP were picked to separate plates and their progeny was tested to be GFP homozygous. L4 hermaphrodite with no GFP were also picked to separate plate for self fertilization. The progeny was then bleached and incubated at 20°C on a rotor. Aliquots were taken out on day 10 to test for L1 survival. **Figure 5** shows the logic diagram of these crosses.

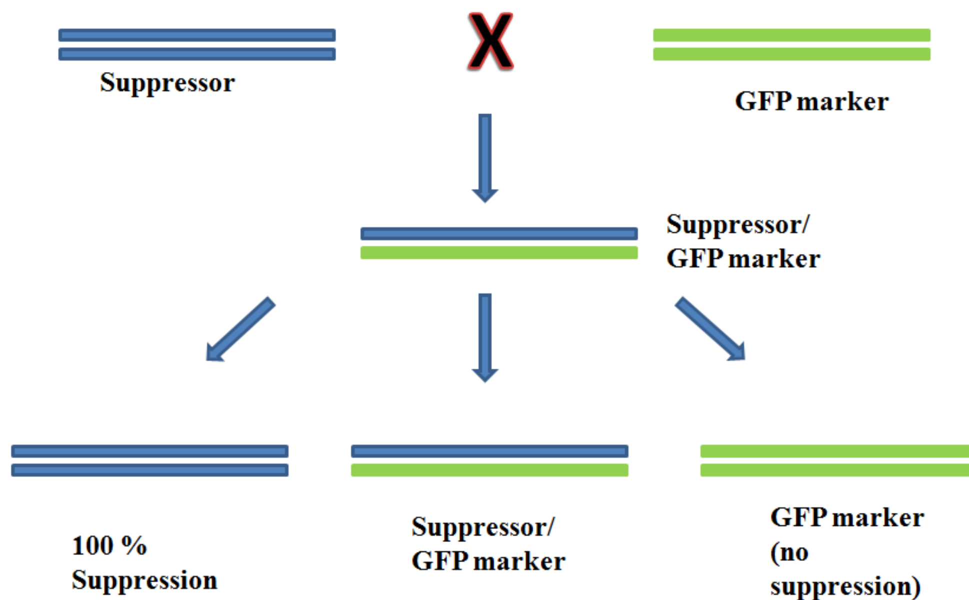


Figure 5: Logical diagram of crosses performed for chromosomal linkage analysis

Note: The 100% suppression or no suppression can only be observed when there is no recombination.

The details of crosses are as follows:

For Chromosome 1:

IC1887 – *quIs18(daf-18 genomic; pRF4) sup (qu44) II; daf-18(ok480)IV; him-5 (e1490) V* X **IC1015** (*zdIs5(P mec-4::GFP) I; daf-18 (ok480) IV; him-5 (e1490) V)*

IC1888 – *quIs18 (daf-18 genomic; pRF4) sup (qu45) (outcross line 1) II; daf-18(ok480)IV; him-5 (e1490) V*X **IC1015** (*zdIs5(P mec-4::GFP) I; daf-18 (ok480) IV; him-5 (e1490) V)*

For Chromosome 2:

IC1887 – *quIs18(daf-18 genomic; pRF4) sup (qu44) II; daf-18(ok480)IV; him-5 (e1490) V* X **IC1304** (*mIn1 [dpy-10(e128); mIs14[myo-2::GFP]] II; daf-18(ok480)IV;him-5(e1490) V*)

IC1888 – *quIs18(daf-18 genomic; pRF4) sup (qu45) (outcross line 1) II; daf-18(ok480)IV; him-5 (e1490) V* X **IC1304** (*min-1 II; daf-18(ok480)IV ;him-5(e1490) V*)

For Chromosome 3:

IC1887 – *quIs18(daf-18 genomic; pRF4) sup (qu44) II; daf-18(ok480)IV; him-5 (e1490) V* X **IC1865** (*daf-18(ok-480);unc-25::GFP (juIs73) III;quIs18 II;him-5(e1490) V)*

IC1888 – *quIs18(daf-18 genomic; pRF4) sup (qu45) (outcross line 1) II; daf-18(ok480)IV; him-5 (e1490) V* X **IC1865** (*daf-18(ok-480);unc-25::GFP (juIs73) III;quIs18 II;him-5(e1490) V).*

For Chromosome 4:

IC1887 – *quIs18(daf-18 genomic; pRF4) sup (qu44) II; daf-18(ok480)IV; him-5 (e1490) V,* X **IC1940** – *quIs18(II);daf-18 (ok480) IV; mIs11[myo2-GFP] IV; him5-(e1490) V*

IC1888 – *quIs18(daf-18 genomic; pRF4) sup (qu45) (outcross line 1) II; daf-18(ok480)IV; him-5 (e1490) V* X **IC1940** – *quIs18(II);daf-18 (ok480) IV; mIs11[myo2-GFP] IV; him5-(e1490) V*

For Chromosome 5:

IC1887 – *quIs18(daf-18 genomic; pRF4) sup (qu44) II; daf-18(ok480)IV; him-5 (e1490) V,* X **IC1939** – *daf-18 (ok480) IV; mIs10 [myo-2-gfp] V ; him-8 (e1489)*

IC1888 – *quIs18(daf-18 genomic; pRF4) sup (qu45) (outcross line 1) II; daf-18(ok480)IV; him-5 (e1490) V* X **IC1939** – *daf-18 (ok480) IV; mIs10 [myo-2-gfp] V ; him-8 (e1489)*

For Chromosome X:

IC1887 – *quIs18(daf-18 genomic; pRF4) sup (qu44) II; daf-18(ok480)IV; him-5 (e1490) V,* X **IC2010** – *daf-18(ok-480)IV; him-5(e1490) V; oxIs12(unc47::GFP) X; quIs18 II*

IC1888 – *quIs18(daf-18 genomic; pRF4) sup (qu45) (outcross line 1) II; daf-18(ok480)IV; him-5 (e1490) V,* X **IC2010** – *daf-18(ok-480)IV; him-5(e1490) V; oxIs12(unc47::GFP) X; quIs18 II*

3.2 L1 Arrest longevity Assay

3.2.1 Embryo preparation for L1 synchronization:

To synchronize worms at L1 arrest stage, embryos were prepared as follows: The desired worm strains were grown on multiple seeded-plates to obtain gravid adult hermaphrodites which actively lay eggs. Each plate was washed with 2 mL of M9 buffer. The M9 wash was then dispensed into a 1.5 mL microcentrifuge tubes then centrifuged at 3000 RPM using a Fisher Scientific Accuspin Micro 17. The worm pellet was formed at the bottom of the tube. The M9 was aspirated from the top and was replaced with 1mL of bleach solution (10% bleach, 1 M NaOH). Each sample was then vortexed for approximately four minutes until such time that the worm bodies disappeared leaving the eggs. The tubes were centrifuged as above and the bleach solution aspirated from the pellet. The pellets were then washed with M9 buffer and centrifuged as above. A minimum of three washes with M9 were performed. The final pellet (eggs) was resuspended in fresh M9 and placed on a Barnstead Thermolyne Labquake Shaker at 20°C to hatch overnight.

3.2.2 Survival Scoring:

Each day, an aliquot containing a minimum of 30 L1 arrested worms was removed and plated on nematode growth media (NGM) agar plates. I scored survival by counting the number of worms that moved (alive) and then divided that number by the total number of worms in the aliquot. Initial viability was visually assessed using a Zeiss compound microscope. Immediately after plating the L1 worms in an M9 droplet, the worms remained suspended in liquid causing them to thrash, thus facilitating the identification of living worms. Worms that did not thrash within ~10 s

of being observed and did not respond to touch stimuli from a worm pick were scored as dead. Each time point on survival curves represents the average of at least 3 independent trials.

A note on contamination: During any trial, if fungal contamination appeared on the unseeded plate within 5 days of the L1 aliquot being plated for counting, the trial was terminated and the data discarded. Fungal contamination has previously resulted in shortened lifespan.

3.3 Genotyping Assay for *daf-18 (ok480)*

3.3.1 Worm lysis for genotyping:

To make the worm lysis buffer, 5% Thermo Scientific proteinase K was added in 1x PCR buffer. From this lysis buffer 10 µl was added to the lid of a 200 µl PCR tube and 3-5 worms of the required worm strain were added to the lysis buffer. These PCR tubes were then centrifuged at 6000 RPM for 5 s using a Qualitron Inc mini-centrifuge, and were frozen in a thermos of liquid nitrogen for a minimum of five minutes. After freezing, the PCR tubes were removed and were either kept at -80°C for later use, or placed in a Biometra T personal PCR machine for a worm lysis cycle (single cycle at 65°C for 75 minutes then 95°C for 15 minutes). The worm DNA was then ready for PCR genotyping. The primers and cycles used for PCR were dependent on the required fragment size.

3.3.2 PCR primers used for confirmation of *daf-18 (ok480)*

To confirm the *daf-18(ok480)* deletion mutants through PCR, the following primers were used:

oIC 641: 5’AACTTACCACTGGCAACGAATGAATAC (forward primer)

oIC642: 5'AATCGTGGAGATAAGGCTGCTAAATC

The deletion showing *daf-18(ok480)* mutation will give a band of ~750 bp whereas wild-type *daf-18* will give a band of ~ 1500 bp.

3.4 RNAi knockdown Assay:

Candidate genes that extended the lifespan of *daf-18 (ok480)* worms were selected from previous screens performed in the Chin-Sang lab. Bacteria containing RNAi constructs obtained from the Ahringer RNAi Library were grown overnight in 2XTY (see Appendix A) containing 100 µg/mL of ampicillin at 37°C. RNAi plates (See Appendix A) were seeded with 200 µL of overnight culture. Seeded plates were left at room temperature for two days so that the bacteria can start growing and dsRNA synthesis can take place. Afterwards, these plates were stored at 4°C for a maximum of one month. Plates older than one month were not used for RNAi knockdown assay. Gravid adults from *daf-18 (ok480), daf-18 (ok480); quIs18*, and *daf-18* (*ok480); quIs24* strains were bleached and eggs were incubated in M9 buffer overnight for L1 synchronization. Synchronized worms were then plated on the seeded RNAi plates and were grown until developed into fully grown gravid adults (about 4-6 days). These plates were then washed and bleached for egg preparation (see section 3.2). Synchronization of worms allow the equal exposure of RNAi to all embryos during growth. The L1 larvae were then incubated in M9 at 20°C. Everyday an aliquot was obtained from these larvae and L1 scoring assay was performed to test increased survival.

3.5 Ethyl methanesulfonate (EMS) Mutagenesis:

Multiple unbiased EMS mutagenesis screens were conducted to isolate suppressors of *daf-18 (ok480)* L1 short lived phenotype. The screens were conducted on *daf-18* (*ok480), daf-18 (ok480);quIs18* (somatic rescue), and *daf-18 (ok480);quIs24* (germline rescue) strains. The worms from all three strains were bleached and synchronized to the L4 stage. The worms were then washed with M9 buffer multiple times and centrifuged at 3000 RPM. After the final wash, worms were resuspended in 4mL of M9 containing 20 µL of EMS making a final concentration of 47 mM. The worms were then incubated at room temperature for 4 h on an ISE GelSurfer Rocker. Exposing the worms to EMS at the L4 stage allowed targeting of the germline and increased the probability of hereditable mutations. After incubation, the worms were washed with M9 multiple times to remove EMS completely. The worms were then placed on plates seeded with OP50 *E. coli* for recovery. Ten parental generation (P0) worms were picked to separate plates (100 plates per strain) and allowed to lay eggs before they were removed. When the F1 worms became gravid adults, the plates were then washed and bleached. The eggs were allowed to hatch in M9 buffer at 20°C. The F2 worms were starved in M9 buffer for a few days. The F2 generation was then plated after 4 days for *daf-18 (ok480)* worms, 8 days for *daf-18 (ok480);quIs24*, and 10 days for *daf-18(ok480);quIs18* worms. The plates were then analyzed for any surviving worm. The candidate worms were picked to new plates to maintain stable lines and were tested using the L1 arrest protocol described above (section 3.2). Only one suppressor was selected from each parental plate. The suppressors were then outcrossed twice to eliminate any unwanted background mutations. The suppressors were also compared with other known suppressors to reduce the probability of isolating the same suppressors again.

3.6 L1 and Dauer constitutive Assay:

To perform the L1 and dauer constitutive assay, the desired strains were bleached using the above protocol. The L1 synchronized worms were placed on NGM worm plates and incubated at 15°C, 20°C or 25°C. The worm plates were visually analyzed after 4-5 days to observe the L1 constitutive or dauer constitutive phenotypes.

3.7 Yeast Two Hybrid Screening:

Yeast two-hybrid screens were used to identify the proteins that physically interact with DAF-18.

