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New genes that extend *Caenorhabditis elegans*' lifespan in response to reproductive signals

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Summary

In *Caenorhabditis elegans* and *Drosophila*, removing germline stem cells increases lifespan. In *C. elegans*, this lifespan extension requires DAF-16, a FOXO transcription factor, and DAF-12, a nuclear hormone receptor. 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 influence the expression of 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. 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 noticeably affect bulk fat storage levels; therefore, we propose a model in which they may influence production of a lifespan-extending signal or metabolite.

Key words: lifespan; germline; steroid; DAF-16; DAF-12; aging.

Introduction

Removing the germline of *Caenorhabditis 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). This lifespan extension appears to be caused specifically by loss of germline stem cells during adulthood (Arantes-Oliveira *et al.*, 2002). Removing germline stem cells in *Drosophila* adults increases the fly's lifespan by up to 50% (Flatt *et al.*, 2008), and in mice, too, signals from the reproductive system can extend lifespan (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 but unanswered question.

Two transcription factors, the FOXO-family transcription factor DAF-16 and the nuclear hormone receptor (NHR) DAF-12, are required for germline loss to extend lifespan in *C. elegans* (Hsin & Kenyon, 1999). DAF-16/FOXO is best known for its ability to extend lifespan in response to reduced insulin/IGF-1 signaling (Kenyon, 2010b). FOXO proteins have been linked to longevity in many animal species, and at least eight gene association studies suggest that they affect human longevity as well (Kenyon *et al.*, 1993; Hwangbo *et al.*, 2004; Giannakou *et al.*, 2004; Taguchi *et al.*, 2007; Willcox *et al.*, 2008; Anselmi *et al.*, 2009; Flachsbart *et al.*, 2009; Li *et al.*, 2009; Pawlikowska *et al.*, 2009; Soerensen *et al.*, 2010). In worms with reduced insulin/IGF-1 signaling, DAF-16 localizes to the nuclei of larval and adult tissues throughout the animal (Henderson & Johnson, 2001; Lee *et al.*, 2001; Lin *et al.*, 2001). 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 and 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 overexpressing dFOXO in adipose tissue extends fly lifespan, and down-regulation of insulin signaling in mouse adipose tissue extends lifespan as well (Bluher *et al.*, 2003; Giannakou *et al.*, 2004; Hwangbo *et al.*, 2004).

Loss of the germ cells also increases the levels of TCER-1, a putative transcription elongation factor, in the intestine (Ghazi *et al.*, 2009). TCER-1 appears to have a rather focused activity in the worm, as its loss prevents germline ablation from extending lifespan, but does not affect 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. Likewise, an intestinal adaptor protein called KRI-1 is required for germline loss, but not insulin/IGF-1 pathway inhibition, to increase lifespan. Together, these and other findings indicate that DAF-16's regulation and activity in the reproductive and insulin/IGF-1 pathways are distinct from one another (Kenyon, 2010a).

The NHR DAF-12 plays at least two distinct roles in germline-less animals. First, in animals that lack germ cells, DAF-12 is partially required for nuclear localization of DAF-16/FOXO (Berman & Kenyon, 2006). 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). DAF-12's second role could be linked to that of another, recently described, NHR, NHR-80, as DAF-12, but not DAF-16, is required for overexpression of *nhr-80* to extend lifespan in germline-deficient animals (Goudeau *et al.*, 2011).

How DAF-12 ultimately influences lifespan is not known. In intact animals, a set of potential longevity genes regulated by DAF-12 has been identified using microarray analysis (Fisher & Lithgow, 2006). Here, at low temperature, loss-of-function *daf-12* alleles shorten lifespan, whereas gain-of-function alleles modestly lengthen lifespan (Gerisch *et al.*, 2007). Interestingly, some of the down-regulated genes identified by Fisher and Lithgow are known to be functionally significant lifespan genes that are also down-regulated by DAF-16 in *daf-2*/insulin/IGF-1 receptor mutants.

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This finding suggests that DAF-12 and DAF-16 regulate at least some genes in common.

In this study, we have used microarray analysis to clarify the roles of DAF-16 and DAF-12 in the germline pathway. 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 get a better idea of how DAF-16 and DAF-12 might interact to activate their target genes in the germline pathway. For example, some genes, such as the superoxide dismutase *sod-3* (Yamawaki *et al.*, 2010) and the lipase K04A8.5 (Wang *et al.*, 2008), are known to be activated by DAF-16 independently of DAF-12 in germline-deficient animals, but the size and composition of this gene class is not known. Nor is it known whether DAF-16 and DAF-12 might co-regulate specific target genes in this pathway. 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 comprises 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 (+) vs. mutant (–) animals would yield a broad range of these transcription factors' targets, not only those germane to the germline regulation of lifespan. 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 Fig. 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 Fig. 1, we then compared the gene expression patterns of these animals to one another in various combinations. We hybridized 60 samples to arrays designed to probe 20 374 predicted *C. elegans* open reading frames (ORFs), as described previously (Cristina *et al.*, 2009).

To validate our method's ability to pick out specific functionally related gene classes, we first compared the gene expression patterns of all the strains grown at high temperature (25 °C, the germline-deficient condition) with the same strains grown at low temperature (the intact condition). 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 S1). 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).

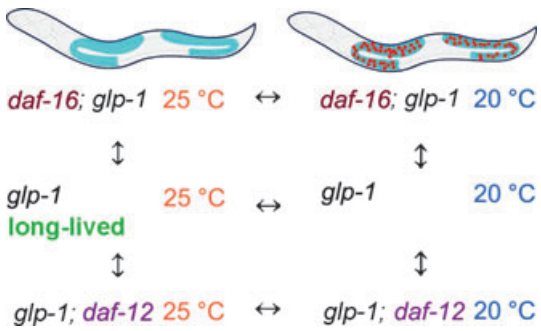


Fig. 1 Block design for microarray experiments. *glp-1*(*e2141ts*) animals lack a germ line and are long-lived at 25 °C, but are germline (+) and have a normal lifespan at 20 °C.

