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New Regulatory and Metabolic Genes that Influence C. elegans' Lifespan in Response to Reproductive Signals

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New Regulatory and Metabolic Genes that Influence *C. elegans*' Lifespan in Response to Reproductive Signals

by

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PREFACE

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ABSTRACT **Mark McCormick**

New Regulatory and Metabolic Genes that Influence *C. elegans'* Lifespan in Response to Reproductive Signals.

In both *C. elegans* and *Drosophila,* removing germline stem cells increases lifespan. In *C. elegans,* this lifespan extension requires the DAF-16/FOXO transcription factor and the DAF-12 nuclear hormone receptor, and the role of DAF-16/FOXO in this process may also be shared in flies. To better understand the regulatory relationships between DAF-16 and DAF-12, we used microarray analysis to identify downstream genes. We found that these two transcription factors regulate distinct but overlapping sets of genes in response to loss of the germline. In addition, we identified several new genes that are required for loss of the germline to increase lifespan in *C. elegans*. One, *phi-62,* encodes a conserved, predicted RNA binding protein. PHI-62 influences DAF-16-dependent transcription, possibly by collaborating with TCER-1, a putative transcription-elongation factor, and FTT-2, a 14-3-3- protein known to bind DAF-16. Three other genes encode proteins involved in lipid metabolism; one is a triacylglycerol lipase, and another is an acyl CoA reductase. These genes do not affect bulk fat storage levels, but rather may influence production of a lifespan-extending signal or metabolite.

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CHAPTER 1: INTRODUCTION

Aging is a fundamental biological process, and has long been considered an unsolved problem of biology (Haldane 1942; Medawar 1952). It has been asked whether aging is a random decline in function due to wear and tear, as might be seen in a piece of machinery. Or might differences in adult life history be regulated, much as those during development are?

Some evolutionary theories of aging suggest that rates of post-reproductive aging may not be subject to natural selection pressure, which leads to the possibility that rate of aging or length of lifespan may not be subject to biological regulation (Kirkwood & Austad 2000). Several lines of evidence suggest that this is not the case, and that aging, at least the rate of aging, is regulated. Some closely related species, which must have evolved from a common ancestor with a particular lifespan, now have diverged through genetic changes to have very different lifespans. Bats and mice of similar size and life history can have lifespans that vary by as much as a factor of 25 (Austad & Fischer 1991; Kenyon 1996).

Additionally, there is a rapidly growing list of single gene mutations that have been discovered to have profound effects on lifespan in organisms from yeast to humans, and many of these genes are themselves parts of signaling pathways that lead to the subsequent differential regulation of many other downstream genes (Gems & Partridge 2001; Tatar *et al.* 2003; Kenyon 2005; Piper *et al.* 2008; Panowski & Dillin 2009). Regardless of the evolutionary provenance of these regulatory pathways, much progress has been made in discovering the individual genes that affect them, including

insulin/IGF-1 signaling (Friedman & Johnson 1988; Kenyon *et al.* 1993; Kimura *et al.* 1997), and the TOR pathway (Vellai *et al.* 2003; Jia *et al.* 2004; Kapahi *et al.* 2004; Kaeberlein *et al.* 2005). In some cases mutation of a single gene in these pathways can double an organism's natural lifespan. This suggests the possibility that understanding these perturbations could lead to insight into the basic mechanisms of aging, and of diseases whose primary risk factor is age alone.

The usefulness of *C. elegans* as a model organism in the study of lifespan

Caenorhabditis elegans was pioneered in the 1960s as a model organism (Brenner 1974). Many genetic pathways that have been subsequently dissected through the use of *C. elegans* have proven to be widely conserved through disparate organisms including humans. One example of such successful work is the elucidation of some of the signaling and mechanism involved in programmed cell death (Ellis & Horvitz 1986; Hengartner 1997). *C. elegans* has also been at the forefront in the study of the genetics of aging and lifespan since its inception (Klass 1977; Johnson & Wood 1982; Klass 1983; Johnson *et al.* 1984; Friedman & Johnson 1988; Kenyon *et al.* 1993; Duhon & Johnson 1995; Vanfleteren & Braeckman 1999). Many of the genes first discovered to profoundly affect lifespan in *C. elegans* have already been later shown to have similar effects in distantly related species (Holzenberger *et al.* 2003; Hwangbo *et al.* 2004; Kenyon 2005; Giannakou *et al.* 2007; Taguchi *et al.* 2007; Berryman *et al.* 2008; Flatt *et al.* 2008; Salih & Brunet 2008; Willcox *et al.* 2008; Anselmi *et al.* 2009; Flachsbart *et al.* 2009; Pawlikowska *et al.* 2009).

C. elegans has a relatively short wild-type lifespan, approximately 15-20 days under normal conditions at 20°C. This allows experiments measuring lifespan, and changes in lifespan, in a population of worms to be completed reasonably quickly. Because *C. elegans* most commonly reproduces by hermaphrodite self-fertilization, isogenic populations of viable mutant strains are easily maintained. However, *C. elegans* also exhibits XO/XX sexual reproduction, which allows easy crossing of strains. The use of RNA interference to disrupt gene function in lieu of mutation is an invaluable tool for *C. elegans* genetics (Fire *et al.* 1998; Timmons *et al.* 2001), and two libraries of dsRNA producing bacteria have been produced which target most of the predicted open reading frames in *C. elegans* (Kamath *et al.* 2003; Rual *et al.* 2004). This genetic tractability and relatively short lifespan, combined with a robust set of tools and techniques, speak to *C. elegans*' suitability as a model for the study of aging. At the same time, previously discovered functionally relevant orthologs, in species from yeast to human, of many lifespan and aging affecting genes first discovered in *C. elegans* suggest the likely broader implications of work first performed in this model organism.

Regulation of lifespan by the germline in *C. elegans*

One of the most intriguing pathways affecting the aging of *C. elegans* involves the reproductive system, which actually controls the rate of aging of the whole animal. The influence of the germline and reproductive system on the lifespan of *C. elegans* is a complex story still very much under active investigation (Mukhopadhyay & Tissenbaum 2007). In *C. elegans*, the entire reproductive system normally arises from four cells present at the time of hatching, Z1, Z2, Z3, and Z4 (Kimble & Hirsh 1979). Z2 and Z3

go on to give rise to the germline of the animal, while Z1 and Z4 eventually give rise to the somatic reproductive tissues (somatic gonad). Work in our lab has shown that removing only Z2 and Z3, the cells that give rise to the germline, by laser ablation, increases the lifespan of the animal by as much as 60%, while removing Z1, Z2, Z3, and Z4, thus the somatic gonad as well as the germline, does not extend lifespan (Hsin & Kenyon 1999), suggesting that opposing signals arise from the germline and the somatic gonad. It has been shown that it is the proliferating germline stem cells (rather than differentiated sperm or oocytes) which must be present to prevent any lifespan extension or absent for lifespan extension to occur (Arantes-Oliveira *et al.* 2002). This lifespan extension by removal of the germline has been recapitulated in flies (Flatt *et al.* 2008), and a link between reproductive state and lifespan has also been shown in mice (Cargill *et al.* 2003; Mason *et al.* 2009). Interestingly, removing the ovaries (a somatic reproductive tissue that contains the oocytes) from women increases the incidence of all-cause mortality during aging (Parker *et al.* 2009). As women normally lose their germ cells (oocytes) due to menopause, this finding has at least superficial similarities with the situation in worms.

In wild type animals, germline cell state is controlled spatially as cells move from the distal tip of the gonad toward the vulva. The distal tip cell, located at the distal end of the gonad, produces a signaling molecule gradient between the distal end of the gonad and the vulva. Near the distal tip, cells are kept in mitosis by signals from the distal tip cell, and as they migrate toward the vulva, they enter meiosis (Kimble & White 1981). *glp-1*, one of two known *C. elegans* members of the LIN-12/Notch family of receptors, is required for this regulation. In particular, if *glp-1* is inactivated, germline precursor

cells enter meiosis prematurely without undergoing any further mitosis and germline is proliferation ceases (Austin & Kimble 1987). The *glp-1* receptor is expressed specifically on the cell membranes of mitotic germline cells (Crittenden *et al.* 1994), and it responds in this regulatory context to *lag-2*, a Delta/Serrate/Lag-2 family transmembrane protein that acts as a ligand for the *glp-1* receptor (Henderson *et al.* 1994). By taking advantage of temperature-sensitive mutations in *glp-1*, such as *glp-1(e2141ts)* (Priess *et al.* 1987), we can maintain viable isogenic strains that can produce large populations of adults completely lacking a germline, without the need for laser ablation microsurgery (Arantes-Oliveira *et al.* 2002).

Whether caused by laser ablation of Z2 and Z3 or temperature-sensitive mutations in *glp-1*, lifespan extension by removal of the *C. elegans* germline is completely dependent on the presence of two transcription factors, the DAF-12 nuclear hormone receptor and the DAF-16/FOXO forkhead transcription factor (Hsin & Kenyon 1999).

Another gene that has been identified which is required for germline removal to extend lifespan is *kri-1,* which encodes a conserved ankyrin-repeat containing protein homologous to human KRIT 1 (Krev interaction trapped/cerebral cavernous malformation 1) (Berman & Kenyon 2006). Upon germline removal, DAF-16 localizes to the nuclei of intestinal cells (Lin *et al.* 2001), and *kri-1* is required for this localization (Berman & Kenyon 2006).

Additionally, *tcer-1*, which encodes a transcription elongation factor homologous to the eukaryotic transcription elongation factor TCERG1 (also known as CA150), has been shown to be required for removal of the germline to extend lifespan (Ghazi *et al.*

2009). *tcer-1* is required specifically for the up-regulation of some, but not all, known *daf-16* regulated genes upon removal of the germline (Ghazi *et al.* 2009).

Finally, a fat metabolism gene, K07A8.5, has been shown to be required for removal of the germline to extend lifespan in *C. elegans* (Wang et al. 2008). This gene is also partially required for a decreased Nile red staining phenotype found in germline-deficient animals (Wang *et al.* 2008), which is thought to indicate an increase in the number or size (not clear) of lysosomes or lysosome-like compartments (Schroeder *et al.* 2007; Soukas *et al.* 2009).

Overview of the thesis

This work hopes to shed light on several closely related questions about how the reproductive system affects lifespan, which will be mentioned in turn. For one, the question of how one tissue may signal to or affect another in the regulation of lifespan is a fascinating one, and some progress has been made on aspects of this question (Apfeld & Kenyon 1998; Wolkow *et al.* 2000; Libina *et al.* 2003; Iser *et al.* 2007; Yamawaki *et al.* 2008). As discussed above, in *C. elegans*, removal of the cells that give rise to the animal's germline, either by laser ablation of the germline precursor cells, or by mutations in genes required for germ cell proliferation, can extend lifespan by up to 60% (Hsin & Kenyon 1999; Arantes-Oliveira *et al.* 2002). How might information indicating the presence or absence of the germline be transduced to the remaining cells and tissues in the animal? We have some clues arising from the aforementioned fact that two previously studied transcription factors, DAF-16/FOXO and the nuclear hormone receptor DAF-12, are known to be required for this lifespan extension, and this

information was central to the design of this study (Hsin & Kenyon 1999). Using whole transcriptome microarrays to look at mRNA levels of over 95% of *C. elegans'* predicted open reading frames simultaneously, we hoped to see what changes in overall transcription were induced by removal of the germline, both in the presence or absence of each of these transcription factors. It could be the case that some of the transcriptionally regulated targets of either DAF-16 or DAF-12 in this context might play a role in this tissue-to-tissue endocrine signaling that can be inferred to occur for the remaining tissues of the worm to live longer in the absence of its germline.

Another immediate question arises from the fact that there are two distinct transcriptional regulators, DAF-16 and DAF-12, each singly required for the lifespan extension of germline-deficient animals. Might some or all of the relevant targets of these two transcription factors coincide? This might shed light on which of their targets are the most functionally relevant. Additionally, DAF-16 and DAF-12 have a known epistatic relationship in a very different context: regulation of the dauer decision (Larsen *et al.* 1995). Dauer is a diapause-like alternative larval stage that is entered in response to low food levels or crowding (Albert *et al.* 1981; Riddle *et al.* 1981; Swanson & Riddle 1981; Golden & Riddle 1982). This leads to the question: how are the functions of DAF-16 and DAF-12 related to one another in this germline regulation of lifespan? They might they regulate completely disparate, or completely identical, sets of genes as described above, but one might also regulate an inclusive subset of the other, suggesting a linear pathway in which one acts downstream of the other. In C. elegans, larvae can enter a diapause-like alternative larval stage called the dauer in response to crowded or low food conditions. Interestingly, *daf-12* and *daf-16* also both play a role in this separate

regulatory pathway, and in this dauer context, if *daf-12* is activated by a gain-of-function mutation, *daf-16* is no longer required (Gerisch *et al.* 2001), suggesting that in the dauer pathway *daf-12* is epistatic to *daf-16*.

With respect to the targets of DAF-16 and of DAF-12, there is more that we can ask. The transcriptional targets of DAF-16 have previously been studied in a different pathway affecting lifespan, the *daf-2* insulin/IGF-1 signaling pathway, which also requires the presence of DAF-16 for lifespan extension (Kenyon *et al.* 1993; Murphy *et al.* 2003). Because DAF-16 is necessary for lifespan extension in both of these pathways, it's interesting to ask what the relevant (most significantly changed) transcriptional targets of DAF-16 in these two contexts have in common. This sheds some light on those targets which may prove to relate to some of the know differences between these two pathways' phenotypes, and also those which could be part of a core conserved set of affected genes in both cases. Similarly, some previous work has been done on transcriptional differences between longer-lived hypermorph and shorter-lived hypomorph alleles of DAF-12, in otherwise normal animals that have not had their germlines removed (Fisher & Lithgow 2006). Again, we can ask what genes may be common to the transcriptional signature of these two processes, and the level of generality, if any, of their requirement for lifespan extension in different contexts.

Once lists of genes that all respond in concert to the loss of DAF-16 or of DAF-12 respectively in the germline regulation of lifespan are known, it is possible to computationally examine the likely regulatory regions for these open reading frames (namely, their immediate upstream sequence regions) en masse in order to ask what sequences if any occur with an improbably high frequency in these putative promoter

regions. These sequences may be regulatory elements relevant to this phenotype, and it's possible that some of them may even eventually prove to be bound by either DAF-16 or DAF-12 respectively. Some overrepresented sequences have been identified from the previous study of DAF-16 targets in the *daf-2* insulin/IGF-1 pathway (Murphy *et al.* 2003), and although not previously reported it is possible to identify overrepresented sequences from the previously published targets of DAF-12 that may relate to lifespan (Fisher & Lithgow 2006), so, as before, it should be possible to ask whether there are similarities between sequences identified in this study and those found in studying these transcription factors in other contexts that do not involve the removal of the germline.

Finally, many of these transcriptional targets themselves, as well as genes found to vary most significantly between long and short-lived conditions in this pathway, may be genes whose function is known or whose probable function can be guessed from their sequence. In addition, using RNAi by bacterial feeding (Kamath *et al.* 2003) it is possible to assay the effect if any of knockdown of these genes of interest on lifespan, both in wild type animals and germline-deficient animals. This can be done for predicted targets of DAF-16, those of DAF-12, and for genes that vary most significantly between long and short-lived in the context of germline removal. For genes found to be of further interest due to RNAi lifespan phenotype and/or predicted function, further characterization may be possible to begin to unravel what role they play in these changes in lifespan.

The results of all of these investigations are reported in Chapter 2.

CHAPTER 2: NEW REGULATORY AND METABOLIC GENES THAT INFLUENCE *C.ELEGANS*' LIFESPAN IN RESPONSE TO REPRODUCTIVE SIGNALS

SUMMARY

In both *C. elegans* and *Drosophila,* removing germline stem cells increases lifespan. In *C. elegans,* this lifespan extension requires the DAF-16/FOXO transcription factor and the DAF-12 nuclear hormone receptor, and the role of DAF-16/FOXO in this process may also be shared in flies. To better understand the regulatory relationships between DAF-16 and DAF-12, we used microarray analysis to identify downstream genes. We found that these two transcription factors regulate distinct but overlapping sets of genes in response to loss of the germline. In addition, we identified several new genes that are required for loss of the germline to increase lifespan in *C. elegans*. One, *phi-62,* encodes a conserved, predicted RNA binding protein. PHI-62 influences DAF-16-dependent transcription, possibly by collaborating with TCER-1, a putative transcription-elongation factor, and FTT-2, a 14-3-3- protein known to bind DAF-16. Three other genes encode proteins involved in lipid metabolism; one is a triacylglycerol lipase, and another is an acyl CoA reductase. These genes do not affect bulk fat storage levels, but rather may influence production of a lifespan-extending signal or metabolite.