3.7.1 Preparation of GFP reporter yeast competent cells:

To assist recombination, *ade*-2 homologous arms of 1420 bp were stitched with GFP-Envy construct (See Appendix B for sequence) (Slubowski et al. 2015). GAl1 UAS-GFP Envy was amplified from pIC1096 (pGBKT7 with GFP ENVY reporter inserted) using primers oIC1780 (5' CATTAATTGCGTTGCGCTCACTG 3') / oIC1781 (5' GCAGCTGGCACGACAGGTTTC 3'). The PCR gave a product of 1420 bp. *ade*-2 region was amplified with upstream region in two PCR products, that were later used in stitch PCR for recombining GFP-Envy within *ade*-2 gene. The first product was 1191 bps long with 565 nucleotides upstream of *ade*-2 gene and 626 nucleotides of *ade*-2 gene (oIC1802: 5' GACTAGCGCACTACCAGTATATCATC 3'/ oIC1803: 5' ATTGGGTAAGAAAACACTAAACCGTTAAC 3'). The second PCR gave a product of 1100 bp of *ade*-2 gene (oIC1804: 5' TGTAGAGACTATCCACAAGGACAATATTTG 3' /oIC1805: 5' GCTTCGTAACCGACAGTTTCTAAC 3'). The stitch PCR to integrate GAL1-UAS-GFP Envy in *ade*-2 gene was performed in two steps. The first stitch was done between the first PCR product of *ade*-2 gene using primers oIC1802/ oIC1781 and the second stitch PCR was performed

using the second product of *ade*-2 gene using primers oIC1780/ oIC1805. This construct was transformed in Y187 cells with a strong positive self-activation properties (pIC1074= DAF-2 C-terminal domain) and screened for GFP positive colonies. The positive integration of GAL1 UAS-GFP Envy in the yeast chromosome was confirmed by PCR using oIC1823 (5' GGAAGACTAGTAACGCCGTATCG 3') / oIC1824 (5' GTAGTAACCAAAGTTGGCCATGG 3'). The insertion at the right position would give a product of 1960 bp and no product would result from lack of insertion. **Figure 6** illustrates the logic diagram of this construct.

With this final construct, I successfully integrated the GFP-Envy construct at the right position. In the modified yeast strains, protein interactions could be observed by evaluating GFP expression levels under a fluorescent microscope (modified protocol of (Chen et al. 2008)). I used this system to study the interactions of DAF-18 and DAF-2 with other proteins.

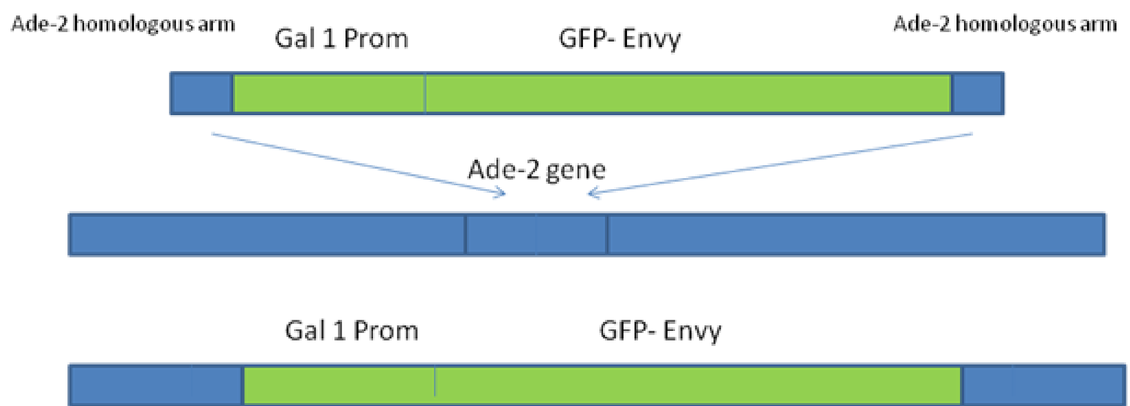


Figure 6: Recombination of GFP reporter tagged with Gal1 promoter within *ade*-2 region of yeast strain Y190.To recombine Envy-GFP in yeast, it was tagged with Gal1 promoter. ~1kb regions, homologous to *ade*-2 gene, were flanked on both sides of the gene construct to facilitate *in vivo* yeast recombination.

I isolated six yeast strains, classified as YIC1 - YIC6 (R1-R6), that exhibit the recombination event. The isolated strains were tested with positive and negative controls. FEM-2 and FEM-3 interaction (Chin-Sang and Spence 1996) was used as positive control and empty vectors (pGBKT7 and pGADT7) were used as negative. After successful testing, I performed multiple yeast two-hybrid screens using the isolated yeast strain YIC6 with pIC129 (C-terminal domain of DAF-18 in pGBKT7 vector). I screened approximately 5 million yeast colonies. The colonies were picked on the basis of their GFP expression levels and were then tested with Filter-lift and X-gal agarose overlay assays. Plasmids were isolated from the colonies that gave positive interactions. Isolated plasmids were retested with pIC129 (binding domain of DAF-18 C-term-bait) to confirm interaction. I also tested the plasmids with empty pGBKT7 vector as a negative control to test for self-activation. Finalized plasmids were sent for sequencing to TCAG DNA Sequencing Facility at Peter Gilgan Centre for Research and Learning, Toronto. The sequencing results for Y2H isolates were analyzed using BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi). The Wormbase website (http://www.wormbase.org/) was used to identify the functions of the resulting genes.

3.7.2 Preparation of Competent bait cells:

Transformation of *Saccharomyces cerevisiae* with bait plasmid (pIC129 : binding domain of DAF-18 C-term):

To make the competent cells with bait plasmid, YIC6 cells were thawed from -80°C aliquots and centrifuged at 5000 RPM. The supernatant was aspirated and following reagents were added to the pellet:

50% PEG 4000	66.7%
1M Lithium acetate	10%
Salmon Sperm DNA	14.7%
Bait Plasmid (pIC129)	1.4%
ddH_2O	6.7%

(Please refer to Appendix A for solutions)

The reaction mixture volume up was made up to 360 µL. The reagents were vortexed well so the cells and the reagents are mixed properly. The tubes were then incubated at 30°C on a rocker for 30 minutes. After incubation, the tubes were transferred to a 42°C water bath for 15 minutes for heat shock. After heat shock, the tubes were centrifuged at 5000 RPM for 1 min and the supernatant aspirated. The pellet was mixed with 80 µL of ddH_2O by gentle pipetting. An aliquot of 100 µL was plated on selective media plates *i.e.* -Trp plates (Appendix A). The plates were then incubated at 30°C for 3-4 days until visible colonies appeared.

Making competent cells:

Once visible colonies appeared on -Trp plates, a single colony was picked to 20 mL -Trp liquid media containing 2µL/mL adenine hemisulfate . The media was incubated for 2 days until the yeast grew to the point of saturation. YPAD (Appendix A) was then added (~250 mL) to adjust the OD_{600} to 0.3. The yeast culture was then incubated for one doubling time (OD_{600} = 0.6). The yeast culture was then centrifuged at 5000RPM for 8 minutes and the pellet washed in 0.4 volume of starting volume of 100 mM LiAC. The cells were centrifuged again and washed in 0.2 volume

of starting volume with 100 mM LiAC. The cells were centrifuged a final time and were re-suspended in 100mM Lithium acetate (LiAC) with 15% Dimethyl sulfoxide (DMSO) or glycerol to a final volume of 0.03 of our starting volume. Aliquots of 100 µL were made in 1.5 mL microcentrifuge tubes and slowly frozen and stored at -80C.

3.7.3 Double Transformation for Yeast Two Hybrid Assay:

To perform the double transformation of yeast cells, frozen competent cells containing bait plasmid were inoculated into 3 mL of -Trp media (Appendix A). The tube was incubated at 30°C on a shaker for 2 days. This 3 mL starter culture was inoculated into 50 mL -Trp media and incubated at 30°C on a shaker for 2 days. Actively growing yeast cells were transferred to 500 mL YPAD media (Appendix A) and concentration adjusted to an OD_{600} of 0.3. The YPAD media was incubated at 30°C on a shaker for one doubling time (~6 h). Actively growing yeast cells were harvested by centrifugation at 5000 Xg for 6 minutes . The cell pellets were resuspended in 100 mL of 100 mM LiAC and centrifuged as above. This step was repeated twice. The final cell pellets were resuspended in 3 mL of 100 mM LiAC and were consolidated into a 40mL centrifuge tube. The tube was then incubated at 30°C for 15 minutes on a rocker and then centrifuged at 4000RPM for 6 minutes. Supernatant was removed and the cell pellet was mixed with transformation reaction components (20X) to perform the second transformation. Following are the details of transformation reaction mixture:

Reaction component	**Percentage**
50% PEG	66.7%
1M LiAC	10%
Salmon sperm DNA	23.13%
cDNA Library	0.2%

The transformation reaction was vortexed vigorously for approximately one minute . Afterwards, 300 µL aliquots were transferred to microcentrifuge tubes. Aliquots were incubated at 30°C on a rocker for 30 minutes, then heat shocked at 42°C for 15 minutes. The tubes were centrifuged at 5000 Xg for 6 minutes and pellets were resuspended in ddH_2O (by gently pipetting) to a final volume of 8 mL. To check the transformation efficiency, the cells were diluted 20X and plated on -Leu, -Trp plates (Appendix A). I took 10 µL of yeast cells from 8 mL of total mixture so efficiency was calculated by multiplying the number of colonies with 800 to get the number of colonies in 8 mL of total mixture. The remaining cells were placed on -Leu -Trp -His (50 mM 3AT) plates (Appendix A) (200 µL on each plate). The plates were then incubated at 30°C for 3-5 days until colonies started to appear (some screens can take 2-14 days).

3.7.4 Yeast Transformation by Mating of Y187 and Y190 cells:

A tube with competent Y190 cells with bait plasmid was thawed and inoculated into 50 mL of -Trp media containing 2 µL/mL adenine hemisulfate. The cells were incubated overnight at 30°C until an OD_{600} of 0.8 was reached. Cells were harvested by centrifugation as above and resuspended in 4-5 mL of -Trp media (Appendix A). Approximately 1.2 mL of Y187 competent yeast cells with 10 µL of cDNA library were then mixed in a 2 L flask with the Y190 bait cells with 45 mL of YPAD containing 0.6 µg/mL Kanamycin (Appendix A). The library vial was rinsed twice with 1 mL of YPAD and added to the flask. The flask was incubated at 30°C overnight with minimal shaking (<50rpm). Next day, the cells were observed under a microscope to check for mating. If budded yeast were observed, cells were centrifuged at 1000 RPM for 10 minutes. The pellet of cells was re-suspended in 8 mL of YPAD containing 0.6µg/mL kanamycin. The total volume of the cells was 9.5 mL. An aliquot of 10 µL from these cells was diluted in 90 µL of H_2O and plated on a -Leu, -Trp plate (Appendix A) to check the efficiency of mating. The remaining cells were plated on -Leu, -Trp, -His plates (Appendix A) (221 µL on each plate) and incubated at 30°C for 3-5 days. As the total volume of cells was 9.5 mL and 10 µL was plated to get efficiency, the total number of colonies on -Leu, -Trp plate was then multiplied with 950 to get the efficiency of mating for 9.5 mL of total volume.

3.7.5 Filter Lift Assay:

Although the GFP expression showed the positive interactions, once yeast colonies appeared on the plates, a filter lift assay was performed to confirm these positive interactions. Colonies were streaked on -Leu -Trp duplicate plates (Appendix A) to create an extra copy after performing the filter lifts.

One of the duplicate plates was covered with a nitrocellulose circle and left to become saturated with yeast cells. Four mL of Z-buffer containing 100 μg/mL X-Gal and 0.5 μL/mL BME was added to each membrane filter in a 15 mL petri dish. The nitrocellulose membrane with yeast colonies was marked for alignment and then the membrane was removed from the plate and dipped in liquid nitrogen. The membrane was then removed from liquid nitrogen once the colonies were frozen and placed in the lid of the plate to let it thaw. Once the membrane was thawed and resumed its flat shape, it was placed on top of the filter paper with yeast colonies facing upwards. The petri dish was closed and was wrapped in parafilm to keep from drying and to contain BME. All yeast plates were also streaked with strong positive interactors as positive controls and negative interactors as negative control. Positive interactors started changing color within an hour or so. Less strong interactors took almost 6 h whereas, weak interactors took 24 h. After 24 h, colonies showing no color change were declared non interactors. **(Please refer to Appendix A for solutions).**

3.7.6 X-Gal Agarose Overlay Assay:

For each plate with patches of yeast colonies, 7 mL working solution was prepared and was cooled. X-Gal (100μg/mL) solution and BME solution (0.5 μL/mL) were added to the working solution. A thin layer of agarose solution (almost 7 mL) was applied on the yeast colonies carefully with a plastic pipette. Once the agar cooled down and solidified, the plate was incubated at 30°C. Strong interactors turned blue within 1-2 h and weak ones in 6-8 h. Colonies could be picked through the top of the agar even after 5 days. **(Please refer to Appendix A for solutions).**

3.7.7 Isolation of plasmids from yeast cells:

Yeast was grown in -Leu, -Trp media (Appendix A) and 1mL of saturated yeast culture was centrifuged at 17,000 x g for 1 min. Yeast cells were re-suspended in 100 µL TESS, frozen in liquid nitrogen for 30 s, then thawed at 37°C for 1 min. This step was repeated once more and cells were incubated at 95°C for 10 min. The tube was centrifuged at 17,000 x g for 5 min. The supernatant was transferred to a Qiagen column containing 100 µL silica slurry and equilibrated with 6 M guanadinium HCL and mixed well. It was then passed through the column and washed with 1-2 mL wash buffer. The column was then centrifuged at high speed to dry the column. The DNA was eluted with 50 µL of H_2O in to new microcentrifuge tube. An aliquot (10 µL) of 1M DTT was added to the DNA (alternatively 2 µL of 1M DTT can be added to competent cells). DNA/DTT (5 µL) mix was used to transform ultra-competent Top 10 bacterial cells which were incubated on ice for 20 min. The cells were then heat shocked at 42°C for 1 minute and 1 mL of 2XTY added. The cells were incubated at 37°C for 1 hour. The tube was then centrifuged and cells plated on LB AMP plates (Appendix A). Once colonies appeared on the plates, a mini prep protocol was used to isolate the plasmid from the bacterial cells. The positive interacting plasmids were re-tested with controls to test for self-activation. The plasmids were also tested again with bait individually to confirm positive interactions. Once the positive interaction was confirmed, the plasmids were sent for sequencing results.