Genes regulated by DAF-16

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*. (*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 cut-off (Table S2). Of these, 38 were also present among 512 previously identified genes regulated by DAF-16 in a *daf-2* mutant background (Murphy *et al.*, 2003), a highly significant overlap ($P < 1E{-}15$). This striking correspondence suggests that DAF-16 may extend lifespan in germline-deficient animals and insulin/IGF-1 mutants via similar sets of downstream genes.

We performed a BiNGO analysis of this list of 230 genes for overrepresented biological processes (Shannon *et al.*, 2003; Maere *et al.*, 2005) (Table S3). This comparison yielded 'aging', 'multicellular organismal aging' and 'determination of adult lifespan' as the three most significantly overrepresented processes. These three categories were all overrepresented on the basis of the same 13 genes (Table S4), of which 10 were annotated as being involved in lifespan in part because they were previously identified as targets of DAF-16 in a *daf-2* mutant background, including *mdt-15*, which will be discussed further later. Other sets of closely related overrepresented categories contained genes involved in monocarboxylic acid metabolism, carboxylic acid metabolism and organic acid metabolism. Genes in these overrepresented sets included *fat-5,* which encodes a fatty acid desaturase, and *hacd-1*, which encodes a hydroxyacyl-CoA dehydrogenase, both of which have been shown previously to be regulated by MDT-15 (Taubert *et al.*, 2006), as well as *gei-7*, which encodes an isocitrate lyase/malate synthase that has been shown to be regulated both by DAF-16 in a *daf-2(–)* background (Murphy *et al.*, 2003) and by MDT-15 (Taubert *et al.*, 2006). In an independent study, Goudeau *et al.* (2011) recently identified *fat-5* as a *daf-16*-regulated gene in *glp-1* mutants as well.

Genes regulated by DAF-12 in response to germline loss

Our list of *daf-12*/NHR-dependent genes contained 130 genes using a $P < 0.01$ significance level cut-off (Table S5). Of these, eight overlapped with 224 genes previously reported to be *daf-12* regulated in intact animals grown at low temperature, again a highly significant overlap ($P < 1E{-}4$) (Fisher & Lithgow, 2006). As with DAF-16, this overlap suggests that downstream targets by which DAF-12 influences lifespan in response to germline loss may be shared in other contexts. A BiNGO analysis of this list of genes showed no overrepresented biological processes.

Genes regulated by both DAF-12 and DAF-16 in animals lacking germ cells

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, seven 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 (Murphy *et al.*, 2003; Fisher & Lithgow, 2006). 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* in this strict analysis encoded a neuropeptide-like protein, a sodium neurotransmitter symporter family protein, a broad-complex domain-containing protein and several proteins of unknown function (Table S6). Interestingly, one of these proteins, C46G7.2, was identified in an RNAi screen for increased paraquat resistance, and C46G7.2 RNAi was shown to increase wild-type lifespan by 44% in a *daf-16*-dependent manner (Kim & Sun, 2007).

In any comparison of strict cut-off lists, the problem exists that genes with a less dramatic degree of regulation that is nonetheless shared may escape notice. To ask whether a more subtle enrichment of those genes might exist than that observed by the overlap of our two $P < 0.01$ cut-off lists, we performed gene set enrichment analysis (Subramanian *et al.*, 2005), testing the individual $P < 0.01$ cut-off list of predicted DAF-16 targets against a list of all *C. elegans* genes present on our array ranked by likelihood of DAF-12 regulation, and vice versa. In these more sensitive analyses, a more subtle enrichment was in fact detected: of 230 predicted DAF-16 targets, 103 were predicted to show DAF-12 regulation (Table S7), and of 130 predicted DAF-12 targets, 69 were predicted to show DAF-16 regulation (Table S8).

To further investigate this overlapping regulation as well as to validate our array results, we performed qPCR of *lips-17*, a gene identified in our long- vs. short-lived comparison; *dod-8*, a gene identified in this study as well as previous work (Murphy *et al.*, 2003) as a DAF-16 target; and the previously-identified DAF-16 targets *gpd-2*, *nnt-1* and *sod-3* (Murphy *et al.*, 2003; Ghazi *et al.*, 2009), in germline-deficient worms that were otherwise wild type or null for either *daf-12* or *daf-16.* Confirming our array results, we found that *lips-17* expression was up-regulated over 2.5-fold in germline-deficient animals and that this increase was partially dependent on both *daf-12* and *daf-16* (Fig. S1). Our array results were also confirmed by the finding that expression of *dod-8* in germline-deficient worms was dependent on the presence of *daf-16* (Fig. S1). Interestingly, for *dod-8* as well as the *daf-16* reporters *sod-3*, *nnt-1* and *gpd-2*, the decrease in expression upon removal of *daf-16* was paralleled by a more modest, although in some cases (*gpd-2*, *nnt-1*) statistically significant, decrease upon removal of *daf-12* (Fig. S1). This again corresponds well with our array results, which show by gene set enrichment analysis that roughly half of those genes most strongly dependent on *daf-16* show a more subtle dependence on *daf-12*, and vice versa.