INTRODUCTION

Removing the germline of *C. elegans*, either by laser ablation of the germline precursor cells, or by mutation of genes required for germ cell proliferation, extends lifespan by approximately 60% (Hsin & Kenyon 1999; Arantes-Oliveira *et al.* 2002). This lifespan extension appears to be a consequence of germline stem-cell loss during adulthood (Arantes-Oliveira *et al.* 2002). Removing germline stem cells in *Drosophila* adults by mutations analogous to those employed in *C. elegans* increases the fly's lifespan by up to 50% (Flatt *et al.* 2008), and in mice, too, the reproductive system can modulate the lifespan of the animal (Cargill *et al.* 2003; Mason *et al.* 2009). How the germ cells, which give rise to the progeny, also control the rate of aging of the body in which they reside is a fascinating, unanswered, question.

Two transcription factors, the FOXO-family transcription factor DAF-16 and the nuclear hormone receptor DAF-12, are required for germline loss to extend lifespan in *C. elegans*. DAF-16/FOXO is best known for its ability to extend lifespan in response to inhibition of insulin/IGF-1 signaling, and FOXO proteins appear to be capable of extending lifespan in many organisms, including humans (Hwangbo *et al.* 2004; Giannakou *et al.* 2007; Taguchi *et al.* 2007; Willcox *et al.* 2008; Anselmi *et al.* 2009; Flachsbart *et al.* 2009; Li *et al.* 2009; Pawlikowska *et al.* 2009). In worms with reduced insulin/IGF-1 signaling, DAF-16 localizes to the nuclei of tissues throughout the animal (Henderson & Johnson 2001; Lee *et al.* 2001; Lin *et al.* 2001), where it alters the expression of a variety of genes that influence lifespan, including antioxidant, metabolic, chaperone, and innate immunity genes (Lee *et al.* 2003; McElwee *et al.* 2003; Murphy *et al.* 2003; Oh *et al.* 2006), as well as genes normally expressed only in the germline

(Curran *et al.* 2009). Loss of the germline has a different effect on DAF-16 nuclear localization, causing DAF-16 to accumulate primarily in the nuclei of one tissue, the intestine, during adulthood (Lin *et al.* 2001). The intestine appears to serve as *C. elegans'* entire endoderm, carrying out functions associated with adipose tissue (fat storage), and the liver and pancreas (yolk production, and production of insulin/IGF-1-like hormones). DAF-16 functions in the intestine and other tissues to extend lifespan in response to inhibition of insulin/IGF-1 signaling, but it appears to function primarily in the intestine to extend lifespan in response to loss of the germ cells (Libina *et al.* 2003). DAF-16's function in the intestine/adipose tissue may potentially be conserved, as dFOXO expression exclusively in adipose tissue extends fly lifespan, and down-regulation of insulin signaling in mouse adipose tissue extends lifespan as well (Hwangbo *et al.* 2004).

Loss of the germ cells also increases the levels of TCER-1, a putative transcription elongation factor, in the intestine. TCER-1 appears to have a rather focused activity in the worm, as its loss prevents germline ablation from extending lifespan without affecting normal lifespan (Ghazi *et al.* 2009). A small set of genes up-regulated by DAF-16 in response to both germline loss and insulin/IGF-1 pathway inhibition has been identified, and TCER-1 is required for expression of some, but not all, of these genes in response to germline loss(Ghazi *et al.* 2009). TCER-1 is not up-regulated in insulin/IGF-1-pathway mutants, and its activity is not required for their DAF-16-dependent gene expression, or for lifespan extension. Together these findings indicate that DAF-16's regulation and activity in the reproductive and insulin/IGF-1 pathways are distinct from one another.

Interestingly, DAF-16 up-regulates an intestinal gene, K04A8.5, involved in lipid metabolism in response to germline loss, and this gene, in turn, is required for lifespan extension (Wang *et al.* 2008). Thus changes in lipid metabolism appear to play a key role in this lifespan extension pathway.

The DAF-12 nuclear hormone receptor has several interesting roles in the determination of *C. elegans* lifespan. At low and intermediate growth temperatures, the *daf-12* null allele *daf-12(rh61rh411)* shortens lifespan, so under these conditions, the gene promotes longevity (Gerisch *et al.* 2007). At low temperature (15°C), mutations that inhibit the cytochrome P450 gene *daf-9,* which in turn prevent the synthesis of DAF-12 ligands, extend lifespan in a *daf-12*-dependent fashion (Gerisch *et al.* 2001; Jia *et al.* 2002; Gerisch *et al.* 2007). Thus, in wild-type animals grown at low temperature, DAF-12 has the potential to further extend lifespan, but is prevented from doing so by its ligand. At high temperature (25°C), the situation is reversed. Here, inhibition of *daf-9* activity shortens lifespan in a *daf-12*-dependent fashion (in response to signals from thermosensory neurons) (Lee & Kenyon 2009). Thus, in wild-type animals grown at high temperature, DAF-12 has the potential to shorten lifespan but is prevented from doing so by its ligand. In animals that lack a germ line, loss of either *daf-9* or *daf-12* prevents lifespan extension. Thus, in this situation, DAF-12 activity extends lifespan, and this activity is promoted, not inhibited, by ligand.

How DAF-12 ultimately influences lifespan is not known. A set of DAF-12-regulated genes was identified recently by comparing gene expression profiles at low temperature between a short-lived *daf-12* null mutant and a long-lived *daf-12* gain-of-function (ligand-insensitive) mutant whose phenotype resembles that of *daf-9* loss-of-

function mutants (Fisher & Lithgow 2006). Interestingly, some of the down-regulated genes they identified are known to be functionally-significant lifespan genes that are also regulated by DAF-16 in *daf-2/*insulin/IGF-1 receptor mutants. This finding suggests that DAF-12 and DAF-16 regulate at least some genes in common.

In animals that lack germ cells, DAF-12 is partially required for the nuclear localization of DAF-16/FOXO(Berman & Kenyon 2006; Gerisch *et al.* 2007). However, DAF-12 must play an additional role in the germline pathway, as a mutant DAF-16 protein that is constitutively localized to the nucleus cannot extend lifespan in response to germline loss without DAF-12 activity (Berman & Kenyon 2006).

In this study, we have sought to clarify the roles of DAF-16 and DAF-12 in the germline pathway by carrying out microarray analysis. In particular, we were interested in learning to what extent the genes regulated by DAF-16 and DAF-12 in the germline pathway were likely to be the same as those regulated by these two transcription factors in other longevity pathways. In addition, we wanted to ask whether DAF-16 and DAF-12 were likely to regulate the same, overlapping, or disparate sets of downstream genes in response to loss of the germline. Finally, we hoped that among the genes whose expression changed in response to germline loss we might identify some required for lifespan extension.

RESULTS

Experimental design and initial validation

Because the germline makes up two thirds of the cells in *C. elegans* adults, a straightforward comparison of the differences in global transcription between intact and

germline-deficient animals might identify mainly genes expressed in the germline, genes that are not necessarily involved in the germline's regulation of lifespan. Similarly, we might also expect that a comparison of *daf-12* or *daf-16* wild-type (+) versus mutant (-) animals would yield a broad range of these transcription factors' targets, not only those germane to the germline regulation of lifespan. In order to generate a comparison of gene expression under conditions that produce short and long lifespan that would not be overwhelmed by the many germline-expressed genes, we used ANOVA analysis of a simple block design (see Figure 2.1) (Kerr *et al.* 2000; Kerr & Churchill 2001). To remove the germ cells, we used a temperature-sensitive *glp-1* mutation, which prevents germline proliferation at high temperature, and we used *daf-12* and *daf-16* null mutations to remove these two transcription-factor gene activities. Using the strategy outlined in Figure 2.1, we then compared the gene expression patterns of these animals to one another in various combinations. We hybridized 60 arrays, each containing 70mer oligos designed to probe 20,374 predicted *C. elegans* ORFs, as described previously (Cristina *et al.* 2009).

To validate our method's ability to pick out specific functionally related classes of genes, we first compared the gene expression patterns of all the strains grown at high temperature (25°C, the germline-deficient condition) to the same strains grown at low temperature (the intact condition). We found that the list of genes generated by this comparison was highly enriched for genes already known to have germline-specific functions related to early development and similar processes (Table 2.1). In addition, 1/3 of the genes we identified were also present in previous microarray experiments designed to identify germline-specific genes ($p<1E-4$) (Reinke *et al.* 2004).

Conservation of targets in different pathways that regulate lifespan

To identify genes that were regulated (directly or indirectly) by DAF-16/FOXO in response to germline loss, we looked for genes whose expression change upon removal of the germline was most dependent on the presence of *daf-16*. (The *daf-12* mutants were excluded from this analysis.) Our list of germline-regulated DAF-16 targets contained 230 genes using a $p<0.01$ significance-level cutoff (Table 2.2). Of these, 38 were also present among 512 previously identified targets of DAF-16 in a *daf-2* mutant background (Murphy *et al.* 2003), a highly significant overlap ($p<E-15$). This striking correspondence suggests that many of the targets through which DAF-16 influences the lifespan of germline-deficient animals are the same targets through which it influences lifespan in response to reduced insulin/IGF-1 signaling. These overlapping targets may be of particular interest as genes central to DAF-16's regulation of lifespan. Because there were also many non-overlapping targets, it is likely that in addition to a shared signature of DAF-16 regulation, there are germline-specific and insulin/IGF-1-signaling specific targets as well.

As an independent assessment of this issue, in our laboratory, Ghazi *et al*. recently used GFP fusions to examine the expression of seventeen genes predicted from previous microarray and other experiments to be regulated by DAF-16 (Ghazi *et al.* 2009). Interestingly, many of these genes were up-regulated in one set of tissues in animals lacking germ cells but in other tissues in *daf-2* mutants. For example, a *dod-8:GFP* reporter was switched on in the intestine in response to germline loss, but in muscles and neurons in *daf-2* mutants. Several genes were regulated in the same way under the two

conditions, and others were up-regulated only in *daf-2* mutants or only in germline-deficient animals. Thus, while there does appear to be considerable overlap between the two sets of *daf-16*-dependent genes under these two conditions, there are distinct differences as well.

Our list of genes whose expression changed in germline-deficient animals in a *daf-12/NHR*-dependent fashion contained 130 genes using a $p<0.01$ significance-level cutoff (Table 2.3). Of these, 8 overlapped with 224 genes previously reported to be *daf-12*-regulated in otherwise wild-type animals grown at low temperature, again a highly significant overlap ($p<1E-4$) (Fisher & Lithgow 2006). As with DAF-16, this overlap suggests that many of the targets by which DAF-12 influences lifespan in response to germline loss may be conserved in other contexts.

Overlap between the targets of DAF-12 and those of DAF-16 in the germline context.

Having established that there was a conserved transcriptional output from each of these two transcription factors alone, in multiple lifespan-regulating contexts, we asked what target genes these two transcription factors might have in common in germline-deficient animals. Of the 230 predicted DAF-16 targets and the 130 predicted DAF-12 targets, 7 were common to both lists, which is statistically significant ($p<1E-3$), although less so than the overlap with previously reported targets of each transcription factor respectively. Although the great majority of genes whose expression was most significantly affected by the loss of DAF-16 were distinct from those most significantly affected by the loss of DAF-12 (a finding that rules out the possibility of placing these

two transcription factors in a simple linear pathway) we can say that they have a greater than random coincidence in transcriptional output. The seven genes regulated by both *daf-16* and *daf-12* were not enriched (in statistical significance) for the "high end" of the two original lists. They encoded encoded a neuropeptide-like protein, a sodium neurotransmitter symporter family protein, a broad complex domain-containing protein, and several proteins of unknown function, and their potential significance is unclear (Table 2.4).

Regulatory analysis

One possible source of additional information about the regulation of these genes could come from an analysis of overrepresented sequences in their promoter regions. To identify such sequences, we used RSAT oligo analysis of 1 kb-upstream regions (van Helden *et al.* 1998). For our predicted DAF-16 targets, the two most significantly overrepresented sequences were, first, CTTATCAGT, and then TTGTTTAC. A portion of the first sequence, CTTATCA, was identified previously as being overrepresented in the promoter sequences of DAF-16 targets in the insulin/IGF-1 signaling pathway (Murphy *et al.* 2003), and was subsequently identified as a predicted ELT transcription factor binding site that was overrepresented in the promoters of several aging related gene sets (Budovskaya *et al.* 2008). DAF-16 has been shown to bind to this sequence *in vitro* (Curran *et al.* 2009). The second sequence was originally identified as a consensus binding sequence for mouse DAF-16/FOXO proteins (Furuyama *et al.* 2000) and it was also found overrepresented in the promoters of DAF-16 targets in the *C. elegans* insulin/IGF-1 signaling pathway (Murphy *et al.* 2003). The fact that the top two

overrepresented sequences found in microarray analysis of two different DAF-16-dependent pathways are the same argues again that many of the DAF-16 target genes are likely to be the same.

For our predicted DAF-12 targets, we found the sequence TTGATAA, which has not been identified in previous aging or *daf-12* studies, to be overrepresented. This sequence was not identified in the previous DAF-12 low-temperature studies (Fisher & Lithgow 2006). We analyzed this previously published list of predicted DAF-12 targets that might affect lifespan (Fisher & Lithgow 2006) using the same method and found a one base-pair variation of the same sequence (CTGATAA), further supporting the idea that this novel sequence may play a role in DAF-12's regulation of lifespan.

In addition to these two analyses, we also looked for overrepresented sequences among genes whose change in expression correlated most significantly with lifespan; that is, whether the strain was long or short-lived. Among these "long-vs short-life" genes, we found a new sequence, AGTAACCC, and another portion of the putative ELT/DAF-16 binding site, TTATCAC, to be overrepresented (Budovskaya *et al.* 2008).

Functional analysis

Next, we used RNAi to test the functional significance of genes identified in our microarray experiments. To do this, we fed intact and germline-deficient animals bacteria expressing the corresponding dsRNA sequences, and measured the worms' lifespans. The most statistically significant genes were tested for each of three categories. The first category included genes whose expression was found to differ most significantly between the long-lived germline-deficient condition and all of the remaining

non-long-lived conditions. These non-long-lived conditions included, in a *glp-1(ts)* background, the following strains: *daf-16(+) daf-12(+)* animals grown at 20°C, and *daf-12*(-), and *daf-16*(-) worms mutants grown at either 20°C or 25°C. We began testing genes once we had carried out our first set of microarrays, which included only Day-1 adults. We tested a total of 19 genes from this set (see Table 2.6). Among the genes we identified in this screen (#12 in our list) was *tcer-1,* the putative transcription elongation factor discussed above. This finding too, validated our microarray strategy, and also indicated that the increase in the TCER-1::GFP signal observed in germline-deficient animals (Ghazi *et al.* 2009) likely results from changes in *tcer-1* mRNA levels. We tested additional strains after completing all the arrays, which included mRNA samples from day 1-3 adults (Table 2.5). The second list we compiled and interrogated contained genes that were predicted to be (direct or indirect) DAF-16 targets in the germline pathway, and the third list contained genes that were predicted to be DAF-12 targets in the germline pathway.

From this analysis, we identified several genes that were required for the increased longevity of germline-defective animals but not for the longevity of wild type. Theses genes are discussed further below.

phi-62 **encodes a conserved RNA-binding protein required for germline-deficient animals to live long**

phi-62 encodes a small, 95-amino acid protein containing a predicted RNA-binding domain. *phi-62* was identified in our initial day-1 "long vs. short-lived" comparison (# 17 in our list), along with *tcer-1*. *phi-62* is *C. elegans'* only member of a highly conserved

protein family that has a single ortholog in many diverse animal species (Rampias *et al.* 2008). Although its biological function is unknown, the *Ceratitis capitata* ortholog has been shown to have ribonuclease activity *in vitro* (Rampias *et al.* 2003). We found that RNAi knockdown of *phi-62* has also previously been shown to accelerate protein aggregation in a worm model of polyglutamine aggregation diseases (Nollen *et al.* 2004). RNAi knockdown of *phi-62* reduced the lifespan of germline-deficient animals approximately to that of wild type, while reducing wild-type lifespan only slightly. This phenotype was similar to that caused by *daf-16* RNAi (Figure 2.2 A,B). With this in mind, we asked whether *phi-62* was required for the expression of known *daf-16*-regulated genes using GFP reporters in transgenic animals. We examined *dod-8* and *sod-3*, both of which are up-regulated in germline-deficient animals in a *daf-16* dependent manner. Interestingly, *dod-8* up-regulation was abolished by RNAi knockdown of *phi-62*, whereas *sod-3* expression was unaffected (Figure 2.2 C-G). This target specificity was reminiscent of that of *tcer-1*, which is also required for germline-deficient animals to live long (Ghazi *et al.* 2009). To investigate this apparent similarity in more closely, we examined 3 more genes that were regulated by *tcer-1*. We found complete coincidence between the effects of *phi-62* and *tcer-1* on these animals (Figure 2.3). These findings raise the interesting possibility that this putative RNA binding protein functions in association (directly or indirectly) with TCER-1.