3.8 Sequencing of *age-1* gene in suppressors :

To test whether the isolated suppressors are alleles of *age-1* gene, I amplified the *age-1* gene from the desired strains and sent the DNA for sequencing.

3.8.1 Primers for amplification of *age-1* gene and Sequencing:

The following primers were used to amplify all the coding region of *age-1* gene.

Primers set 1:

oIC1885: 5' GTGCTCAGCGTTTCTATCGC 3'

oIC1886: 5' TCGCGTCGAGACCAGGAATC 3'

These primers gave a 2683 bp product.

Primer set 2:

oIC1887 : 5' CTCGTCAAGCACCGAAGTAC 3'

oIC1888 : 5' GTGGAGGATTTGAAGAGAGGATTG 3'

These primers gave a 2018 bp product with an overlap of 648 bp with PCR product from set 1.

Primer set 3:

oIC1889 : 5' GGTGGTGATGATGCTGATGC 3'

oIC1890: 5' AATAAACACAGGGAGAGAGGAGAG 3'

These primers gave a 2170 bp PCR product.

Primer set 4:

oIC1906: 5' AAGATCGTCGTTGGATGCTG 3'

oIC1907: 5' AGCTCAGGCAACTCCATTCC 3'

These primers gave a product of 1305 bp. The product was overlapping with primer set 3.

Primer set 5:

oIC1978: 5' CCCGTCAAAGATACGATTCCTG 3'

oIC1979: 3' GCAGGTTGTGATGATGTACGTC 5'

These primers gave a product of 2037 bp with overlapping region from primer set 2.

Sequencing primers for *age-1* gene:

The following primers were used for sequencing of *age-1* gene:

oIC1891: 5' GTGCTCAGCGTTTCTATCGC 3' (forward primer of set 1)

oIC1892: 5' CTCCACGGCACTTTCCCAATG 3'

oIC1893: 5' CTGGATTCGTTGTTCGCCGAC 3'

oIC1894: 5' CTCGTCAAGCACCGAAGTAC 3' (forward primer of set 2)

oIC1895: 5' GTCGTGTCTGTCGTGTCGAG 3'

oIC1896: 5' ACCTTCCGTTCTGTGCAATG 3'

oIC1897: 5' GGTGGTGATGATGCTGATGC 3' (forward primer of set 3)

oIC1898: 5' TCTGTGCGCCTTTAAGATGG 3'

oIC1899: 5' CCTTAAAGGCGCACACTTCC 3'

oIC1906: 5' AAGATCGTCGTTGGATGCTG 3' (Forward primer of set 5)

All these primers were designed so that they will cover the coding region of both isoform-A and isoform-B of *age-1*. Moreover, the sequencing results of these primers overlapped to avoid any gaps.

3.9 Primordial germ cell proliferation analysis:

To determine if isolated suppressors control the proliferation of primordial germ cells, L1 arrest worms were visualized using PGL-1 (P granule abnormality protein 1) antibodies. The "L1 arrest longevity assay" was performed as described above. An aliquot of 4 µL/mL of 95% Ethanol was added to M9 buffer before incubating eggs. L1 worms were stained using the PGL-1 antibodies on the sixth day of incubation. The staining protocol was followed as described by Chin-Sang et al. (1999).

3.10 Statistical Analysis

All the acquired data was stored in excel spreadsheet and analysis was performed using appropriate formulas. Mean for all the experiments was calculated using AVERAGE function in excel and standard deviation was calculated using STDEV function. T-test was performed for individual strains compared to N2 wild-type using TTEST function (Two tailed distribution, Un-equal variance).

Chapter 4

Results

4.1 L1 arrest and Longevity assays:

Previous work in our lab modified *daf-18 (ok480)* by injecting the worms with *daf-18* genomic DNA construct as an extrachromosomal array (*quEx518 [Pdaf-18::daf-18::gfp::daf-18 3'UTR ; odr-1::mcherry])* (Chamberlain 2014). As extrachromosomal arrays are silenced in germline, this strain was classified as somatic rescue. Subsequently an integrated copy of the array on Chromosome 2 was isolated and called *daf-18* (*quIs18*). To express *daf-18* only in germline, the *daf-18* genomic DNA was expressed under germline specific gene, *pie-1*, promoter and was integrated using the MosSCi technique (Zeiser et al. 2011). This single integrated copy of germline specific *daf-18* was called *quIs24*. Previous L1 arrest longevity studies showed that neither somatic nor germline *daf-18* expression alone brings the L1 arrest to wild type levels , however, when *daf-18* is put back in both germline and soma, only then the L1 worms live up to the wild type level (Chamberlain, 2014). **Figure 7** represents the work of Gabriel Chamberlain on the effects of somatic and germline rescue of *daf-18* (genomic DNA). Our lab has shown that dauer and neuronal phenotype can be restored by only somatic expression of DAF-18 (*quIs18*). This shows that germline and somatic DAF-18 must work together to maintain the L1 arrest in a different pathway than dauer formation.

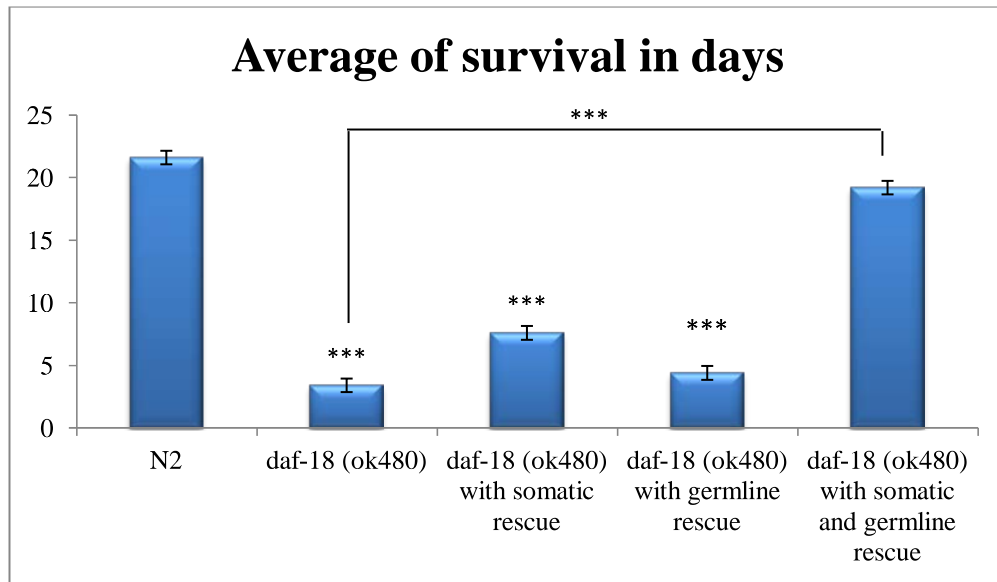


Figure 7: Graph showing the survival in days during L1 arrest for *daf-18 (ok480)* mutants with somatic and germline rescue (Chamberlain, 2014). n=**5.** Significance compared to N2, *** significant at p<0.0005.

4.2 Genetic modifier screens to identify the suppressors of L1 arrest phenotype of *daf-18 (ok480)* mutants:

Genetic modifier screens have been proven to be a great technique to identify components of a genetic pathway. To achieve the aim of identifying the suppressors of *daf-18 (ok480)* mutants, I decided to use systematic reverse genetics screen (RNAi knockdown) and random forward genetics mutagenesis screen (EMS mutagenesis). The *daf-18 (ok480)* mutants have L1 arrest longevity of 4 days, using these approaches I screened for genes that when knocked down or mutated, could significantly increase this longevity up to at least 10 days. Along with the *daf-18 (ok480)* mutants, germline and somatic rescue mutants were also tested in both screens.

4.2.1 Reverse genetics screen using RNAi Knockdown:

Reverse genetics screens using RNAi has been a successful approach in these kind of studies. RNAi in worms can be as simple as feeding worms bacteria engineered to produce double-stranded RNA (dsRNA) to induce a robust RNAi response. Worms are screened for candidate genes for their ability to suppress the *daf-18(ok480)* L1 arrest longevity defect. The previous RNAi screen from our lab used *daf-18(ok480)* mutant worms and generated a list of genes that induced a slight change in longevity of L1 arrested worms. Some of the resulting genes looked interesting because of their roles in related pathways. I further tested those genes for their suppression on L1 arrest phenotype of *daf-18 (ok480)* mutants with germline and somatic rescue. The genes that were further tested in the screens are listed below:

- *sup-17*
- *hrdl-1*
- *dpl-1*
- *tag-165*
- *lin-12*
- *glp-1*
- *jamp-1*
- *let-363*
- *asna-1*
- *mpk-1*
- *hum-1*
- *mgl-2*
- *agef-1*

- K05F1.6
- *pld-1*

4.2.1.1 No significant longevity extension in *daf-18 (ok480)* mutant worms:

The L1 arrest longevity assay was performed after the worms were fed RNAi containing bacteria (HT115 competent cells). Although an increase of 1-2 days was observed in most cases, none of them showed an extended increase in life span of L1 arrested worms (*i.e.* beyond 6-10 days). It was observed in some cases that the life span did increase significantly but the results were not reproducible. **Figure 8** shows the RNAi screening results for *daf-18 (ok480)* with germline rescue (IC1589) and *daf-18 (ok480)* with somatic rescue (IC817).

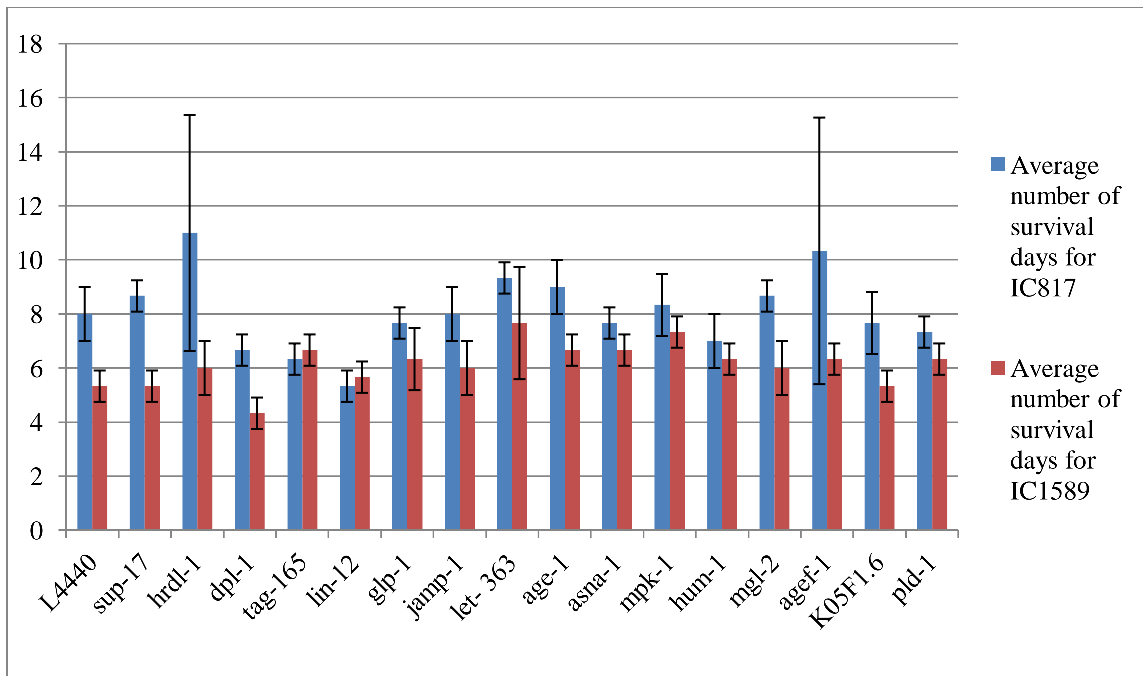


Figure 8: Results from RNAi screens showing increase in longevity by insignificant number of days. The screen was performed on both IC817 – *quIs18(daf-18 genomic; pRF4)* II; *daf-18(ok480)* IV (Blue bars) and IC1589 – *quIs24 (Ppie-1::daf-18 cDNA::pie-1 3'UTR) II*; *daf-18(ok480) IV; him-5(e1490) V* (Red bars).