We then subjected these predicted overlapping subsets of genes again to BiNGO analysis for overrepresented gene ontology categories. For the predicted *daf-12* targets, as before, neither the *daf-16* enriched nor *daf-16* un-enriched subsets showed any gene ontology categories overrepresented. Among the *daf-16* targets, those showing *daf-12* enrichment no longer showed overrepresentation for aging and lifespan-related biological processes, while those un-enriched still did. As discussed earlier, this is largely because the *daf-16* targets that showed *daf-12* enrichment from our array results in germline-deficient animals do not overlap significantly with previously published targets of *daf-16* in the *daf-2* pathway. Additionally, the *daf-12* un-enriched *daf-16* targets, that is, those *daf-16* targets that we predict are not dependent on *daf-12*, also contained all of the genes responsible for overrepresentation of fatty acid metabolism in this group, and as a result, fatty acid metabolism is reported as overrepresented in the *daf-12* un-enriched subset, but not in the *daf-12* enriched subset.

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 erythroid-like transcription factor family (ELT) transcription-factor binding site that was overrepresented in the promoters of several aging-related gene sets (Budovskaya *et al.*, 2008). 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).

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. It is worth noting here that the sequence TTGATAA has a reverse complement (TTATCAA) that differs by one base pair from the above-mentioned TTATCAC-predicted ELT-binding sequence previously found to be overrepresented in the promoters of predicted *daf-16* targets (Murphy *et al.*, 2003) and in the promoters of several aging-related gene sets (Budovskaya *et al.*, 2008). Because our unseeded overrepresented oligo sequence results did not include either of the two previously reported *daf-12* binding sites (Ao *et al.*, 2004; Shostak *et al.*, 2004), we asked specifically whether either of these sequences was overrepresented in the promoters of our 130 predicted DAF-12 targets and found that neither was. Finally, we note that, very recently, chromatin immunoprecipitation was used to identify DAF-12 targets from intact larvae and adults (Hochbaum *et al.*, 2011). Again their overrepresented DAF-12 binding sites did not coincide with ours.

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

contained genes whose expression differed 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 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 S10). Among the genes we identified in this screen (#12 in our list) was *tcer-1,* the putative transcription elongation factor discussed earlier. This finding, too, validated our microarray strategy and also indicated that the increase in the TCER-1::green fluorescent protein (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 S9). 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. These genes are discussed below.

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

phi-62 was identified in our initial day-1 'long- vs. short-lived' comparison (#17 in our list), along with *tcer-1*. This gene encodes a small, 95-amino acid protein containing a predicted RNA-binding domain. *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). The *Ceratitis capitata* ortholog has been shown to have ribonuclease activity *in vitro* (Rampias *et al.*, 2003, and RNAi knockdown of *phi-62* has been shown to accelerate protein aggregation in a worm model of polyglutamine-aggregation disease (Nollen *et al.*, 2004). We found that 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 (Fig. 2A). 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 (Fig. 2B). 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 more closely, we examined three more genes that were regulated by *tcer-1*. We found complete coincidence between the effects of *phi-62* and *tcer-1* on these genes (Fig. S2). 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 the lifespan increases produced by *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). Because of the similarity between the *tcer-1*(–) and *phi-62*(–) phenotypes, we tested whether loss of *phi-62* activity might affect other lifespan pathways similarly to the way they are affected by loss of *tcer-1*. 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. S3). *phi-62* was partially required for lifespan extension caused by e*at-2* and *isp-1* (respiration-defective) mutations.

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

Using Gene Orienteer (http://www.geneorienteer.org) (Zhong & Sternberg, 2006), we discovered that PHI-62 is predicted to bind FTT-2. This prediction is based on the interaction between the *Drosophila* orthologs of FTT-2 and PHI-62 in a yeast two-hybrid assay (Giot *et al.*, 2003). FTT-2 is 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 found that RNAi knockdown of *ftt-2* reduced the long lifespan of germline-defective animals to that of wild type (Fig. 3), while not shortening wild-type lifespan. 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.

In an attempt to further characterize the regulatory relationships between TCER-1, PHI-62, FTT-2 and DAF-16, we asked whether the previously-reported lifespan extension of *tcer-1*-overexpressing worms was dependent on expression of the other three genes. As previously reported (Ghazi *et al.*, 2009), we saw a modest but statistically significant increase in the lifespan of *tcer-1*-overexpressing worms relative to wild-type, and this increase did not occur upon RNAi knockdown of *daf-16* (Table S10). We further saw that this increase of lifespan in *tcer-1*-overexpressing worms did not occur upon RNAi knockdown of *ftt-2* or *phi-62* (Table S10), suggesting that *ftt-2* and *phi-62* act downstream of, or in parallel to, *tcer-1*. To find out more about possible relationships between *daf-16*, *ftt-2* and *phi-62*, we asked whether RNAi knockdown of *ftt-2* or *phi-62* could further shorten the already shortened lifespan of germline-deficient *daf-16*; *glp-1* worms, and found that it could not in either case (Fig. S4). This finding is consistent with the possibility that these three genes play related roles in the lifespan extension of germline-deficient animals.

We also asked whether the nuclear localization of DAF-16 in the intestines of germline-deficient animals was affected by RNAi knockdown of either *phi-62* or *ftt-2*. We found that RNAi knockdown of *phi-62* greatly reduced DAF-16 intestinal nuclear localization in germline-deficient animals, and knockdown of *ftt-2* reduced it as well, although more modestly.