TCER-1 activity is not required for all longevity pathways: for example, it is not required for *daf-2* (insulin/IGF-1-receptor) mutation, *eat-2* mutation (a condition that mimics caloric restriction) (Lakowski & Hekimi 1998) or *clk-1* mutation (which affects ubiquinone biosynthesis) (Wong *et al.* 1995) to increase lifespan. Because of the

similarity between the *tcer-1(-)* and *phi-62(-)* phenotypes, we were interested to ask whether loss of *phi-62* activity might affect other lifespan pathways similarly to the way they are affected by loss of *phi-62.* Like knockdown of *tcer-1,* RNAi knockdown of *phi-62* had no effect on the lifespans of *daf-2* mutant animals in one trial, and caused only a slight reduction in lifespan in a second trial (Fig. 2.4). *phi-62* was partially required for lifespan extension by *eat-2* and *isp-1* mutations.

FTT-2 is predicted to bind to PHI-62 and is required for the increased lifespan of germline-deficient animals.

Using Gene Orienteer (www.geneorienteer.org) (Zhong & Sternberg 2006), we discovered that PHI-62 is predicted to bind FTT-2. FTT-2 a 14-3-3 protein that has previously been shown to bind to DAF-16 (Berdichevsky *et al.* 2006; Wang *et al.* 2006; Li *et al.* 2007). We thus asked whether RNAi knockdown of *ftt-2* affected the lifespan of germline-defective animals, and found that it reduced the long lifespan of germline-defective animals to that of wild type (Figure 2.5), while not shortening wild-type lifespan further. Because FTT-2 has also been shown to interact with SIR-2.1 (Berdichevsky *et al.* 2006; Wang *et al.* 2006), we tested the lifespan of a *glp-1(e2141ts); sir-2.1(ok434)* double mutant. These animals were long lived, indicating that SIR-2.1 is not part of the germline longevity pathway (data not shown). These results suggest the possibility that PHI-62, TCER-1, FTT-2 and DAF-16 may act together in the regulation of DAF-16 target gene expression.

***lips-17* and *rrl-1* are lipid-metabolism genes required for the longevity of germline-deficient animals.**

lips-17 encodes a predicted triacylglycerol lipase, and was present in our list of genes differentially regulated in long vs. short lived animals. Y71H10A.2, which we named *rrl-1* (for "reproductive regulation of lifespan"), encodes a predicted fatty acyl reductase, and was identified as a gene up-regulated in germline-deficient animals in a *daf-12*-dependent manner in our microarrays. RNAi knockdown of either *lips-17* or *rrl-1* shortened the lifespan of germline-deficient animals roughly to that of wild type, while not shortening wild-type lifespan (Figure 2.6 A-D). Another triacylglycerol lipase, K04A8.5, was shown previously to be required for germline-deficient animals to live long (Wang *et al.* 2008).

Animals lacking a germline have been shown to have greatly reduced staining with Nile red, and RNAi knockdown of K04A8.5 suppresses this abnormal Nile-red phenotype (Wang *et al.* 2008). We asked what effect RNAi knockdown of *lips-17* and *rrl-1* might have on Nile red staining, and found that knockdown of *lips-17*, and to a lesser extent knockdown of *rrl-1*, increased Nile red staining in germline-deficient animals while not increasing staining in wild-type animals, a phenotype reminiscent of the K04A8.5-knockdown phenotype (Figure 2.6 E). Nile red has been thought to stain fat in *C. elegans* but it was recently shown instead to stain intestinal lysosomes (Schroeder *et al.* 2007; O'Rourke *et al.* 2009). Recently published work has suggested that oil-red-O staining is a better indicator of fat levels than is Nile red, as confirmed by solid-phase lipid extraction followed by GC/MS (O'Rourke *et al.* 2009; Soukas *et al.* 2009). Using oil-red-O staining, we found that overall fat storage was clearly increased in germline-

defective animals relative to wild type, as first observed by O'Rourke *et al*. (O'Rourke *et al.* 2009). This increase was not affected by RNAi knockdown of *lips-17*, *rrl-1*, or K04A8.5 (Figure 2.6 F). This finding suggests that these lipid-metabolizing genes are unlikely to influence lifespan by mechanisms that involve gross changes in total lipid stores. Because it is still not known how *daf-16*, which acts specifically in the intestine in this pathway, is able to affect the longevity of non-intestinal tissues, we propose that *lips-17*, *rrl-1* and K04A8.5 may play roles in the synthesis or processing of a lipophilic longevity signal that allows intestinal DAF-16 to influence the lifespan of other tissues. Consistent with this model, KO4A8.5 is expressed in the intestine, though we do not know where *lips-17* or *rrl-1* are expressed.

***mdt-15(RNAi)* worms are short-lived**

mdt-15 is a transcriptional mediator subunit orthologous to human MED15. In *C. elegans, mdt-15* is required for expression of fatty-acid desaturase genes, genes induced during fasting, and genes involved in metabolic adaptation to ingested material (Taubert *et al.* 2006; Taubert *et al.* 2008). *mdt-15* was identified as a gene up-regulated in germline-deficient animals in a *daf-16*-dependent manner in our microarrays, and was also previously identified as a target of *daf-16* in the insulin signaling pathway (Murphy *et al.* 2003). In one trial, RNAi knockdown of *mdt-15* was found to reduce the lifespan of germline-deficient animals. However, unpublished data in our lab (Peichuan Zhang, personal communication) suggests that in several trials RNAi knockdown of *mdt-15* had a similarly dramatic effect on the lifespan of wild-type worms, and that the worms exhibited a sick phenotype, which suggests that *mdt-15* may not act in a germline-specific

fashion. It is not clear whether *mdt-15* might play a role in the expression or action of the lipid metabolism genes discussed previously, but that is an interesting possibility.

DISCUSSION

In this study, we used a block-design microarray approach to identify genes differentially expressed in long-lived germline-deficient animals. We further identified genes whose altered expression most strongly depended on either the DAF-16/FOXO transcription factor or the DAF-12 nuclear hormone receptor. We found that many of the genes dependent on DAF-16 in this germline context were the same genes previously identified in a *daf-2(-)* context, suggesting that there exists a transcriptional signature common to these two lifespan-extending pathways. This interpretation is strengthened by the finding that the same upstream-DNA sequences were overrepresented in DAF-16-regulated genes in these two longevity pathways.

We also found a statistically very significant overlap between genes dependent on DAF-12 in the germline context and genes that are differentially expressed in *daf-12* loss-of-function versus *daf-12* gain-of-function mutants, which are short-lived and long-lived at low temperature, respectively (Fisher & Lithgow 2006). These findings suggest that DAF-12 can activate the same genes in more than one longevity pathway. As with DAF-16, this interpretation is strengthened by the finding that similar upstream DNA sequences were overrepresented in DAF-12-regulated genes in different longevity pathways.

One of the most interesting questions we wanted to address in this study was the question of the regulatory relationship between DAF-12 and DAF-16. Because DAF-12

is partially required for DAF-16 nuclear localization, one could imagine that DAF-12 would be required for all the targets of DAF-16 that are up or down regulated following loss of the germ cells. However, we found no evidence of this. Our microarrays revealed a smaller overlap (7 genes, p<1E-3) between genes influenced by DAF-16 and those influenced by DAF-12 than one would expect if their target genes were all the same. Overall, these common genes were not enriched (in statistical significance) for the "high end" of the two original lists, but may be interesting to test their functions in the future. It is possible that at least some genes regulated by DAF-16 have a partial dependence on DAF-12, and vice versa, and in fact that is what we see using GFP fusions to some of these genes *in vivo* (Yamawaki *et al.,* submitted).

We tested candidate genes for RNAi lifespan phenotypes and identified several new genes that were completely required for loss of the germline to extend lifespan. One of these, *phi-62*, encodes a protein predicted to bind to a known DAF-16-binding protein, FTT-2, which we showed was also required for loss of the germline to extend lifespan. (We note that *ftt-2* has a very close homolog in worms predicted to cross react with *ftt-2* RNAi, called *par-5*. So we cannot predict whether this other homolog, or both proteins, function in this system.) *phi-62* also influenced reporter expression of known *daf-16* target genes. This suggests the possibility that DAF-16, PHI-62 and FTT-2 may act together to extend the lifespan of germline deficient animals (Figure 2.7). One of our most intriguing findings was that the target specificity of PHI-62 seemed quite similar to the target specificity of TCER-1. The genes encoding both of these proteins were required for *dod-8* expression, but not for *sod-3* expression, and the two genes exhibited similar target specificity for each of the three additional genes we examined. This

suggests the interesting possibility that this putative RNA binding protein is part of the machinery that activates the expression of those DAF-16-regulated genes that utilize TCER-1 for transcription elongation. As such, this conserved protein could perform an essential function involving RNA metabolism during transcription.

The genes *lips-17* and *rrl-1* are predicted to be involved in lipid metabolism, and are required for loss of the germline to extend lifespan. Like K07A8.5, which was previously shown to be required for the longevity of germline-deficient animals, *lips-17* encodes a triacylglycerol lipase (Wang *et al.* 2008). Thus, in germline-deficient animals, these two gene products presumably liberate long-chain fatty acids from triglycerides (forming fatty acyl CoA in the process). We considered the possibility that these two genes are both knocked down by the same RNAi clone, but this was not predicted to be the case using the DEQOR software (Henschel *et al.* 2004). *rrl-1* encodes an acyl CoA reductase, which is a class of enzyme that reduces fatty acyl CoA molecules to aldehydes or alcohols. This enzyme could potentially act on the long-chain acyl-CoA molecules liberated by the triacylglycerol lipases. Inhibition of these three genes appears to have little or no effect on overall fat levels (as measured by the fat indicator oil-red O), suggesting that they exert their effects on lifespan in a more subtle way; for example, by producing signaling molecules or important metabolites for the cell.

Loss of these genes produces an interesting Nile red phenotype. Nile red has recently been shown to stain lysosomes in the *C. elegans* intestine, and whereas fat levels rise, Nile red levels fall in *glp-1* mutants. The significance of this reduced staining is not clear; but one possibility is that in *glp-1* mutants, the level of xenobiotics and other

damaged macromolecules normally sent to the lysosomes for degradation is reduced, or these substances are more rapidly degraded within lysosomes. As a consequence, *glp-1* mutants have reduced Nile red staining and a long lifespan. If the lipid products of *lips-17* and *rrl-1* activity are required for a beneficial lysosomal-linked process, then their requirement for longevity can be explained. A different possibility is that the lysosomal phenotype is not directly related to the lifespan-extending function of these genes. Reduced long-chain lipids can act as pheromones in some species, and they, or their derivatives, could potentially be part of a signaling pathway within the worm. The K07A8.5 gene is known to be expressed in the intestine under the control of DAF-16, so one could imagine that, in association with LIPS-17 and RRL-1, it generates a signaling molecule that relays information about the state of the reproductive system from the intestine to other tissues, triggering events that extend lifespan (Figure 2.7). Finally, we note that inhibition of a transcriptional mediator protein called MDT-15 shortens the lifespan of *glp-1* mutants as well as wild type. This protein has previously been found to regulate many genes involved in fat metabolism, so it would be interesting to learn whether it regulates the expression of *lips-17* and *rrl-1*.

EXPERIMENTAL PROCEDURES

Strains. All strains were maintained as described previously (Brenner 1974). CF1903: *glp-1(e2141ts) III*, CF1658: *glp-1(e2141ts) III*; *daf-12(rh61rh411) X*, CF1880: *daf-16(mu86) I*; *glp-1(e2141) III*, CF2562: *glp-1(e2141ts) III*; sIs10314[P*dod-8*::*GFP* + pCeh361], DA1116: *eat-2(ad1116) II*, CF1041: *daf-2(e1370) III*, CF1929: *glp-1(e2141ts) II*; muIs84[P*sod-3::GFP*], CF3201A: *glp-1(e2141ts) III*; sEx10466[P*nnt-1*::*GFP* + pCeh361], CF3204: *glp-1(e2141) III*; sEx14516[pCes T21D12.9::GFP + pCeh361], CF3124: *glp-1(e2141) III*; sEx11128[P*gpd-2::GFP*]

Microarray Analysis. Data were analyzed using MAANOVA (Wu *et al.* 2003). For the long/short lived lists, a linear model with block-specific terms for *daf-12, daf-16,* germline(+)/germline(-), and long-lived was generated and the long-lived term was tested. For the specific target lists, a linear model with block-specific terms for *daf-12* (or *daf-16*) and germline(+)/germline(-) was generated and the *daf-12*:germline(-) (or *daf-16*:germline(-)) interaction term was tested. For the *daf-16* targets analysis, *daf-12* mutant containing arrays were excluded, and vice-versa. All lists used an Fs:Ptab 0.01 cutoff.

Gene Ontology Analysis. Data were analyzed using the BiNGO 2.3 plugin (Maere *et al.* 2005) for Cytoscape 2.6 (Shannon *et al.* 2003), using a hypergeometric probability with Benjamini-Hochberg false discovery rate correction at a $p<0.05$ cutoff.

RNAi clone analysis. The identity of all RNAi clones was verified by sequencing the inserts using the M13-forward primer. pAD43 was used as the *daf-16* RNAi clone (Dillin *et al.* 2002); all other clones were from Julie Ahringer's RNAi library (Kamath *et al.* 2003).

Lifespan analysis. Lifespan analysis was conducted at 20°C as described previously unless otherwise stated (Hsin & Kenyon 1999). RNAi treatments were either performed as whole-life treatments or adult-only treatments (Dillin *et al.* 2002). In the whole-life analysis, eggs were added to plates seeded with the RNAi bacteria of interest. In the adult-only analysis, eggs were added to plates seeded with control RNAi bacteria, and adult animals were transferred to gene-specific RNAi-bacterial plates. The chemical 2'fluoro-5'deoxyuridine (FUDR, Sigma) was added to adult worms (100 μM) in some experiments to prevent their progeny from developing. Strains were grown at 20°C under optimal growth conditions for at least two generations before use in lifespan analysis. STATA software was used for statistical analysis and to determine means and percentiles. In all cases, p-values were calculated using the Log-rank (Mantel-Cox) method.

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Table 2.1. germline(+) vs germline(-) comparison is enriched for gene categories involved in early development.

Description	Corrected p-value
anatomical structure development	1.23E-02
positive regulation of biological process	1.92E-02
positive regulation of growth	1.92E-02
growth	1.92E-02
positive regulation of growth rate	1.92E-02
regulation of growth rate	1.92E-02
regulation of growth	1.92E-02
developmental process	2.34E-02
nematode larval development	2.89E-02
larval development	2.89E-02
post-embryonic development	2.90E-02
anatomical structure morphogenesis	2.90E-02
multicellular organismal development	4.15E-02
embryonic development	4.65E-02
ribosome biogenesis	4.92E-02

Categories of genes differentially expressed between germline(+) and germline(-) animals, using BiNGO 2.3, hypergeometric distribution with Benjamini-Hochberg false discovery rate correction, $p < 0.05$.

Table 2.2. Genes regulated by DAF-16 in the germline pathway.