4.2.2 Forward genetics mutagenesis screen:

EMS is a strong mutagen. It is used to mutate the DNA as it introduces G/C to A/T point mutations at a frequency of ~7 X 10^{-6} per mutagenised G/C base pair, approximately 1 mutation per 2000 copies of a gene screened (Anderson 1995, Jorgensen and Mango 2002). Multiple unbiased EMS mutagenesis screens were performed in our lab previously, to identify novel suppressors of *daf-18(ok480)* L1 arrest phenotype. None of those screens were successful to isolate any suppressors that allowed *daf-18(ok480)* worms to survive L1 arrest beyond 8 days. I also performed multiple mutagenesis screens using *daf-18 (ok480)* mutants, and *daf-18 (ok480)* mutants with germline and somatic *daf-18* (genomic DNA) rescue. I screened almost 10,000 worms for each strain, looking for any worm that would live longer than 8 days in L1 arrest, for *daf-18 (ok480)* mutants (L1 longevity = 4 days), and *daf-18 (ok480)* mutants with germline rescue L1 longevity = 5 days) and 10 days for *daf-18 (ok480)* mutants with somatic rescue (L1 longevity = 7-8 days).

4.2.2.1 Isolation of suppressors increasing the longevity to a significant number of days:

Screens of *daf-18 (ok480)* (**RB712** – *daf-18 (ok480)*) and *daf-18 (ok480)* with germline rescue (**IC1731**: *daf-18 (ok480) IV; quIs24;him-5 V*) were not able to generate any novel suppressors. Although *daf-18(ok480)* with germline rescue were able to produce multiple L1 worms that survived longer than 8 days but when they were put on food source (*E. coli* OP50), they would either show a sterile phenotype with vulval defects or were too sick and died without producing progeny.

From the screens of *daf-18 (ok480)* mutant with somatic rescue (**IC817** –*daf-18(ok480)* IV; *quIs18(daf-18 genomic ;pRF4)* II), I was able to isolate two suppressors, named as *sup (qu44)* and *sup (qu45)*. L1 arrest was rescued from 7-8 days to 14 day for both, *sup (qu44)* and *sup*

(qu45). The L1 worms with *sup (qu44)* were able to produce progeny as adults up to 14 days, however, L1 worms with *sup (qu45)* were only able to produce progeny until day 13, and showed either an L1 constitutive or dauer constitutive phenotype after coming out of L1 arrest on day 14.

4.2.2.2 Out crossing of the suppressors to eliminate background mutations

The suppressors were then outcrossed twice with **IC1015** (*zdIs5 I; daf-18 (ok480) IV*; *him-5 (e1490) V)* to get rid of background mutations. All the worms that lived longer than 10 days had *quIs18* (somatic *daf-18* genomic DNA rescue) in them, suggesting that *quIs18* is required by the suppressors to extend the life span. After outcrossing the mutants four times each, it was observed that the longevity of the worms increased significantly. Longevity of *sup (qu44)* was increased to 19 day (**IC1887**: *sup (qu44)* out-crossed) and *sup (qu45)* was increased to 28 days (**IC1888**: *sup (qu45)* out-crossed and **IC1889**: *sup (qu45)* out-crossed with *zdIs5* reporter gene) (**Figure 9**). PCR was performed again to confirm the presence of *daf-18(ok480)* mutation.

4.3 Characterization of suppressors:

The following experiments were performed to characterize the suppressor:

4.3.1 Chromosomal linkage analyses of suppressors:

Chromosome 1: To test the position of the suppressors, a cross was performed with **IC1015** (*zdIs5(P mec-4p*::*GFP) I; daf-18 (ok480) IV; him-5 (e1490) V).* The *zdIs5* has a *mec-4p*::GFP reporter gene on chromosome 1. If the suppressors were on chromosome 1, none of the homozygous GFP worms would show the long lived phenotype. However, F2 progeny produced both GFP and non-GFP worms that lived longer that 10 days and homozygous GFP (with stronger expression) progeny was also observed. This suggested that the suppressors are not on chromosome 1. The results are as listed in **Table 1**.

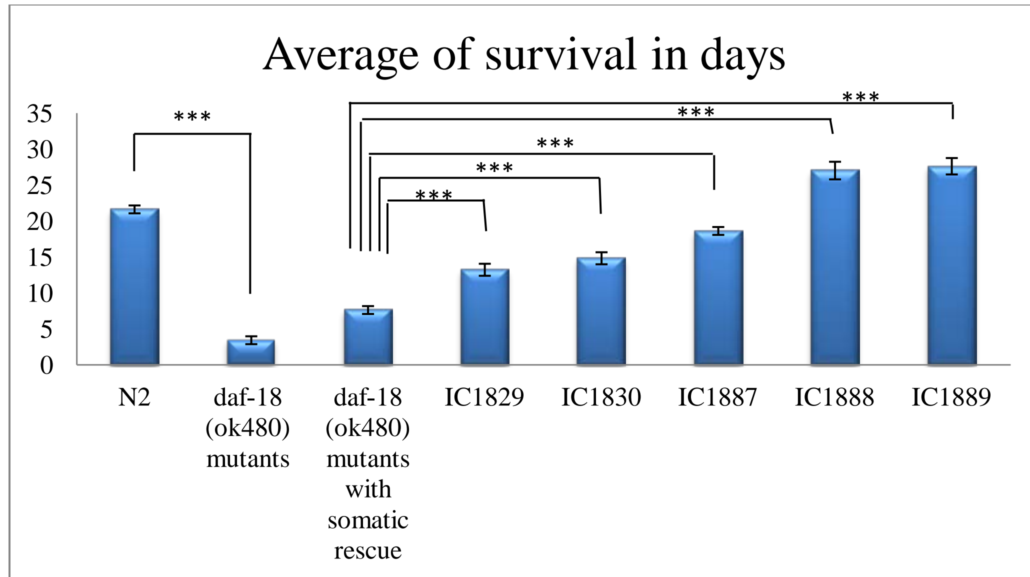


Figure 9: Increased survival of Suppressors compared to *daf-18 (ok480)* mutants. Graph showing the survival in days during L1 arrest for **N2** (wild-type), *daf-18 (ok480)* mutants and **IC817** – *daf-18 (ok480)* mutants with somatic rescue compared to suppressors, *sup (qu44)* and *sup (qu45),* and out-crossed suppressors. A significant increase in longevity can be observed in suppressor strains. **IC1829 –** *sup (qu44)* suppressor of *IC817 (daf-18(ok480; quIs18 (daf-18 genomic; pRF4) II)) Line E7.7,* **IC1830** – *sup (qu45)* suppressor of *IC817 (daf-18(ok480); quIs18 (daf-18 genomic; pRF4) II) Line E1.1,* **IC1887**: *quIs18(daf-18 genomic; pRF4) sup(qu44)* out-crossed *II; daf-18(ok480)IV; him-5 (e1490) V*, **IC1888:** *quIs18(daf-18 genomic; pRF4) sup(qu45)* out-crossed *II; daf-18(ok480)IV; him-5 (e1490) V*, **IC1889:** *zdIs5 I; quIs18(daf-18 genomic; pRF4) sup(qu45)* out-crossed *II; daf-18(ok480)IV; him-5 (e1490) V*. **n**= 5. *** significant at p<0.0005.

Chromosome 2: The suppressors were crossed to **IC1304** (*mIn1 II; daf-18(ok480)IV ;him-5(e1490) V*) to test their presence on chromosome 2. The GFP dumpy worms in F2progeny were too sick to produce any progeny. The non-GFP worms were bleached to test their L1 longevity. These results confirm that the suppressors require *quIs18* (somatic *daf-18),* which is linked to

Chromosome 2 and although the sample size is small it suggests that the suppressor mutation is on Chromosome 2. The results for non-GFP progeny are listed in **Table 1**.

Chromosome 3: The suppressors were crossed with **IC1865** (*daf-18(ok-480); unc-25::GFP (juIs73) III;quIs18 II;him-5(e1490) V)* to check their localization on chromosome 3. The GFP and non-GFP worms were picked from F2 progeny. If the suppressors were on chromosome 3, all non-GFP should live longer than 10 days. However, the results show that suppressors are either not present on chromosome 3 or have a higher recombination frequency with the GFP marker. The results are as listed in **Table 1**.

Chromosome 4: The suppressors were crossed with **IC1940** – *quIs18(II);daf-18 (ok480) IV; mIs11[myo2-GFP] IV; him5 (e1490) V,* to check their position on chromosome 4. The GFP and non-GFP worms were picked from F2 progeny. If the suppressors were on chromosome 4, all non-GFP should live longer than 10 days. However, the results show that suppressors are either not present on chromosome 4 or have a higher recombination frequency with the GFP marker. The results are as listed in **Table 1**.

Chromosome 5: The suppressors were crossed with **IC1939** – *daf-18 (ok480) IV; mIs10 [myo-2-gfp] V ; him-8 (e1489) IV* to check their localization on chromosome 5. The GFP and non-GFP worms were picked from F2 progeny. If the suppressors were on chromosome 5, all non-GFP should live longer than 10 days. However, the results suggested that suppressors are either not present on chromosome 5 or have a higher recombination frequency with the GFP marker. The results are as listed in **Table 1**.

Chromosome X: The suppressors were crossed with **IC2010** – *daf-18(ok-480)IV; him-5(e1490) V; oxIs12(unc47::GFP) X; quIs18 II* to check if they are present of chromosome X. The GFP and non-GFP worms were picked from F2 progeny. If the suppressors were on chromosome X, all

non-GFP should live longer than 10 days. However, the results suggested that suppressors are either not present on chromosome X or have a higher recombination frequency with the GFP marker. The results are as listed in **Table 1**.

Suppressor	Chromosome	GFP / Non-GFP	Total	Alive on day 10
sup (qu44)	I	GFP	15	8
		Non-GFP	15	7
	II	Non-GFP	12	12
	III	GFP	5	4
		Non-GFP	10	7
	IV	GFP	10	10
		Non-GFP	10	9
	V	GFP	14	6
		Non-GFP	20	16
	X	GFP	11	8
		Non-GFP	13	5
sup (qu45)	I	GFP	15	10
		Non-GFP	15	8
	II	Non-GFP	5	5
	III	GFP	11	10
		Non-GFP	8	4
	IV	GFP	14	12
		Non-GFP	8	5
	V	GFP	12	6
		Non-GFP	16	4
	X	GFP	7	5
		Non-GFP	10	4

Table 1: Table showing the results for chromosomal linkage analysis for Suppressors *sup (qu44)* and *sup (qu45)*.

4.4 Comparison of the newly isolated suppressors to already known suppressors of DAF-18

4.4.1 Comparison between DAF-2 and the Suppressors:

As discussed earlier, DAF-18/PTEN works as an antagonist in insulin-like signaling pathway and assists the nuclear localization of DAF-16/FOXO. Our lab has also shown the DAF-2/IR and DAF-18/PTEN directly interact and DAF-2 down regulates the expression of DAF-18/PTEN (Liu et al. 2014). Studies have shown the *daf-2* mutants are long lived as adults and L1 arrested animals and show a dauer constitutive phenotype (Kimura et al. 1997, Gems et al. 1998b). Moreover, *age-1/PI3K* mutants also known to increase the life span at L1 and adults and also show a dauer constitutive phenotype (Friedman and Johnson 1988, Weinkove et al. 2006, Zhang et al. 2011). So far, only *daf-2* and *age-1* mutants have been shown to suppress the short lived L1 arrest phenotype of *daf-18 (ok480)* mutants to some extent (**Figure 10**). According to my proposed hypothesis, the pathway branches somewhere after *daf-2* and *daf-18.* To further support this hypothesis, I tested the double-mutants for *daf-2 (e1370); daf-18(ok480)* with and without somatic rescue. I further tested the null allele *daf-2 (e979)*; *daf-18(ok480)* with and without somatic rescue as well to check the effect on longevity. **It appears that *daf-2* insulin receptor mutants can extend the L1 longevity of the *daf-18* null animals that carry *daf-18(+)* in the soma (*quIs18(daf-18(+)* soma); *daf-18 (ok480)*** (**Figure 10**). Although my suppressors behave like *daf-2* mutants, the mapping data suggest they are not new *daf-2* alleles as they do not to map to chromosome 3 where *daf-2* is found.