This latter finding was unexpected. Previous groups have shown an *increase* in DAF-16 nuclear localization upon RNAi knockdown of *ftt-2* and no effect upon RNAi knockdown of its close homolog *par-5* (Berdichevsky *et al.*, 2006; Li *et al.*, 2007). However, these studies examined intact rather than germline-deficient worms and also employed an *ftt-2* or *par-5*-specific RNAi construct, whereas the RNAi clone we used (from the Ahringer library) is likely to target both homologs. Therefore, we obtained the *ftt-2*- and *par-5*-specific constructs and repeated these measurements in both *glp-1*(–) and wild-type worms. Again, we saw that DAF-16 intestinal nuclear localization was decreased in *glp-1*(–) mutants and not increased in the wild-type (Fig. S5). Why do our findings differ? In our experiments, we initiated RNAi treatment at the time of hatching and examined the worms when they became adults, at 20 °C, after shifting the animals at the L2–L4 stage to 25 °C [as this removes the germline stem cells of *glp-1*(*e2141ts*) mutants]. Li *et al.* initiated RNAi in L4 larvae

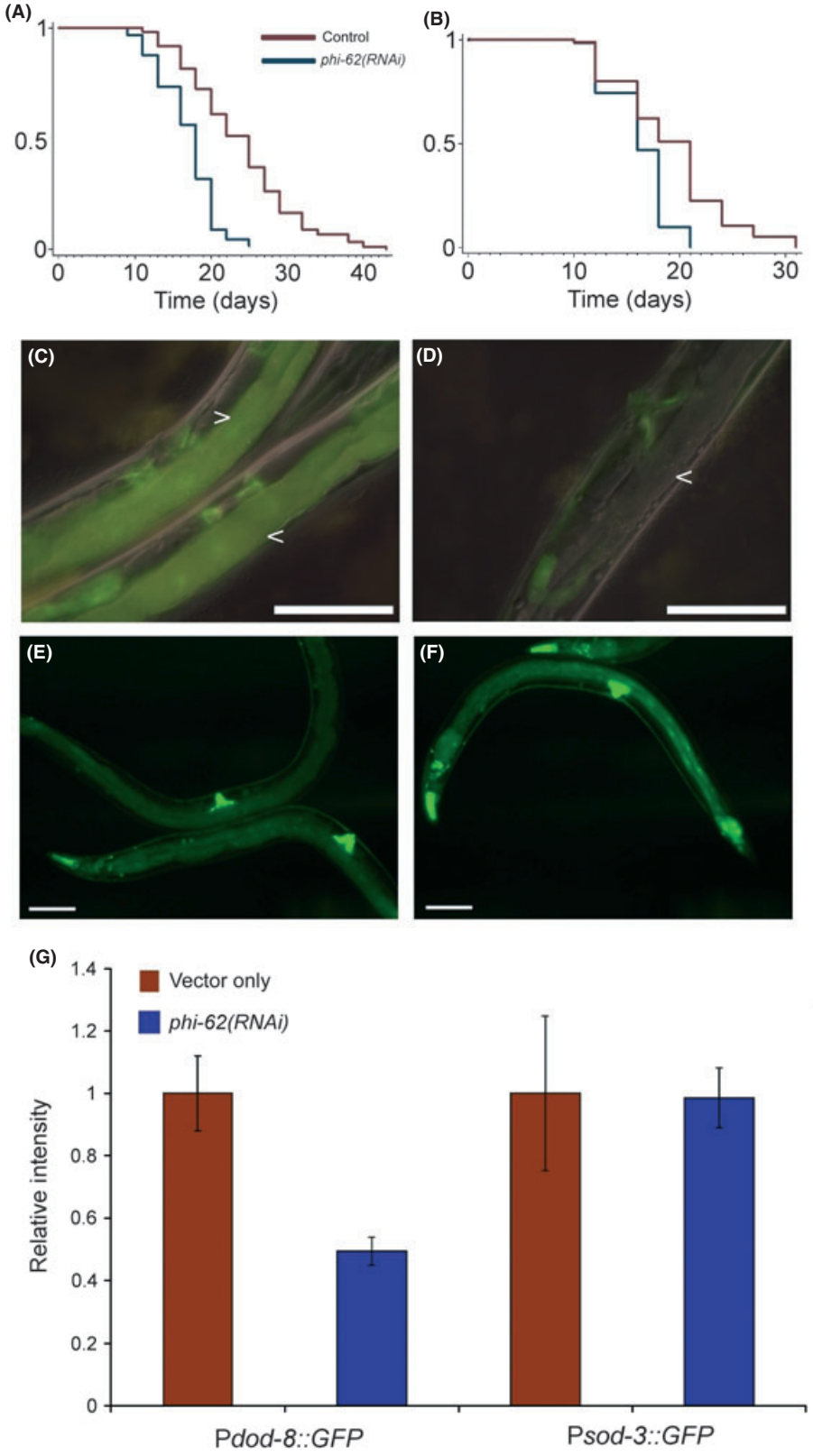


Fig. 2 *phi-62* is required for removal of the germline to increase lifespan. It is also required for up-regulation of *dod-8*, a known *daf-16*-regulated gene, but not for *sod-3*, another *daf-16*-regulated gene, in animals that lack the germline. (A) *glp-1*(*e2141ts*): vector-only control $n = 96$, $m = 23.8$ 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*). (G) Relative GFP intensities. P*dod-8::GFP* vector-only vs. *phi-62*(*RNAi*), $P < 0.01$. Scale bar = 200 μm.