Gene Name	P-value	Description
Y40H4A.1	0.0000	*gar-3* encodes a muscarinic acetylcholine receptor
W07B8.1	0.0001	Cysteine protease Cathepsin L
R12B2.5b	0.0005	*mdt-15* - (MeDiaTor), Positive cofactor 2 (PC2)
Y75B8A.14	0.0006	Putative transcription factor FET5
K04G7.3	0.0006	*ogt-1*; mutations can suppress constitutive dauer formation in *daf-2*
B0523.3	0.0007	*pgl-2* - (P GranuLe abnormality), 80% sim to yeast RAD50
F15B9.1	0.0009	*far-3* - (Fatty Acid/Retinol binding protein)
W09B6.1	0.0009	*pod-2* acetyl-coa carboxylase
F23B12.6	0.0009	Farnesyltransferase, farnesylates Prelamin-A
T08B1.1	0.0009	Organic cation/carnitine transporter 1
F41E6.2	0.0010	*C. elegans* GRD-5 protein; contains similarity to Pfam domain PF04155
ZK637.13	0.0012	Myoglobin, contains similarity to Pfam domain PF00042 (Globin)
F48D6.4c	0.0012	contains similarity to Cotton leaf crumple geminivirus BV1.; TR:Q80A41
K11G9.6	0.0013	*mtl-1*; contains similarity to copper-binding (detoxifying) metallothionein
F25B4.9	0.0013	*clec-1* - (C-type LECtin)
T24H7.5a	0.0014	*tat-4* - (Transbilayer Amphipath Transporters (subfamily IV P-type ATPase))
T20G5.7	0.0014	*dod-6*, secreted surface protein; 2-hybrid hit to *ima-3*
Y48B6A.2	0.0015	*C. elegans* RPL-43 protein; contains similarity to Pfam domain PF01780
F22E12.1	0.0015	Serine proteinase inhibitor (KU family)
K08D8.5	0.0016	Uncharacterized protein
K07E1.1	0.0016	contains similarity to Pfam domain PF03079 (ARD/ARD' family)
K08B4.6	0.0016	*cpi-1* (cysteine protease inhibitor) aka *cli-2*
M01H9.3a	0.0016	Similar to GPI-anchored cell surface glycoproteins
F48D6.4b	0.0017	contains similarity to Simian virus 40 Large T antigen
R13A1.2	0.0017	K+/Cl- cotransporter KCC1 and related transporters
Y43F8C.1	0.0018	*nlp-25* - (Neuropeptide-Like Protein)
T21E3.3	0.0018	*lrp-2* - (Low-density lipoprotein Receptor Related)
R166.5a	0.0018	No concise description or NCBI KOG info available.
F11E6.5	0.0019	*elo-2* encodes a palmitic acid elongase
F01F1.12a	0.0019	Fructose-biphosphate aldolase, homolog of the human gene ADHUB
C07G2.2b	0.0020	*C. elegans* ATF-7 protein; contains similarity to Interpro domain IPR004827
W06H8.6	0.0021	contains similarity to *Staphylococcus aureus* Hypothetical protein SAV2654
F48D6.4a	0.0021	contains similarity to *Macroptilium* mosaic virus BV1
Y48G1BR.1	0.0022	contains similarity to *Anopheles gambiae* str PEST AgCP8278
F47B8.4	0.0022	Glutaredoxin-related protein (repair of oxidative protein damage)
T24H7.2	0.0023	hsp-70 family ER resident protein
T04D3.1	0.0025	Uncharacterized coiled-coil containing protein
ZC410.5	0.0025	No concise description or NCBI KOG info available.
F11A6.1a	0.0025	*kpc-1* targets *daf-7*; encodes two isoforms of a Kex2/subtilisin-like protein
D1086.3	0.0026	Uncharacterized protein
F10D2.11	0.0026	*ugt-41* - (UDP-GlucuronosylTransferase)
Y53G8B.1	0.0026	Glutathione S-transferase
F13D11.4	0.0026	Similar to plant dihydroflavonol-4-reductase
F56D12.6b	0.0026	Predicted proline-serine-threonine phosphatase-interacting protein

D2023.2	0.0027	*C. elegans* PYC-1 protein; contains similarity to Pfam domains PF00682
Y39B6A.1	0.0027	contains similarity to *Plasmodium lophurae* Histidine-rich glycoprotein
H19N07.4	0.0027	H19N07.4 encodes an integral membrane protein
C36A4.9	0.0028	contains similarity to Pfam domain PF00501 (AMP-binding enzymes)
C24A11.8a	0.0028	*frm-4* encodes a predicted transmembrane protein
H32C10.3	0.0029	Ankyrin repeat and DHHC-type Zn-finger domain containing protein
C06B3.4	0.0030	*stdh-1* / *dod-8* 17 beta-hydroxysteroid dehydrogenase type 3
F58G6.5b	0.0030	*C. elegans* NHR-34 protein; contains similarity to Pfam domains
M02D8.4a	0.0030	Asparagine synthase (glutamine-hydrolyzing)
D2045.1	0.0031	*atx-2* is required for early embryonic patterning
ZC518.3c	0.0031	*C. elegans* CCR-4 protein; contains similarity to Pfam domains PF00560
C06E8.3c	0.0031	Serine/threonine protein kinase
C05E4.9b	0.0032	*gei-7* encodes a predicted isocitrate lyase/malate synthase
W08G11.4	0.0032	contains similarity to Pfam domain PF01603
F18C5.10	0.0032	contains similarity to Interpro domain IPR001865 (Ribosomal protein S2)
M01F1.4	0.0032	Uncharacterized membrane protein
ZC64.3a	0.0033	*C. elegans* CEH-18 protein; contains similarity to Pfam domain PF00157
C54G6.1a	0.0033	Unnamed protein
ZK488.10	0.0033	*C. elegans* PQN-97 protein; contains similarity to Pfam domain PF02520
F16B4.8	0.0034	*cdc-25.2* encodes a putative homolog of Cdc25 phosphatase protein family
F59B10.6	0.0034	sim to Dmel neu3 metallopeptidase
B0024.8	0.0034	Uncharacterized conserved protein
R53.7a	0.0035	5'-AMP-activated protein kinase, gamma subunit
ZK1058.1	0.0035	*mmcm-1* encodes an ortholog of human methylmalonyl-CoA mutase
C48E7.1	0.0035	contains similarity to Interpro domain IPR009030 (Growth factor, receptor)
C24B9.9	0.0035	*dod-3*
Y62E10A.2	0.0036	Y62E10A.2 encodes an ortholog of Pop7 (Rpp20)
C54F6.14	0.0036	*ftn-1* activity is essential for normal lifespan under iron stress
K08F8.1b	0.0037	contains similarity to Pfam domain PF00069
F35E12.5	0.0038	No concise description or NCBI KOG info available.
M02B1.3	0.0038	Uncharacterized conserved protein
M02D8.4b	0.0038	Asparagine synthase (glutamine-hydrolyzing)
T22H2.5b	0.0038	*plsc-2* - (PhosphoLipid SCramblase)
T19H5.4	0.0039	No concise description or NCBI KOG info available.
C15F1.6	0.0039	*C. elegans* ART-1 protein; contains similarity to Pfam domain PF00240
F19H8.1	0.0040	*tps-2* encodes one of two trehalose-6-phosphate synthase proteins
F56A4.3	0.0040	Glutathione-S-transferase
F59B10.5	0.0041	similar to B0207.5 and *D. pseudoobscura* Q299L3
F01F1.12b	0.0041	Fructose-biphosphate aldolase
F23F1.2	0.0043	similar to *C. albicans* mitochondrial carrier family and EF hand protein
C17G10.9c	0.0043	No concise description or NCBI KOG info available.
C05C8.4	0.0043	No concise description or NCBI KOG info available.
ZK909.2b	0.0044	*C. elegans* KIN-1 protein; contains similarity to Pfam domain PF00069
C32H11.4	0.0044	Uncharacterized protein
T03E6.2	0.0045	contains similarity to Pfam domain PF01461 (7TM chemoreceptor)
C37A5.8	0.0045	Unnamed protein
M01D7.7b	0.0045	The *egl-30* gene encodes an ortholog of the heterotrimeric G protein alpha
ZK856.10	0.0045	DNA-directed RNA polymerase subunit E'

T06A4.1	0.0045	No concise description or NCBI KOG info available.
F55H12.4	0.0045	contains similarity to *Brugia malayi* 24 kDa secreted protein.; TR:O76497
ZK430.8	0.0046	contains similarity to Pfam domains PF01549 (ShTK domain)
F31D4.8	0.0046	Unnamed protein
T24H10.5	0.0046	contains similarity to *Anopheles gambiae* str PEST EbiP7291 (Fragment)
T04H1.2	0.0046	Uncharacterized protein
K04A8.10	0.0046	UDP-glucuronosyl and UDP-glucosyl transferase
ZK637.5	0.0046	contains similarity to Pfam domain PF02374 (Anion-transporting ATPase)
ZK270.1	0.0046	*C. elegans* PTR-23 protein; contains similarity to Pfam domain PF02460
Y46G5A.6	0.0046	contains similarity to Pfam domain PF00270 (DEAD and DEAH box helicases)
ZK682.4	0.0046	*hlh-10* (helix-loop-helix 10) Transcription factor
F41H8.2	0.0047	Unnamed protein
C40H1.7	0.0047	Predicted lipase
C08A9.1	0.0047	*sod-3* encodes a iron/manganese superoxide dismutase
Y4C6B.3	0.0048	contains similarity to Interpro domain IPR007114
F46B6.8	0.0048	Triglyceride lipase-cholesterol esterase [KOG2624]
F44G3.8	0.0049	*fbxa-144* - (F-box A protein)
F39C12.3b	0.0049	*C. elegans* TSP-14 protein; contains similarity to Pfam domain PF00335
T25G3.4	0.0049	T25G3.4 encodes a mitochondrial glycerol-3-phosphate dehydrogenase
Y38H6C.14	0.0049	contains similarity to *Drosophila melanogaster* Flybase gene name is CG6004
F41C3.2	0.0049	contains similarity to *Anopheles gambiae* str PEST AgCP6255 (Fragment)
C05D11.11b	0.0049	Glycine/serine hydroxymethyltransferase
M28.6	0.0049	*lact-3* - (beta-LACTamase domain containing), predicted esterase
F08F1.8	0.0050	*tth-1* encodes a thymosin beta ortholog
C34F6.10	0.0050	No concise description or NCBI KOG info available.
R07B1.3	0.0051	Plasma membrane glycoprotein CD36 and related membrane receptors
F26H9.6	0.0051	*C. elegans* RAB-5 protein; contains similarity to Pfam domain PF00071
C34D10.2	0.0051	CCCH-type Zn-finger protein
Y53G8B.2	0.0051	Acyl-CoA:diacylglycerol acyltransferase (DGAT)
C25E10.7	0.0052	encodes a putative secreted TIL-domain protease inhibitor
Y55F3C.1	0.0053	contains similarity to Pfam domain PF01748
W02D3.1	0.0053	contains similarity to Pfam domain PF00173
F59G1.1b	0.0054	contains similarity to Pfam domain PF00535 (Glycosyl transferase)
T07C4.9	0.0054	*nex-2* encodes an annexin
Y54E10BR.5	0.0055	contains similarity to Pfam domain PF00461 (Signal peptidase I)
Y46G5A.31	0.0055	*gsy-1* is orthologous to the human gene GLYCOGEN SYNTHASE 1 GYS1
R12B2.5a	0.0055	*mdt-15* - (MeDiaTor), Positive cofactor 2 (PC2)
T27A1.7	0.0056	*srh-105* - (Serpentine Receptor, class H)
F15E6.4	0.0056	Unnamed protein
T19E7.3	0.0056	*bec-1* encodes a coiled-coil protein orthologous to beclin1
K06B4.12	0.0057	Tandem pore domain K+ channel
Y32H12A.4	0.0058	Protein phosphatase 1, regulatory (inhibitor) subunit PPP1R2
Y34F4.2	0.0060	sim to uncharacterized B. subtilis membrane protein yoaS
K02D7.5	0.0060	Multitransmembrane protein contains similarity to Pfam domain PF03083
Y39B6A.3	0.0061	ISA1 mitochondrial matrix protein
C17C3.1c	0.0062	Acyl-CoA thioesterase; Unnamed protein
F58E10.4	0.0063	*aip-1* encodes an AN-1-like zinc finger-containing protein
ZC395.5	0.0064	contains similarity to *Medicago truncatula* Pathogenesis-related transcript

T08A9.12	0.0065	*spp-2*; (SaPosin-like Protein family)
Y54G2A.18	0.0065	contains similarity to Pfam domain PF05529
R03D7.1	0.0065	R03D7.1 is orthologous to the human gene METHIONINE SYNTHASE (MTR)
C10G11.5a	0.0065	*pnk-1* - (PaNtothenate Kinase)
R08D7.6	0.0065	Cyclic nucleotide phosphodiesterase
D2063.3	0.0066	Homologous to yeast superoxide-radical responsive glutathione synthase
Y57A10B.3	0.0066	contains similarity to Pfam domain PF00651 (BTB/POZ domain)
B0250.1	0.0066	*rpl-2* encodes a large ribosomal subunit L8 protein.
C46G7.2	0.0067	No concise description or NCBI KOG info available.
T05B4.3	0.0068	contains similarity to Pfam domain PF01549 (ShTK domain)
R01B10.5	0.0069	contains similarity to Pfam domain PF05571 (Protein of unknown function)
C35B1.5	0.0070	contains similarity to Pfam domain PF00085 (Thioredoxins)
W01B6.4	0.0071	contains similarity to Interpro domain IPR008962 (PapD-like)
F56G4.4	0.0071	Spliceosomal protein FBP21
Y4C6B.5	0.0072	Uncharacterized protein
ZC518.2	0.0073	*sec-24.2* Vesicle coat complex COPII, subunit SEC24/subunit SFB2
K07C6.4	0.0073	Cytochrome P450 CYP2 subfamily
ZK353.9	0.0073	contains similarity to Pfam domain PF06201 (Domain of Unknown Function)
Y46C8AL.8	0.0073	contains similarity to Pfam domain PF00059 (Lectin C-type domain)
ZC196.2	0.0073	contains similarity to Pfam domain PF00635 (Major sperm protein)
F54F3.3	0.0074	Triglyceride lipase-cholesterol esterase [KOG2624]
T19A6.1a	0.0074	contains similarity to Pfam domain PF04193 (PQ loop repeat)
F55G11.2	0.0074	contains similarity to Pfam domain PF02408 (Domain of unknown function)
C52E4.1	0.0074	*cpr-1* encodes a cysteine protease of the cathepsin B-like cysteine protease
R02F2.8	0.0075	Amino acid transporter
F20B6.7	0.0075	No concise description or NCBI KOG info available.
F48C1.9	0.0075	contains similarity to *Bradyrhizobium japonicum* Blr6682 protein
F26G1.2	0.0075	No concise description or NCBI KOG info available.
C30C11.4	0.0075	C30C11.4 encodes a member of the Hsp70 family of heat shock proteins
F42A10.4a	0.0076	*C. elegans* EFK-1 protein; contains similarity to Pfam domain PF02816
F54D5.12	0.0076	Proteins containing the FAD binding domain
F35E8.8	0.0076	Glutathione S-transferase
ZK721.2	0.0076	*C. elegans* UNC-27 protein; contains similarity to Pfam domain PF00992
T20G5.8	0.0077	Secreted surface protein, contains similarity to Pfam domain PF01549
F49C12.5b	0.0077	Extracellular protein with conserved cysteines
Y67D8C.10a	0.0077	*C. elegans* MCA-3 protein; contains similarity to Pfam domains PF00690
F08F1.3	0.0077	No concise description or NCBI KOG info available.
E04F6.11b	0.0078	The *clh-3* gene encodes a chloride channel
F42C5.2	0.0079	7 transmembrane receptor
C10G11.5b	0.0079	*pnk-1* - (PaNtothenate Kinase)
F49E2.5c	0.0079	contains similarity to Pfam domain PF06392 (Acid shock protein repeat)
F49H12.6b	0.0079	*acl-4* encodes a phosphate acyltransferase
C08B6.8	0.0080	Oligoribonuclease (3'->5' exoribonuclease)
R07G3.2	0.0080	Triacylglycerol lipase [LSE0529]
F41D3.8	0.0080	Extracellular protein with conserved cysteines
ZK180.4	0.0081	contains similarity to Pfam domain PF00025
K10B3.10	0.0081	*spc-1* encodes the *C. elegans* alpha spectrin ortholog
W10G11.2	0.0081	contains similarity to Pfam domain PF01579 (Domain of unknown function)