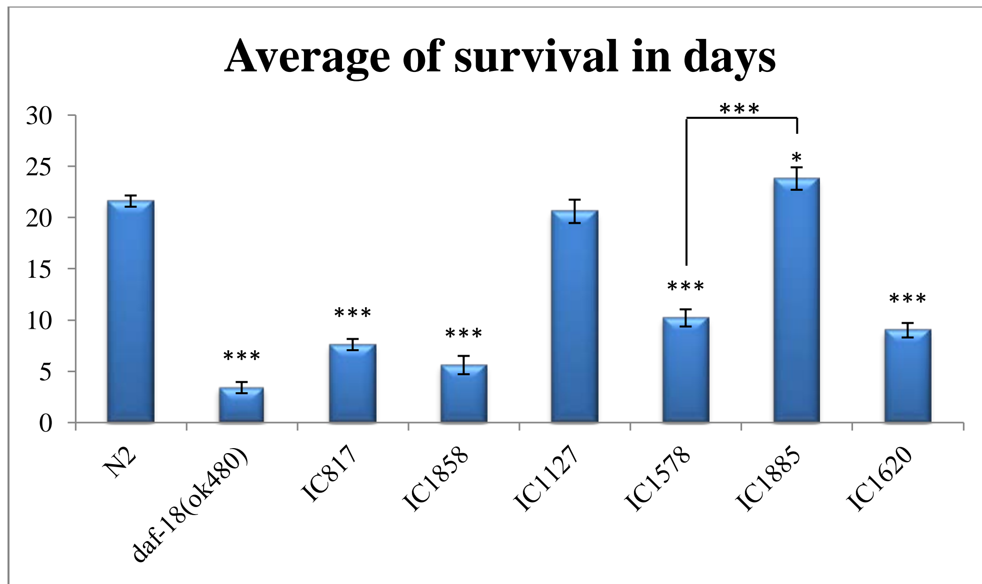


Figure 10: Graph showing the L1 longevity results for *daf-2 (e979* and *e1370); daf-18(ok480)* with and without somatic rescue. IC817: (*daf-18 (ok480)* with somatic rescue): *daf-18(ok480),quIs18,* **IC1858**: quIs18 *II ; daf-2 (e1370) III; daf-18 (ok480) IV*, **IC1127:** *zdIs5 I ; daf-2 (e1370) III ; daf-18 (ok480) IV*, **IC1578:** *zdIs5 (1); daf-2 (e979) (III); daf-18 (ok480) (IV)*, **IC1885:** *zdIs5 I ; quIs18 II ; daf-2 (e979) III ; daf-18 (ok480) IV*, **IC1620:** *age-1(m333) II ; daf-18 (ok480) IV*, **n**=5. Significance compared to N2, * significant at p<0.05, *** significant at p<0.0005.

4.4.1.1 Comparison of constitutive L1 arrest and dauer constitutive phenotype of *daf-2* mutants and suppressors

To further compare the phenotypic differences of *daf-2* mutants and suppressors, I observed the difference of phenotype at different temperatures. *daf-2* (ts) mutants show a constitutive L1 arrest or dauer phenotype at 25°C and the strain cannot be maintained at 20°C or 25°C. The results showed that the suppressor strain, *sup (qu44),* didn't show predominant dauer constitutive phenotype although there were some dauer constitutive worms. However, *sup (qu45),* showed dauer constitutive phenotype at 25°C but unlike *daf-2* mutants, you can maintain *sup (qu45)* at 20°C. **Table 2** shows the results from the experiment.

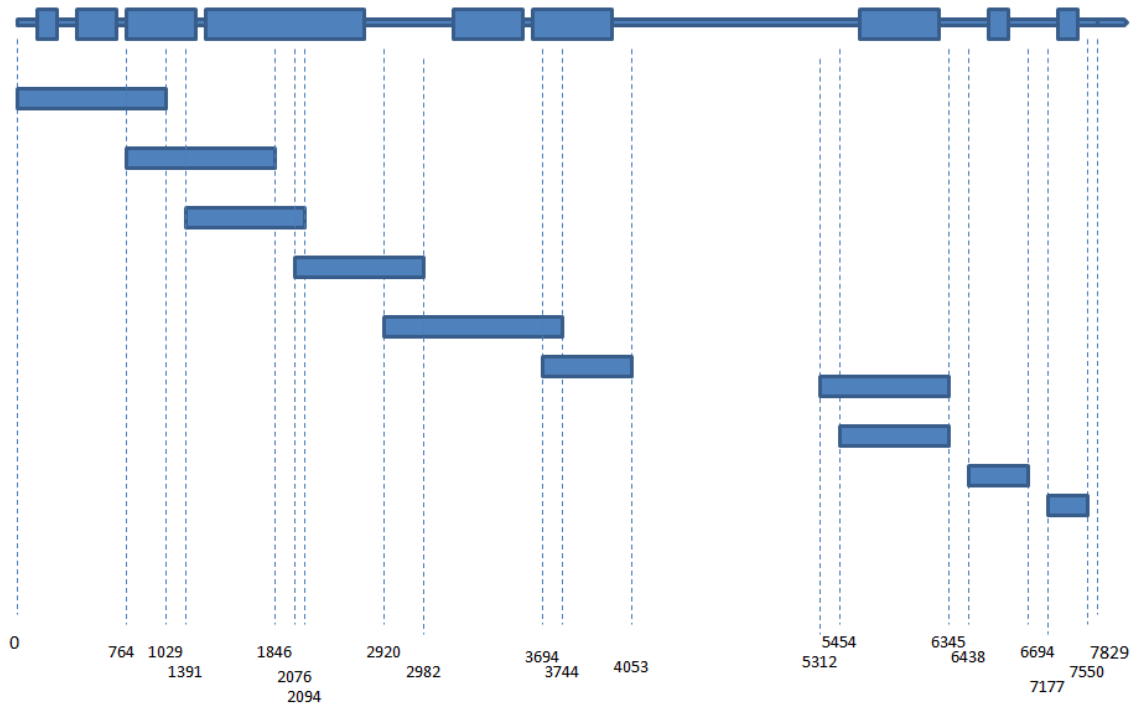
Genotype	25°C	20°C	15°C
N2	All Adults	All Adults	All Adults
daf-18(ok480)	Mostly sick but all adults	All Adults	All Adults
daf-2 (e979)	Embryonic lethal / L1 Constitutive	L1 Constitutive	All Adults
daf-2 (e979); daf-18(ok480)	All Adults but sterile	All Adults	All Adults
daf-2 (e979); quIs18;daf-18(ok480)	L1 Constitutive	L1 Constitutive	All Adults
IC1829 – *sup (qu44) suppressor of IC817 (daf-18(ok480; quIs18 (daf-18 genomic; pRF4) II)) Line E7.7,* (Original suppressor)	Some dauer most Adults	All Adults	All Adults
IC1830 – *sup (qu45) suppressor of IC817 (daf-18(ok480); quIs18 (daf-18 genomic; pRF4) II) Line E1.1,*	Dauer Constitutive	All Adults	All Adults
IC1887: *quIs18(daf-18 genomic; pRF4) sup(qu44)* out-crossed *II; daf-18(ok480)IV; him-5 (e1490) V*	Most sterile adults some dauer	All Adults	All Adults
IC1888: *quIs18(daf-18*	Dauer Constitutive	All Adults	Some Adults rest dauer

genomic; pRF4) sup(qu45) **out-crossed** ***II; daf-18(ok480)IV; him-5 (e1490) V***			constitutive

Table 2: Comparison between temperature sensitivity for L1/ Dauer constitutive phenotypes of suppressors and *daf-2* mutants at 25°C, 20°C and 15°C.

4.4.2 Comparison between AGE-1 and the Suppressors:

As the suppressors showed a probable localization on chromosome 2, I wanted to rule out the possibility that they are just another allele of *age-1*. As mentioned above, *daf-2* and *age-1* are so far the known suppressors of *daf-18*. Since *age-1* and *quIs18* are on the same chromosome *i.e.* chromosome II, I was not able to perform the same crosses I performed for testing *daf-2*. So to test the possibility of *age-1* alleles being the suppressors, I sequenced the *age-1* gene in the suppressors. As the results show, when compared to wild-type un-spliced DNA (See Appendix B for sequence), the suppressors also show wild-type *age-1* sequence **(Figure 11 and Figure 12)**.



AGE-1 Sequencing results for IC1829

Figure 11: Comparison of sequencing results of IC1829 with wild-type un-spliced DNA (Appendix B). IC1829 – *sup (qu44)* suppressor of *IC817 (daf-18(ok480; quIs18 (daf-18 genomic; pRF4) II)) Line E7.7.* All the coding region is sequenced using the specific primers. The sequencing results from different primers are also overlapping. The dotted lines show the position of overlaps in the un-spliced DNA sequence (See Appendix B for sequence).

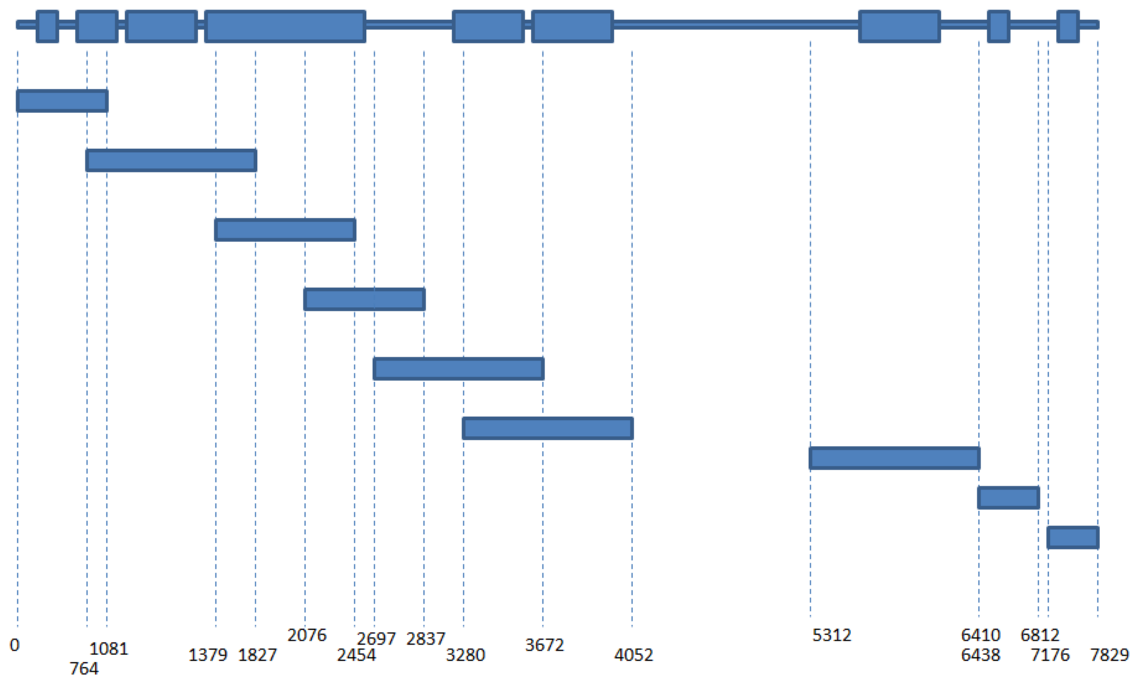


Figure 12: Comparison of sequencing results of IC1830 with wild-type un-spliced DNA (Appendix B). IC1830 – *sup (qu45) suppressor of IC817 (daf-18(ok480); quIs18 (daf-18 genomic; pRF4) II) Line E1.1.* All the coding region is sequenced using the specific primers. The sequencing results from different primers are also overlapping. The dotted lines show the position of overlaps in the un-spliced DNA sequence (See Appendix B for sequence).

4.5 Effect of suppressors on germline proliferation

During L1 arrest, *C. elegans* larvae arrest all their energy consuming developmental processes, most importantly germline proliferation, keeping germ cells (Z2/ Z3) from dividing (**Figure 14**). In addition to dauer defects and short lived L1 arrest phenotype, null mutants of DAF-18 also have protruding vulval defects and proliferation and germline during L1 arrest (Fukuyama et al. 2012). Previous work from our lab showed that somatic expression of DAF-18 in *daf-18 (ok480)* mutants can rescue vulval defects and dauer formation (Chamberlain 2014). However, expressing DAF-18 just in somatic cells cannot rescue germline proliferation and L1 arrest longevity to wild-

type. As the previous results show, *sup (qu44)* and *sup (qu45)* are able to rescue the L1 arrest longevity to wild-type, I wanted to test if they can also rescue the germline proliferation of *daf-18* null mutants with somatic expression of DAF-18.

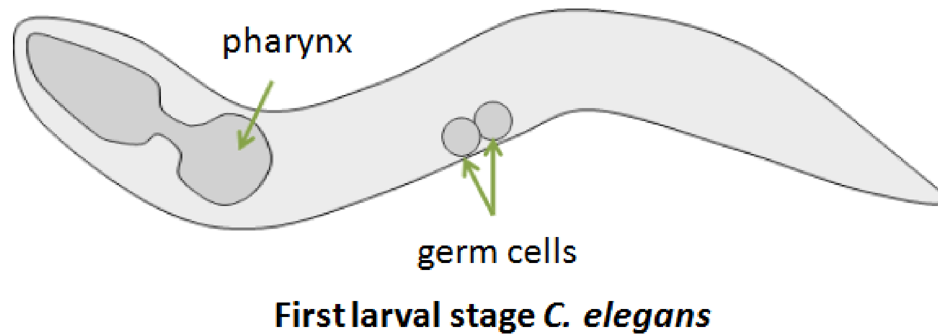


Figure 13: First larval stage of *C. elegans* (L1): The wild-type L1 arrested worms have two germ cells (Z2/Z3) throughout their L1 arrest. However, *daf-18 (ok480)* mutants show proliferation in germ cells giving rise to up 8 cells.