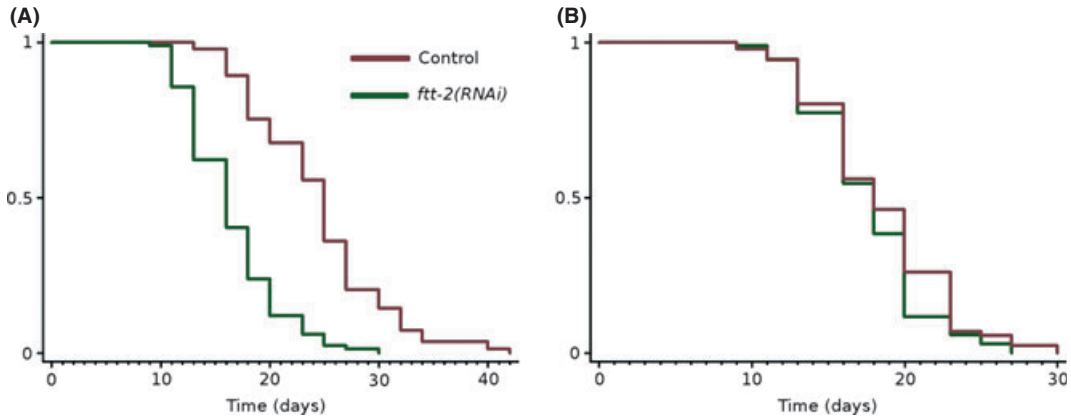


Fig. 3 *ftt*-2 RNAi prevents loss of the germline from increasing lifespan. (A) *glp-1*(*e2141ts*): vector-only control $n = 90$, $m = 24.4$, *ftt-2*(*RNAi*) $n = 87$, $m = 16.4$, $P < 0.0001$, (B) wild-type (N2): vector-only control $n = 89$, $m = 18.4$, *ftt-2*(*RNAi*) $n = 85$, $m = 17.6$, $P < 0.1$.

at 16 °C. They then examined L3 progeny, rather than same-generation adults. Berdichevsky *et al.* also initiated RNAi in L4 larvae and observed nuclear DAF-16 localization in their L3 and adult progeny. Whether the timing of treatment, temperature or another variable is responsible for the difference, the results reported here are relevant to regulation of DAF-16 in the germline context because (i) our treatment regime mimics exactly that used in the lifespan assays, whose outcomes we seek to understand, and (ii) work in our laboratory has shown that the up-regulation of DAF-16 reporters in germline-deficient animals is not noticeable until adulthood.

Finally, we asked whether RNAi knockdown of *ftt-2* might affect the expression of genes that are normally switched on in a *daf-16*-dependent fashion in germline-defective mutants. We assayed GFP reporters for *sod-3,* whose induction requires *daf-16*, and *dod-8,* whose induction not only requires *daf-16* but also *tcer-1* and *phi-62.* Surprisingly, we did not see any change in the up-regulation of these two genes (Fig. S6).

lips-17 and *fard-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 *fard-1*, 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 *fard-1* shortened the lifespan of germline-deficient animals roughly to that of wild-type, while not shortening wild-type lifespan (Fig. 4). Another triacylglycerol lipase, K04A8.5, was shown previously to be required for germline-deficient animals to live long (Wang *et al.*, 2008), as were genes required for the synthesis of oleic acid (Goudeau *et al.*, 2011).

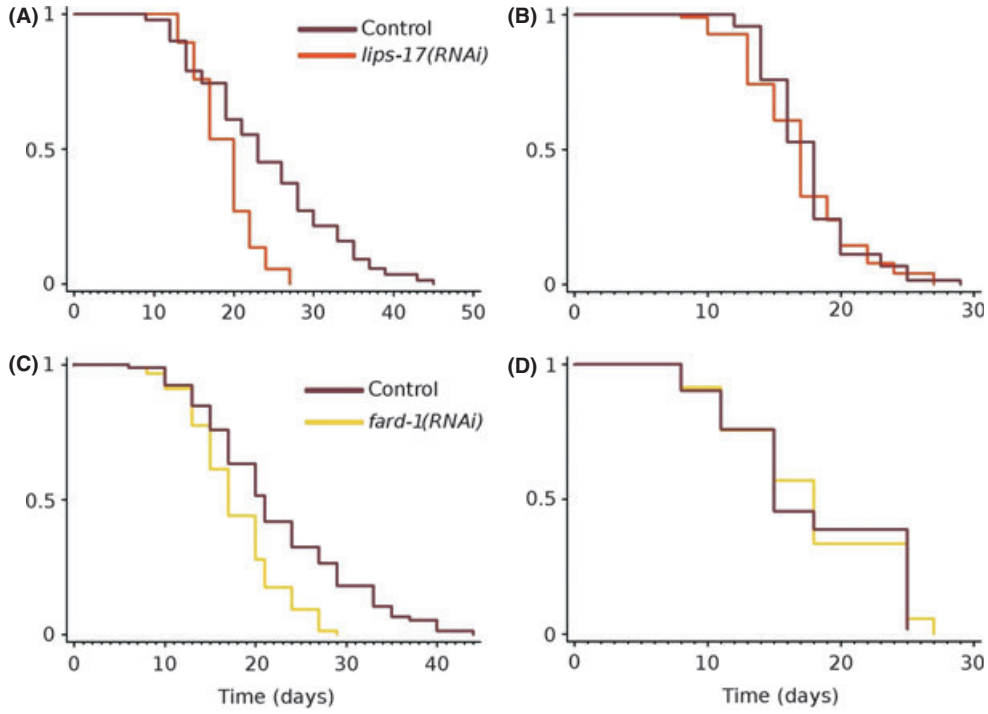
Animals lacking a germline have been shown to have greatly reduced staining with the dye Nile red, and RNAi knockdown of K04A8.5 suppresses this abnormal Nile red phenotype (Wang *et al.*, 2008). We asked what effect RNAi knockdown of *lips-17* and *fard-1* might have on Nile red staining and found that knockdown of *lips-17*, and to a lesser extent knockdown of *fard-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 (Fig. 5). Nile red has been thought to stain fat in *C. elegans*, but it was shown recently to more specifically 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.* (2009). This increase was not affected by RNAi knockdown of *lips-17*, *fard-1* or K04A8.5 (Fig. 4). This finding suggests that these lipid-metabolizing genes are unlikely to influence lifespan by mechanisms that involve gross changes in total lipid stores.