Y51H7C.4	0.0082	Uncharacterized protein
T27D12.3	0.0083	C-type lectin contains similarity to Pfam domain PF00059
R09B5.6	0.0083	*hacd-1* (hydroxyacly-CoA dehydrogenase 1)
F28F8.6	0.0083	*atx-3* Ataxin 3/Josephin
Y38C1AA.1b	0.0084	contains similarity to *Brachydanio rerio* Hypothetical protein.; TR:Q7SXT8
R107.7	0.0084	*gst-1* encodes a putative glutathione S-transferase of the pi class
K01A2.6	0.0084	Uncharacterized protein
Y51H1A.3a	0.0085	Mitochondrial complex I NADH-ubiquinone oxidoreductase ASHI subunit
M01H9.3b	0.0086	contains similarity to *Drosophila melanogaster* Flybase gene name is Dhc16F
W08A12.2	0.0086	homologous to Cytochrome c oxidase subunit 4 isoform 2
T13B5.1	0.0086	Sodium-neurotransmitter symporter
T24A11.3	0.0086	*toh-1* encodes an astacin-like metalloprotease
Y57G11C.16	0.0086	*C. elegans* RPS-18 protein; contains similarity to Pfam domain PF00416
ZK637.7b	0.0086	*C. elegans* LIN-9 protein; contains similarity to Pfam domain PF06584
F53H4.3	0.0088	No concise description or NCBI KOG info available.
W06H8.7	0.0088	*C. elegans* STR-206 protein; contains similarity to Pfam domain PF01461
D1086.1	0.0089	Uncharacterized protein
F08C6.5	0.0090	No concise description or NCBI KOG info available.
C30B5.4	0.0091	Predicted RNA-binding protein (RRM superfamily)
F46B3.9	0.0091	Fibrillins and related proteins containing Ca2+-binding EGF-like domains
W06D12.3	0.0092	*fat-5* encodes a delta-9 fatty acid desaturase
F53B1.8	0.0092	Organic anion transporter
ZK185.1	0.0094	contains similarity to Interpro domain IPR007087
C07A12.1	0.0094	The *ham-2* gene encodes a C2H2 zinc finger-containing protein
K07B1.8	0.0094	No concise description or NCBI KOG info available.
F53A10.2a	0.0094	F53A10.2 encodes a Rap1GAP homolog
T01C3.4	0.0095	Lipase, best studies homolog is yeast steryl ester hydrolase
R13.1	0.0095	contains similarity to Pfam domain PF02891
F32B5.6a	0.0095	contains similarity to *Plasmodium falciparum* Gene 11-1 protein precursor
B0213.14	0.0095	Cytochrome P450 CYP2 subfamily
T16G1.7	0.0096	contains similarity to Pfam domain PF02958 (Domain of unknown function)
C30G7.1	0.0096	*C. elegans* HIL-1 protein; reported to control longevity in *C. elegans*
R04B3.3	0.0097	Coeffector of mDia Rho GTPase
F14F9.4	0.0098	40% similar to DNA double-strand break repair rad50 ATPase
ZK673.7	0.0098	*C. elegans* TNC-2 protein; contains similarity to Pfam domain PF00036
C02F4.1	0.0099	The *ced-5* gene a homolog of the human protein DOCK180
Y54F10AM.7	0.0099	contains similarity to Pfam domain PF01744 (GLTT repeat (6 copies))
F23F12.3	0.0099	Synaptic vesicle transporter SVOP and related transporters
C54C6.1	0.0099	*rpl-37* encodes a large ribosomal subunit L37 protein
C06H2.6	0.0099	No concise description or NCBI KOG info available.
C17E7.9a	0.0100	Unnamed protein
T28C6.8	0.0100	Widely conserved protein homologous to human BRI3

Table 2.3. Genes regulated by DAF-12 in the germline pathway.

Gene Name	P-value	Description
C34F6.3	0.0007	Collagens (type IV and type XIII), and related proteins
H12C20.3	0.0007	Predicted hormone receptor
F56H6.2	0.0007	Uncharacterized protein
ZK354.5	0.0009	*C. elegans* MSP-51 protein; contains similarity to Pfam domain PF00635
Y71H10A.2	0.0010	contains similarity to Pfam domain PF03015 (Male sterility protein)
Y63D3A.7	0.0012	contains similarity to Pfam domain PF05047 (Mitochondrial ribosomal)
R09E10.6	0.0013	Unnamed protein
T13F2.11	0.0015	*C. elegans* MSP-78 protein; contains similarity to Pfam domain PF00635
EGAP2.3	0.0015	*pho-1* encodes the major *C. elegans* intestinal acid phosphatase
C06A8.1a	0.0018	C06A8.1 is orthologous to human MSH HOMEO BOX HOMOLOG 1
F38B7.1a	0.0018	CCCH-type Zn-finger protein, similar to tis11
K08D8.1	0.0019	Uncharacterized protein
B0198.1	0.0019	Tetraspanin family integral membrane protein
W05B2.6	0.0019	*col-92* - (COLlagen); contains similarity to Pfam domain PF01484
F42G10.1	0.0019	Encodes a neprilysin
F38H4.10	0.0020	Cell cycle-associated protein Mob1-1
T05A1.5	0.0022	No concise description or NCBI KOG info available.
ZK228.4	0.0022	contains similarity to Pfam domain PF00583 (Acetyltransferase)
T21C9.3b	0.0023	contains similarity to Pfam domain PF00858 (Amiloride-sensitive channel)
C04G6.1a	0.0023	*mpk-2* encodes a mitogen activated protein (MAP) kinase
F35B12.7	0.0024	*nlp-24* - (Neuropeptide-Like Protein)
W05E7.1	0.0025	*grd-3* innate immune response protein encodes a hedgehog-like protein
Y59A8B.2	0.0026	contains similarity to Pfam domain PF00443
F53H4.3	0.0027	No concise description or NCBI KOG info available.
W06F12.3	0.0028	Casein kinase (serine/threonine/tyrosine protein kinase)
ZK994.4	0.0029	contains similarity to Pfam domains PF00560
F14H3.5	0.0029	Unnamed protein
Y5H2B.2	0.0032	*C. elegans* NHR-13 protein; contains similarity to Pfam domain PF00104
K07F5.3	0.0033	*C. elegans* MSP-56 protein; contains similarity to Pfam domain PF00635
R11D1.11	0.0034	*dhs-21* encodes a member of the short-chain dehydrogenases/reductases
Y69E1A.5	0.0034	Phosphatidylethanolamine binding protein
C44C10.1	0.0035	Collagens (type IV and type XIII), and related proteins
Y51A2D.18	0.0035	contains similarity to Pfam domain PF00083 (Sugar transporters)
EEED8.1	0.0036	contains similarity to Pfam domain PF00567 (Tudor domain)
T28H11.1	0.0036	Unnamed protein
T05A10.2	0.0036	CLC-4 (claudin-like in *C. elegans* 4) protein
F29D10.5	0.0036	contains similarity to Pfam domains PF00096
F58A6.9	0.0039	contains similarity to Pfam domain PF00635 (Major sperm protein)
C54G6.5	0.0039	*spp-17* - (SaPosin-like Protein family)
C18E9.4	0.0039	NADH:ubiquinone oxidoreductase, NDUFB3/B12 subunit
F37A8.1	0.0040	Unnamed protein
T08B1.1	0.0040	Organic cation/carnitine transporter 1
Y53C12B.2	0.0041	contains similarity to Interpro domain IPR004087 (KH domain)
R07B1.4	0.0041	Glutathione S-transferase

K11B4.2	0.0042	contains similarity to *Anopheles gambiae* str PEST AgCP15219
Y71H2AM.18	0.0042	contains similarity to Pfam domain PF00270 (DEAD box helicases)
W06B11.2	0.0043	The *puf-9* gene encodes a predicted RNA binding protein
R13A5.6	0.0043	transthyretin-like protein involved in secretion
C40H5.1	0.0044	*nspa-9* - (Nematode Specific Peptide family/group A)
ZK180.2	0.0045	Gamma-aminobutyric acid type B receptor, subunit 2 precursor
ZK353.6	0.0045	*C. elegans* LAP-1 protein; contains similarity to Pfam domain PF00883
C08H9.13	0.0045	Chitinase
R07E5.4	0.0046	No concise description or NCBI KOG info available.
F42A8.1	0.0046	Unnamed protein
F58B3.1	0.0047	*C. elegans* LYS-4 protein
K02A2.3	0.0048	K+/Cl- cotransporter KCC1 and related transporters
Y59C2A.3	0.0049	contains similarity to *Babesia bigemina* 200 kDa antigen p200
Y71A12B.5	0.0050	contains similarity to Pfam domain PF00059 (Lectin C-type domain)
F01D5.5	0.0050	contains similarity to Pfam domain PF01549 (ShTK domain)
ZK1321.1	0.0051	CCCH-type ZN finger protein, ortholog of TIS11
D1054.8	0.0051	Similar to reductases with broad range of substrate specificities
F35E12.5	0.0054	No concise description or NCBI KOG info available.
C32F10.8	0.0055	Alanine aminotransferase
T27E7.1	0.0056	Unnamed protein
B0238.14	0.0057	No concise description or NCBI KOG info available.
C36F7.5	0.0058	contains similarity to Pfam domain PF03057
ZK816.5	0.0058	*dhs-26* (dehydrogenases, short-chain 26)
Y51A2D.10	0.0059	contains similarity to Pfam domain PF01060 (Transthyretin-like family)
T24C12.2	0.0059	*C. elegans* GAP-1 protein; contains similarity to Pfam domain PF00616
F58G6.7	0.0061	Copper transporter
Y17G7B.7	0.0061	*C. elegans* TPI-1 protein; contains similarity to Pfam domain PF00121
C02F5.8	0.0062	Tetraspanin family integral membrane protein
T08G11.5	0.0062	*unc-29* encodes a subunit of the nicotinic acetylcholine receptor
C06A1.3	0.0062	Serine/threonine specific protein phosphatase PP1, catalytic subunit
F21C3.3	0.0063	Zinc-binding protein of the histidine triad (HIT) family
K05F1.3	0.0065	Medium-chain acyl-CoA dehydrogenase
Y104H12D.2	0.0065	contains similarity to *Clostridium acetobutylicum* Arginine biosynthesis
C30G12.2	0.0066	Predicted 11-cis retinol dehydrogenase
T13B5.1	0.0068	Sodium-neurotransmitter symporter
F55C5.1	0.0069	contains similarity to Pfam domain PF00635 (Major sperm protein)
Y39G10AR.5	0.0072	ZYG-11 family member; component of cul-2 ubiquitin ligase like complex
D2062.6	0.0073	Unnamed protein
T13F3.6	0.0074	No concise description or NCBI KOG info available.
ZK1151.3	0.0075	No concise description or NCBI KOG info available.
D2089.1b	0.0075	*C. elegans* RSP-7 protein; contains similarity to Pfam domain PF00076
F21D9.4	0.0075	Uncharacterized protein
ZK512.7	0.0076	Uncharacterized protein
T15B7.3	0.0076	COL-143; cuticle collagen
Y57A10B.3	0.0077	contains similarity to Pfam domain PF00651 (BTB/POZ domain)
C10G11.9	0.0078	Unnamed protein
ZC155.4	0.0078	Glycerophosphoryl diester phosphodiesterase
Y57G11C.2	0.0078	contains similarity to Pfam domain PF02931

F40H7.4	0.0078	*srx-101* - (Serpentine Receptor, class X) 7-transmembrane receptor
K07A9.2	0.0078	*C. elegans* CMK-1 protein; contains similarity to Pfam domain PF00069
K05F1.2	0.0079	*C. elegans* MSP-142 protein; contains similarity to Pfam domain PF00635
F20G2.3	0.0079	Phosphatidylinositol transfer protein SEC14 and related proteins
R10E9.2	0.0080	Unnamed protein
W03D2.8	0.0082	No concise description or NCBI KOG info available.
Y57G11C.31	0.0082	contains similarity to Pfam domain PF03407 (Protein of unknown function)
C46G7.2	0.0082	No concise description or NCBI KOG info available.
W10G11.19	0.0082	No concise description or NCBI KOG info available.
B0336.7b	0.0083	Expression is seen in two components predominantly in young larvae
F53A9.10	0.0083	Troponin
T11F9.12	0.0084	No concise description or NCBI KOG info available.
Y19D10B.1	0.0084	contains similarity to *Mycoplasma penetrans* Conserved hypothetical protein
K09D9.1	0.0086	Uncharacterized protein
T09F5.5	0.0086	*srh-55* - (Serpentine Receptor, class H)
K08D10.8	0.0087	Phospholipid scramblase; encodes a homolog of human scramblase
T08G11.2	0.0087	*C. elegans* EGL-32 protein; contains similarity to Interpro domain IPR00098
C09E8.1b	0.0087	Sodium-neurotransmitter symporter
F47B8.11	0.0089	*C. elegans* SSS-2 protein; contains similarity to *Mus musculus* Matrilin-2
C32D5.11	0.0089	Homolog of TOPORS (topoisomerase I interacting)
F45E12.5b	0.0090	contains similarity to Pfam domain PF00238 (Ribosomal protein L14)
F28H1.2	0.0092	*C. elegans* CPN-3 protein; contains similarity to Pfam domain PF00307
R53.3a	0.0093	*egl-43* encodes a zinc finger protein that affects HSN cell migration
F07F6.5	0.0093	Unnamed protein
W06A7.2	0.0093	Uncharacterized protein
M03F4.2a	0.0094	*C. elegans* ACT-4 protein; contains similarity to Pfam domain PF00022
Y43F8C.1	0.0094	*nlp-25* - (Neuropeptide-Like Protein)
ZK525.2	0.0094	*C. elegans* AQP-11 protein
F15A2.1	0.0095	Collagens (type IV and type XIII), and related proteins
F45G2.5	0.0095	*bli-5* encodes a protease inhibitor that affects cuticle integrity
F58A3.5	0.0095	Uncharacterized protein with conserved cysteine
T24A6.3	0.0095	*C. elegans* GRL-30 protein; contains similarity to Pfam domain PF04155
ZK899.8f	0.0096	*gap-2* encodes a Ras GTPase-activating protein (RasGAP)
C34E10.9	0.0097	No concise description or NCBI KOG info available.
T28C12.6	0.0097	contains similarity to Pfam domain PF00095 (Whey Acidic Protein)
C09G12.3	0.0099	7-transmembrane receptor
C35A5.3	0.0100	No concise description or NCBI KOG info available.

Table 2.4. Overlap between DAF-16 and DAF-12 targets.

Gene Name	**Description**
F53H4.3	Unstudied gene of unknown function
snf-3	Sodium neurotransporter symporter family gene
C46G7.2	Unstudied gene of unknown function
nlp-25	Neuropeptide-like protein
F35E12.5	Unstudied gene of unknown function
T08B1.1	Major Facilitator Superfamily domain containing predicted membrane transporter
btb-14	Broad complex / Tramtrack / Bric-a-brac- domain containing protein.

Table 2.5. Genes differentially regulated between long and short-lived animals in the germline context.