When compared to the *daf-18 (ok480)* worms and *daf-18 (ok480)* worms with somatic rescue, it was observed along with the longevity rescue, that germline proliferation was restored to wild-type (N2) in both *sup (qu44)* and *sup (qu45)* (**Figure 15**).

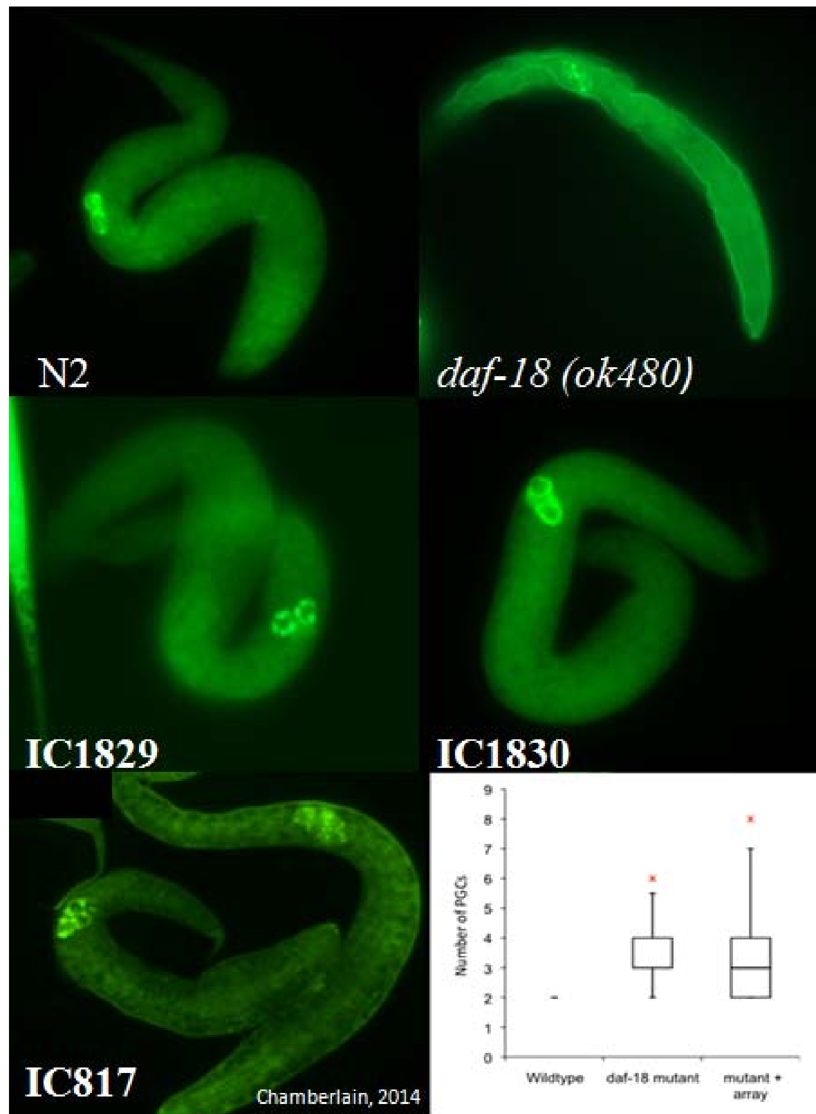


Figure 14: Germline proliferation rescued in *daf-18 (ok480)* worms by suppressors: anti-PGL-1 staining was performed to observe Z2/Z3 proliferation. L1 arrested worms arrest their germline proliferation in wild-type (N2) worms (top left). *daf-18 (ok480)* worms show a proliferating germline where germline cells Z2/Z3 can dived further up to 8 cells (top right). Somatic rescue of *daf-18 (ok480)* worms cannot rescue the germline proliferation (Bottom left and right) (Chamberlain 2014). The isolated suppressors not only increase the longevity of *daf-18 (ok480); quIs18* worms to wild-type, they also rescue the germline proliferation (IC1829 – *sup (qu44) suppressor of IC817 (daf-18(ok480; quIs18 (daf-18 genomic; pRF4) II)) Line E7.7*: middle left, IC1830 – *sup (qu45) suppressor of IC817 (daf-18(ok480); quIs18 (daf-18 genomic; pRF4) II) Line E1.1,*: middle right, IC817: *daf-18(ok480) IV; quIs18 (daf-18 genomic; pRF4) II).*

4.6 Yeast two hybrid screening of DAF-18 C' terminal interactors

The Y2H system is an *in vivo* genetic screen to detect the interaction between two proteins of interest using the functional reconstitution of the distinct DNA binding and activation domains of a transcription factor. This results in the subsequent activation of reporter expression, which is controlled by the transcription factor. The traditional Y2H system uses GAL1-lacZ fusion gene as the reporter for a protein-protein interaction. This reporter gene encodes the enzyme beta-galactosidase which labels the yeast cell when using a colorimetric substrate (X-Gal) (Fields and Song 1989). To make the procedure simpler, we decided to insert a non-enzymatic visual reporter *i.e.* Green Fluorescent Protein (GFP) under control of the GAL-1 promoter. In this modified version, we aimed to integrate the Envy green fluorescent protein gene (Envy GFP) (Chen et al. 2008) tagged with 4X GAL-1 UAS binding sites (GAL-1 promoter), into the ADE2 locus of the Y187 yeast strain (See material and methods for protocol).

After successful integration of Envy GFP at the *ade-2* locus, the integrated yeast strain was used to perform Y2H screening. The DAF-18 ' terminal domain was used as the bait and worm protein library was used for potential interactions. The sequencing results for Y2H isolates were analyzed using BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi). The Wormbase website (http://www.wormbase.org/) was used to identify the functions of the encoded products of cDNA that emerged from the screen **(Table 3)**.

Database name	Y2H Number	Gene	Function
pIC1161	28	*drd-5*	Dietary restriction down regulated
pIC1162	30	*eef-1A.1*	Eukaryotic translation Elongation Factor
pIC1163	42	*cts-1*	CiTrate Synthase
pIC1164	44	H21P03.2	Enriched in germline, regulated by *lin-35*
pIC1165	46	*copb-1*	COP (COat Protomer) Beta subunit
pIC1166	**54**	***daf-21***	**Required for dauer development, down regulates *age-1***
pIC1167	77	*atg-13*	Autophagy
pIC1168	113	F26F4.6	Expressed in the hypodermis, nervous system, head, and the intestine.
pIC1169	124	*rps-2*	Ribosomal Protein, Small subunit
pIC1170	148	*tba-2*	Encodes an alpha-tubulin
pIC1171	158	R07H5.8	Larval development, receptor-mediated endocytosis, reproduction and secretion by cell etc
pIC1172	165	R07G3.8	Expressed in the nervous system and the reproductive system
pIC1173	194	F57F5.1	Involved in embryo development, locomotion, nematode larval development and receptor-mediated endocytosis
pIC1174	**208**	***daf-21***	**Required for dauer development, down regulates *age-1***
pIC1175	243	*spd-2*	Spindle Defective
pIC1176	**262**	***daf-21***	**Required for dauer development, down regulates *age-1***

pIC1177	268	*tnt-2*	Expressed in the mu_int_R, mu_int_L, anal depressor muscle, reproductive system, and the body wall musculature
pIC1178	283	C54D10.4	Shown to interact with AAK-2
pIC1179	295	C27A12.7	Predicted to have ubiquitin-protein transferase activity, based on protein domain information
pIC1180	334	*msh-6*	Involved in post-replicative mismatch DNA repair

Table 3: Positive interactors of DAF-18 (C-terminal), isolated from Y2H screens, with the gene names and their functions. *daf-21* isolates are colour coded red.

The results from the Y2H screens of DAF-18 C-terminal region showed some very interesting proteins like H21P03.2, DAF-21, and C54D10.4. Because of known functions of these proteins, they are more likely to be good candidate for our novel pathway. However, I plan to focus on DAF-21 as it was isolated three times and *daf-21(p673)* mutants have a dauer constitutive phenotype, a phenotype opposite to *daf-18* (Vowels and Thomas 1994) and like DAF-18 has is expressed in the germline cells (Inoue et al. 2003).DAF-21 is a Hsp90-homolog that is critical for gonad and vulva development as well as oocyte maturation in *C. elegans* (Inoue et al. 2006, Gaiser et al. 2009, Gillan et al. 2009). **Figure 16** shows the sequencing results of the *daf-21* isolates.

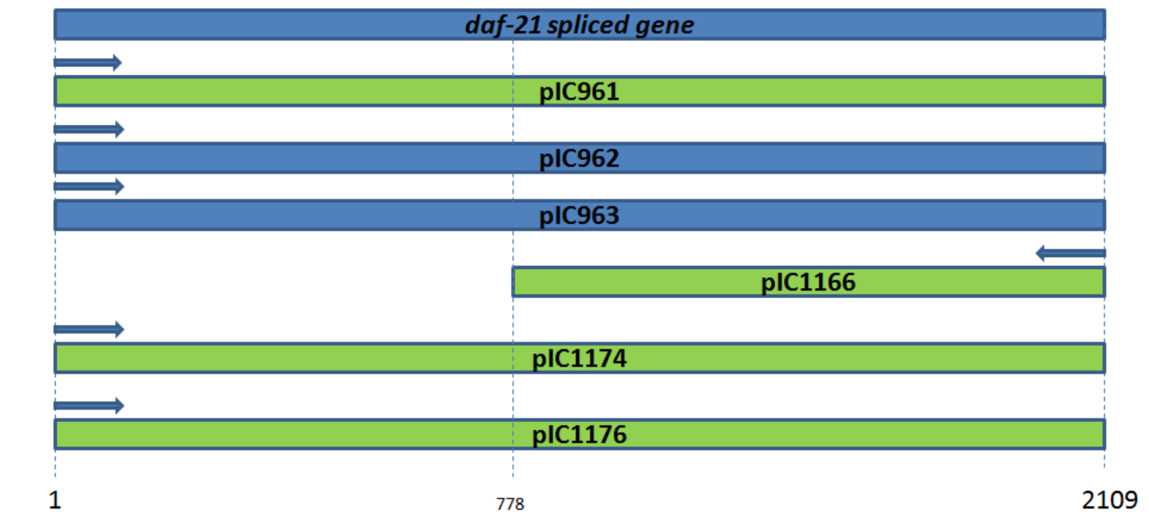


Figure 15: Comparison of overlapped region of DAF-21 Y2H isolates with the previously isolated DAF-21 vectors. The green bar represents the interacting plasmid sequences while the blue bar represent the *daf-21* spliced gene. The blue arrows represent the direction of the sequence in the library.

4.7 Absence of DAF-18 suppresses the embryonic lethality and L1 constitutive phenotype of *daf-2 (e979)* mutants

Insulin signaling is a key component in regulating L1 arrest. Insulin signaling receptor DAF-2/IR gets activated by insulin-like signaling peptides and regulates growth and development. Null mutants of *daf-2 (e979)* are temperature sensitive and are embryonic lethal or are in constitutive L1 arrest at 25°C (Gems et al. 1998b, Patel et al. 2008). Although DAF-18 is downstream of insulin signaling pathway, it was observed that double null mutants *i.e. daf-2(e979); daf-18(ok480)*, were able to grow to adults at 25°C, although the adults were sterile and the strain cannot be maintained at 25°C (**Table 2**). The previous work in our lab showed that *daf-2; daf-16* double-mutants were not able to rescue this phenotype of *daf-2 (e979)* mutants, although DAF-16 is downstream of DAF-18 in IIS pathway. This suggests that *daf-18 (ok480)* can rescue the

embryonic lethal and L1 constitutive phenotype of *daf-2 (e979)* mutants in a DAF-16 independent manner and DAF-2 has terminal effectors other than DAF-16.