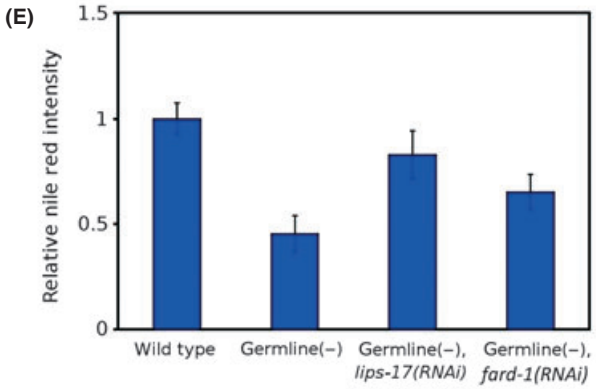
mdt-15(*RNAi*) worms are short-lived

One final RNAi knockdown, of *mdt-15*, produced a noteworthy phenotype. *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, 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 *daf-16*-regulated gene 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, as reduction of *mdt-15* function also shortens wild-type lifespan (Taubert *et al.*, 2008), *mdt-15* may not act in a germline-specific fashion. It is not clear whether *mdt-15* plays a role in the expression or action of the lipid metabolism genes discussed earlier, 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 NHR. We found that many of the genes dependent on DAF-16 in this germline-deficient 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. These overlapping targets may be of particular interest as genes central to DAF-16's regulation of lifespan. Because there were also





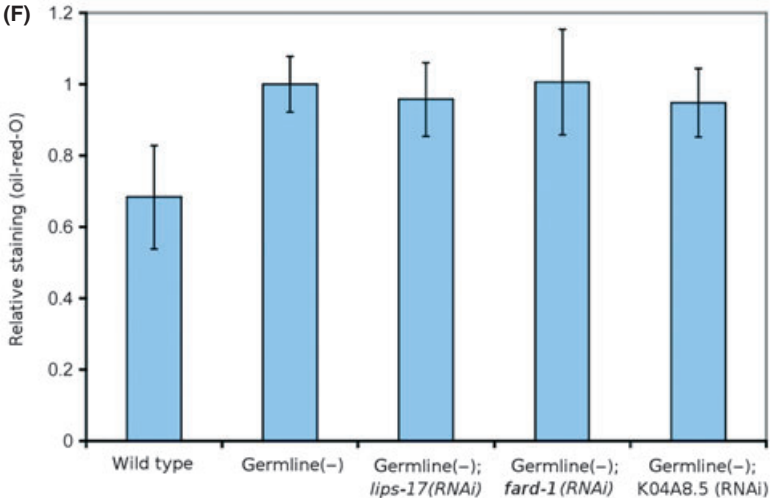


Fig. 4 *lips-17* and *fard-1* are required for removal of the germline to increase lifespan. In addition, *lips-17* and *fard-1* RNAi alter Nile red lysosomal staining, but not Oil-red-O lipid staining. (A,B) *lips-17*: (A) *glp-1*(*e2141ts*) vector-only control $n = 89$, $m = 23.9$; *lips-17*(*RNAi*) $n = 77$, $m = 18.9$, $P < 0.0001$. (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) *fard-1*. (C) *glp-1*(*e2141ts*). Vector-only control $n = 85$, $m = 22.3$; *fard-1*(*RNAi*) $n = 87$, $m = 17.8$, $P < 0.0001$. (D) Wild-type (N2): vector-only control $n = 63$, $m = 17.8$; *fard-1*(*RNAi*) $n = 78$, $m = 17.9$, $P > 0.6$. (E) Nile red staining, *P*-values: wild-type vs. germline-deficient $P < 1E{-}6$, germline-deficient vector-only control vs. *lips-17*(*RNAi*) $P < 1E{-}5$, germline-deficient vector-only control vs. *fard-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(−); *fard-1*(*RNAi*) $P = 0.02$. germline(−) vs. all RNAi treatments, N.S.

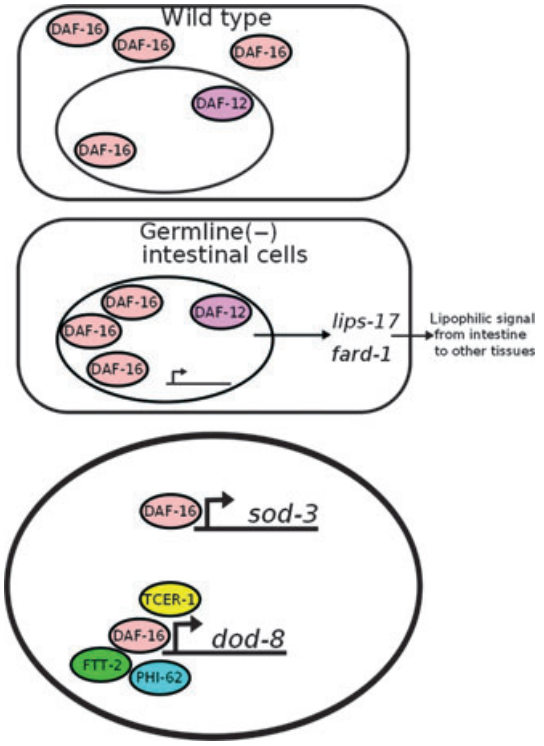


Fig. 5 Summary and a hypothetical model. Upon removal of the germline, DAF-16 localizes to the nuclei of intestinal cells. Together with DAF-12, this causes up-regulation of *fard-1* and *lips-17*, which may be involved in the production of a hypothetical lipophilic signal from the intestine to other tissues. DAF-16, PHI-62 and FTT-2 (or their orthologs in other species) have been shown to interact physically and are all required for lifespan extension by germline ablation, suggesting the possibility that they may act together in this process. PHI-62 and TCER-1 are required for changes in the expression of some but not all DAF-16 target genes.

many nonoverlapping 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.* (2009) used GFP fusions to examine the expression of 17 genes predicted from previous microarray and other experiments to be regulated by DAF-16. Several genes were regulated in the same way in *daf-2* mutants and germline-defective animals, and others were up-regulated only in *daf-2* mutants or only in germline-deficient animals. Interestingly, some 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. 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.