Gene Name	P-value	Description
F13D11.4	0.0010	Similar to plant dihydroflavonol-4-reductase.
F56A4.3	0.0010	Glutathione-S-transferase. Contains similarity to Pfam domain PF02798
M28.10	0.0018	Uncharacterized protein
C54F6.14	0.0025	*ftn-1* activity is essential for normal lifespan under iron stress
F54F3.3	0.0031	Triglyceride lipase-cholesterol esterase [KOG2624]
C06B3.4	0.0032	*stdh-1* / *dod-8* 17 beta-hydroxysteroid dehydrogenase type 3
C34F6.2	0.0033	COL-178 protein; contains similarity to Pfam domains PF01484
Y51H1A.3a	0.0036	Mitochondrial complex I NADH-ubiquinone oxidoreductase ASHI subunit
C52D10.1	0.0043	No concise description or NCBI KOG info available.
R07G3.2	0.0045	Triacylglycerol lipase [LSE0529]
F35E12.5	0.0046	No concise description or NCBI KOG info available.
T09E11.3	0.0048	similar to chondroitin 6-sulfotransferase and related sulfotransferases
M88.5	0.0049	IGF-II mRNA-binding protein IMP, contains RRM and KH domains
C38C3.8	0.0049	Unnamed protein
F48D6.4a	0.0049	contains similarity to Macroptilium mosaic virus BV1.; TR:Q8B5S2
K02D7.5	0.0053	Multitransmembrane protein contains similarity to Pfam domain PF03083
K11G9.6	0.0060	*mtl-1*; contains similarity to copper-binding (detoxifying) metallothionein
F15B9.1	0.0062	*far-3* - (Fatty Acid/Retinol binding protein)
R11A5.7	0.0063	Homlogous to carboxypeptidase A2
K03H1.4	0.0066	Uncharacterized protein with conserved cysteine
C03C11.1	0.0066	Uncharacterized protein
Y111B2A.20	0.0068	UDP-galactose transporter related protein
ZK507.4	0.0069	Uncharacterized protein
T20G5.7	0.0070	*dod-6*, secreted surface protein; 2-hybrid hit to ima-3
F48D6.4b	0.0073	contains similarity to Simian virus 40 Large T antigen
F49C12.7	0.0074	Predicted small molecule kinase
F28C12.2	0.0075	*sra-18* - (Serpentine Receptor, class A (alpha))
F48D6.4c	0.0075	contains similarity to Cotton leaf crumple geminivirus BV1.; TR:Q80A41
W05B2.6	0.0075	*col-92* - (COLlagen); contains similarity to Pfam domains PF01484
W07B8.5	0.0077	*cpr-5* - (Cysteine PRotease related)
W03F11.1	0.0077	three chitin-binding peritrophin-A domains
ZK1320.3	0.0079	No known protein domains, only one homolog (*C. briggsae* ortholog)
F56C9.8	0.0080	Likely INTEGRIN LIGAND; 58.8% similar to human Isoform 1 of ADAM 28
ZK813.1	0.0081	contains similarity to Pfam domain PF03964 (Chorion family 2)
Y113G7A.16	0.0081	contains similarity to *Drosophila melanogaster* RhoGEF
F26F12.1	0.0081	*col-140* - (COLlagen)
C01B12.1	0.0082	*sqt-2* encodes a collagen required for normal alae formation
F57F5.1	0.0086	Cysteine proteinase Cathepsin L
K07C6.13	0.0086	*srx-69* - (Serpentine Receptor, class X)
H25K10.4	0.0088	similar *Clostridium acetobutylicum* ATP-dependent Lon protease
W08D2.6	0.0088	*col-123* is homologous to the human gene A TYPE IV COLLAGEN
F08F8.9b	0.0089	contains similarity to *Anopheles gambiae* str PEST AgCP11053

F13G3.5	0.0090	*ttx-7* encodes a myo-inositol monophosphatase (IMPase)
F16B4.7	0.0091	Unnamed protein
F15E6.8	0.0093	Unnamed protein
Y48G9A.1	0.0097	Protein containing adaptin N-terminal region
C49C3.12	0.0097	C-type lectin contains similarity to Pfam domain PF00059
F11G11.11	0.0100	*col-20* - (COLlagen)

Table 2.6. Lifespan data.

RNAi	In germline-deficient: Mean	n	% change vs. control	p-value vs. control	In wild type: Mean	n	% change vs. control	p-value vs. control
Long vs. Short lived comparison								
C03C11.1	24.7	78	3.5	0.359	17.0	91	-2.9	0.354
C38C3.8	23.6	83	-1.2	0.670	18.7	85	6.8	0.100
C52D10.1	23.6	86	-1.5	0.506	18.4	77	5.1	0.205
F13D11.4	24.1	84	0.6	0.940	16.1	88	-7.9	0.015
F35E12.5	23.1	85	-3.4	0.227	15.6	89	-10.8	0.005
F48D6.4	23.4	79	-2.4	0.254	13.3	95	-23.9	<0.0001
F49C12.7	21.4	83	-10.6	0.008	16.3	89	-6.9	0.047
F56A4.3	23.3	83	-2.6	0.971	16.9	93	-3.3	0.356
M28.10	20.8	65	-12.9	0.001	15.1	99	-13.9	<0.0001
T09E11.3	23.4	84	-2.4	0.653	17.5	80	-0.2	0.958
Y51H1A.3	24.3	91	1.4	0.492	17.5	76	0.2	0.701
col-178	23.1	96	-3.5	0.612	17.6	83	0.9	0.862
daf-16	16.0	85	-33.2	<0.0001	15.8	88	-9.7	0.0003
dod-6	22.7	93	-5.2	0.149	14.9	95	-14.5	<0.0001
dod-8	22.9	82	-4.2	0.268	17.0	90	-2.7	0.486
ftn-1	23.9	74	0.1	0.801	18.4	90	5.3	0.094
lips-17	18.9	77	-21.1	<0.0001	16.9	92	-3.3	0.437
mtl-1	22.5	72	-5.8	0.131	15.7	90	-10.0	<0.0001
control	23.9	89	0.0		17.5	90	0.0	
Long vs. Short Day 1 comparison								
C03C11.1	27.9	73	7.8	0.093	19.4	62	-2.0	0.495
col-178	23.4	78	-9.5	0.063	18.4	51	-7.2	0.146
F48G7.8	24.1	81	-6.7	0.042	19.6	52	-1.1	0.786
T18H9.7	21.9	70	-15.5	0.0002	18.2	59	-8.3	0.030
ZK1320.3	23.4	72	-9.7	0.033	18.8	50	-5.0	0.212
ZK632.9	20.4	75	-21.1	0.000	19.2	63	-3.0	0.383
daf-16 1	15.0	72	-42.1	<0.0001	17.1	67	-13.7	0.0003
control 1	25.9	85	0.0		19.8	56	0.0	
F07G11.9	23.8	89	0.1	0.862	19.7	46	3.1	0.657
F07H5.6	22.0	78	-7.5	0.090	18.9	39	-0.9	0.742
F42A9.5	22.5	89	-5.4	0.134	20.1	46	5.0	0.198
K07H8.2	23.3	100	-2.3	0.463	19.1	68	-0.3	0.511
T10D4.3	22.8	83	-4.1	0.169				
Y25C1A.8	23.8	84	-0.2	0.810	17.7	48	-7.4	0.042
clc-4	21.5	75	-9.9	0.005				
cts-1	20.0	80	-16.2	<0.0001	15.9	66	-17.0	<0.0001
daf-16 2	16.4	72	-30.9	<0.0001	17.4	64	-9.1	0.044
fbxc-33	24.0	79	0.8	0.818	18.8	58	-1.7	0.658
pes-9	21.6	91	-9.2	0.020	19.6	63	2.6	0.642
phi-62	16.9	72	-28.8	<0.0001	16.2	62	-15.4	<0.0001

tag-175	21.6	84	-9.3	0.014	17.9	65	-6.2	0.054
tcer-1	18.2	96	-23.6	<0.0001	18.1	65	-5.5	0.069
control 2	23.8	96	0.0		19.1	70	0.0	
***daf-16* interacting genes**								
T08B1.1	22.5	84	-3.6	0.369	20.5	69	13.2	0.001
Y75B8A.1	23.8	76	2.1	0.482	20.7	56	14.3	0.001
clec-1	21.4	68	-8.2	0.135	16.1	35	-10.9	0.003
daf-16	15.6	100	-33.2	<0.0001				
glb-1	22.3	90	-4.3	0.396	16.5	55	-9.0	0.098
grd-5	25.3	83	8.2	0.149	19.0	65	5.1	0.237
mdt-15	15.2	86	-34.8	<0.0001	15.8	52	-12.8	0.009
mtl-1	21.2	64	-9.0	0.041	19.2	80	5.9	0.086
ogt-1	20.8	57	-10.8	0.011	18.3	61	1.2	0.821
pod-2	23.3	82	-0.2	0.979	17.7	70	-2.5	0.863
tat-4	22.9	58	-1.8	0.586	17.7	45	-2.5	0.519
control	23.3	71	0.0		18.1	63	0.0	
***daf-12* interacting genes**								
C06A8.1	20.6	91	-7.6	0.127	20.6	65	15.8	0.001
F38B7.1	24.1	88	7.7	0.249	18.6	85	4.4	0.290
F38H4.10	23.1	90	3.3	0.947	18.5	56	3.6	0.229
F56H6.2	23.4	92	4.7	0.415	17.7	64	-0.4	0.941
K08D8.1	23.8	92	6.7	0.249	19.3	74	8.1	0.052
T05A1.5	22.1	89	-1.3	0.322	18.9	90	6.3	0.112
W05B2.6	23.2	85	3.6	0.694				
Y63D3A.7	21.0	86	-6.2	0.181	18.0	66	0.8	0.873
rrl-1	17.8	87	-20.2	<0.0001	17.9	78	0.6	0.815
col-179	22.8	97	2.2	0.883	17.3	71	-2.7	0.888
col-92	22.2	81	-0.7	0.857	18.4	51	3.1	0.467
daf-16	16.1	73	-28.0	<0.0001				
msp-51	22.3	92	-0.2	0.708	16.5	43	-7.1	0.244
msp-78	23.6	88	5.6	0.380	19.2	65	7.8	0.235
nhr-68	22.9	90	2.7	0.903	17.6	83	-1.2	0.980
pho-1	24.0	91	7.2	0.218	17.1	68	-4.0	0.579
tsp-20	25.3	84	13.4	0.034	17.2	67	-3.5	0.680
control	22.3	85	0.0		17.8	63	0.0	
ftt-2								
ftt-2	16.4	87	-32.7	<0.0001	17.6	85	-4.5	0.094
control	24.4	90			18.4	89		
ftt-2	13.0	84	-36.1	<0.0001	17.3	71	-3.9	0.146
daf-16	13.3	86						
control	20.3	64	0.0		18.3	61	0.0	
***phi-62* in other long-lived strains**								
daf-2								
phi-62	40.2	59	-8.9	0.176				

control	44.1	89	0.0	
isp-1				
phi-62	20.5	39	-34.1	<0.0001
control	31.1	90	0.0	
eat-2				
phi-62	19.0	52	-30.5	<0.0001
control	27.4	23	0.0	

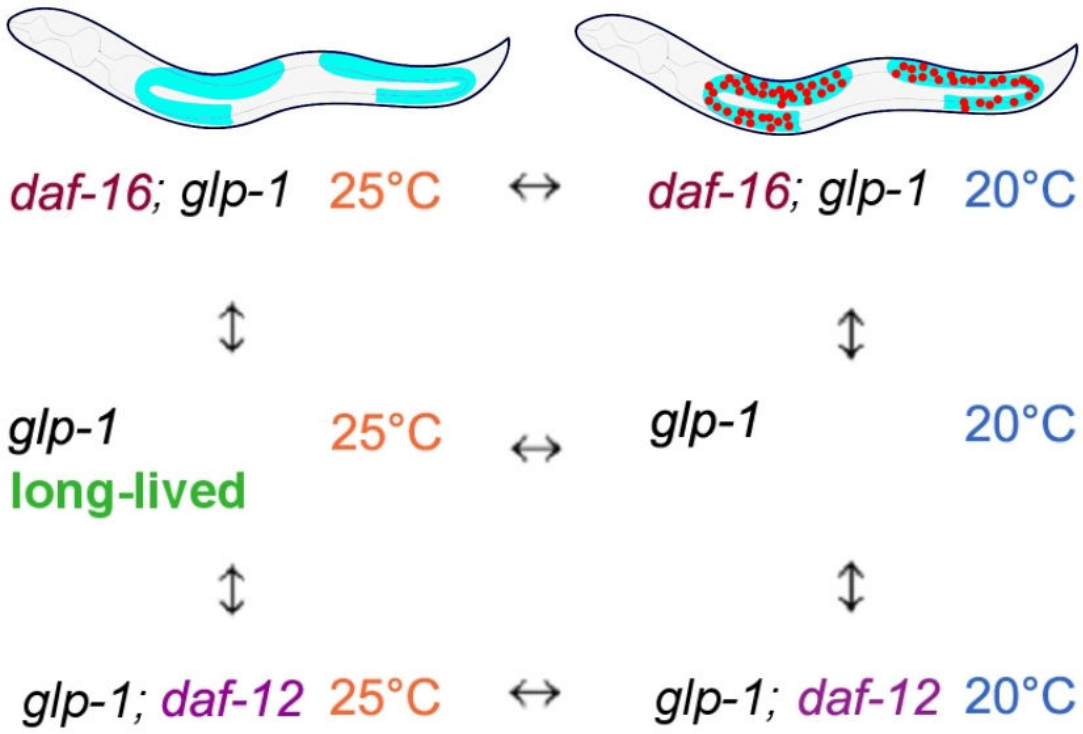


Figure 2.1. Block design for microarray experiments. *glp-1(e2141ts)* animals lack a germ line and are long lived at 25°C, but are germline(+) and have a normal lifespan at 20°C.

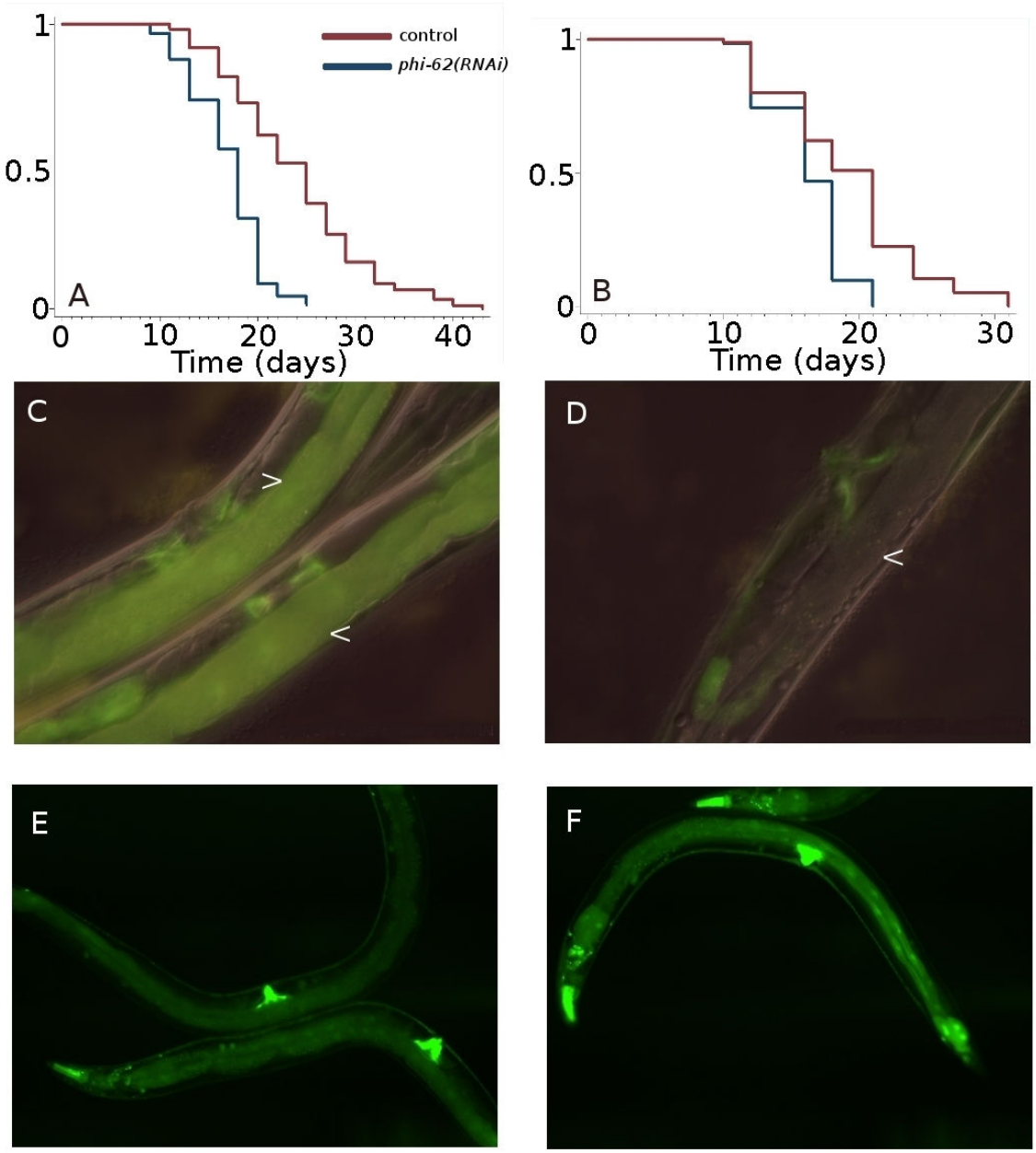


Figure 2.2 (A)-(F). *phi-62* is required for removal of the germline to increase lifespan, and for up-regulation of *dod-8*, a known *daf-16*-regulated gene, but not for *sod-3*, another *daf-16*-regulated gene, in animals that lack the germline. A) *glp-1(e2141ts)*: vector-only control n = 96, m = 23.8 days, *phi-62(RNAi)* n = 72, m = 16.9 days, $p < 0.0001$. B) wild type (N2): vector-only control n = 70, m = 19.1 days, *phi-62(RNAi)* n = 62, m = 16.2 days, $p < 0.0001$. C,D) *glp-1(e2141ts)*; P*dod-8::GFP*, which is expressed in the intestine, C, vector-only control (two worms are shown), D, *phi-62*(RNAi), arrows indicate intestine. E,F) *glp-1(e2141ts)*; P*sod-3::GFP*, E, vector, F, *phi-62(RNAi)*.