Chapter 5

Discussion

C. elegans worms use L1 arrest as the first starvation survival responses after hatching. Among other developmental pathways, the IIS pathway plays a very crucial role in maintaining this L1 arrest. Sensory neurons in conjunction with the IIS signalling pathway sense the availability of nutrients which initiates growth after L1 arrests. Mutations in IIS lead to L1 arrest defects ranging from constitutive L1 arrest (e.g. *daf-2*) to a mere 3-4 days L1 arrest (e.g. *daf-18*). DAF-18 is the homolog of second most mutated tumour suppressor PTEN, making it extremely interesting to understand its roles in different developmental pathways. Mutation in DAF-18/PTEN lead to a severely reduced L1 arrest (3-4 days). DAF-18/PTEN is an antagonist of Insulin-like signaling pathway, but it has been suggested that it diverges into a different pathway to control L1 arrest. DAF-18/ PTEN has functions in developmental pathways independent of DAF-16/ FOXO, the ultimate target of IIS pathway. Although, DAF-16/FOXO plays important roles in maintaining cellular quiescence during L1 arrest, *daf-18* mutants show a more severe phenotype than *daf-16* mutants. As DAF-18/PTEN is upstream of DAF-16/FOXO, it led me to hypothesize that there is a DAF-16/FOXO-independent pathway downstream from DAF-18/PTEN, that is playing a more crucial role in maintaining L1 arrest **(Figure 17).**

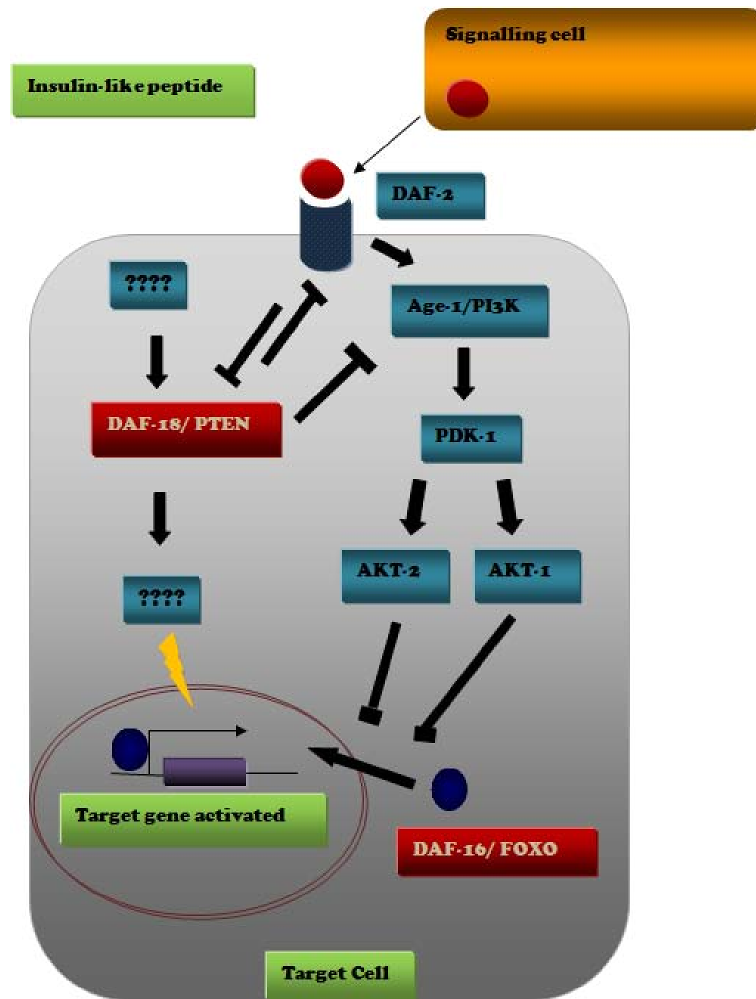


Figure 16: Proposed model of alternate pathways with reference to insulin-like signaling pathway- On the basis of the observation that the ultimate target of IIS pathway, DAF-16/FOXO, shows a less severe L1 arrest phenotype than that of upstream DAF-18/PTEN, I proposed the hypothesis that DAF-18/PTEN is working in a diverged pathway to maintain L1 arrest. In the nucleus DAF-18/PTEN is probably regulating DAF-16 independent genes.

5.1 DAF-18 controls L1 arrest longevity through an alternate pathway, possibly branching after DAF-2 in insulin signaling pathway

DAF-18/PTEN works as an antagonist of insulin-like signaling pathway. DAF-18/PTEN inhibits the activity of AGE-1/PI3K by dephosphorylation of the lipid substrate PIP3 to PIP2 (Ogg and

Ruvkun 1998). DAF-2/IR also inhibits DAF-18/PTEN by phosphorylation at Y27 and Y174 by kinase region of IRβ subunit (Liu et al. 2014). Previous results showed that double-mutants with *daf-2(e979); daf-18(ok480)* showed an increased life span of L1 arrested worms as compared to *daf-18 (ok480)* mutants (**Figure 10**) (Zanetti 2014). However the double-mutants did not rescue the longevity to wild type. This suggests that DAF-18/PTEN may function downstream of other receptors independent of DAF-2 for L1 arrest longevity. *daf-2(e979); daf-18(ok480)* mutants with somatic expression of DAF-18 were able to rescue the L1 arrest longevity to wild type (**Figure 10**), suggesting that the alternate pathway to control L1 arrest branches somewhere in Insulin signaling pathway after *daf-2* and *daf-18.* It is still not clear if AGE-1 is involved in the alternate pathway or not as the somatic rescue gene of *daf-18* is present on the same chromosome as *age-1*. From the sequencing results of the suppressors it was clear that the suppressors are not *age-1* alleles, suggesting that the suppressors are novel in this regard. To get a better understanding of the role of *age-1,* the double-mutants *age-1; daf-18* with somatic DAF-18 expression should be tested. Since the somatic *daf-18* (*quIs18)* is on chromosome II, the same chromosome as *age-1,* we can use the *daf-18 genomic* extrachromosomal array *quEx53* to make the strain *age-1(0); daf-18(ok480); quEx53* and test for L1 arrest longevity. I predict that it should suppressor similar way to *daf-2.*

5.2 Somatic expression of DAF-18/PTEN is sufficient to rescue L1 arrest longevity to wild-type via alternate pathway

Previous work from our lab showed that DAF-18 in somatic tissues extends the life span of L1 arrested worms to 3-4 days with a total L1 longevity of 8 days (Chamberlain 2014). Somatic expression of DAF-18 can rescue other phenotypes of *daf-18 (ok480)* mutants like dauer formation, and adult longevity (Brisbin et al.), but L1 arrest longevity and germline proliferation

is not rescued. The results from the suppressor screens suggested that with the help of the isolated suppressors, that DAF-18 expression in just somatic tissues can rescue the L1 longevity to wild-type. Although it is not clear whether the rescue will be observed even in the absence of somatic DAF-18 expression and if the suppressors will have the ability to maintain L1 arrest longevity without the sensitized background. So far, the efforts I made to isolate the suppressors and maintain the L1 arrest longevity without the somatic expression of DAF-18 were not successful. It could be either because the suppressors need the expression of DAF-18 in somatic tissues and/or the suppressors are linked to the integrated *quIs18* gene for somatic expression of DAF-18. To observe the phenotype of suppressors on their own, the suppressors can be crossed to **IC1639** – *dpy-9 (e12) mIs11 IV; him-5 (e1490) V,* to lose *daf-18 (ok480)* mutation.

5.3 DAF-18/PTEN can control germline proliferation cell-non autonomously

DAF-18/PTEN has already been known to work independent of DAF-16/FOXO to control the germline proliferation in L1 arrested worms (Fukuyama et al. 2006). Another important pathway that controls germline proliferation independent of DAF-18/PTEN, is the AMPK pathway (Fukuyama et al. 2012). Previous work from our lab showed an increase in DAF-18/PTEN levels in primordial germ cells, Z2/Z3 during L1 (Brisbin et al. 2009). However, *daf-18* mutants with germline expression of DAF-18 were unable to either suppress the germline proliferation during L1 arrest, nor increase the life span of L1 arrested worms to wild-type, suggesting that DAF-18 is required both in the soma and germline to stop germline divisions during L1 arrest (Chamberlain 2014). During the suppressors screening, the isolated suppressors have *daf-18 (ok480)* mutation with somatic DAF-18 expression. Although the suppressor strains do not have DAF-18/PTEN expressed in germline, the PGL-1 germline staining shows an arrest in germline proliferation

during L1 arrest. This suggests that DAF-18/PTEN in the soma alone, with the suppressor mutation, have the ability to control the germline proliferation cell-nonautonomously. This could also suggest that DAF-18/PTEN controls the germline proliferation through the diverged pathway. Insulin-like signaling is known to work cell-nonautonomously to control L1 arrest (Zhang et al. 2011, Kasuga et al. 2013). Moreover, DAF-18/PTEN has been observed to control dauer formation and life span extension both cell autonomously and nonautonomously (Masse et al. 2005).

5.4 Maintaining germline quiescence does not lead to a wild-type L1 longevity

DAF-18/PTEN maintains germline quiescence during L1 arrest. Primordial germ cells Z2/Z3 arrest at the L1 stage by a different mechanism than the somatic cell arrest. The Z2/Z3 cells halt their growth after DNA synthesis at the G2 stage during L1 arrest (Fukuyama et al. 2006). *daf-18* mutants show a defect in germline quiescence during L1 arrest but DAF-18 does not require *daf-16* to maintain this quiescence (Fukuyama et al. 2006). To maintain this quiescence, *daf-18* works redundantly with α-subunits of AMPK, encoded by *aak-1* and *aak-2* (Fukuyama et al. 2012, Lee et al. 2012). The two pathways converge to inhibit TOR complex 1 which is required for the ectopic germline proliferation (Fukuyama et al. 2012). Although *aak-2 (ok524)* mutants do not show germline proliferation in L1 arrest, the longevity is still decreased, and TOR mutants can suppress the germline proliferation of *daf-18* but not the L1 arrest longevity (Fukuyama et al. 2012). This shows that the defects in L1 arrest longevity of *daf-18* mutants is not solely because of proliferating germline and *daf-18* is controlling other aspects of L1 arrest as well. The suppressors of L1 arrest longevity with *daf-18 (ok480)* carrying somatic DAF-18 expression also showed a rescue in germline proliferation. This suggest that suppressor mutations can replace the loss of *daf-18* in the germline to extend longevity as well as inhibit germline proliferation.

5.5 *daf-18(ok480)* can rescue embryonic lethality and L1 constitutive phenotype in *daf-2 (e979)* mutants at 25°C

The insulin signaling receptor DAF-2 is one of the key components in controlling growth and development through IIS pathway. Mutants with defective *daf-2* display constitutive dauer formation, an increase L1 arrest and increased lifespan, depending on the mutant allele (Gems et al. 1998a). One of the most severe phenotypes is exhibited by *daf-2 (e979),* resulting in embryonic lethality and constitutive L1 arrest at 25°C (Vowels and Thomas 1992). *daf-2 (e979)* mutation results in C146Y substitution. *daf-2 (e979)* belongs to class 2 *daf-2* alleles which is a phenotypically severe class (Gems et al. 1998a). Previous work in our lab showed that despite DAF-16 being the terminal affecter of IIS pathway, *daf-2 (e979); daf-16(mu86)* mutants cannot rescue the L1 constitutive phenotype of *daf-2 (e979)* mutants at 25°C, showing that the L1 arrest phenotype is not controlled by mere nuclear localization of DAF-16. I showed that *daf-2(e979); daf-18(ok480)* mutants were able to grow to adulthood at 25°C (**Table 2**). However, these adults were sterile and the strain could not be maintained at 25°C. These results support the hypothesis that DAF-18 is controlling the L1 arrest and embryonic lethality through an alternate pathway than DAF-16.

5.6 DAF-21 is a possible interactor of DAF-18

DAF-21 is a member of the heat-shock protein family and is one of the 90 kDa heat shock protein (HSP90) homologs in *C. elegans.* Heat-shock proteins are a universally occurring class of proteins which are expressed under stress conditions. These proteins work as molecular chaperones with essential roles in folding of newly translated proteins (Csermely et al. 1998). DAF-21 is expressed predominantly in germline cells Z2/Z3 (Inoue et al. 2003). The abundance

of DAF-21 expression in germline cells could be because of the continuous development in germline throughout life and the need of molecular chaperones for newly folded proteins in germline. Loss of function of DAF-21 mutants (*daf-21 (p673))* have a smaller brood size showing it has an important function in maintaining and maturation of germline cells (Vowels and Thomas 1994). RNAi experiments with *daf-21* also show an embryonic lethal phenotype making it essential for growth during embryogenesis and early larval stages (Piano et al. 2000). Although DAF-21 is present in germ cells, it is also located in somatic cells during L1 and adult stages (Inoue et al. 2003). DAF-18/PTEN has been studied for its role in maintaining germline quiescence during L1 arrest (Fukuyama et al. 2006). Brisbin et al. (2009) reported that DAF-18 is also expressed in Z2/Z3 cells. The Y2H results show an apparent positive interaction between DAF-18 and DAF-21 and the localization of both proteins in germline also suggest an interaction in germline maintenance. Interestingly, when DAF-21 cDNA, was inserted in opposite orientation in the plasmid (pIC1166), it also showed an interaction with DAF-18. This could suggest that *daf-21* DNA probably interacts with DAF-18. However so far we do not have any other proof of a positive interaction between DAF-18 and DAF-21 but these results give us a hint of an interaction in a separate pathway. Future binding experiments should confirm the interaction.