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 vs. *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 in germline-deficient animals, as is its known ligand dafachronic acid (Berman & Kenyon, 2006; Gerisch *et al.*, 2007), one could imagine that DAF-12 would be required for DAF-16 to affect the expression of any gene in response to loss of the germline. However, consistent with studies of a few specific *daf-16*-regulated genes (Wang *et al.*, 2008; Yamawaki *et al.*, 2010), we did not find this. A strict overlap comparison from our microarrays revealed a smaller overlap (seven genes, $P < 1E{-}3$) between genes strongly influenced by both DAF-16 and DAF-12 than one would expect if their target genes were all the same. We identified a much larger but not complete overlap when considering more subtle regulation of targets by both DAF-16 and DAF-12, which makes it clear that some, but not all, genes regulated by DAF-16 have a partial dependence on DAF-12, and vice versa, and in fact that is what we see using both qPCR (Fig. S1) and GFP fusions to some of these genes *in vivo* (Yamawaki *et al.*, 2010).

Some of the genes whose expression changed were known to have interesting effects on lifespan, including *tcer-1* (Ghazi *et al.*, 2009) and C46G7.2 (Kim & Sun, 2007), one of the seven genes co-regulated by *daf-16* and *daf-12*. We tested additional 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 highly conserved putative RNA-binding protein. One of our most intriguing findings was that the target specificity of PHI-62 seemed quite similar to the target specificity of TCER-1. This suggests the possibility that PHI-62 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. However, we did observe differences between these gene functions: knocking down *tcer-1* changes DAF-16-dependent gene expression, but not DAF-16 nuclear localization (Ghazi *et al.*, 2009); whereas knocking down *phi-62* reduces DAF-16 nuclear localization as well as DAF-16-dependent gene expression.

PHI-62 was predicted to bind to a known DAF-16-binding protein, the 14-3-3 protein FTT-2, so we tested FTT-2's role in the germline pathway. Like loss of PHI-62, loss of 14-3-3 activity does not shorten the lifespan of *daf-2* mutants (Berdichevsky *et al.*, 2006; Wang *et al.*, 2006; Li *et al.*, 2007), but it did completely abolish the longevity of germline-defective animals. (We note that because *ftt-2* and its close homolog *par-5* are predicted to cross-react with the *ftt-2* RNAi clone we used to assay longevity, we do not know which is involved.) This finding suggests the possibility that DAF-16, PHI-62 and FTT-2 may act together to extend the lifespan of germline-deficient animals (Fig. 5). The role of 14-3-3 proteins in longevity is complex. In flies, loss of 14-3-3-protein function can be sufficient to trigger FOXO nuclear localization and extend wild-type lifespan (Nielsen *et al.*, 2008). In contrast, in worms, loss of 14-3-3 proteins slightly shortens wild-type lifespan (Berdichevsky *et al.*, 2006; Wang *et al.*, 2006; Li *et al.*, 2007). However, in our study, we observed suppression of the *glp-1*-mutant longevity phenotype accompanied by a decrease in the level of DAF-16 nuclear localization, but no obvious decrease in the expression of the two DAF-16-regulated genes we examined. This makes the role of 14-3-3 proteins in this pathway enigmatic, suggesting that their further study could lead to new insights about their roles in FOXO-dependent signaling pathways.

Two of the genes that are required for the longevity of germline-deficient animals, *lips-17* and *fard-1*, are involved in fat metabolism. 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 when examined using the DEQOR software (Henschel *et al.*, 2004). *fard-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 appeared to have little or no effect on overall fat levels (as measured by the fat indicator oil-red O). 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 nonintestinal tissues, we propose that these three lipid metabolic genes may function 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. These lipid metabolic genes could be part of a pathway that also involves oleic acid, a monounsaturated fat that appears to be required for the longevity of germline-defective animals (Goudeau *et al.*, 2011). The regulation of these fat metabolic genes is unexpectedly diverse. KO4A8.5 is regulated by *daf-16*, but not *daf-12*, whereas we identified *fard-1* as a *daf-12*-regulated gene. The genes that synthesize oleic acid in response to germline loss are regulated by NHR-80, but not by DAF-12 or DAF-16. It will be interesting to learn whether lipid metabolic genes regulated by different branches of the complex germline pathway might actually have distinct functions in lifespan determination.

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 *fard-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*, CF2032: *muEx307* (*Ptcer-1::tcer-1::gfp, Podr-1::rfp*), CF2562: *glp-1*(*e2141ts*) *III*; *sIs10314*[*Pdod-8*::GFP + *pCeh361*], DA1116: *eat-2*(*ad1116*) *II*, CF1041: *daf-2*(*e1370*) *III*, CF1929: *glp-1*(*e2141ts*) *II*; *muIs84*[*Psod-3*::GFP], CF1935: *daf-16*(*mu86*) *I*; *glp-1*(*e2141*) *III*; *muIs109* (*Pdaf-16::daf-16::gfp*, *Podr-1::rfp*), CF3201A: *glp-1*(*e2141ts*) *III*; *sEx10466*[*Pnnt-1*::GFP + *pCeh361*], CF3204: *glp-1*(*e2141*) *III*; *sEx14516*[*pCes* T21D12.9::GFP + *pCeh361*], CF3124: *glp-1*(*e2141*) *III*; *sEx11128*[*Pgpd-2*::GFP].