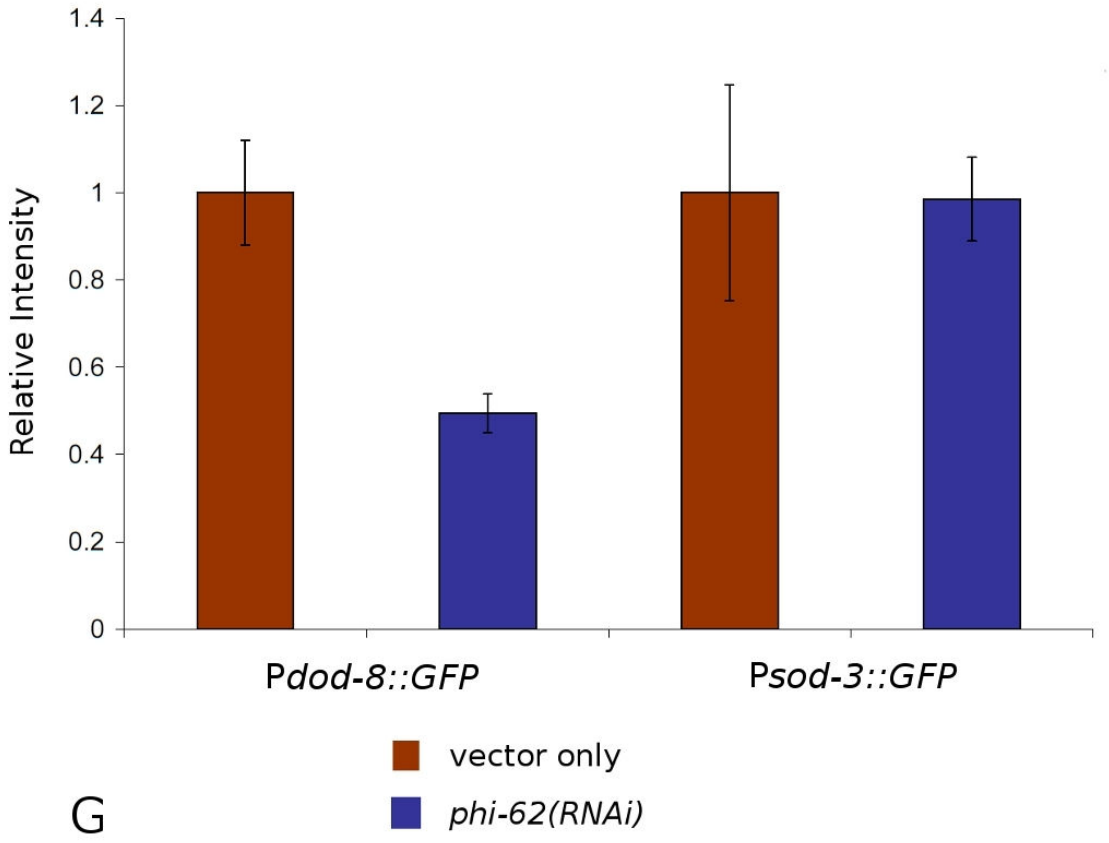


Figure 2.2 (G). *phi-62* is required for up-regulation of *dod-8*, a known *daf-16*-regulated gene, but not for *sod-3*, another *daf-16*-regulated gene, in animals that lack the germline. G) Relative GFP Intensities. P*dod-8::GFP* vector only vs. *phi-62(RNAi)*, $p < 0.01$.

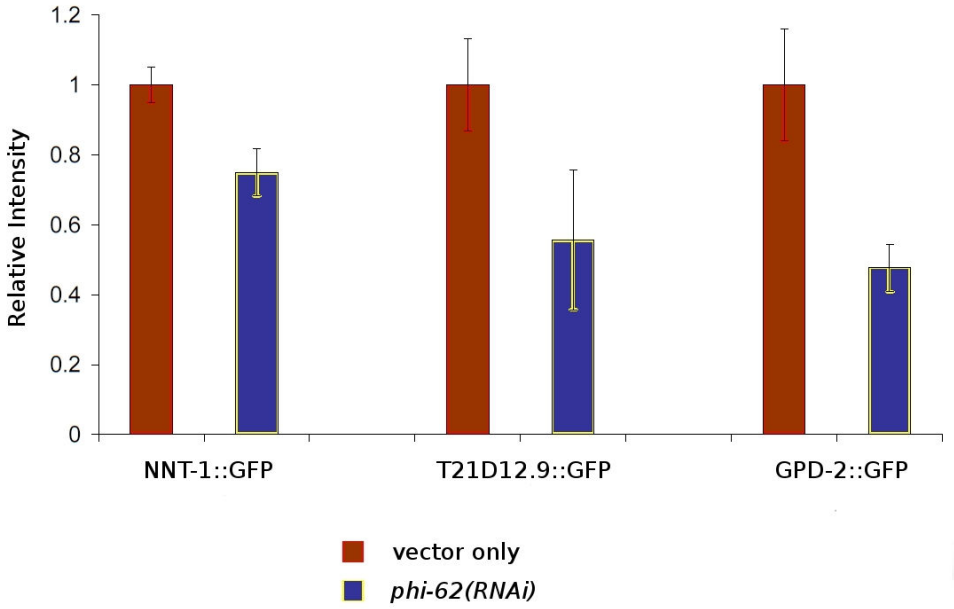
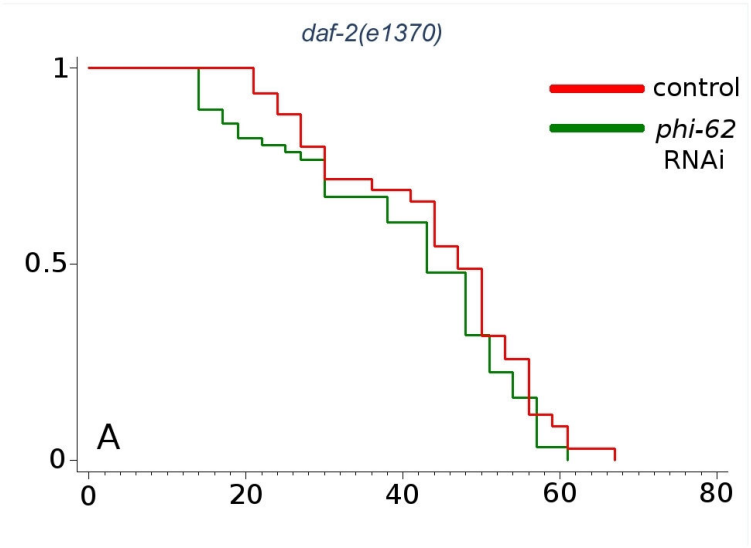
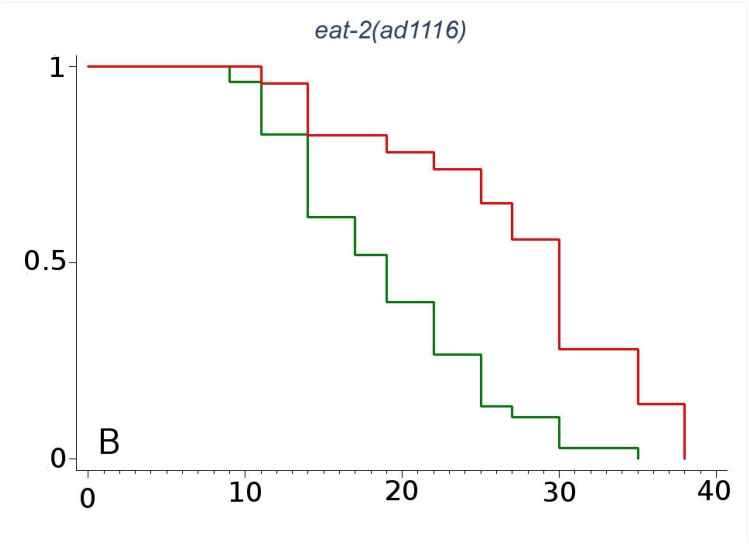
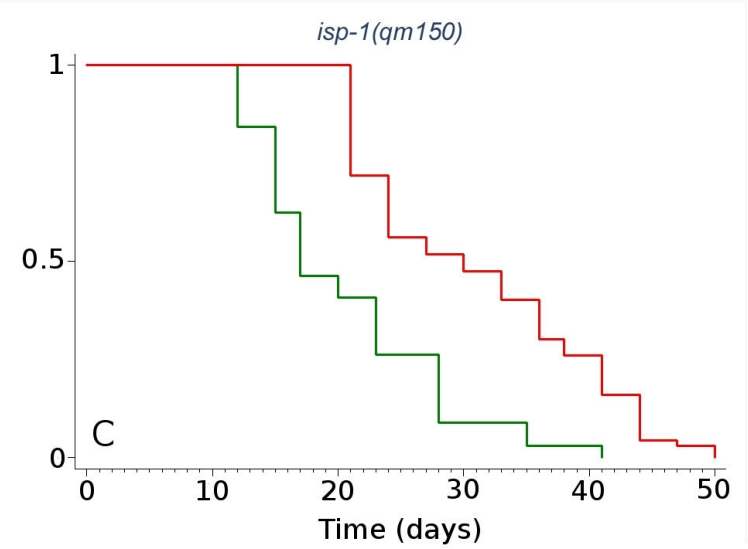


Figure 2.3. Effects of *phi-62* RNAi knockdown on other *daf-16* reporters also reported by Ghazi *et al.* to be affected by *tcer-1* RNAi knockdown. $p < 0.01$ for control vs. *phi-62(RNAi)* in all three cases.

Figure 2.4. Effects of *phi-62* RNAi knockdown on other long-lived mutants. A) *daf-2(e1370)*, vector-only control, n = 89, m = 44.1; *phi-62(RNAi)*, n = 59, m = 40.2, $p < 0.2$. B) *eat-2(ad1116)*, vector-only control, n = 23, m = 27.4; *phi-62(RNAi)*, n = 52, m = 19.0, $p < 0.0001$. C) *isp-1(qm150)*, vector-only control, n = 90, m = 31.1; *phi-62(RNAi)*, n = 39, m = 20.5, $p < 0.0001$. Second trial (not shown), *daf-2(e1370)*, vector-only control, n = 93, m = 31.3; *phi-62(RNAi)*, n = 78, m = 27.2, $p < 0.25$.) *eat-2(ad1116)*, vector-only control, n = 90, m = 28.1; *phi-62(RNAi)*, n = 81, m = 19.9, $p < 0.0001$. *isp-1(qm150)*, vector-only control, n = 92, m = 29.0; *phi-62(RNAi)*, n = 81, m = 22.5, $p < 0.0001$.


daf-2(e1370)
control
phi-62
RNAi
1
0.5
0
A
0
20
40
60
80


eat-2(ad1116)
1
0.5
0
B
0
10
20
30
40


isp-1(qm150)
1
0.5
0
C
0
10
20
30
40
50
Time (days)

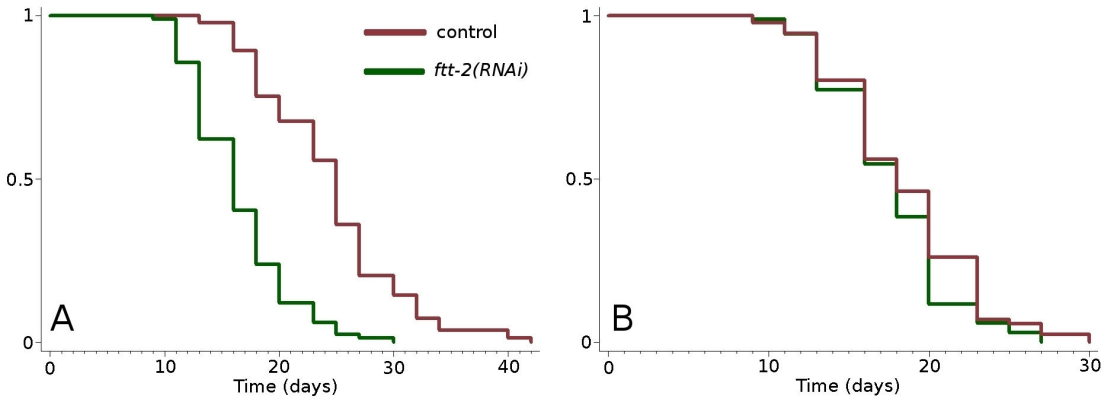


Figure 2.5. *ftt-2* is required for removal of the germline to increase lifespan. A) *glp-1(e2141ts)*: vector-only control n = 90, m = 24.4, *ftt-2(RNAi)* n = 87, m = 16.4, $p < 0.0001$, B) wild type (N2): vector-only control n = 89, m = 18.4, *ftt-2(RNAi)* n = 85, m = 17.6, $p < 0.1$.

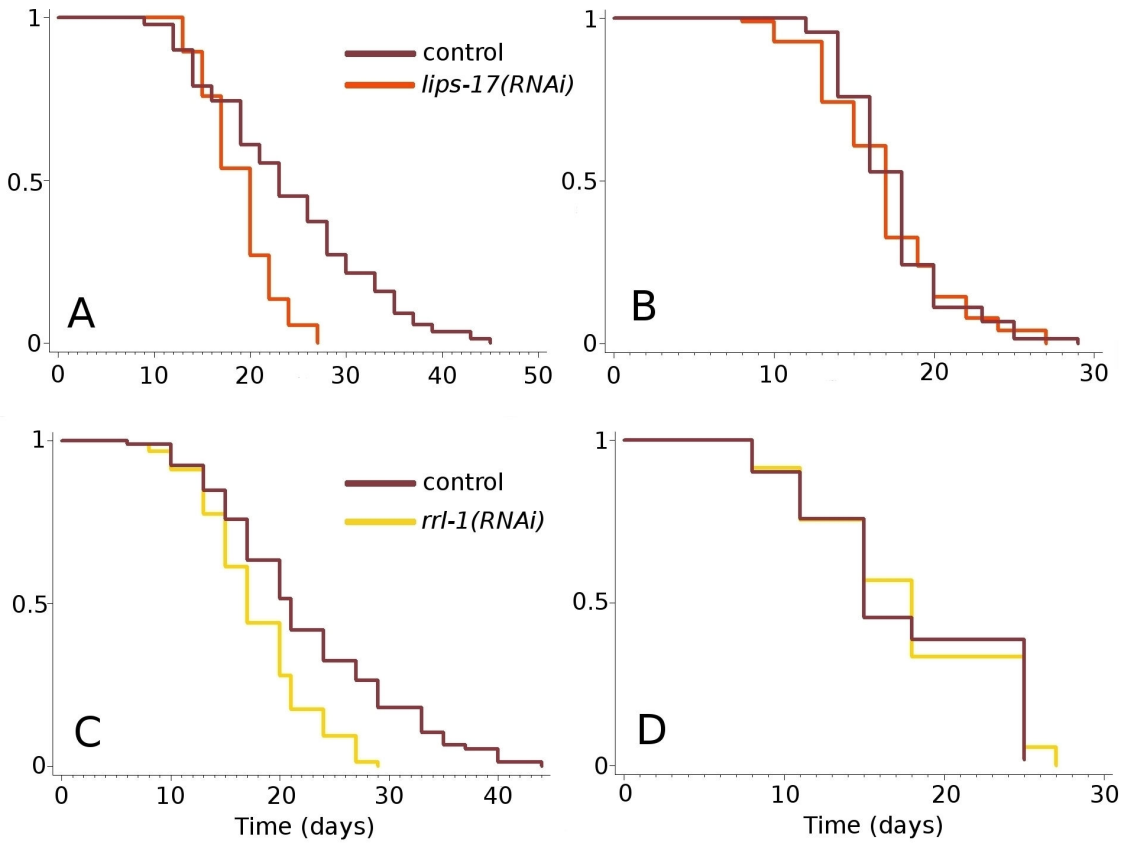


Figure 2.6 (A)-(D). *lips-17* and *rrl-1* are required for removal of the germline to increase lifespan. A,B) *lips-17*: A, *glp-1(e2141ts)* vector-only control n = 89, m = 23.9, *lips-17(RNAi)* n = 77, m = 18.9, $p < 0.0001$. B, wild type (N2). vector-only control n = 90, m = 17.5, *lips-17(RNAi)* n = 92, m = 16.9, $p > 0.4$. C,D) *rrl-1*. C, *glp-1(e2141ts)*. vector-only control n = 85, m = 22.3, *rrl-1(RNAi)* n = 87, m = 17.8, $p < 0.0001$. D, wild type (N2): vector-only control n = 63, m = 17.8, *rrl-1(RNAi)* n = 78, m = 17.9, $p > 0.6$.