5.7 Conclusions and future directions:

The L1 larval stage has a great significance when studying controlled growth and development at the cellular level. DAF-18 has a major role in controlling this growth and development. Results from previous studies showed that DAF-18 controls the L1 arrest though an IIS independent pathway. My research supports this hypothesis where *daf-18 (ok480)* L1 larvae were able to survive in days comparable to wild-type life span with the help of unknown suppressors. Although these suppressors seem to need somatic expression of DAF-18 (*quIs18)*, this could

either suggest that the suppressors are linked to somatic expression array of DAF-18 on chromosome II or the suppressors need somatic DAF-18 to control the L1 arrest. Future experiments can help to address this and possibly the isolation of suppressors from an integrated somatic expression array of DAF-18 can be created by providing extrachromosomal DAF-18 array. This will help us understand if suppressors are physically linked to *quIs18* or they need somatic expression of DAF-18. We can also outcross the suppressors to observe a phenotype on their own *i.e.* without *daf-18 (ok480).*

It was observed that *daf-2 (e979); daf-18 (ok480); quIs18* mutants were able to rescue the L1 arrest longevity to wild type. This shows that without DAF-2 and with somatic expression of DAF-18 the L1 arrest longevity can be rescued. For future work, we can test different insulin like peptides to see if any of them is specifically involved in maintaining L1 arrest longevity. We can also test **IC1589** – *quIs24(Ppie-1::daf-18cDNA::pie-1 3'UTR) II*; *daf-18 (ok480) IV; him-5 (e1490) V* with *daf-2 (e979),* to see if the germline rescue of DAF-18 would also have some effect on L1 arrest longevity in the absence of insulin receptor. We can also try to use *daf-2* RNAi as a positive control for L1 arrest longevity in such experiments. Although *age-1* RNAi had no effect on *daf-18 (ok480); quIs18* L1 longevity, it could be either AGE-1 is not involved in the proposed pathway or RNAi cannot work properly when studying L1 arrest longevity. To test if AGE-1 is a component of the proposed pathway, we can cross the extrachromosomal array of *daf-18* genomic expression (*quEx53*) in *age-1; daf-18 (ok480)* mutants and see the effects on L1 arrest longevity.

From my chromosomal linkage experiments, the suppressors appear to be present on chromosome II. Future experiments can screen related genes on chromosome II to confirm if any of those genes will help in suppression of *daf-18 (ok480)* L1 arrest phenotype. If the suppressors have a phenotype on their own it may help us clone the gene by transformation rescue of

candidate genes on chromosome II. The germ cells Z2/Z3 antibody staining also show that suppressors have the ability to control the germline proliferation in *daf-18 (ok480)* mutants with the help of somatic DAF-18 expression. This suggests a cell non-autonomous function of DAF-18 in controlling germline proliferation during L1 arrest. Future experiments could include the germline staining of **IC1885***: zdIs5 I; quIs18 II; daf-2 (e979) III; daf-18 (ok480) IV*, to see if *daf-2* can inhibit the germline proliferation in *daf-18; quIs18* mutants.

My research further demonstrated that *daf-18 (ok480)* can suppress the embryonic lethality and constitutive L1 arrest phenotype of *daf-2 (e979)* (ts) mutants. However, the double-mutants *daf-2 (e979); daf-18 (ok480)* are sterile at 25°C and cannot be maintained at 25°C. These findings suggest a two way interaction between DAF-2 and DAF-18 and provide a powerful genetic tool to isolate suppressors of *daf-2 (e979); daf-18 (ok480)* sterility. The isolation of these new suppressors will further help in the understanding of these complex pathways.

My Y2H results revealed an apparent positive interaction between DAF-21 and C' terminal of DAF-18. DAF-21 is a member of HSP-90 complex and is abundant in germline cells. Brisbin et al. (2009) showed that DAF-18 is also abundantly expressed in the germline cells. Future experiments can be directed towards finding a correlation between the two proteins. This will also help to shed light on pathways other than the IIS pathway, where DAF-18 plays a crucial role in controlling growth and development. The other positive interactors from Y2H screen can also be further tested. The formation of the GFP reporter yeast strains will also help in simplifying Y2H screens in future studies.

In summary, my work supports the hypothesis of an alternate pathway branching either inside or outside of IIS pathway, through which DAF-18 controls L1 arrest. The identification of the isolated suppressors will be a major step in unveiling the hidden pathway which will help in

understanding the complexities of tumor suppressor homolog, DAF-18/PTEN, in regulation of growth and development.

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Appendix A

Media recipes

2XTY

For 1 Litre mix

-Tryptone	16g
-Yeast Extract	10g
-NaCl	5g

-dH_2O to 1 litre

(2XTY Amp add 100ul (75 mg/mL Amp) for every 100mL 2XTY)

Aliquot to 125 mL bottles

Autoclave on liquid cycle for 40 minutes

(Recipe from chin-sang.ca)

RNAi Plates

For 2 Litres mix

-Agar	40 g
-NaCl	6 g
-Bacto-Peptone	5 g

-dH2O to 1 litre

Autoclave on liquid cycle for 40 min

Let media cool to 55°C

After cooling add and mix together:

- KPO_4	50 mL
- $MgSO_4$	2 mL
- $CaCl_2$	2 mL
- Cholesterol (5 mg/mL in 95% EtOH)	2mL
- Ampicillin (100 mg/mL)	2mL
- 20% β-lactose in ddH2O	20mL

Pour into small worm plates

Store at 4°C

LB plates

To make 4 L:

40 g Bacto Tryptone

20 g Bacto Yeast extract

40 g NaCl

Make up in 3200 mL dH_2O.

Adjust pH from ~6.9 to 7.5 with 12-14 mL of 1 M NaOH

Bring volume to 4 L.

Add 60 g Bacto-agar, mix, autoclave.

When agar has cooled to 55°C, pour plates (~25 mL per large plate). If you can keep your hand on the side of the flask then it is cool enough.

Mark LB sides with GREEN stripe.

LB Amp plates (75 mg/mL Ampicillin)

Follow protocol for LB plates until after autoclaving. When agar has cooled to 55°C, add 4 mL (2 vials) of 75 mg/mL Ampicillin stock (1000X stock, kept at -20°C). Pour plates as above. Amp plates marked with RED stripe.

Ampicillin is unstable. Plates older than four months cannot be used--so don't make up too much at any one time.

YPAD plates

To make 1 liter

10 g yeast extract

20 g peptone

48 mg Adenine hemisulfate (For YPD plates leave this out)

20 g dextrose

20 g agar

dH2O to 1 liter

Autoclave

Synthetic media plates (-LEU, -TRP, etc.)

To make 1 liter

10 g yeast nitrogen base (without amino acids with ammonium sulfate)

20 g dextrose

20 g agar

dH_2O to 1 liter

Autoclave for 40 minutes and take out as soon as cycle over; immediately add the appropriate synthetic powder (amount on side of bottle) and mix.

Note you can make drop out mixes from existing synthetic media, for example you can make -LEU, -TRP media by adding HIS to a -LEU -TRP -HIS triple drop out synthetic media.

For Yeast 2 Hybrid Screening (not for general use)

3-AT (3-amino, 1,2,4-triazole) addition:

Let agar cool to 50°C then add 3-AT powder to media.

For 25 mM 3-AT, add 2.1 g/L

For 50 mM 3-AT, add 4.2 g/L

Solutions for Yeast Transformations:

1M Lithium Acetate:

Add 10.2 g Lithium Acetate salt in 100 mL of ddH2O and autoclave

50% Polyethylene glycol (PEG)

Dissolve 50g PEG (4000) in 30 mL ddH2O and mix. Bring the ddH2O to 100 mL. Continue heating and stirring until dissolved properly. Filter sterilize afterwards.

Solutions for filter lift assay:

Z-Buffer (1L)

$Na_2HPO_4.7H_2O$ 16.1 g

$NaH_2PO_4.H_2O$ 5.5 g

KCl 0.75g

$MgSO_4.7H_2O$ 0.25 g

Upon using Z-Buffer add:

X-Gal (100 mg/mL) 10 uL/mL

BME 2.7 uL/mL

Solutions for X-Gal Agarose overlay Assay:

Stock solution:

0.5M Potassium Phosphate Buffer pH 7.0

For 200 mL = Mix 61 mL of 1M K_2HPO_4 and 39 mL of 1M KH_2PO_4 and add 100 mL of H_2O

6% Dimethyl Formamide

0.1% SDS (For 100 mL of working solution add 93 mL of Phosphate buffer, 6 mL of DMF and 1 mL of 10% SDS)

Low Melt-Agarose: To 100 mL working solution add 0.5 g low melt agarose and microwave for 1 min with intervals.

Appendix B

DNA Sequences

Sequence of the insert to make GFP reporter yeast strains (YIC1-YIC6) (*ade-2* homology arms of 1 kb with UAS-GFP-Envy-yDH1 terminator):

AAACCACTCAAAAGGAAGACTAGTAACGCCGTATCGTGATTAACGTATTACATAAGT

TACAGGATTCATGCTTATGGGTTAGCTATTTCGCCCAATGTGTCCATCTGACATTACT

ATTTTGCATTTTAATTTAATTAGAACTTGACTAGCGCACTACCAGTATATCATCTCAT

TTCCGTAAATACCAAATGTATTATATATTGAAAGCTTTTGACCAGGTTATTATAAAAG

AAACTTCATGCTCGAAAAAGATCATTTCGAAAAGTTGCCTAGTTTCATGAAATTTTA

AAGCAGTTTATATAAATTTTACCTTTTGATGCGGAATTGACTTTTTCTTGAATAATAC

ATAACTTTTCTTAAAAGAATCAAAGACAGATAAAATTTAAGAGATATTAAATATTAG

TGAGAAGCCGAGAATTTTGTAACACCAACATAACACTGACATCTTTAACAACTTTTA

ATTATGATACATTTCTTACGTCATGATTGATTATTACAGCTATGCTGACAAATGACTC

TTGTTGCATGGCTACGAACCGGGTAATACTAAGTGATTGACTCTTGCTGACCTTTTAT

TAAGAACTAAATGGACAATATTATGGAGCATTTCATGTATAAATTGGTGCGTAAAAT

CGTTGGATCTCTCTTCTAAGTACATCCTACTATAACAATCAAGAAAAACAAGAAAAT

CGGACAAAACAATCAAGTATGGATTCTAGAACAGTTGGTATATTAGGAGGGGGACA

ATTGGGACGTATGATTGTTGAGGCAGCAAACAGGCTCAACATTAAGACGGTAATACT

AGATGCTGAAAATTCTCCTGCCAAACAAATAAGCAACTCCAATGACCACGTTAATGG

CTCCTTTTCCAATCCTCTTGATATCGAAAAACTAGCTGAAAAATGTGATGTGCTAACG

ATTGAGATTGAGCATGTTGATGTTCCTACACTAAAGAATCTTCAAGTAAAACATCCC

AAATTAAAAATTTACCCTTCTCCAGAAACAATCAGATTGATACAAGACAAATATATT

CAAAAAGAGCATTTAATCAAAAATGGTATAGCAGTTACCCAAAGTGTTCCTGTGGAA

CAAGCCAGTGAGACGTCCCTATTGAATGTTGGAAGAGATTTGGGTTTTCCATTCGTCT
TGAAGTCGAGGACTTTGGCATACGATGGAAGAGGTAACTTCGTTGTAAAGAATAAG
GAAATGATTCCGGAAGCTTTGGAAGTACTGAAGGATCGTCCTTTGTACGCCGAAAAA
TGGGCACCATTTACTAAAGAATTAGCAGTCATGATTGTGAGATCTGTTAACGGTTTA
GTGTTTTCTTACCCAATACGGATTAGAAGCCGCCGAGCGGGTGACAGCCCTCCGAAG
GAAGACTCTCCTCCGTGCGTCCTCGTCTTCACCGGTCGCGTTCCTGAAACGCAGATGT
GCCTCGCGCCGCACTGCTCCGAACAATAAAGATTCTACAATACTAGCTTTTATGGTTA
TGAAGAGGAAAAATTGGCAGTAACCTGGCCCCACAAACCTTCAAATGAACGAATCA
AATTAACAACCATAGGATGATAATGCGATTAGTTTTTAGCCTTATTTCTGGGGTAAT
TAATCAGCGAAGCGATGATTTTTGATCTATTAACAGATATATAAATGCAAAAACTGC
ATAACCACTTTAACTAATACTTTCAACATTTTCGGTTTGTATTACTTCTTATTCAAATG
TAATAAAAGTATCAACAAAAAATTGTTAATATACCTCTATACTTTAACGTCaaggagaaaa
aaccATGTCCAAGGGTGAAGAATTATTCACTGGTGTTGTTCCAATCTTGGTTGAATTGG
ATGGTGATGTTAACGGTCACAAGTTTTCTGTTAGAGGTGAAGGTGAAGGTGATGCTA
CTAATGGTAAATTGACCTTGAAGTTCATCTGTACCACAGGTAAATTGCCAGTTCCATG
GCCAACTTTGGTTACTACTTTGTGTTATGGTGTTCAATGCTTCGCTAGATACCCAGAT
CATATGAAGCAACACGATTTCTTCAAATCCGCTATGCCAGAAGGTTACGTTCAAGAA
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