Microarray analysis

Worms were grown synchronously at 20 °C from eggs produced by bleaching mixed populations. They were transferred from 20 to 25 °C between L2 and L4 to cause sterility in *glp-1*(–) strains and harvested as early adults. Adults from these same harvested plates were set aside to confirm the expected lifespan phenotypes for all strains. Sixty samples were hybridized, consisting of 20 wild-type, 12 *daf-16*, 9 *daf-12*, 9 *glp-1*, 3 *glp-1*; *daf-12* and 7 *daf-16*; *glp-1* samples. 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. Additional enrichment analysis was performed using GSEA v2.0 (Subramanian *et al.*, 2005).

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. The *ftt-2*- and *par-5*-specific RNAi constructs used to assay DAF-16 localization were kindly provided by the Lee lab (Li *et al.*, 2007). 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). For all germline(–) worms and parallel controls, animals were transferred from 20 to 25 °C after 24 h at 20 °C and transferred back to 20 °C as late L4/early adult. RNAi treatments were performed as whole-life treatments, in which eggs were placed on 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, St. Louis, MO, USA) was added to experimental and control 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 (StataCorp, College Station, TX, USA) 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.

Microscopy

All fluorescence images were captured using a Retiga EXi Fast1394 CCD digital camera (QImaging, Burnaby, BC, Canada) attached to a Zeiss Axioplan 2 compound microscope (Zeiss Corporation, Jena, Germany). OPENLAB 4.0.2 software (Improvision, Coventry, UK) was used for image acquisition. GFP assays were conducted on a Leica MZ16F (Wetzlar, Germany) stereomicroscope with fluorescence filter sets or the Zeiss Axioplan 2 compound microscope mentioned earlier.

Quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Grand Island, NY, USA) and purified using Qiagen (Valencia, CA, USA) RNAeasy Mini kit. cDNA was generated using Protoscript First Strand cDNA Synthesis kit (New England Biolabs, Ipswich, MA, USA). SybrGreen real-time Q-PCR reactions were performed on an Applied Biosystems (Carlsbad, CA, USA) 7300 Real-Time PCR System. Oligos were used at a final concentration of 0.55 μM, with a 60 °C annealing temperature for 30 s. *ama-1* was used

as a reference gene. *P*-values were calculated using a two-tailed Student's *t*-test. Primers used were as follows: *gpd-2*, 5′-AAG GCC AAC GCT CAC TTG AA-3′ and 5′-GGT TGA CTC CGA CGA CGA AC-3′, *lips-17*, 5′-ATC TGT TGC TGG AGC CAA TCG-3′ and 5′-TAT CCA ACT TTA TCG TCT CC-3′, *nnt-1*, 5′-CAG TAG AAA CTG CTG ACA TGC TTC-3′ and 5′-GAG CGA TGG GAT ATT GTG CCT GAG-3′, and *sod-3*, 5′-AAA GGA GCT GAT GGA CAC TAT TAA GC-3′ and 5′-AAG TTA TCC AGG GAA CCG AAG TC-3′. *phi-62* has a very small ORF (95aa), and we were unable to amplify it for quantification successfully with the following four sets of primers: 5′-GCT TTC TGC ATG GTC ATG TCG G-3′ and 5′-CTC GAT GGA ACC TTT CCG TG-3′, 5′-CCA TCG AGT GTA ATC GAC GC-3′ and 5′-CGT GTT GTA TTT ATT CTG CC-3′, 5′-TCG GCT TTC TGC ATG GTC ATG TCG-3′ and 5′-ATG AAG ATC TGG GAA GAG AG -3′, and 5′-GGT TCC ATC GAG TGT AAT CGA CGC-3′ and 5′-AAG AGT AAC GCA TAG AGT CC-3′.

DAF-16::GFP localization

Eggs were picked from parents raised on OP-50 bacteria at 20 °C and placed on isopropyl β-D-1-thiogalactopyranoside-induced RNAi bacterial lawns on NG-carbenicillin plates to hatch. *glp-1*(*e2141ts*) worms (and controls) were transferred to 25 °C after 24 h, then to 20 °C at the late L4 stage. Adult worms were scored using a Zeiss SteREO Lumar.V12.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 Quantitative PCR of *lips-17*, *dod-8*, *sod-3*, *gpd-2* and *nnt-1* in germline-deficient animals with and without *daf-12* and *daf-16* mutations.

Fig. S2 Effects of *phi-62* RNAi knockdown on *daf-16* target genes known to be affected by *tcer-1* RNAi knockdown (Ghazi *et al.*, 2009).

Fig. S3 Effects of *phi-62* RNAi knockdown on insulin/IGF-1-pathway, respiration-defective and calorically restricted mutants.

Fig. S4 Lifespan of *daf-16*; *glp-1* germline-deficient animals subjected to RNAi knockdown of *phi-62* or *ftt-2* using RNAi constructs from the Ahringer library.

Fig. S5 DAF-16::GFP localization in *glp-1*(–) and wild-type animals upon knockdown of *ftt-2*, *par-5* and *phi-62*.

Fig. S6 Effects of *ftt-2* RNAi knockdown on expression of *dod-8* and *sod-3*. We note that this RNAi clone, from the Ahringer library, cross-reacts with the 14-3-3 protein *par-5*.

Table S1 The germline (+) vs. germline (–) comparison is enriched for gene categories involved in early development.

Table S2 Genes regulated by DAF-16 in the germline pathway.

Table S3 Overrepresented biological processes in genes regulated by DAF-16 in the germline pathway.

Table S4 Twelve genes make up the total overrepresentation for all three aging-related biological processes for DAF-16 targets in germline-deficient animals.

Table S5 Genes regulated by DAF-12 in the germline pathway.

Table S6 Strict overlap between DAF-16 and DAF-12 targets.

Table S7 Gene set enrichment of DAF-16 targets for DAF-12 regulation.

Table S8 Gene set enrichment of DAF-12 targets for DAF-16 regulation.

Table S9 Genes differentially regulated between long- and short-lived animals in the germline context.

Table S10 Lifespan data.

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