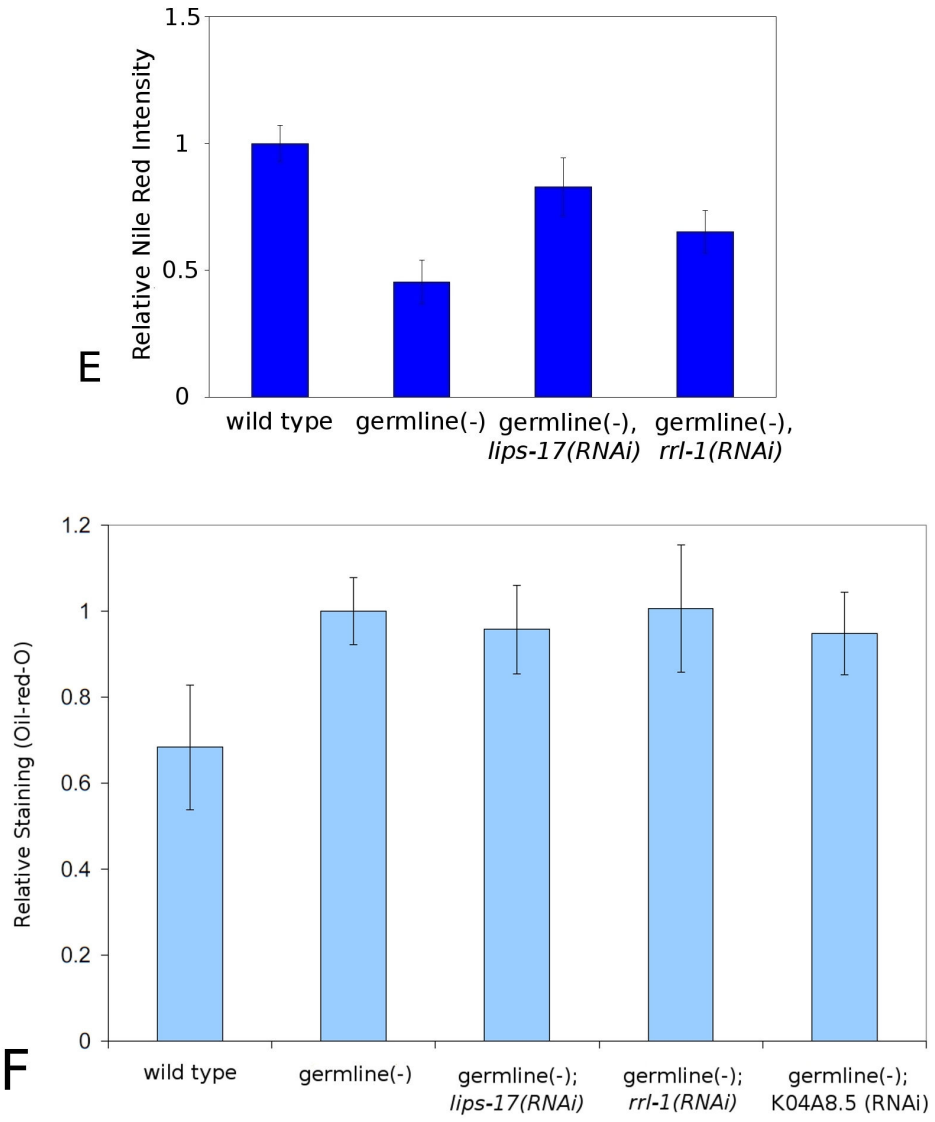
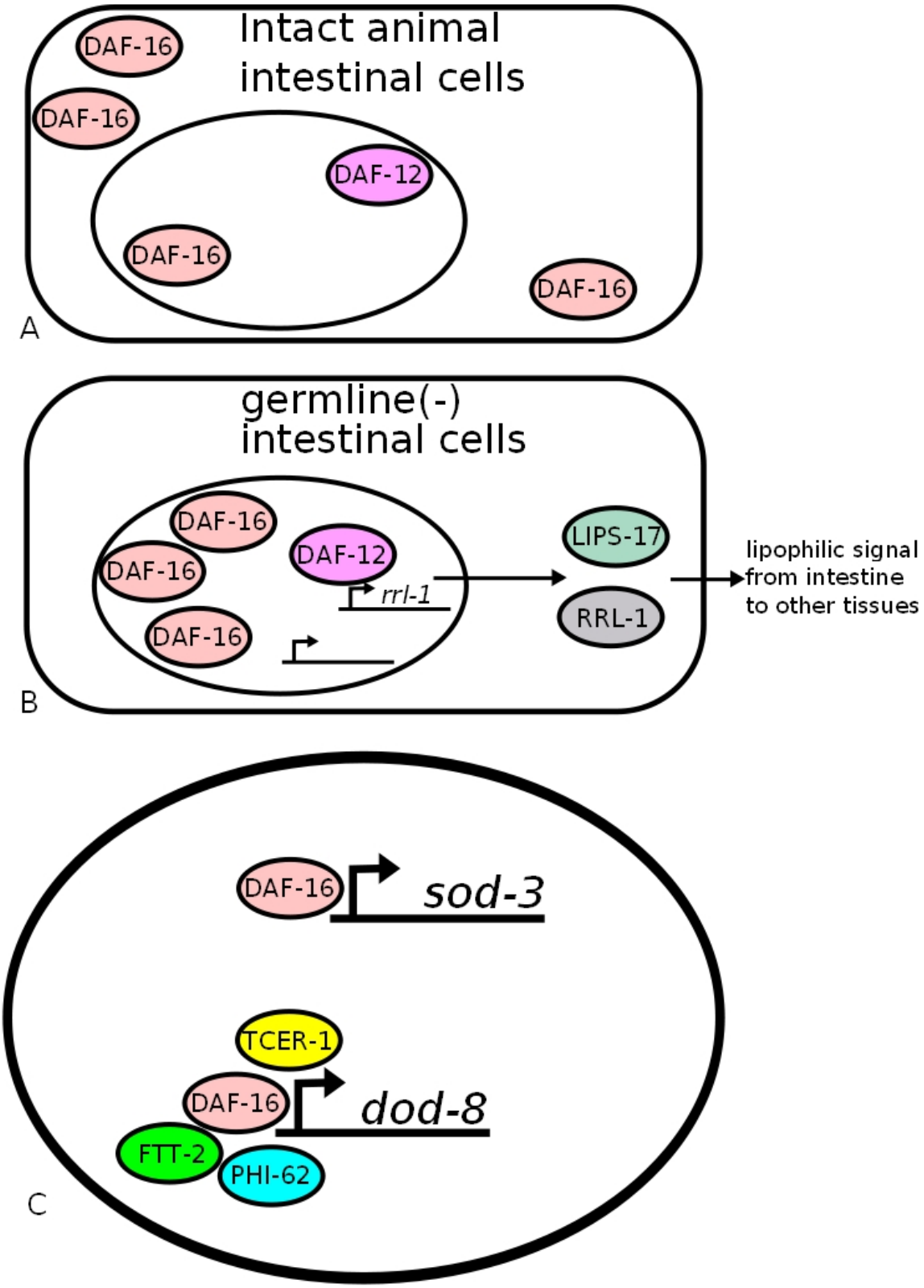


Figure 2.6 (E-F). *lips-17* and *rrl-1* cause an increase in Nile red staining in germline-deficient animals but do not affect Oil-red-O staining. E) Nile red staining, p-values: wild type vs. germline-deficient $p < 1E\text{-}6$, germline-deficient vector only control vs. *lips-17(RNAi)* $p < 1E\text{-}5$, germline-deficient vector only control vs. *rrl-1(RNAi)* $p < 0.01$. F) Oil-red-O staining, p-values: wild type vs. germline(-), germline(-); *lips-17(RNAi)*, and germline(-); K04A8.5(RNAi), all $p < 0.01$. wild type vs. germline(-); *rrl-1(RNAi)* $p = 0.02$. germline(-) vs. all RNAi treatments, N.S.

Figure 2.7. Model. A) In wild-type animals with an intact germline, DAF-16 is not strongly localized to the nucleus of the cell, and animals do not live long. (B,C) Upon removal of the germline, DAF-16 localizes to the nucleus of the intestinal cells. Many genes are transcriptionally up-regulated. B) *rrl-1* (whose transcriptional up-regulation is dependent on DAF-12) and *lips-17* are both involved in fatty acid metabolism but do not affect bulk fat storage in the animal. It is possible that they may be involved in the production or processing of a lipophilic signal which could transmit lifespan-affecting information about the status of the germline from the intestine to other tissues of the animal. In this hypothetical model, the DAF-12 regulation of *rrl-1* may help to explain DAF-12's requirement for lifespan extension in germline-deficient animals. We do not know where in the animal DAF-12 acts to extend lifespan in response to germline removal, but it could potentially act in the intestine, as drawn. C) TCER-1, PHI-62, DAF-16, and FTT-2 are all required for lifespan extension of germline-deficient animals. As FTT-2 is predicted to bind to PHI-62 and has been shown to bind to DAF-16, it is possible that PHI-62 and FTT-2 may act together with DAF-16 to increase lifespan of germline-deficient animals and to influence expression of known DAF-16 germline targets. Some known DAF-16 targets shown to be up-regulated in response to removal of the germline, such as *sod-3*, do not require TCER-1 or PHI-62 for their up-regulation, while others, such as *dod-8*, do require both TCER-1 and PHI-62 in addition to DAF-16. Although these proteins are shown in the nucleus, where DAF-16's transcriptional regulation of genes including *sod-3* and *dod-8* occurs, it is not known where these proteins interact, or where PHI-62 or FTT-2 may be localized, under these conditions.


Intact animal
intestinal cells
DAF-16
DAF-16
DAF-12
DAF-16
DAF-16
A
germline(-)
intestinal cells
DAF-16
DAF-16
DAF-12
rrl-1
DAF-16
LIPS-17
RRL-1
lipophilic signal
from intestine
to other tissues
B
DAF-16
sod-3
TCER-1
DAF-16
dod-8
FTT-2
PHI-62
C

CHAPTER 3: CONCLUDING REMARKS

In this final chapter, we summarize our findings and discuss their implications for future research.

Briefly, we sought to understand what transcriptional differences existed between long and short-lived worms in the context of lifespan extension by removal of the germline, and in the presence or absence of DAF-16 and DAF-12 transcription factors in these worms. We were able to identify a group of genes whose transcriptional level varied distinctly for each of these categories, as listed in Tables 2.2, 2.3, and 2.5. The sparse overlap between our DAF-16 and DAF-12 targets, and lack of enrichment for the overlapping targets among the most significant targets of either transcription factor alone, suggest that the majority of the genes most significantly influenced by the presence of DAF-16 differ from those most significantly influenced by the presence of DAF-12. Nevertheless, those few genes that do appear to be influenced by both transcription factors (Table 2.4) may warrant further study.

Although the overlap between the targets of DAF-16 and DAF-12 was slight though significant, there was a much more significant overlap between each transcription factor's targets in the germline removal context and its targets identified in previous studies. Our predicted DAF-16 targets showed a very large overlap with DAF-16's previously identified targets (Murphy *et al.* 2003), suggesting that there may be a core set of conserved genes representing a common transcriptional signature for DAF-16 in two distinct pathways that affect lifespan. This concords with the fact that our promoter analysis also found the same upstream, presumptive regulatory, sequences to be

overrepresented that had previously been found from DAF-16's targets in the daf-2 insulin/IGF-1 signaling context. This suggests that these common targets may warrant additional interest in the future.

Again with DAF-12, we found a statistically significant overlap between our predicted targets and previously reported targets (Fisher & Lithgow 2006), suggesting as in the case of DAF-16 that those targets of DAF-12 that have now been found to be transcriptionally regulated in two distinct contexts that both affect lifespan may be of increased interest for further study. In the case of DAF-12, promoter analysis of the previously identified targets had not been published, but as with DAF-16, our own analysis of both our DAF-12 targets and the previously identified DAF-12 targets showed the same sequences most overrepresented in the upstream, putative promoter, regions, suggesting that these sequences may be important cis-elements for DAF-12 regulation, possibly DAF-12 binding sites. This may warrant additional study of these promoter sequences for their possible DAF-12 binding properties if any. Interestingly, our functional analysis described below identified a target of DAF-12, *rrl-1*, which is required for lifespan extension in germline-deficient animals. As this DAF-12-regulated gene may help to explain some or all of the DAF-1 dependence of lifespan extension in germline-deficient animals, it would be interesting in the future to further characterize *rrl-1*'s DAF-12 dependence and regulation. This could include such experiments as gel-shift promoter binding assays to detect possible direct regulation of *rrl-1* by DAF-12, as well as RT-PCR or western blot assays to further quantify changes in *rrl-1* mRNA and protein levels in the presence or absence of DAF-12.

We studied the lifespan phenotypes of the RNAi knockdown of several genes identified in our microarrays, and identified several that had an effect on the extended lifespan of germline-deficient animals. *phi-62*, a predicted RNA binding protein, was required for removal of the germline to extend lifespan. Because *phi-62* is predicted to bind to *ftt-2*, a known *daf-16* binding partner, we tested the effect of RNAi knockdown of *ftt-2* on the lifespan of germline deficient animals and found that it was also required. This suggests the possibility that these proteins may act together to extend the lifespan of germline-deficient animals. Biochemical experiments to test for direct binding between PHI-62 and FTT-2, and possibly between PHI-62 and DAF-16, could shed further light on their possible interactions in the future.

Two genes, *lips-17* and *rrl-1,* which we identified as required for lifespan extension in germline-deficient animals, are known to be involved in lipid metabolism, presumably in liberating fatty acids from stored triglycerides. This is of interest because another gene involved in fat metabolism, K07A8.5, has also been shown to be required for lifespan extension in germline-deficient animals (Wang *et al.* 2008). Like K07A8.5, *lips-17* is a triglyceride lipase; however, the RNAi clones for these two genes are not predicted to cross react. Because RNAi knockdown of *rrl-1* and *lips-17* does not affect bulk triglyceride storage, as measured by oil-red O staining, it is interesting to consider the possibility that they may play a role in the production of a lipophilic signal. Acyl reductases such as *rrl-1* reduce fatty acids to fatty aldehydes and alcohols. These type of reduced fatty acid molecules have been found to be pheromones in some species. This leads to a hypothetical model in which these two genes play a role in the production of a signal that allows the remaining tissues of the animal to receive information about the

presence or absence of the germline. We also found that MDT-15, a regulatory protein which has been shown to affect levels of many genes known to be involved in fat metabolism, is required for the long lifespan of germline-deficient animals. Although MDT-15 also significantly affects the lifespan of wild type animals, it is still possible that it may be found to play a role in the same process in which rrl-1 and lips-17 may be acting. It would be interesting in the future to consider these possibilities in more detail.

lips-17 and *rrl-1* also exhibit an effect on Nile red staining, as does K07A8.5 (Wang *et al.* 2008). Nile red is thought to stain lysosome-like compartments in *C. elegans* (Schroeder *et al.* 2007; Soukas *et al.* 2009). Germline-deficient animals exhibit decreased Nile red staining relative to wild type, and RNAi knockdown of *rrl-1* or *lips-17* partially rescues this effect. This leads to some interesting possibilities. It may be that increased efficiency of either lysosomal processing, or some upstream processing of or protection from xenobiotics, causes the decreased staining seen in germline-deficient animals, and that this is linked to their longer lifespan. Thus RNAi knockdown of *rrl-1* or *lips-17* may return this increased efficiency toward wild-type, and the lifespan along with it. It is also possible that the effect on lifespan and the effect on Nile red staining of these genes is not related. It would be interesting in the future to ask if there are genes that affect this Nile red staining phenotype that do not affect the extended lifespan of germline-deficient animals.

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